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In vitro synergistic activities of cefazolin and nisin A against mastitis pathogens

by

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In vitro における **cefazolin** と **nisin A** の
乳房炎原因菌に対する相乗的殺菌効果

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２０１９

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INTRODUCTION

Bovine mastitis is an inflammatory condition of the mammary gland, most often caused by intramammary microbial infection. It might necessitate treatment, culling, discarding of milk, and reduced milk quality and yield, which can lead to increased production costs and impose a huge economic strain on the industry [1, 38]. Over 150 different types of bacteria have been isolated from animals with bovine mastitis [6]. On the basis of the bacterial etiologic agents, bovine mastitis can be classified as the contagious or environmental type. While *Staphylococcus aureus* and *Streptococcus agalactiae* are categorized as contagious pathogens [37], coagulase-negative staphylococcus (CNS) [16], *Escherichia coli* [37], *S. dysgalactiae* [37], and *Enterococcus faecalis* [39] are categorized as environmental pathogens. Many intramammary infections caused by these pathogens lead to subclinical and chronic mastitis. Intramammary infection caused by CNS is especially common in many dairy farms around the world; it has caused herd problems such as elevated bulk milk somatic cell count (SCC) and decreased milk quality [35, 44]. In the dairy industry, mastitis is one of the major reasons for the use of antibiotics [29, 33]. Intramammary infusion of antibiotics is the most common therapy for mastitis in dairy farms worldwide [6, 32].

Cephalosporins, a type of β-lactam antibiotics, are considered to be the most important semisynthetic antibiotics for mastitis treatment in dairy cattle [6, 15]. Cephalosporins exert bacteriocidal effects by inhibiting bacterial cell wall synthesis [11, 15]. First-generation cephalosporins are most frequently used for intramammary treatment of mastitis [6, 15], where they generally exhibit good activity [15, 17, 19]. However, the emergence of antibiotic-resistant bacteria due to overuse of antibiotics against mastitis pathogens has been reported previously [3, 34]. Moreover, antibiotic usage results in the presence of antibiotic residues in milk, which is used for human consumption [2, 20, 29]. Therefore, in order to minimize the use of antibiotics, there is an urgent need for alternative antibiotic therapy approaches for bovine mastitis.

Nisin A, a class-I bacteriocin produced by *Lactococcus lactis*, is an antibacterial peptide comprising 34 amino acids. It has been used as a food preservative for over 50 years [23]. Nisin Z, variant of nisin A possessing a different amino acid residue at position 27, has recently been revealed to be a possible alternative for treatment of bovine mastitis caused by Gram-positive bacteria [10, 46]. However, nisin A and Z are generally more active against Gram-positive bacteria than Gram-negative bacteria and exert their bactericidal effect at the cytoplasmic membrane of the target organism [7, 25]. Therefore, the combination of cephalosporin and nisin A might provide an extended activity spectrum against mastitis pathogens and reduce the antibiotic dose for mastitis treatment.

This study aimed to evaluate the combined effect of a first-generation cephalosporin and nisin A against mastitis pathogens using the checkerboard and time-kill assays.

MATERIALS AND METHODS

Bacterial strains

All mastitis pathogen isolates including *S. aureus* (n = 20), *S. intermedius* (n = 20), *S. agalactiae* (n = 10), *S. dysgalactiae* (n = 18), *E. faecalis* (n = 18), and *E. coli* (n = 18) were obtained from clinical or subclinical cases in commercial dairy herds located in different geographical areas of Fukuoka Prefecture, Japan, between 2006 and 2012. These isolates were identified on the basis of colony morphology, Gram-staining properties, and catalase and coagulase test findings and by using commercial kits (API® STAPH, API® 20 STREP, and API® 20 E, bioMérieux Industry, Marcy-l'Étoile, France). Isolates maintained at –80°C in tryptic soy broth with 20% glycerol were retrieved and plated onto Mueller–Hinton agar (MHA) plates supplemented with 5% sheep blood and incubated at 37°C for 24 hr [15].

Antimicrobial substance

Cefazolin (CEZ), which is a first-generation cephalosporin, was purchased from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). For use in this study, CEZ stock solutions were prepared at a concentration of 10 mg/ml.

Nisin A was manufactured by Omu Milk Products Co., Ltd. (Fukuoka, Japan). The nisin A-producing *L. lactis* strain was grown in MRS broth (Oxoid Ltd., Basingstoke, UK) for 16 hr at 30°C. Nisin A was recovered from the culture supernatant and purified as previously described, with some modifications [47]. Briefly, the culture supernatant was applied to a hydrophobic resin column, and the adsorbed nisin A was eluted with 40–70% ethanol and separated by evaporation. The compound was further purified by reverse-phase high-performance liquid chromatography (HPLC). The concentration of purified nisin A was determined from the HPLC peak area with a standard curve prepared by commercial nisin preparation (Sigma-Aldrich, St. Louis, MO, U.S.A.) [13].

Determination of minimum inhibitory concentration (MIC)

The MICs of CEZ and nisin A against mastitis pathogens were determined by the microdilution method, in accordance with the Clinical and Laboratory Standards Institute guidelines [14]. Briefly, target strains were inoculated onto MHA plates supplemented with 5% sheep blood and incubated overnight

at 37°C. The cells were diluted in Mueller–Hinton broth (MHB) to an approximate final concentration of 5×10^5 colony forming units (CFUs) per ml. Fifty microliters each of the antimicrobial agent dilutions and bacterial inocula were dispensed into individual wells of a 96-well microtiter plate. The plate was then incubated at 37°C for 24 hr. Minimum inhibitory concentration was defined as the lowest concentration of each antibiotic that completely inhibited bacterial growth, as apparent to the unaided eye.

Checkerboard assay

The interactions between CEZ and nisin A against mastitis pathogens were evaluated by the microbroth checkerboard method in 96-well microtiter plates containing MHB, as described in previous reports [28, 43]. Briefly, CEZ and nisin A were serially diluted along the y and x axes, respectively. The final antimicrobial substance concentrations (after two-fold dilution) ranged from 1/16 to 4 times the MIC for CEZ and from 1/256 to 4 times the MIC for nisin A. The checkerboard plates were inoculated with bacteria at an approximate concentration of 5×10^5 CFU/ml and incubated at 37°C for 24 hr, following which bacterial growth was assessed visually. To evaluate the effect of the combination treatment, the fractional inhibitory concentration (FIC) index for each combination was calculated as follows: FIC index = FIC of CEZ + FIC of nisin A, where FIC of CEZ (or nisin A) was defined as the ratio of MIC of CEZ (or nisin A) in combination and MIC of CEZ (or nisin A) alone. The FIC index values were interpreted as follows: ≤ 0.5, synergistic; > 0.5 to ≤ 1.0, additive; >1.0 to ≤ 2.0, indifferent; and > 2.0, antagonistic effects [18, 30].

Time-kill assay

The findings of *in vitro* interaction determined through the checkerboard assay were confirmed through the time-kill assay, which was performed by the broth dilution method, as described in previous studies [36, 41]. Briefly, the experiment included the control, CEZ, nisin A, and combination (CEZ–nisin A) groups. For bacterial inoculation, three isolates were arbitrarily selected from the most dominant FIC index group for each mastitis pathogen. Equal proportions of the three isolates from each group were combined to obtain a three-isolate mixture of each mastitis pathogen species [1]. The MICs of the three-isolate mixtures were measured by the same method as described above. Five milliliters of MHB without antimicrobial substances was used as the control, while MHB (5 ml) with CEZ or nisin A alone at concentrations of 0.5 ×, 1 ×, and 2 × MIC was added separately into the

corresponding tubes. The two antimicrobial substances were added in combination to the corresponding tubes at the following concentrations: 0.5 × MIC CEZ + 0.5 × MIC nisin A; 1 × MIC CEZ + 1 × MIC nisin A; and 2 × MIC CEZ + 2 × MIC nisin A. The bacterial inocula were diluted to approximately 5×10^5 CFU/ml in a 5-ml final volume of MHB. At 0, 3, 6, 9, and 24 hr of incubation with agitation at 37°C, 100-µl aliquots of culture supernatant were collected from each tube, diluted tenfold, and inoculated onto MHA plates with 5% sheep blood. The plates were incubated at 37°C for 24 hr, following which the colony counts were determined. The lower limit for detection of bacterial count was 2.3 $\log_{10}$ CFU/ml (i.e., 200 CFU/ml). Experiments were performed in triplicate. A growth curve was plotted with the average bacterial count at each time point. Bactericidal effect was defined as a reduction of ≥ 3 $\log_{10}$ CFU/ml relative to the starting inoculum. Synergism was defined as a reduction of ≥ 2 $\log_{10}$ CFU/ml observed at 24 hr post-incubation with the CEZ–nisin A combination relative to that observed with either antimicrobial substance. Additive effect was defined as a reduction of 1 to < 2 $\log_{10}$ CFU/ml observed at 24 hr post-incubation with the CEZ–nisin A combination relative to that observed with either antimicrobial substance. Indifferent effect was defined as an increase or decrease of < 1 $\log_{10}$ CFU/ml, and antagonism was defined as an increase of > 2 $\log_{10}$ CFU/ml observed at 24 hr post-incubation with the CEZ–nisin A combination relative to that observed with either antimicrobial substance [36, 41].

RESULTS

MIC and checkerboard assay

Table 1 presents a summary of MICs of CEZ and nisin A, alone and in combination, against mastitis pathogens. Cefazolin was very active against *S. aureus*, *S. intermedius*, *S. agalactiae*, and *S. dysgalactiae*, with MIC_{50} values ranging from 0.13 to 0.5 μg/ml. While CEZ exhibited good activity against *E. coli* (MIC_{50}, 2 μg/ml), it was less active against *E. faecalis* (MIC_{50}, 32 μg/ml). Nisin A was very active against *S. intermedius* and *S. agalactiae* (MIC_{50}, 0.06 and 0.25 μg/ml, respectively); while the compound exhibited good activity against *S. aureus* (MIC_{50}, 1 μg/ml), *S. dysgalactiae* (MIC_{50}, 1 μg/ml), and *E. faecalis* (MIC_{50}, 4 μg/ml), it was not active against *E. coli* (MIC_{50}, 128 μg/ml). The MIC_{50} values of CEZ and nisin A in combination with each other were 2- to 8-times lower than those of either antimicrobial substance alone.

Table 2 presents results of the checkerboard assay of CEZ and nisin A against mastitis pathogens. Nisin A and CEZ exhibited synergistic or additive interactions, with FIC index values ranging from 0.19 to 1. There were no instances of indifferent or antagonistic interaction between the two compounds. The CEZ–nisin A interaction in *S. aureus* (100%) and *E.* faecalis (72.2%) cultures was predominantly synergistic, while that in *S. intermedius* (60%), *S. agalactiae* (100%), *S. dysgalactiae* (100%), and *E. coli* (77.8%) was mostly additive.

Time-kill assay

Figure 1 presents the results of the time-kill assay. The MICs of CEZ and nisin A against the three-isolate mixtures of both *S. aureus* and *S. intermedius* were 0.5 and 1 μg/ml, respectively. Against the *S. aureus* strains, CEZ, alone, exhibited no bactericidal effect at any time point in the incubation period (Fig. 1-1a). In contrast, nisin A, alone, exhibited transient bactericidal effects at 2 × MIC (Fig. 1-1b). However, the CEZ–nisin A combination at ≥ 1× MIC exhibited bactericidal activity within 3 hr of incubation, with effects lasting for 24 hr (Fig. 1-1c). Against the *S. intermedius* strains, CEZ, alone, exhibited bactericidal activity at ≥ 1 × MIC (Fig. 1-2d). In contrast, incubation with nisin A, alone, resulted in a clear concentration-dependent decrease in bacterial count within 6 hr after exposure; this, however, did not prevent the regrowth of the surviving bacteria (Fig. 1-2e). However, the CEZ–nisin A combination at ≥ 0.5 × MIC exhibited bactericidal activity within 3 hr of incubation, with effects lasting for 24 hr (Fig. 1-2f).

The MICs of CEZ and nisin A against the three-isolate mixture of *S. agalactiae* were 0.13 and

0.25 μg/ml, respectively. Cefazolin, alone, exhibited no bactericidal effect at any time point in the incubation period (Fig. 1-3g). In contrast, nisin A, alone, exhibited transient bactericidal effects at 2 × MIC (Fig. 1-3h). The CEZ–nisin A combination exhibited concentration-dependent bactericidal activity; at ≥ 1 × MIC, the antimicrobial substance combination exhibited bactericidal activity within 6 hr of incubation, with effects lasting for 24 hr (Fig. 1-3i).

The MICs of CEZ and nisin A against the three-isolate mixture of *S. dysgalactiae* were 0.25 and 32 μg/ml, respectively. Cefazolin, alone, exhibited no bactericidal effect at any time point in the incubation period (Fig. 1-4j). In contrast, nisin A, alone, at 2 × MIC exhibited bactericidal effects within 3 hr of incubation, which lasted for 24 hr (Fig. 1-4k). The CEZ–nisin A combination at ≥ 0.5 × MIC exhibited bactericidal activity within 3 hr, with effects lasting for 24 hr (Fig. 1-4l).

The MICs of CEZ and nisin A against the three-isolate mixture of *E. faecalis* were 32 and 8 μg/ml, respectively. Cefazolin, alone, exhibited no bactericidal effect at any time point in the incubation period (Fig. 1-5m). In contrast, treatment with nisin A, alone, resulted in a concentration-dependent decrease in bacterial count within 6 hr after exposure; at 2 × MIC, nisin A exhibited bactericidal effects that lasted for 24 hr post-incubation (Fig. 1-5n). The CEZ–nisin A combination at ≥ 0.5× MIC exhibited bactericidal activity within 3 hr of incubation, with effects lasting for 24 hr (Fig. 1-5o).

The MICs of CEZ and nisin A against the three-isolate mixture of *E. coli* were 2 and 128 μg/ml, respectively. Cefazolin, alone, exhibited bactericidal activity at 2 × MIC, while nisin A, alone, exhibited transient bactericidal effects at 1 × and 2 × MIC; this effect, however, did not prevent the regrowth of the surviving bacteria (Fig. 1-6p,q). The CEZ–nisin A combination at ≥ 0.5 × MIC exhibited bactericidal activity within 3 hr of incubation, with effects lasting for 24 hr (Fig. 1-6r).

Table 3 presents a summary of the changes in bacterial count in the time-kill assay at 24 hr post-incubation. Incubation with CEZ or nisin A, alone, at 0.5 × or 1 × MIC resulted in increased bacterial counts at 24 hr post-incubation. However, incubation with the CEZ–nisin A combination at 0.5 × or 1 × MIC resulted in a reduction in bacterial count by over 3 $\log_{10}$ CFU/ml relative to the initial count at 0 hr; at 24 hr post-incubation, this combination also resulted in a synergistic reduction of bacterial counts by 3.39 to 6.18 $\log_{10}$ CFU/ml relative to the count observed with either antimicrobial substance alone.

Table 1. Summary of the MIC_{50} values of cefazolin (CEZ) and nisin A, alone and in combination, against bovine mastitis pathogens.

starains	n	MIC_{50} (range) of compound (μg/ml)							
		alone				in combination			
		CEZ		nisin A		CEZ		nisin A	
Staphylococcus aureus	20	0.5	(0.25-1)	1	(0.25-16)	0.06	(0.03-0.13)	0.25	(0.03-1)
Staphylococcus intermedius	20	0.5	(0.25-4)	0.06	(0.02-4)	0.13	(0.03-0.5)	0.03	(0.002-2)
Streptococcus agalactiae	10	0.13	(0.06-0.25)	0.25	(0.03-0.25)	0.06	(0.02-0.13)	0.13	(0.008-0.13)
Streptococcus dysgalactiae	18	0.13	(0.03-1)	1	(0.06-32)	0.06	(0.02-0.13)	0.5	(0.03-4)
Enterococcus faecalis	18	32	(1-64)	4	(0.5-8)	8	(0.25-16)	0.5	(0.02-4)
Escherichia coli	18	2	(1-8)	128	(64-128)	1	(0.06-2)	32	(4-64)

MIC_{50} values were defined as the lowest concentration of the antimicrobial substance at which 50% of the each pathogen were inhibited.

Table 2. Combined effect of cefazolin (CEZ) and nisin A determined by checkerboard assay.

Organism	n	FIC index	No. of strains (%)			
			synergism (≤0.5)	additive (>0.5-≤1.0)	indifferent (>1.0-≤2.0)	antagonism (>2.0)
Staphylococcus aureus	20	0.19 - 0.5	20 (100)	0 (0)	0 (0)	0 (0)
Staphylococcus intermedius	20	0.19 - 1.0	8 (40)	12 (60)	0 (0)	0 (0)
Streptococcus agalactiae	10	0.63 - 1.0	0 (0)	10 (100)	0 (0)	0 (0)
Streptococcus dysgalactiae	18	0.56 - 1.0	0 (0)	18 (100)	0 (0)	0 (0)
Enterococcus faecalis	18	0.19 - 1.0	13 (72.2)	5 (27.8)	0 (0)	0 (0)
Escherichia coli	18	0.5 - 1.0	4 (22.2)	14 (77.8)	0 (0)	0 (0)

The FIC index values were interpreted as follows: ≤ 0.5, synergistic; > 0.5 to ≤ 1.0, additive; > 1.0 to ≤ 2.0, indifferent; and > 2.0, antagonistic effects.

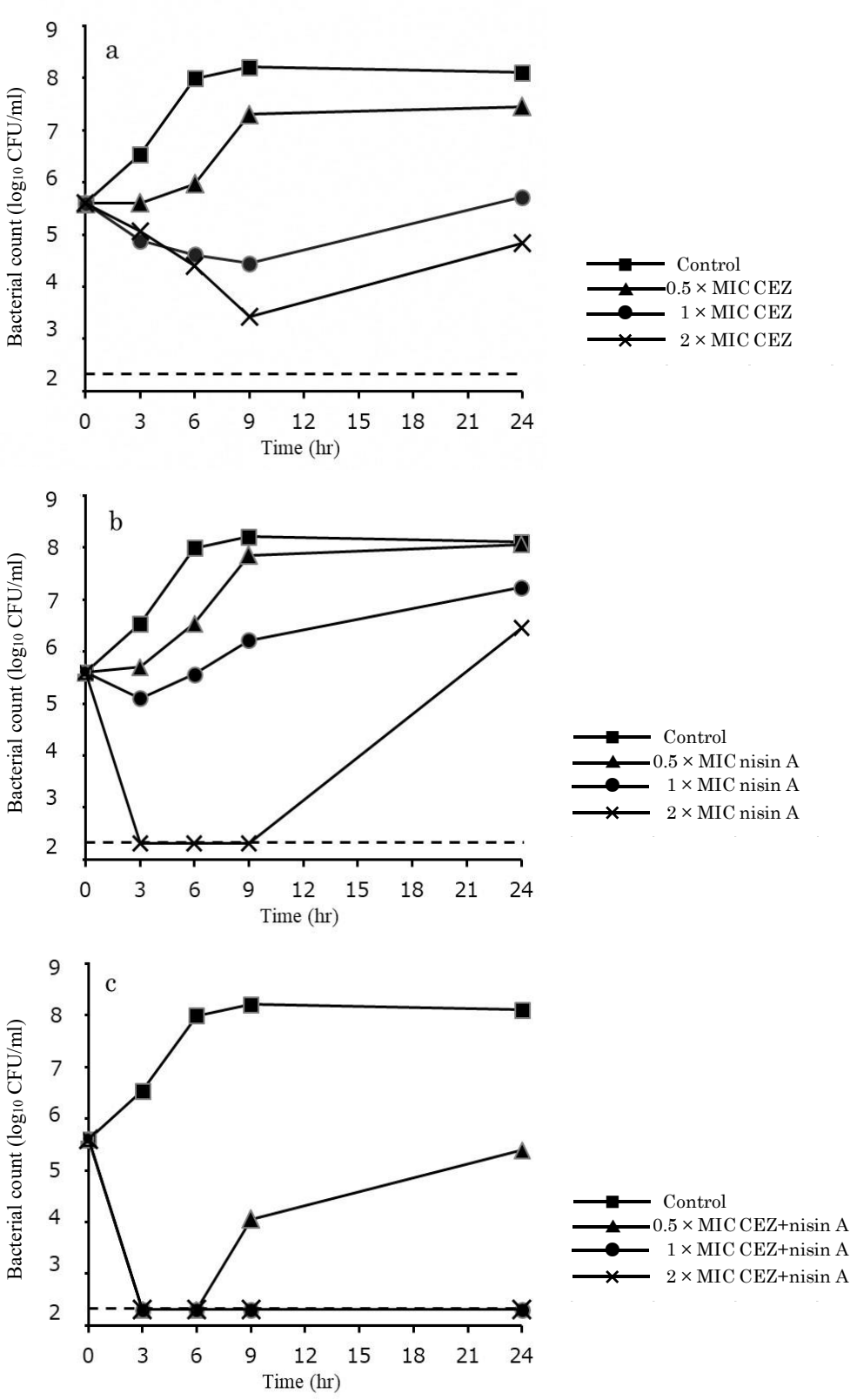

Fig. 1-1. Time-kill curves for cefazolin (CEZ) and nisin A, alone and in combination, against *Staphylococcus aureus*.

a, CEZ alone; **b**, nisin A alone; **c**, CEZ－nisin A combination. Thin dotted line indicates the limit of detection.

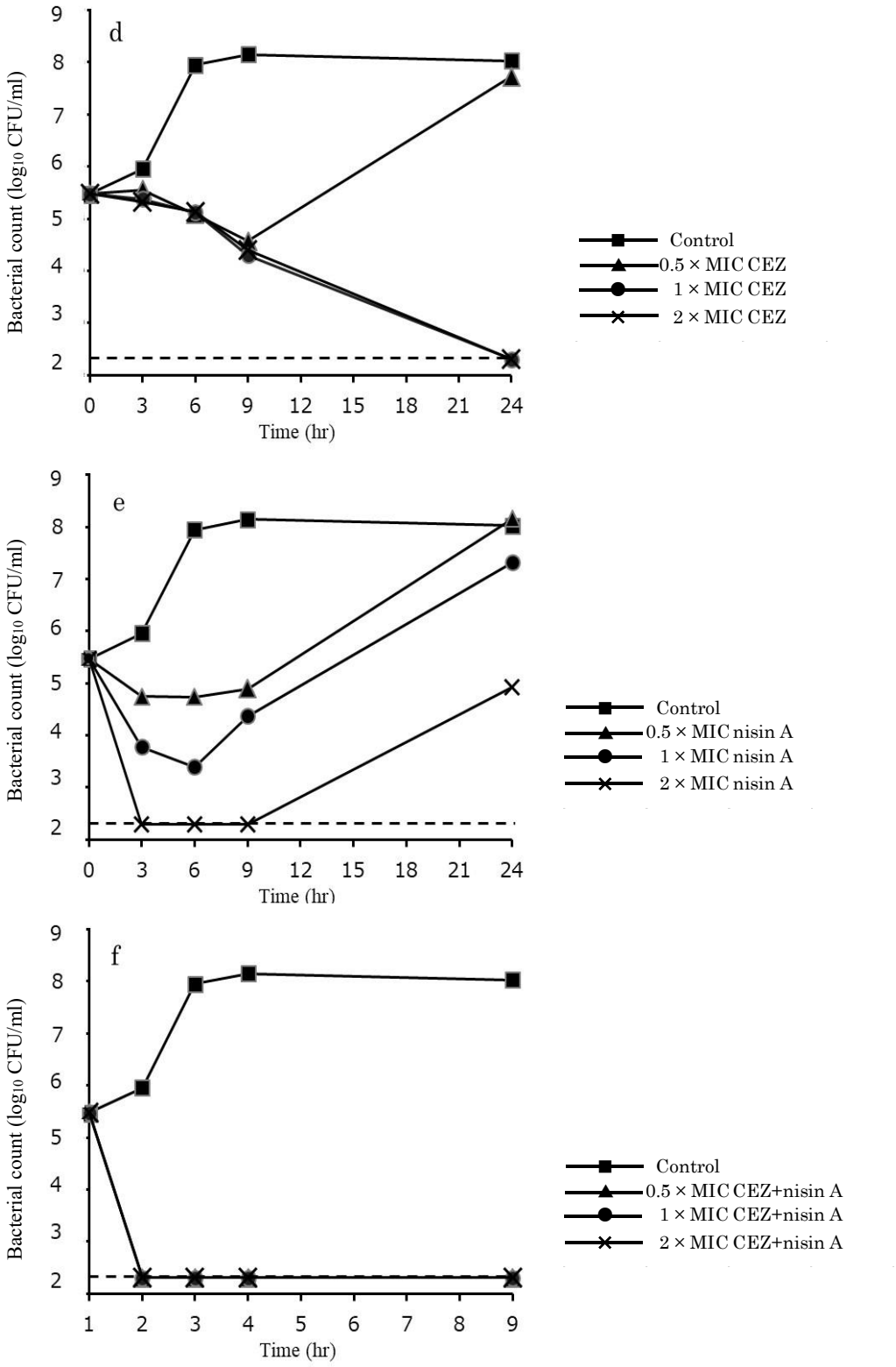

Fig. 1-2. Time-kill curves for cefazolin (CEZ) and nisin A, alone and in combination, against *Staphylococcus intermedius*.

d, CEZ alone; **e**, nisin A alone; **f**, CEZ－nisin A combination. Thin dotted line indicates the limit of detection.

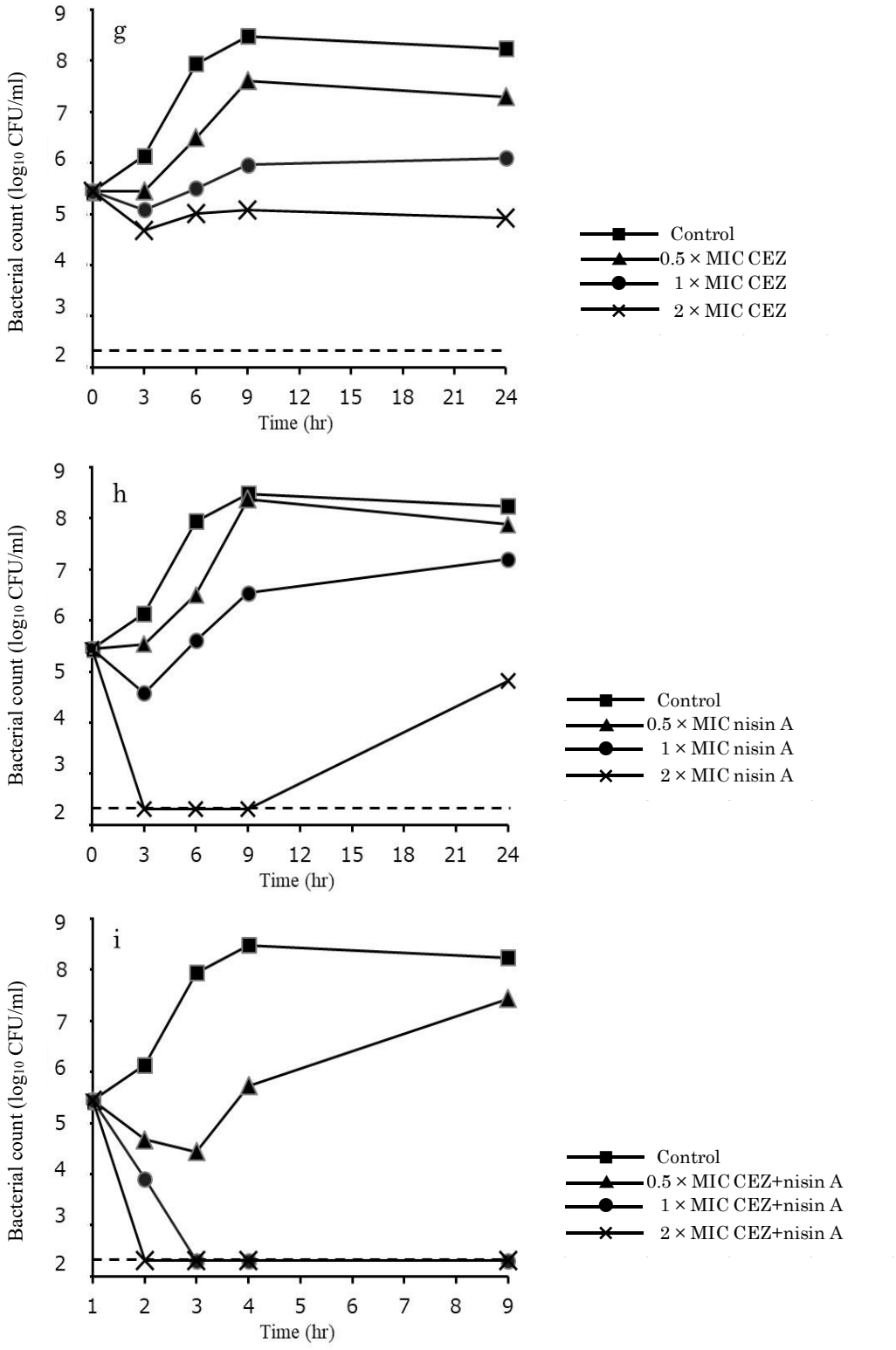

Fig.1-3. Time-kill curves for cefazolin (CEZ) and nisin A, alone and in combination, against *Streptococcus agalactiae*.

g, CEZ alone; **h**, nisin A alone; **i**, CEZ－nisin A combination. Thin dotted line indicates the limit of detection.

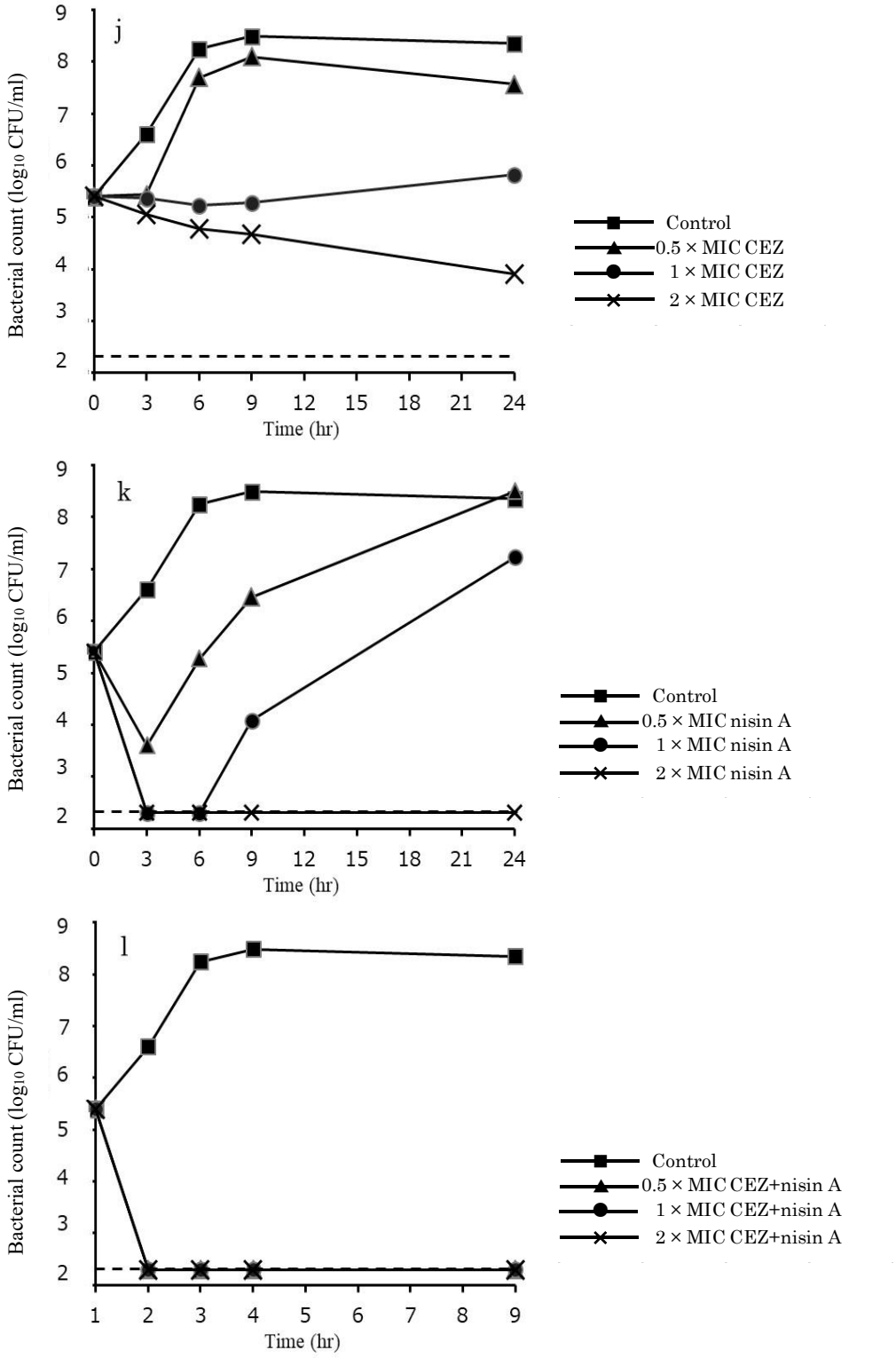

Fig.1-4. Time-kill curves for cefazolin (CEZ) and nisin A, alone and in combination, against *Streptococcus dysgalactiae*.

j, CEZ alone; **k**, nisin A alone; **l**, CEZ－nisin A combination. Thin dotted line indicates the limit of detection.

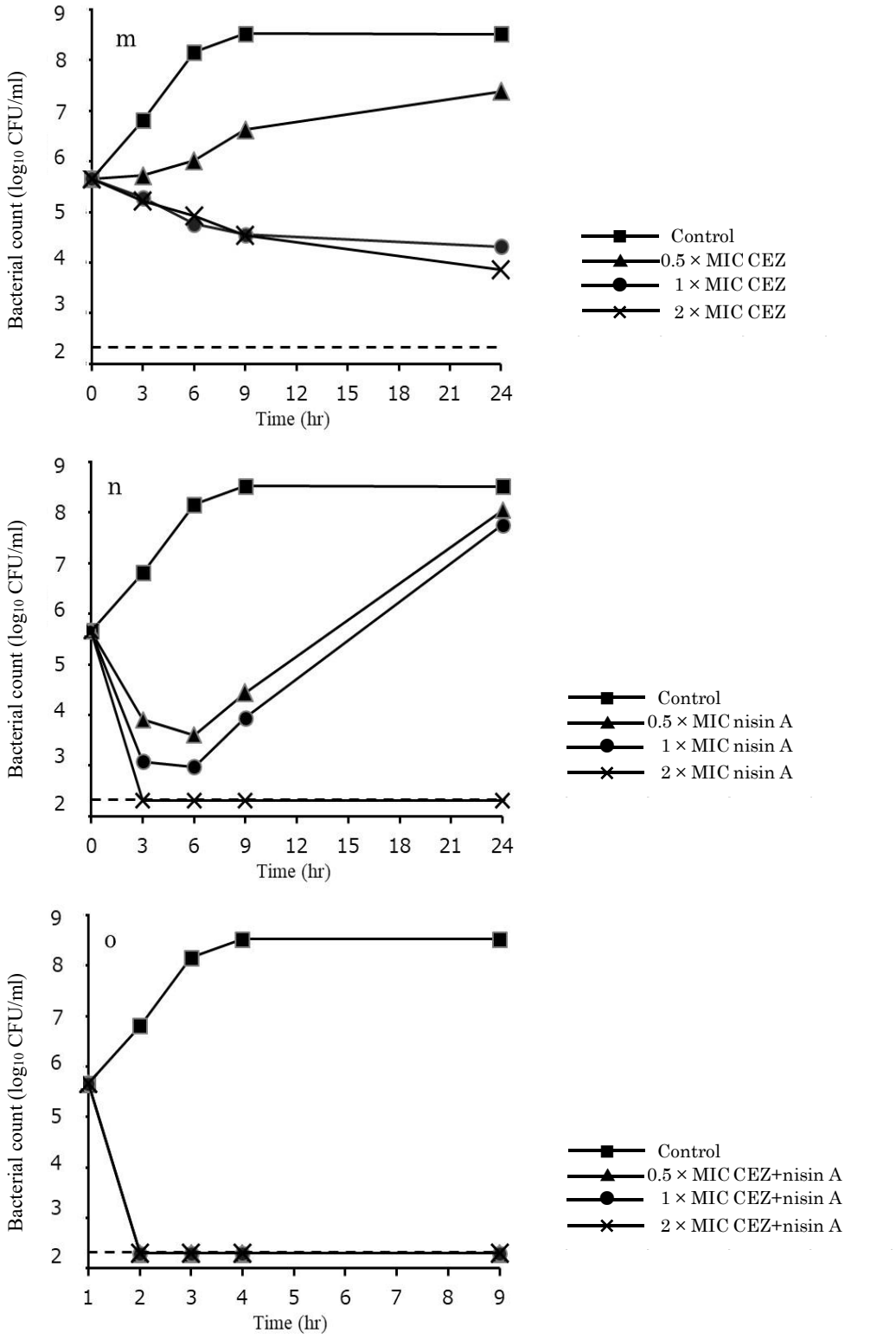

Fig.1-5. Time-kill curves for cefazolin (CEZ) and nisin A, alone and in combination, against *Enterococcus faecalis*.

m, CEZ alone; **n**, nisin A alone; **o**, CEZ－nisin A combination. Thin dotted line indicates the limit of detection.

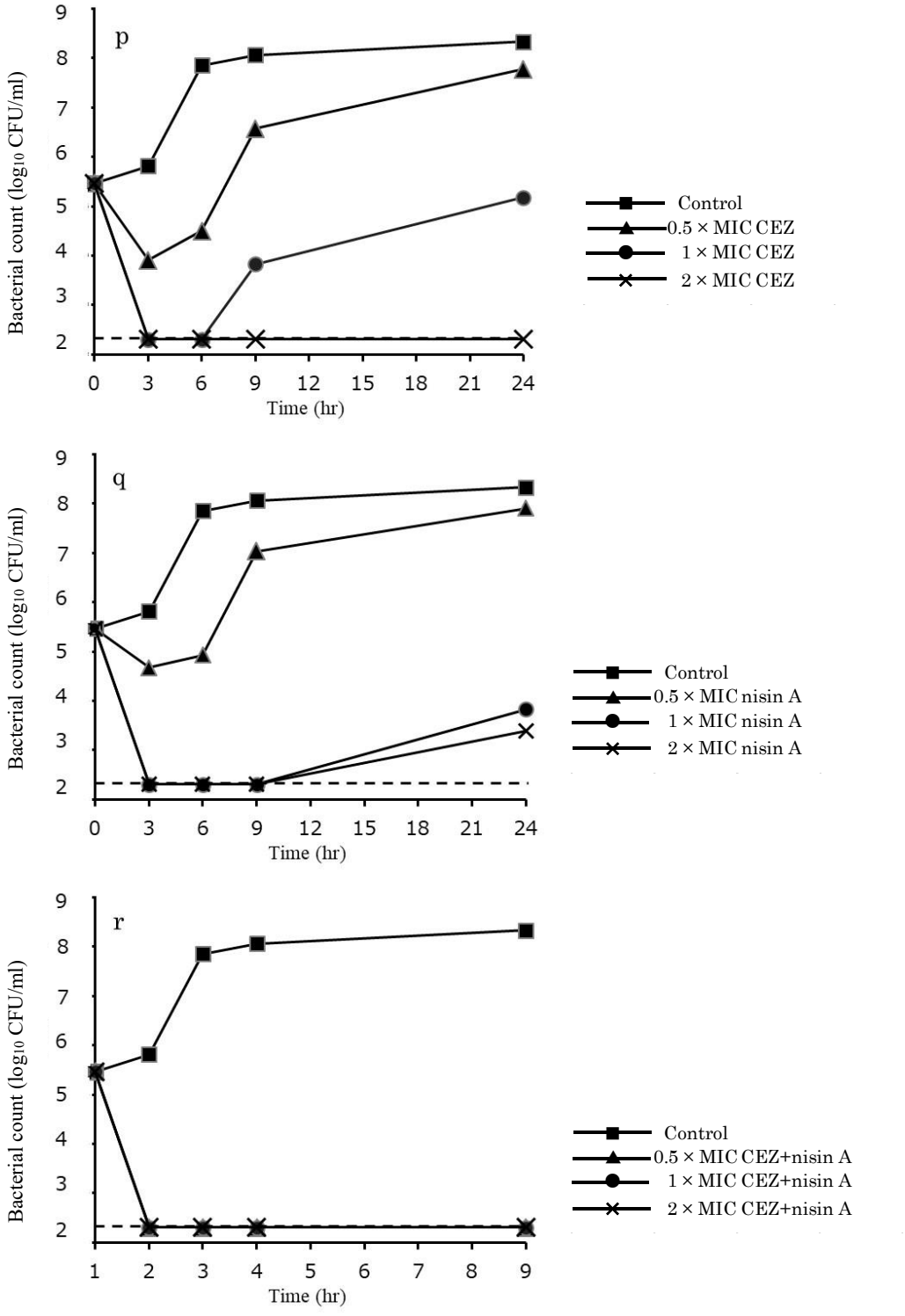

Fig.1-6. Time-kill curves for cefazolin (CEZ) and nisin A, alone and in combination, against *Escherichia coli*.

p, CEZ alone; **q**, nisin A alone; **r**, CEZ－nisin A combination. Thin dotted line indicates the limit of detection.

Table 3. Changes in bacterial count at 24 hr post-incubation with cefazolin (CEZ) and nisin A, alone and in combination.

Organism	× MIC	Changes in bacterial count (log_{10} CFU/ml) from 0 h			a − c	b − c	Interpretation
		CEZ (a)	Nisin A (b)	CEZ−Nisin A (c)			
Staphylococcus aureus	1	0.10	1.64	-3.29	3.39	4.93	S
Staphylococcus intermedius	0.5	2.26	2.68	-3.17	5.43	5.85	S
Streptococcus agalactiae	1	0.50	1.69	-3.31	3.81	5.00	S
Streptococcus dysgalactiae	0.5	2.14	3.08	-3.10	5.24	6.18	S
Enterococcus faecalis	0.5	1.72	2.67	-3.36	5.08	6.03	S
Escherichia coli	0.5	2.31	2.45	-3.16	5.47	5.61	S

Bacterial count was measured by the change in log_{10} CFU/ml from 0 to 24 hr post-incubation, obtained from the data in Fig.1. (i.e., log change = log_{10} CFU24–log_{10} CFU0). The results are calculated at 0.5×MIC except in the case of *S. aureus* and *S. agalactiae* (1×MIC). S, synergism

DISCUSSION

First-generation cephalosporins such as cefazolin have been used for the treatment of bovine mastitis caused by Gram-positive and negative bacteria [6, 32]. The antibacterial peptide nisin, including nisin A, has generally been used in food preservatives because of its high antibacterial activity and nontoxicity [21, 23]. As such, some studies have suggested that nisin is effective against mastitis pathogens [10, 40, 46]. The CEZ–nisin A combination can, therefore, be expected to reduce the antibiotic dose for mastitis treatment by extending the activity spectrum by means of synergistic effects against mastitis pathogens. However, there is no information on the synergistic effect of CEZ and nisin A. The present *in vitro* study investigated the antibacterial activity of the CEZ–nisin A combination against mastitis pathogens for bovine mastitis treatment.

First-generation cephalosporins have generally been reported as exhibiting good bactericidal activity against mastitis bacterial pathogens [15, 17, 19]. However, Tong *et al*. [43] suggested that *E. faecalis* possesses both intrinsic and acquired resistance to a variety of antibiotics. In our study, CEZ exhibited MIC_{50} values of 0.13–2 μg/ml against all mastitis pathogens, except *E. faecalis* (32 μg/ml). These results reasonably correspond with those of previous studies [15, 17, 19, 43].

Although early studies have reported the MICs of nisin against major Gram-positive mastitis pathogens, the reported values were high and exhibited a wide range (10–250 μg/ml) [8]. Nisin has since then been incorporated into commercially available teat-dipping formulations for mastitis prevention. It has been reported to exhibit mean log reductions of 3.90 and 4.22 against *S. aureus* and *E. coli*, respectively, after exposure for 1 min [40]. Furthermore, Cao *et al*. [10] and Wu *et al*. [46] reported the therapeutic efficacy of intramammary nisin infusion in lactating dairy cows with clinical or subclinical mastitis caused by several mastitis pathogens, including staphylococci and streptococci. Although these reports suggest the efficacy of nisin against mastitis pathogens, objective evidence of its antibacterial activity against various mastitis pathogens seems to be insufficient. In our study, the MIC_{50} values of nisin A against staphylococci and streptococci ranged from 0.06 to 1 μg/ml. Even against cephalosporin-resistant enterococci, nisin A exhibited relatively low MIC_{50} values (4 μg/ml). These results demonstrate the antimicrobial efficacy of nisin A against Gram-positive mastitis pathogens and support the results of previous studies [10, 40, 46]. On the other hand, nisin A exhibited very low activity against *E. coli*. An accumulating body of evidence shows that nisin exhibits high antibacterial activity against Gram-positive bacteria but not against Gram-negative species. However, previous studies have suggested that nisin A [22] and Z [27] do exhibit MICs against *E. coli*. Although further research is required to confirm these findings, the present results support the relevance of these previous findings. *E. coli* can cause mammary gland inflammation in dairy cows around the time of parturition and during early lactation, with striking local, and sometimes severe, systemic clinical

symptoms [9]. Understandably, it is desirable that therapeutic substances used for mastitis treatment possess bactericidal properties against both Gram-positive and negative bacteria. Accordingly, we hypothesized that a combination of CEZ and nisin A would be effective against both Gram-positive and negative mastitis pathogens. Furthermore, we expected that the synergistic effect of this combination would reduce the antibiotic dose for mastitis treatment in dairy cows.

In the present study, the synergistic effect was evaluated on the basis of MIC values obtained by the checkerboard and time-kill assays. The MIC_{50} of the CEZ–nisin A combination against mastitis pathogens was 2- to 8-fold lower than that observed with either compound alone. In the checkerboard assay, all interactions between CEZ and nisin A were synergistic or additive, with FIC index values ranging from 0.19 to 1. In the checkerboard assay, the CEZ–nisin A interactions against *S. aureus* and *E. faecalis* strains were mainly synergistic, while those against the other strains were mainly additive. In the time-kill assay, incubation with the CEZ–nisin A combination for 24 hr resulted in a 10^3-fold greater synergistic reduction in bacterial count relative to that observed with either compound at 0.5 × or 1 × MIC. Furthermore, at 0.5 × or 1 × MIC, the CEZ–nisin A combination exhibited bactericidal effects against all pathogen strains within 6 hr of incubation. The checkerboard and time-kill assay methods are among the most widely used techniques for *in vitro* assessment of synergistic effects [45]. However, some studies have reported discordance between the findings of these two methods [12, 45]. In the present study, *S. aureus* and *E. faecalis* strains, which were selected from the synergistic-effect group in the checkerboard assay, were also susceptible to the synergistic effect of CEZ–nisin A in the time-kill assay. However, the *S. intermedius*, *S. agalactiae*, *S. dysgalactiae*, and *E. coli* strains that were selected from the additive-effect group in the checkerboard assay exhibited susceptibility to the synergistic effect in the time-kill assay. Despite this discordance in results, both assays revealed synergistic or additive effects of CEZ–nisin A, thus indicating the efficacy of this combination.

Cephalosporins are β-lactam antimicrobials that exert bactericidal properties by disruption of bacterial cell-wall synthesis [11, 15]. The mode of action of nisin A involves interaction with the membrane-bound cell-wall precursor lipid II concomitant with pore formation in the cytoplasmic membrane of the target organism, resulting in loss of membrane potential and leakage of intracellular metabolites [7, 25].

In the present study, the CEZ–nisin A combination exhibited synergistic or additive effects against both Gram-positive and negative mastitis pathogens. It may, therefore, be inferred that the bactericidal effect of CEZ–nisin A against Gram-positive bacteria results from the interference of CEZ with bacterial cell-wall synthesis and cytoplasmic membrane pore formation by nisin A. On the other hand, the poor sensitivity of Gram-negative bacteria to nisin A might be attributed to the large size of the peptide, which would restrict its passage through the outer membrane of Gram-negative bacteria [24]. In this respect, some reports [5, 24] suggest the use of the metal-chelating agent

ethylenediaminetetraacetic acid (EDTA) for the enhancement of nisin A sensitivity; EDTA removes stabilizing cations from the outer membrane and destroys the membrane function as a penetration barrier. Although the mechanism of action of nisin A against Gram-negative bacteria is not completely understood, it is supposed that CEZ initially mediates the inhibition of Gram-negative bacterial cell-wall synthesis, following which nisin A causes pore formation in the cytoplasmic membrane and leakage of intracellular metabolites.

In the present study, the CEZ–nisin A combination exhibited synergistic or additive effects, which suggests that the two antimicrobial substances can together achieve mastitis pathogen control even at low concentrations. Furthermore, this combination might provide extended activity spectrum against mastitis pathogens such as enterococci or *E. coli*, which are not sufficiently inhibited by either compound individually. An early study [26] had reported that milk fat inhibits the antibacterial effect of nisin. However, Bhatti *et al*. [4] did not observe any decrease in the antibacterial effect of nisin in non-homogenized milk products such as raw milk. Although Szweda *et al*. [42] reported a decreased susceptibility to nisin in antibiotic-resistant *S. aureus* isolated from bovine mastitis, Okuda *et al*. [31] suggested that nisin A that forms stable pore on biofilm cells is highly potent for the treatment of biofilm-associated infections. These findings, together with the present results, suggest that the CEZ–nisin A combination can serve as an alternative therapy for bovine mastitis in the form of intramammary infusion, with lower antibiotic concentrations than normal.

In conclusion, the results of the checkerboard and time-kill assays in the present study indicated that CEZ and nisin A exert synergistic or additive bactericidal effects against bovine mastitis pathogens. These results suggest that the CEZ–nisin A combination is effective in reducing the antibiotic dose in intramammary infusions formulated for mastitis treatment in dairy cattle. Further studies are required for *in vivo* assessment of microbial response to this antimicrobial substance combination.

ABSTRACT

First-generation cephalosporins such as cefazolin (CEZ) have been widely used for mastitis treatment in dairy cattle. However, the use of antibiotics results in the presence of antibiotic residues in milk, which is used for human consumption. Nisin A, a bacteriocin produced by *Lactococcus lactis*, has been used as a broad-spectrum food preservative for over 50 years. Therefore, a combination of CEZ and nisin A might provide an extended activity spectrum against mastitis pathogens and reduce the antibiotic dose for mastitis treatment. This study aimed to evaluate the combined effect of CEZ and nisin A against mastitis pathogens using the checkerboard and time-kill assays. In the checkerboard assay, the CEZ–nisin A combination exhibited a synergistic effect against *Staphylococcus aureus* (n = 20/20) and *Enterococcus faecalis* (n = 13/18), and meanwhile exhibited a mostly additive effect against *Staphylococcus intermedius* (n = 12/20), *Streptococcus agalactiae* (n = 10/10), *Streptococcus dysgalactiae* (n = 18/18), and *Escherichia coli* (n =14/18). There were no indifferent or antagonistic effects between CEZ and nisin A. In the time-kill assay, the CEZ–nisin A combination at 0.5 × or 1 × minimum inhibitory concentration exhibited synergistic reduction of bacterial growth by over 3 $\log_{10}$ colony forming units per ml relative to that observed with either antimicrobial substance alone. These results suggest that the CEZ–nisin A combination can be used for developing an intramammary infusion for mastitis treatment, with lower antibiotic concentrations than normal.

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和文摘要

乳牛の乳房炎は主に乳房内への細菌感染によって発症する。本病は急性に乳房の腫脹・硬結や乳汁性状の異常などの局所症状が認められ，重症の場合は体温上昇や食欲不振などの全身症状を呈する。さらに慢性化すると再発を繰り返し，次第に乳量の低下や乳質悪化をまねくなど酪農業界に大きな損失をもたらす。治療方法は抗生物質への依存度が極めて高く，乳房内注入が広く行われているが，近年は大量使用による耐性菌の出現や生乳中への薬剤残留などによる食品汚染が問題視されている。第一世代セファロスポリンであるセファゾリン（CEZ）は乳房炎治療の第一選択薬として世界中で使用され，細胞壁ペプチドグリカンの合成阻害によりグラム陽性・陰性の両方の原因菌に殺菌的に作用する。一方，クラスIバクテリオシンであるナイシン A は，乳酸菌 *Lactococcus lactis* が産生する 34 残基のアミノ酸で構成される抗菌ペプチドで，安全性の高い食品保存料として我が国をはじめ世界中で利用されている。ナイシン A は細菌の細胞壁前駆体 lipid Ⅱに付着し細胞膜に孔を形成することで殺菌的に作用する。グラム陽性細菌に強い抗菌活性を示すため，*Staphylococcus* 属，*Bacillus* 属，*Clostridium* 属，*Listeria* 属などの食品汚染菌が問題となるチーズ，乳製品，缶詰などの保存料として使用されている。しかし，細胞壁外膜を有するグラム陰性菌に対する殺菌効果は乏しいことも知られている。このため，CEZ とナイシン A を併用すれば，その相互作用により乳房炎原因菌に対する抗菌スペクトルの拡大や乳房炎治療における抗生物質投与量の低減が期待できる。そこで，本研究は CEZ とナイシン A の乳房炎原因菌に対する併用効果を *in vitro* で評価した。

2006 年から 2012 年の間に県内で発生した臨床型・潜在性乳房炎から分離した原因菌を試験に供した。伝染性病原菌として *Staphylococcus aureus* (n=20)および *Streptococcus agalactiae* (n=10)を，また，環境性病原菌として *Staphylococcus intermedius* (n=20)，*Streptococcus dysgalactiae* (n=18)，*Enterococcus faecalis* (n=18)および *Escherichia coli* (n=18)を用いた。CEZ は市販の動物用医薬品を，ナイシン A はオーム乳業（株）が作成したものを用いた。乳房炎原因菌に対する CEZ とナイシン A の最小発育阻止濃度（MICs）は Muller-Hinton broth（MHB）を用いた微量液体希釈法で測定し，菌種ごとに MIC_{50} を算出した。乳房炎原因菌に対する CEZ とナイシン A の併用効果は，MHB と 96 穴マイクロプレートを用いた Checkerboard assay により FIC index 値を算出し，その値が 0.5 以下の場合を相乗効果，0.5 より大きく 1.0 以下を相加効果，1.0 より大きく 2.0 以下を無関係，2.0 より大きい場合を拮抗効果と評価した。また，Checkerboard assay による評価を補強するために，CEZ，ナイシン A の単独および併用時それぞれについて 0.5 倍 MIC，1 倍 MIC および 2 倍 MIC における各乳房炎原因菌の生存細菌数の経時変化（0，3，6，9，24 時間後）を，MHB を用いた Time-kill assay で評価した。

S. aureus，*S. intermedius*，*S.agalactiae* および *S. dysgalactiae* に対する CEZ の MIC_{50}

の値は 0.13-0.5μg/ml と低く，*E. coli* も 2μg/ml だったが，*E. faecalis* では 32μg/ml と高かった。ナイシン A の MIC_{50} は *S. intermedius*，*S.agalactiae* で 0.06 および 0.25μg/ml と低く，*S. aureus*，*S. dysgalactiae* が 1μg/ml，*E. faecalis* では 4μg/ml であったが，*E. coli* では 128μg/ml と極めて高かった。しかし，いずれの菌種においても CEZ とナイシン A 併用時における MIC_{50} の値は，単独時に比べて 1/2 から 1/8 に低下した。Checkerboard assay では，CEZ と nisin A 併用により *S. aureus* (n=20/20)，*E. faecalis* (n=13/18)において相乗効果が優勢であったのに対し，*S. intermedius* (n=12/20)，*S. agalactiae* (n=10/10)，*S. dysgalactiae* (n=18/18)および *E. coli* (n=14/18)では相加効果が優勢であった。また，いずれの供試菌においても無関係および拮抗効果は認められなかった。Time-kill assay では，*S. aureus* と *S. agalactiae* において CEZ と nisin A の 1 倍 MIC による併用で，*S. intermedius*，*S. dysgalactiae*，*E. faecalis*，*E. coli* では 0.5 倍 MIC による併用で培養 3～6 時間以内に生存細菌数が検出限界以下まで低下し，24 時間持続した。

本研究により，CEZ と nisin A を併用すると，グラム陽性・陰性の両方の乳房炎原因菌に対して相乗・相加的な殺菌効果を有することが明らかとなった。また，両者を併用すると単独では MIC_{50} の値が高く殺菌効果が乏しかった *E. faecalis* や *E. coli* に対しても，その値が 1/2～1/8 まで低下したことから，その他の乳房炎原因菌に対する抗菌スペクトルの拡大も期待される。また近年は，ナイシン A のバイオフィルムに対する有効性も報告されていることから，慢性乳房炎に対する治療効果も期待できる。

以上のことから，CEZ とナイシン A を併用することで，従来よりも抗生物質濃度が低く，抗菌スペクトルの広い新たな乳房内注入剤の開発が可能であることが示唆された。

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In vitro synergistic activities of
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