Effectiveness and clinical applications of a safe edible antimicrobial peptide produced by lactic acid bacteria

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1. Introduction

Recently, dental caries and periodontal diseases (Figure 1) have been decreasing by the efforts made by forerunners of the field,[1)] but they still remain to be one of the most frequent infectious diseases that human have. Bacteria that causes oral infections do not exist alone to resist their own hosts. The bacteria colonize on the oral mucous membrane (e.g., tooth surface, periodontal tissues, tongue), dentures, and implants while forming a community with other resident microbiota, and form mushroom-shaped biofilms like a fortress to mature into pathogenic flora. Recent researches have started to reveal that bacteria associated with biofilms have an effect on systemic illnesses (e.g., articular rheumatism, diabetes).[2)3)] In addition, when hosts experience an immunity deficiency due to any changes including aging, diseases, or medical treatments, opportunistic infections that lead to resident microbiota which do not usually exert pathogenicity in ordinary settings or an onset of attenuated microorganisms will occur. Problems in the mucous membrane due to oral resident microbiota can cause pain and uncomfortable sensation, resulting in influencing diet (nutrition intake). Aspiration pneumonia, which is often seen among the elderly, has characteristics of multiple recurrences, leading to the emergence of resistant strains of bacteria and having resistivity against antimicrobial agents as a consequence;[4)5)] however, not only weakened cough reflex, but oral resident microbiota is also related to the onset of this disease.[6)] In Japan, which is now a super-aging society, it is expected to have an increase of systemic infectious diseases that are triggered by oral resident microbiota; this is attributed to an increase of the population who are not even capable of gargling, and thus requiring nursing care.

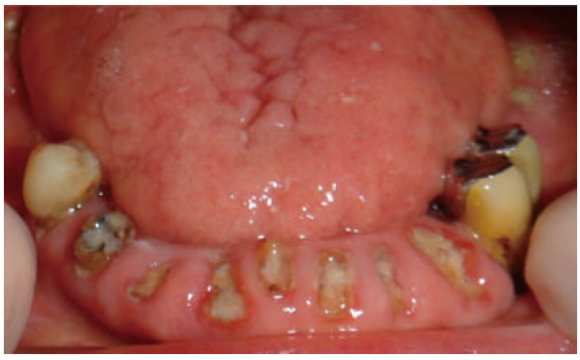

Figure 1. Dental caries and periodontal diseases of the elderly who need nursing care

Periodontal diseases and dental caries are not only resulting in loss of teeth. Due to the progress of periodontal diseases, teeth with exposed cementum are prone to dental caries on root surface, leading to fractured teeth. The fractured teeth remain in the jawbone for a long time and become a site to form a biofilm to be a route for infection transmission of bacteria to enter into the body.

On the other hand, Yoneyama et al. reported that giving oral care to the elderly population, who requires nursing care, decreased the occurrence of aspiration pneumonia,[7)] so it was shown that there is a possibility that oral care can be a means for preventive therapy for systemic illnesses. Akutsu et al. reported a high occurrence of post-operative pneumonia in cases where pathogenic bacteria were detected in dental plaque among those who underwent an operation of esophagus cancer;[8)] however, the occurrence of post-operative complications decreases with introducing oral care especially at the time of the operation of head and neck.[9)] It has been frequently shown that oral care is important for oral resident microbiota to continue to prevent systemic illnesses and coexist without turning into pathogenicity.[10)-12)] In addition, it has been reported that oral care is superior in terms of health economics.[13)]

So, what are the requirements to prepare a

variety of oral care products then? Oral care products must be “user friendly” for both people who need nursing care and caregivers who support them, especially when products are for those who have a compromised immune system, those who require nursing care (for instance, those who are confined to bed, those who are unable to brush their own teeth, and those who are unable to spit).

In Japan, people enjoy fermented food including miso and soy sauce for everyday diet, but this is something Japanese people leverages the characteristics of lactic acid bacterium for flavor and preservation. Fermented food including dairy products has taken hold as dietary culture all over the world, and thus lactic acid bacterium has been eaten and has coexisted with humans. This fact proves the high safety and efficacy of lactic acid bacteria. In this paper, we sought a possibility of utilizing lactic acid bacteria as an oral care agent, which has been used for diet and health enhancement since ancient time.

2. Efficacy of bacteriocin produced by lactic acid bacteria for medical purposes

Bacteriocin is a collective term of antimicrobial peptide or proteins that are synthesized on ribosome by various bacteria. By producing organic acid including lactic acid and antimicrobial substances such as alcohol and bacteriocin, lactic acid bacteria eliminate other bacteria, inhibit the growth of them, and win the struggle for survival. Nisin A, as a representative of bacteriocins produced by lactic acid bacteria (Figure 1), shows an antimicrobial effect against closely positioned Gram-positive microorganism and is highly active in lower concentration than general antimicrobial substances by showing a reaction on the cell membrane instantly. On the other hand, it has characteristics that there is no harm to humans and is digested with digestive enzymes in the intestinal tract of humans and animals so that the burden on the environment is minimized, and thus the chance for resistant bacteria to emerge is extremely small.[14)-16)] Bacteriocin produced by lactic acid bacteria is a natural and safe antimicrobial substance and has been used and researched as food preservative and is greatly expected to be applied in the field of medicine.[17)]

Table 1. Efficacy of nisin A for medical purposes

1	It is a peptide that is composed of 34 amino acids and is degraded with digestive enzymes in the intestinal tract. It is easily degraded and does not remain in the environment.
2	It has a broad antimicrobial spectrum against Gram-positive microorganisms that are analogous bacteria (MRSA, which is a multiply-resistant organism, and VRE (including vancomycin-resistant enterococci)).
3	It shows antimicrobial activity instantly.
4	Generally, it functions in lower concentration than antibiotics.
5	Targeting precursor substances on cell walls, it forms pores in the cell membrane; thus, it has no influence on humans or animals.
6	It is acid- and heat-resistant and shows a high safety profile with low pH.
7	No antimicrobial activities exist against fungi or Gram-negative bacteria when it is utilized alone.
8	There has been no report of resistant bacterium at a practical level.
9	It has neither taste nor smell.

3. Nisin, the representative of bacteriocins produced by lactic acid bacteria

3-1. Nisin A

Nisin, the representative of bacteriocins produced by lactic acid bacteria, is a peptide, which is composed of 34 amino acids that are produced from a part of strains of *Lactococcus lactis* subsp. *lactis* of lactic acid bacteria. There are nisin A, Q, and Z, the analogous bacteria with different amino acid sequences (Figure 2), and a thorough research on the biosynthesis and mechanism of action have been ongoing.[16)] Interestingly, nisin A was found in cheese by a British dairy farmer in 1928, in the same era as when Penicillin, the first antibiotics, was found. It has a broad antimicrobial activity against Gram-positive microorganisms.[18)19)] It has especially a high antimicrobial activity against *Bacillus* and *Clostridium* bacteria

and is effective for the control of trophocytes and the sprout inhibition of endospore. It functions on *Listeria* as well as on *Staphylococcus* bacteria so that nisin A has been leveraged especially as food preservation.[16] Following approvals from WHO (World Health Organization) and FAO (Food and Agriculture Organization of the United Nations); it was approved as GRAS (Generally Recognized As Safe) by FDA (Food and Drug Administration) in the U.S.[20] It was also appointed as a food additive in Japan in 2009[21] and has been already used as a food preservative in cans, cheese, and so forth in over 50 countries in the world.[22]

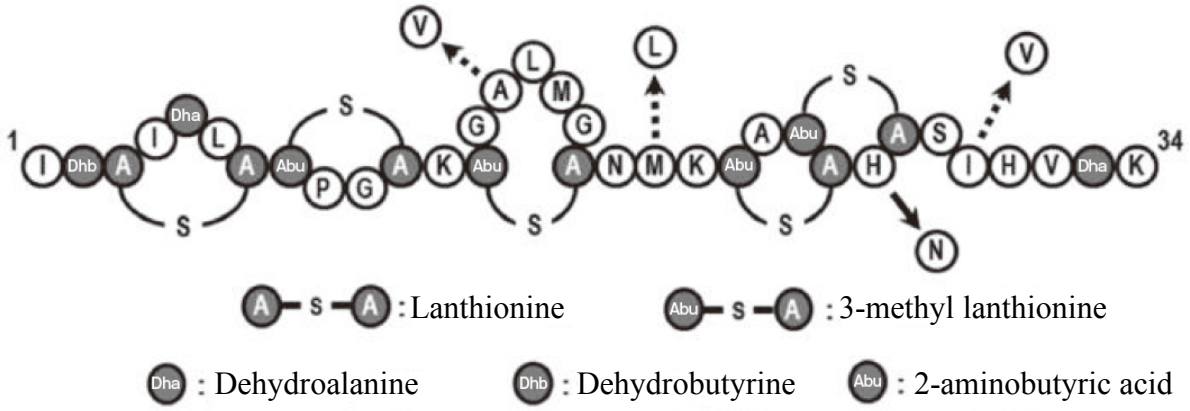

Figure 2. The structure of nisin A

The gray-colored amino acid residues indicate unusual amino acids (amino acids that are not contained in general proteins) generated by post-translational modification. The solid arrow indicates an amino acid that is replaced with nisin Z and Q. The dashed arrows indicate amino acids that are replaced with nisin Q alone (the figure cited from Ishibashi et al., (2014)[36]).

3-2. Sterilization mechanism of nisin A

The sterilization effect by nisin A occurs when pores are formed on the bacterial cell membrane and relatively large-sized molecules including ions or ATP in cells are emigrated[16][23] (Figure 3). To give a detailed description, as a lipid II target molecule that is the precursor of cell walls that are present on the cell membrane of bacteria, nisin N-terminus binds with pyrophosphoric acids of lipid II by seemingly wrapping around to enter into the cell membrane.[24] The sterilization effect is seen when pores in 2.5 nm diameter are formed on the cell membrane by forming a complex with eight nisin molecules and four lipid II molecules, and intracellular substances are emigrated[25] (Figure 4). Affinity to lipid II, which is a precursor of this fundamental skeleton, is the reason why nisin shows the antimicrobial activity in such a low concentration of nM level (Table 2). Lipid II broadly exists on the surfaces of Gram-positive microorganism, and nisin shows the sterilization effect against MRSA

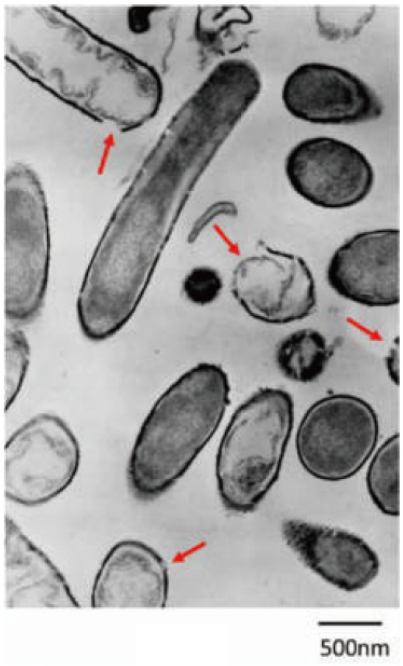

Figure 3. Nisin A's pore formation on *bacillus subtilis* (the arrows show pore formation) (S. M. Asaduzzaman, et al., (2009)[23] modified and cited)

(methicillin-resistant staphylococcus aureus), VRE (vancomycin resistant enterococci), and so forth.[26)-28)] On the other hand, nisin does not show the sterilization effect on human cells that do not have lipid II. In recent years, a possibility of antimicrobial effect on biofilms has also been reported.[29)30)]

Table 2. Nisin A's minimum growth inhibitory concentration (MIC) against main Gram-positive microorganisms (Fujita, et al., (2007)[18)] cited)

Bacterial strain	Strain name	MIC (ng/mL)
Bacillus cereus	JCM 2152^T	84.9
Bacillus coagulans	JCM 2257^T	30.2
Listeria innocua	ATCC 33090^T	231
Lactococcus lactis	ATCC 19435^T	265
Streptcoccus salivarius	JCM 5707^T	2520
Staphylococcus aureus	ATCC 12600^T	158
Enterococcus faecalis	JCM 5803^T	295

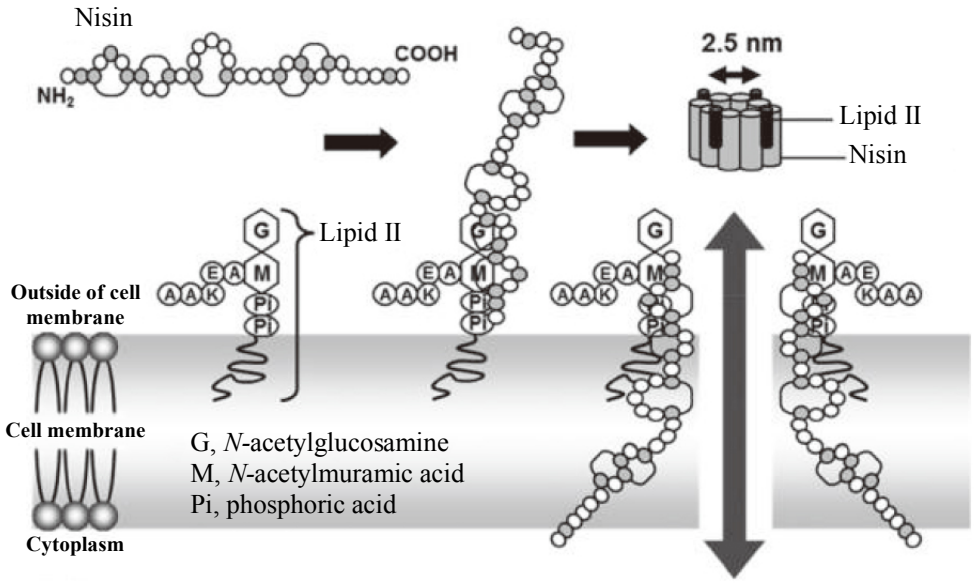

Figure 4. Nisin's mechanism of action
(Figure modified and cited from Breukink and de Kruijff (2006)[25)])

4. NeoNisin®

4-1. Medical compound of highly purified nisin A and the plum extracts

Commercially available nisin products are produced with a salting-out method using a large amount of salts so that purity is as low as 2.5% (w/w) with salts and media components being mixed in, and thus it used to be difficult to utilize nisin as is except for the use as a food preservative. Amid the circumstances, we initiated a development of highly purified technology of nisin A with Sonomoto, et al., from Faculty of Agriculture, Graduate School, Kyushu University, using *Lactococcus lactis* QU53 of lactic acid bacteria detached from "okara (soybean curd refuse)" produced in Fukuoka. As a result, we were able to establish an industrial production system for highly purified nisin A that is 90% or higher (w/w).[31)] The highly purified nisin A was superior to the conventional nisin products in terms of quality (purity) and preservative safety (Figure 5).

Nisin A has high purity, but unlike Gram-positive bacteria, it cannot pass through "exterior walls" of Gram-negative bacteria, which consist of lipopolysaccharide and are present at the outer side of cell walls, so that nisin does not show any effect when it is used by itself.[32)] However, it is known that nisin A will gain a sterilization effect even against Gram-negative bacteria by using in combination with chelating agents and so

forth that destroy and eliminate the exterior walls.[33] On the other hand, the plum extracts show antimicrobial activity in a certain concentration, but it has a strong sour taste, and thus is not suitable for oral use. As a result of conducting a combination test between highly purified nisin A and various plant-derived constituents, Nagatoshi found a unique compounding ratio of nisin A and the plum extracts, which has a synergy effect with nisin A and shows antimicrobial activity against Gram-negative bacteria and does seldom influence the taste, leading to an improvement of the weakness against Gram-negative bacteria.[34] The number of viable bacteria was measured after adding 10 μL of *Escherichia coli* (JCM 1649 strains: 10^3 - 10^4 CFU/mL), which is Gram-negative bacteria, to nisin A or NeoNisin® test solution (10 mL) and contacting for 24 hours. With this, it was found that NeoNisin® could almost completely sterilize *Escherichia coli* (Figure 6).

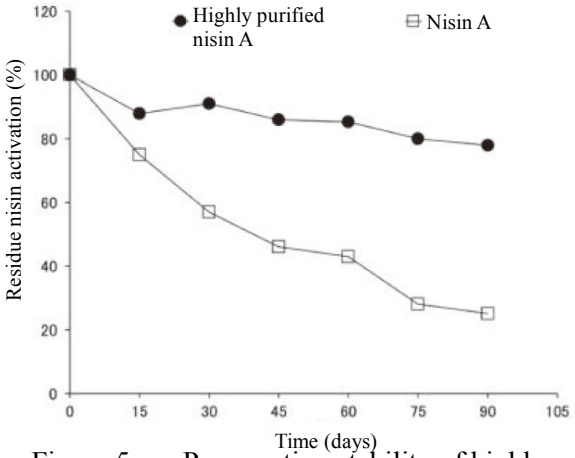

Figure 5. Preservation stability of highly purified nisin A and conventional nisin A (40°C)

4-2. Efficacy of NeoNisin® on oral bacteria

In order to confirm the efficacy of newly developed NeoNisin®, tests were conducted against the following: 1) *Streptococcus mutans* (*S. mutans,* Gram-positive microorganism) that is a causative microorganism for dental caries; 2) *Staphylococcus aureus* (*S. aureus* , Gram-positive microorganism) that can be a causative microorganism for pneumonia, food poisoning, epidermis infection, and so forth; and 3) *Aggregatibacter actinomycetemcomitans* (*A.a.* bacteria, Gram-negative bacteria) that is a periodontal disease-related microorganism. After the above three types of test microorganisms (10^5 CFU/mL) were added to NeoNisin® test solution (200 μL) and contacted for 10 minutes at 37°C, they were cultured at the agar medium, and the number of viable bacteria was measured to calculate the survival rate. As a result, almost all *S. mutans* and *A.a. bacteria* were sterilized, and an obvious sterilization effect against *S. aureus* was also observed (Figure 7).

Next, we examined the sterilization effect against *Porphyromonas gingivalis* (*P. gingivalis*, Gram-negative bacteria) that belongs to red complex,[35] a group with the highest pathogenicity among periodontal disease causative microorganisms. After injecting *P. gingivalis* into NeoNisin® test solution and culturing for 48 hours, the turbidity of the culture medium (600 nm) was measured to calculate the anti-proliferative effect. NeoNisin® showed a great effect even in a very low concentration (Figure 8).

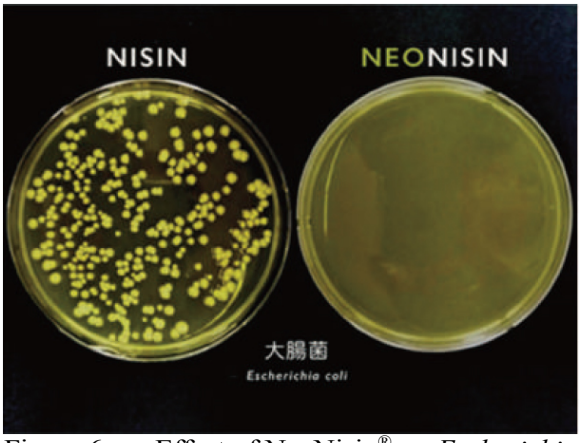

Figure 6. Effect of NeoNisin® on *Escherichia coli*

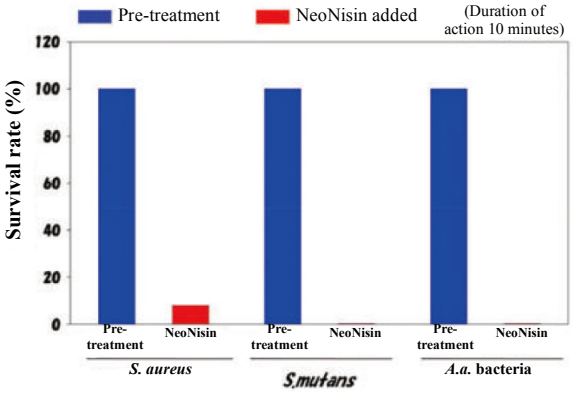

Figure 7. Effect of NeoNisin® on *S. aureus, S. mutans*, and *A.a.* bacteria

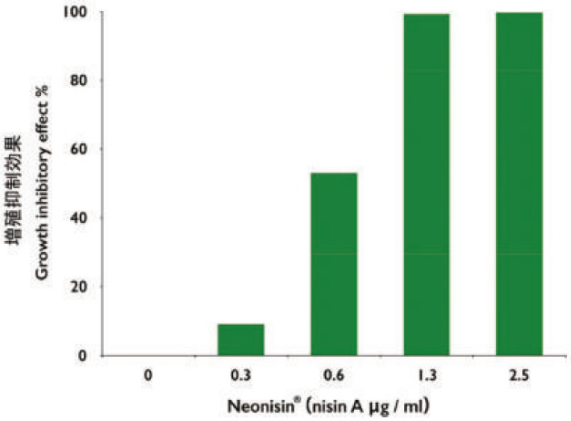

Figure 8. Anti-proliferative effect of NeoNisin® against *P. gingivalis*

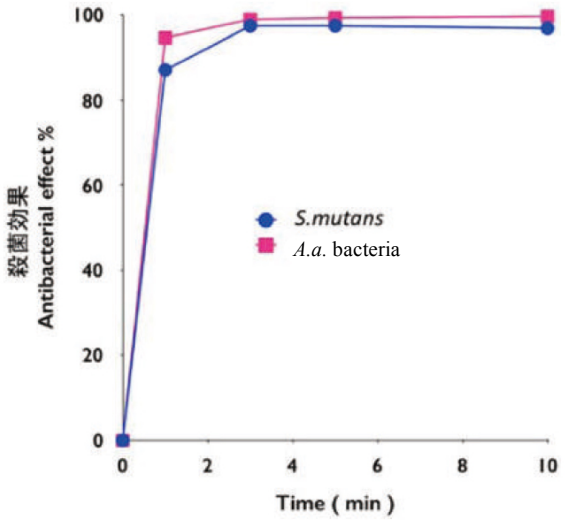

Figure 9. Sterilization rate and speed of NeoNisin® on *S. mutans* and *A.a.* bacteria

Figure 10. Gel type and spray type of Oralpeace®

Furthermore, after making *S. mutans* and *A.a.* bacteria contact NeoNisin® for a certain period of time, they were cultured in the agar medium, and the number of viable bacteria was measured to calculate the sterilization rate. As a result, almost all bacteria were annihilated within a minute, a short duration of action, and NeoNisin® showed the sterilization effect instantly (Figure 9).

With these results, it was showed that NeoNisin® has a possibility of having an instant and robust sterilization effect on both Gram-positive and Gram-negative microorganisms that can become bacteria to compose biofilms in the oral cavity. Especially, the obvious sterilization effect on *S. mutans, P. gingivalis*, and *A.a.* bacteria indicates a great possibility to become a preventative agent for dental caries and periodontal diseases, which are two major diseases in dentistry.

5. Novelty and clinical applications of Oralpeace®

Oralpeace® developed by Trife Inc. is an oral care product that contains NeoNisin® and is available in gel and spray. All ingredients besides NeoNisin® are also edible, so it is harmless even when swallowed, and thus it is available for patients who are not capable of spitting out, including those who need nursing care and people with disability. By applying at the back of dentures, not only it can prevent infections, but also it can protect the mucous

membrane by moistening and so forth. It has a sterilization effect on bacteria but has no effect on human cells. It is also degraded in the intestinal tract but does not disturb intestinal flora. Continuous and recurrent usage is accepted during not only post-operative periods, but also wound healing periods, and it is safe to swallow. Oral care products with these characteristics did not exist so far. There are three types of flavors and they are available in gel and spray (Figure 10). The spray type, in particular, has characteristics where patients can directly spray it into the mouth and swallow, and thus making oral care possible in places where gargling is not feasible, such as, in the office or on transportation (e.g., in airplanes or on trains), and also on the bed or futon mattress. Together with the daily use with toothbrushing, Oralpeace® will add a custom of performing oral care anytime anywhere so that it is thought that biofilm formation triggered by oral resident microbiota can be inhibited more effectively.

In addition, "Oralpeace for Pet" was launched as an oral care product for pets. NeoNisin® is expected to have the same effect on animal oral cavities as in human due to its mechanism of action. With this, it is interesting not only that a custom to conduct oral care for pets on a daily basis can be proposed as it is safe for pets that cannot gargle or spit even if they lick or swallow the product, but also that people who have pets can feel close to their own pets since the lineup packaging used for pets is same as the one used for humans.

6. Conclusion

It is considered that the possibilities of using bacteriocins for medical purposes will expand to the fields where direct spraying contacts can be achieved, such as nasal cavity, pharynx, and skin. Going forward, we would like to examine these possibilities and clinical benefits both in humans and animals while thoroughly considering the characteristics, the mechanism of action, and so forth of bacteriocins.

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Effectiveness and clinical applications of a safe edible antimicrobial peptide produced by lactic acid bacteria

***Abstract*:** Recently many cases have been reported that biofilms created by oral resident microbiota trigger not only dental caries and periodontitis but also various systemic illnesses. Nisin A, a bacteriocin peptide produced by lactic acid bacteria, has properties to instantly sterilize Gram-positive bacteria. In addition, it is harmless to human cells and degradable by digestive enzymes, and has neither taste nor smell. Nisin A is widely used in over 50 countries as a food preservative, and at the practical level no acquired resistant bacteria against nisin A have been reported. Neonisin®, a newly developed antimicrobial agent based on plum extracts and nisin A, sterilizes Gram-negative bacteria also, which are tolerant to nisin A alone, as a result of chelating action caused by the plum extracts. Utilizing Neonisin®, Trife Inc. further developed a product for oral care, named Oralpeace®. Trife succeeded in production of highly purified nisin A, with very low levels of impurities and salts, which is applied to produce Neonisin® and Oralpeace®. Since Oralpeace® consists only of edible ingredients, patients that have difficulties in gargling and spitting can use it safely. Bacteriocins from lactic acid bacteria are being found to have great potential for medical purposes such as prevention of bacterial infections in body parts with possible skin contact as well as food preservation.

Key words: nisin, antimicrobial peptide, food preservative, lactic acid bacteria, oral care, opportunistic infection, oral diseases, antibiotic-resistant bacteria



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