辛子明太子中におけるListeria monocytogenesの増殖に及ぼすナイシン（ニサプリン）の影響

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Note

Effect of Nisin (Nisaplin) on the Growth of *Listeria monocytogenes* in Karashi-mentaiko (Red-pepper Seasoned Cod Roe)

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The influence of Nisaplin, which contains 2.5% nisin, on the growth of *Listeria monocytogenes* in Karashi-mentaiko (red-pepper seasoned cod roe) was investigated. The MICs of Nisaplin for *L. monocytogenes* (10^8 CFU/mL) were measured; seven isolates showed a value of 1,600 μg/mL and one isolate showed a value of 800 μg/mL. All *L. monocytogenes* isolates had a MIC of 800 μg/mL at 10^6 CFU/mL. The number of *L. monocytogenes* in Karashi-mentaiko stored at 4℃ was decreased by Nisaplin added at 60 and 600 μg/g. These results indicated that Nisaplin effectively inhibits the growth of *L. monocytogenes* in Karashi-mentaiko.

Key words: Nisaplin; nisin; *Listeria monocytogenes*; Karashi-mentaiko

Introduction

Food-borne listeriosis is rare in Japan, compared with Europe and the USA. However, it can be life-threatening, having a high fatality rate. There are many reports of the isolation of *Listeria monocytogenes* from milk, dairy products, meat and meat products. Furthermore, it has been reported that *L. monocytogenes* is present in the environment, such as in river water[1] and estuarine water[2]. Recently, *L. monocytogenes* was isolated from raw and processed seafoods[3)-14)], and listeriosis caused by smoked mussels[15] and rainbow trout[16] has been reported. Okutani *et al.*[17] reviewed *L. monocytogenes* contamination of fresh and processed seafood, finding that the proportion was less than 10%. In Japan, raw and processed seafoods are consumed frequently, and processed seafoods, especially Karashi-mentaiko, are popular. Karashi-mentaiko is red-pepper seasoned cod roe. However, *L. monocytogenes* has been isolated from Karashi-mentaiko[13), 14)], and since there is no heat treatment in the manufacturing process of Karashi-mentaiko, the control of bacteria is very important.

Nisin is produced by lactic acid bacteria and has already been permitted as a food additive in many countries, including the USA and EU. There is a report on the antibacterial activity of nisin against bacteria that cause food poisoning such as *Staphylococcus aureus*, *Clostridium botulinum*, *C. perfringens*, and *Bacillus cereus*[18]. It has also been reported that nisin inhibits the growth of *L. monocytogenes*[14]. These reports indicate that nisin is effective to control bacteria responsible for food poisoning. Recently, the influence of nisin on the growth of *L. monocytogenes* in Karashi-mentaiko has been examined[14], and it was indicated that nisin can effectively inhibit growth of *L. monocytogenes* in Karashi-mentaiko. Nisaplin (Danisco, Copenhagen, Denmark) contains 2.5% nisin and is used to control gram-positive bacteria in food. Furthermore, it is allergen-free and complies with CODEX, FCC, and EU standards. In this study, the effect of Nisaplin on the growth of *L. monocytogenes* in Karashi-mentaiko was investigated.

Materials and Methods

1. Bacterial strains and measurement of minimum inhibitory concentrations

Eight isolates (Table 1) of *L. monocytogenes* were used in the Nisaplin susceptibility test. The minimum inhibitory concentrations (MICs) were determined by the standard method of the Japanese Society of Chemotherapy[19] with some modification. Isolates were cultured in Brain Heart Infusion broth (BHI, BD, MD, USA) at 37℃ for 24 hr. After incubation, 0.1 mL of these cultures was re-inoculated into BHI and incubated in the same manner as described above. After incubation, the cultures were diluted 10-fold (approximately 10^6 and 10^8 colony-forming units (CFU)/mL) in 0.1 M

Table 1. Minimum inhibitory concentrations of materials for the *Listeria monocytogenes* isolates[a]

Isolates of *L. monocytogenes*	Origin	Serotype	Number of bacteria inoculated (CFU/mL)	Nisaplin	Sankeeper No. 381	Sankeeper No. 657	Protamine sulfate
Lm-F1	Food	1/2a	10^6	800	200	3,200	200
			10^8	1,600	200	3,200	200
Lm-F2	Food	1/2b	10^6	800	200	3,200	200
			10^8	1,600	200	3,200	200
Lm-F3	Food	4b No. 1	10^6	800	200	3,200	200
			10^8	1,600	200	3,200	200
Lm-F4	Food	4b No. 2	10^6	800	200	3,200	200
			10^8	1,600	200	3,200	200
Lm-P1	Patient	1/2a	10^6	800	200	3,200	200
			10^8	1,600	200	3,200	200
Lm-P2	Patient	1/2b	10^6	800	200	3,200	200
			10^8	1,600	400	3,200	200
Lm-P3	Patient	4b No. 1	10^6	800	200	3,200	200
			10^8	1,600	400	3,200	200
Lm-P4	Patient	4b No. 2	10^6	800	200	3,200	200
			10^8	1,600	400	3,200	200

[a] MIC, μg/mL.

Table 2. Effect of Nisaplin on the growth of *L. monocytogenes* in Karashi-mentaiko stored 4℃[a]

Isolates of *L. monocytogenes*	Nisaplin concentration	Day 0	Day 7	Day 14	Day 21	Day 28
Lm-F1	0 μg/g	1.6×10^2	1.0×10^2	1.5×10^2	1.9×10^2	2.3×10^3
	60 μg/g	1.6×10^2	UD[b]	UD	UD	UD
	600 μg/g	1.6×10^2	UD	UD	UD	UD
Lm-F2	0 μg/g	5.5×10^3	1.5×10^2	2.0×10^2	5.5×10^2	6.7×10^2
	60 μg/g	5.5×10^3	UD	1.0×10^2	2.0×10^2	UD
	600 μg/g	5.5×10^3	UD	UD	UD	UD
Lm-F3	0 μg/g	1.3×10^2	1.0×10^2	2.0×10^2	2.5×10^2	1.9×10^2
	60 μg/g	1.3×10^2	UD	UD	UD	UD
	600 μg/g	1.3×10^2	UD	UD	UD	UD
Lm-F4	0 μg/g	1.8×10^3	1.0×10^2	2.5×10^2	UD	UD
	60 μg/g	1.8×10^3	UD	1.0×10^2	1.0×10^2	1.0×10^2
	600 μg/g	1.8×10^3	UD	UD	UD	UD
Lm-P1	0 μg/g	1.5×10^3	UD	UD	UD	UD
	60 μg/g	1.5×10^3	UD	UD	UD	UD
	600 μg/g	1.5×10^3	UD	UD	UD	UD
Lm-P2	0 μg/g	1.0×10^3	1.5×10^2	2.0×10^2	UD	UD
	60 μg/g	1.0×10^3	1.0×10^2	UD	UD	UD
	600 μg/g	1.0×10^3	UD	UD	UD	UD
Lm-P3	0 μg/g	1.0×10^3	UD	UD	UD	UD
	60 μg/g	1.0×10^3	UD	UD	UD	UD
	600 μg/g	1.0×10^3	UD	UD	UD	UD
Lm-P4	0 μg/g	3.8×10^3	1.1×10^3	4.9×10^3	2.1×10^3	3.3×10^3
	60 μg/g	3.8×10^3	UD	UD	UD	UD
	600 μg/g	3.8×10^3	UD	UD	UD	UD

[a] Data are expressed as CFU/g of sample or CFU/mL of BHI broth.
[b] UD: Undetectable; Detection limit was 10^2 CFU/g of sample.

phosphate buffer (pH 6.5), respectively, and 5 μL aliquots of the dilutions were placed on Mueller–Hinton agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing Nisaplin and incubated at 37℃ for 24 hr. Nisaplin solution was made using sterile distilled water. Sankeeper No. 381 (San-Ei Gen F.F.I., Inc., Osaka, Japan) containing 50% ε-polylysine, and Sankeeper No. 657 (San-Ei Gen F.F.I) containing 100% lysozyme and protamine sulfate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), were also used as solutions in sterile

Table 3. Effect of Nisaplin on the growth of *L. monocytogenes* in Karashi-mentaiko stored at 15℃[a]

Isolates of *L. monocytogenes*	Nisaplin concentration	Day 0	Day 14	Day 21	Day 28
Lm-F1	0 μg/g	5.4×10^3	2.2×10^3	3.0×10^3	2.9×10^3
	60 μg/g	5.4×10^3	UD	UD	3.0×10^3
	600 μg/g	5.4×10^3	UD[c]	UD	UD
Lm-F2	0 μg/g	7.5×10^3	2.2×10^4	3.0×10^4	3.2×10^4
	60 μg/g	7.5×10^3	UD	UD	3.1×10^3
	600 μg/g	7.5×10^3	UD	UD	UD
Lm-F3	0 μg/g	9.9×10^3	1.0×10^4	7.1×10^4	1.3×10^4
	60 μg/g	9.9×10^3	UD	UD	UD
	600 μg/g	9.9×10^3	UD	UD	UD
Lm-F4	0 μg/g	7.6×10^3	2.9×10^3	1.1×10^3	1.0×10^3
	60 μg/g	7.6×10^3	UD	UD	2.0×10^3
	600 μg/g	7.6×10^3	UD	UD	UD
Lm-P1	0 μg/g	1.9×10^4	2.7×10^4	2.0×10^4	4.4×10^4
	60 μg/g	1.5×10^3	UD	UD	3.3×10^2
	600 μg/g	1.9×10^4	UD	UD	UD
Lm-P2	0 μg/g	1.9×10^4	2.3×10^4	2.2×10^4	3.1×10^4
	60 μg/g	1.9×10^4	UD	UD	UD
	600 μg/g	1.9×10^4	UD	UD	UD
Lm-P3	0 μg/g	6.3×10^3	2.2×10^4	UD	UD
	60 μg/g	6.3×10^3	UD	UD	UD
	600 μg/g	6.3×10^3	UD	UD	UD
Lm-P4	0 μg/g	1.2×10^3	2.0×10^4	3.1×10^4	2.9×10^4
	60 μg/g	1.2×10^3	UD	UD	UD
	600 μg/g	1.2×10^3	UD	UD	UD

[a] Data are expressed as CFU/g of sample or CFU/mL of BHI broth.
[b] UD: Undetectable; Detection limit was 10^2 CFU/g of sample.

distilled water.

2. Inhibition of L. monocytogenes in Karashi-mentaiko

Karashi-mentaiko used in this study was produced by a single manufacturer in Japan. Isolates of *L. monocytogenes* were cultured in BHI at 37℃ for 24 hr. After incubation, these cultures were re-inoculated into BHI and incubated using the same method as described above. Nisaplin was added to Karashi-mentaiko and mixed well. The cultures were diluted in 0.1 mol/L phosphate buffer (pH 6.5), and 0.3 mL aliquots of the dilutions were added to 3 g of Karashi-mentaiko, which was then stored at 4℃ for 7, 14, 21, and 28 days or 15℃ for 7, 14, and 21 days. The number of *L. mocytogene* in samples was measured using Oxford-Listeria-Selective agar (Merck, Darmstadt, Germany). The number of *L. monocytogenes* in Karashi-mentaiko used in this study was under 10^2 CFU/g.

3. Measurement of pH of samples

The pH of samples was measured with a pH meter, model D-25 (Horiba Ltd., Kyoto, Japan).

Results

1. MICs of Nisaplin and other materials

The MICs of Nisaplin for eight isolates of *Listeria monocytogenes* are listed in Table 1. At 10^6 CFU/mL, all isolates had a MIC of 800 μg/mL. At 10^8 CFU/mL, seven isolates showed a value of 1,600 μg/mL and one isolate showed a value of 800 μg/mL. The MICs of other materials are also shown in Table 1. With Sankeeper No. 381, MICs at 10^6 CFU/mL were 200 μg/mL and those at 10^8 CFU/mL were 200 to 400 μg/mL. With Sankeeper No. 657, MICs at 10^6 CFU/mL and those at 10^8 CFU/mL were 3,200 μg/mL. The MICs of protamine sulfate were 200 μg/mL, for all isolates at 10^6 CFU/mL and 10^8 CFU/mL.

2. Effect of Nisaplin on the growth of L. monocytogenes in Karashi-mentaiko and pH of samples

The numbers of *L. monocytogenes* inoculated in Karashi-mentaiko were 10^2–10^4 CFU/g. Regarding the effect of Nisaplin on *L. monocytogenes* in Karashi-mentaiko, decreases of the numbers of *L. monocytogenes* in samples containing both 60 and 600 μg/g on storage at 4℃ were seen (Table 2). In the samples without Nisaplin, none of the tested isolates grew clearly: strains Lm-P1 and Lm-P3 were undetected after the 7th day, and strain Lm-P2 was undetected after the 21st day. In the samples containing 60 μg/g Nisaplin, most of the isolates were undetected, though, Lm-F2 strain was detected on the 14th day and the 21st day, and Lm-F4 strain was detected after the 14th day. All the isolates were undetectable in the samples with 600 μg/g

Table 4. The pH values of Karashi-mentaiko containing 600 μg/g Nisaplin[a]

Stored at	Karashi-mentaiko[b]	Day 0	Day 7	Day 14	Day 21
4℃	5.8	5.8	5.8	5.9	5.9
15℃	5.8	5.8	5.8	6.0	6.0

[a] Samples inoculated with *L. monocytogenes* 1/2a from food were measured.
[b] The pH value of Karashi-mentaiko before addition of Nisaplin and *L. monocytogenes*.

Nisaplin.

At 15℃, most of the isolates were detected in the samples without Nisaplin, though Lm-P3 strain was undetectable after the 14th day (Table 3). In the samples with 60 μg/g Nisaplin, most of the isolates were undetectable, except that strains Lm-F1, Lm-F2, Lm-F4 and Lm-P1 were detected on the 21st day. All the isolates were undetectable in the samples with 600 μg/g of Nisaplin.

The pHs of samples were unchanged, as shown in Table 4.

Discussion

Karashi-mentaiko is a processed food eaten by many Japanese. It does not undergo heat treatmen during manufacture, so bacterial control is very important. There are many reports on the control of *L. monocytogenes* in food. However, there are few reports on the treatment of *L. monocytogenes* in processed seafood. Nisin has not yet been officially accepted as a food additive in Japan.

The results obtained in this study indicate that Nisaplin is effective for controlling *L. monocytogenes* in Karashi-mentaiko, although several isolates grew in Karashi-mentaiko containing 60 μg/g Nisaplin. The effective concentration of Nisaplin in Karashi-mentaiko was lower than the MIC. This suggests that ingredients of Karashi-mentaiko, storage temperature and a_w influence the efficacy of Nisaplin. Although there are many reports on the antibacterial activity of nisin, there are few reports on the control of food poisoning bacteria in processed seafoods using nisin. Recently, the effect of nisin on *L. monocytogenes* in Karashi-mentaiko was examined, and it was indicated that nisin A has an inhibitory effect on growth of *L. monocytogenes* in Karashi-mentaiko containing 1,500 μg of nisin A[14]. However, further studies using other isolates of *L. monocytogenes* and Karashi-mentaiko containing various concentrations of nisin are needed to identify the appropriate concentration of nisin in Karashi-mentaiko for control of *L. monocytogenes*. It has been reported that nisin has a remarkable antibacterial activity against gram-positive bacteria[18]. Nisin is digested by digestive enzymes in the human intestine, and lactic acid bacteria which produce nisin are used to produce cheese. Therefore, nisin is considered to be safer than antibiotics. There have been no reports that nisin is harmful to human health. It has been reported that the pH and the water activity (a_w) of seasoning liquid and the storage temperature of products are important for controlling *L. monocytogenes* in Karashi-mentaiko[20]. However, the use of food additives is essential, because the manufacture of Karashi-mentaiko might be modified in response to changing consumer preferences in the future; a_w and pH might be changed to suit various tastes. The use of nisin for the inhibition of *L. monocytogenes* and other harmful bacteria in foods, especially non-heat treated foods, appears to be beneficial. Furthermore, The MIC values of the tested materials indicate that nisin is more effective to inhibit the *L. monocytogenes* than growth inhibitors such as ε-polylysine, lysozyme and protamine sulfate, because the concentration of nisin in Nisaplin is low (2.5%) as compared with that of other materials tested. In summary, our results indicate that Nisaplin is effective for the control of *L. monocytogenes* in Karashi-mentaiko.

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