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Determination of the Antibacterial Constituents Produced by Lactobacilli against a Periodontal Pathogen: Sodium Lactate and a Low Molecular Weight Substance

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Abstract

Background and objectives: Probiotics are living bacteria which can improve the balance of microbiota. There are many recent studies on the effects of probiotics, including oral health promotion and the prevention of oral diseases. However, the mechanisms that underlie the activity of probiotic bacteria against periodontal pathogens have not been clearly elucidated. The purpose of this study was to examine the effects of lactic acid bacteria as probiotics in the prevention and treatment of periodontal disease.

Material and Methods: The growth inhibitory effects of the culture supernatants of 50 strains of lactobacilli on the periodontal pathogen *Porphyromonas gingivalis* ATCC33277 were examined. To obtain a substance with antibacterial properties under neutral pH conditions, each culture supernatant was neutralized and purified by gel filtration column chromatography and reverse-phase HPLC. The molecular weight of purified substances was analyzed with LC-MS.

Results: The results showed that two strains of *Lactobacillus plantarum* 122 (derived from the oral cavity) and *L. fermentum* ALAL020 (derived from fermented soy milk food products) had strong growth inhibition effects. The major antibacterial substance produced by *L. plantarum* 122 was thought to be sodium lactate. On the other hand, the molecular weight of the major antibacterial substance produced by *L. fermentum* ALAL020 we purified was 226.131 Da. An LC-MS analysis revealed that it had the following composition: $C_{11}H_{18}O_3N_2$.

Conclusion: The antibacterial substance of *L. plantarum* 122 against *P. gingivalis* was sodium lactate, and that of *L. fermentum* ALAL020 we purified was a novel low molecular substance. This antibacterial substance has a possibility for using periodontal disease prevention.

Keywords: *Lactobacillus plantarum*; *Lactobacillus fermentum*; Probiotics; Antibacterial activity

Introduction

The oral cavity is a very complex ecosystem which harbors more than 700 bacterial species [1,2]. These bacteria form specific microbial flora in several different habitats, including the tooth surface, the gingival sulcus and the dorsum of the tongue. The onset and development of periodontitis are associated with various factors. The bacterial factor is specific Gram-negative anaerobic bacteria such as *Porphyromonas gingivalis* [3].

Periodontitis is a recurrent disease with acute and chronic phases. In general, non-surgical periodontal treatments such as scaling and root planning are used for the chronic phase of periodontitis. In the acute phase, systematic and topical antibiotic therapies are applied to reduce the causative bacteria and prevent recurrence. However, antibiotic therapy has some harmful side effects, including possible allergic reactions, and antibiotic-resistant bacteria may appear after the prolonged use of antibiotics [4-6]. To develop an alternative therapy, there has been a focus on the usefulness of *Lactobacillus* and *Bifidobacteria* as probiotics [7]. Probiotics are defined by Fuller as, "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance" [8]. The effects of probiotics in the prevention of gastrointestinal infections and the improvement of allergy symptoms have been reported [9-11]. Recently, in the dental field, there have been several attempts to examine the possibility that probiotics may act directly in the oral cavity to prevent oral diseases such as dental caries and periodontal diseases. A number of clinical studies have already reported promising findings [12-14]. However, the antibacterial mechanism of probiotic bacteria against periodontal pathogens has not been clearly elucidated. The purpose of this study was to identify new probiotic strains that exhibit antibacterial activity against the representative periodontal pathogen, *P. gingivalis*, and to identify the antibacterial substances produced by the strains that may be applied to the prevention or treatment of periodontal disease.

Material and Methods

The purification procedure of the antibacterial constituents against a periodontal pathogen produced by lactobacilli was shown in a supplemental file.

Bacterial strains and culture conditions

The present study used 50 strains of lactic acid bacteria (Table 1). All of the lactic acid bacteria strains were cultured in Man-Rogosa-Sharpe (MRS) broth (Difco, Becton Dickinson and Company, Sparks, MD, USA) at 37°C for 24 h under anaerobic conditions (N_2: 80%, CO_2: 10%, H_2: 10%). *P. gingivalis* ATCC33277 was cultured according to the previous study [15].

Screening of probiotic strains by antibacterial activity

Each lactic acid bacterial culture broth was centrifuged to obtain a culture supernatant. After adjusting the pH to 7.0 with 10N NaOH, the supernatant as test samples were serially diluted (two-fold) to 1/256 and *P. gingivalis* culture (10^7 CFU/ml) was inoculated. The growth inhibition was determined as the absorbance value below 0.1 at 620 nm. Antibacterial activity against *P. gingivalis* was detected by the dilution folds as Units.

No.	Strain/species	Site of isolation	Highest inhibitory dilution (Units)
1	*Lactobacillus fermentum* ALAL020	Fermented soy milk	16
2	*L. plantarum* 122	Human oral cavity#	8
3	*L. animalis* ATCC35046	Alimentary canal of animal	4
4	*L. casei* 110	Human oral cavity*	4
5	*L. murinus* ATCC35020	Rat digestive tract	4
6	*L. reuteri* DSM 17938	Human breast milk	4
7	*L. reuteri* ATCC PTA 5289	Human oral cavity	4
8	*L. salivarius* LS1	Human oral cavity	4
9	*Lactobacillus* spp. 11	Yogurt	4
10	*Lactobacillus* spp. 118	Human oral cavity#	4
11	*L. casei* ATCC393	Dairy products	2
12	*L. casei* YIT9029-L 13L	Yogurt	2
13	*L. casei* YIT9029-S 13S	Yogurt	2
14	*L. casei* ALAL003	Fermented soy milk	2
15	*L. fermentum* 103	Human oral cavity*	2
16	*L. gasseri* 102	Human oral cavity*	2
17	*L. mali* ALAL014	Fermented soy milk	2
18	*L. paracasei* 112	Human oral cavity#	2
19	*L. paracasei* 117	Human oral cavity#	2
20	*L. plantarum* 108	Human oral cavity*	2
21	*L. plantarum* ALAL006	Fermented soy milk	2
22	*Lactobacillus* spp. 17L	Red cheddar cheese	2
23	*Lactobacillus* spp. 17S	Red cheddar cheese	2
24	*Lactobacillus* spp. 19L	Red cheddar cheese	2
25	*Lactobacillus* spp. 19S	Red cheddar cheese	2
26	*Lactobacillus* spp. 21	Mozzarella cheese	2

27	*Lactobacillus* spp. 113	Human oral cavity#	2
28	*Lactobacillus* spp. 114	Human oral cavity#	2
29	*Lactobacillus* spp. 115	Human oral cavity#	2
30	*Lactobacillus* spp. 116	Human oral cavity#	2
31	*Lactobacillus* spp. 121	Human oral cavity#	2
32	*Lactococcus lactis* subsp. lactis ALAL019	Fermented soy milk	-
33	*Leuconostoc lactis* ALAL016	Fermented soy milk	-
34	*Leuc. lactis* ALAL017	Fermented soy milk	-
35	*L. acidophilus* ALAL005	Fermented soy milk	-
36	*L. crispatus* 104	Human oral cavity*	-
37	*L. crispatus* 107	Human oral cavity*	-
38	*L. gasseri* 106	Human oral cavity*	-
39	*L. reuteri* ALAL001	Fermented soy milk	-
40	*L. rhamnosus* ALAL004	Fermented soy milk	-
41	*L. salivalius* 105	Human oral cavity*	-
42	*L. salivalius* 109	Human oral cavity*	-
43	*L. johnsonii* ALAL015	Fermented soybean milk	-
44	*Lactobacillus* spp. 14	Mozzarella cheese	-
45	*Lactobacillus* spp. 20	Gouda cheese	-
46	*Lactobacillus* spp. 23	Brie Hermitage cheese	-
47	*Lactobacillus* spp. 24	Foume d'Ambert cheese	-
48	*Lactobacillus casei* 101	Human oral cavity*	-
49	*Lactobacillus* spp. 119	Human oral cavity#	-
50	*Lactobacillus* spp. 120	Human oral cavity#	-

Table 1: Antibacterial activity of lactobacilli expressed as highest inhibitory dilution.

-; Antibacterial activity was not detected.

*; Reference: Hojo K, *et al.* (2007) Distribution of salivary *Lactobacillus* and *Bifidobacterium* species in periodontal health and disease. Biosci Biotechnol Biochem 71: 152-157.

#; Reference: Wakui T, *et al.* (2004) Bacterial investigation of root caries lesions. Jpn J Conserv Dent 47: 673-683.

Fractionation of the *L. plantarum* 122 and *L. fermentum* ALAL020 culture supernatants.

After shaking an equal amount of ethyl acetate or acetone with the culture supernatants of each strains, the water soluble phase was applied to a Sephadex G-25 gel filtration column (100 mmφ × 350 mm) and eluted with deionized water (flow rate 5 ml/min). The total carbohydrate level (420 nm) of the peak fraction was detected using the phenol–sulfuric acid assay method [16]. The acetone supernatant of *L. fermentum* ALAL020 Sephadex G-25 fraction was analyzed by HPLC (JASCO Corporation, Tokyo, Japan) in a reverse-phase preparative ODS (C18) column (Wakoshil 5C18 AR Prep 20.0 mmφ × 250 mm), using gradients of (B) 50% acetonitrile in (A) 10% acetonitrile, both containing 0.05% trifluoroacetic acid: 10-30% of B over 20 min, in a 50% step-wise manner over 10 min, at a flow rate of 5 ml/min.

Determination of the antibacterial activities in the chromatography fractions of *L. fermentum* ALAL020.

After adjusting the pH to 7.0, 20 µl of each fractionated sample were aliquoted into a 96-well plate. Subsequently, 160 µl of GAM broth (Nissui, Tokyo, Japan) supplemented with 5 µg/ml hemin, 1 µg/ml menadione and 20 µl of the *P. gingivalis* culture (1×10^6-10^7 CFU/ml) were added into the wells. The mixtures in the 96-well plates were anaerobically cultured at 37°C for 48 h. The cultures were measured at an absorbance of 620 nm with a microplate reader. The antibacterial activity was measured as the Units used in screening or by the sodium

lactate equivalence (SLU). The SLU was determined by dividing (L) mg/ml by (C) mg/ml, where (L) was the minimum inhibitory concentration (MIC) of sodium lactate against *P. gingivalis* and (C) was the concentration of the fraction that inhibited *P. gingivalis* to below the detection limit. The detection limit was determined as the absorbance value below 0.1 at 620 nm. To determine the antibacterial activity, the reciprocal value of (C) was divided by the reciprocal value of (L), using the following formula: (1/C)/(1/L)=L/C=SLU.

Quantification of sodium lactate and an analysis of the amino acids

Sodium lactate was quantified using an HPLC NANOSPACE SI-1 system (Shiseido, Tokyo, Japan) on a CAPCELL PAC C18 TYPE MG column (1.5×250 mm; Shiseido, Tokyo, Japan) equilibrated with 50 mM $NH_4H_2PO_4$-H_3PO_4 solution (pH 4.2) at a flow rate of 100 µl/min. The absorbance at 210 nm was monitored.

For the amino acid analysis, the sample was heat-hydrolyzed at 110°C with 6 N HCl for 24 h, fluorescently derivatized by 4-fluoro-7-nitrobenzofurazan (NBD-F), and eluted by gradients of (B) CH_3CN/ MeOH/50 mmol/l KH_2PO_4=1/2/2 (v/v/v) in (A) CH_3CN/ 75 mmol/l H_3PO_4=16/84 (v/v). The gradient condition was 0% of B over 5 min, 0-70% over 20 min, and 70-100% over 20 min at a flow rate of 100 µl/min. The eluates were subjected to fluorescence detection (λ ex=470 nm, λ em=540 nm).

The LC-MS analysis of the purified product

An HPLC-purified sample was analyzed using an Ultimate 3000 UHPLC System (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and a Shim-pack VP-ODS column (150 mm × φ4.6 mm, Shimadzu, Tokyo, Japan). Gradient elution was performed using 10-50% acetonitrile with 0.05% formic acid. An MS analysis was performed with a Mass Spectrometry Q Exaction™ Quadrupole/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific). Ionization was performed using the ESI method and detected in the positive mode.

Results

Screening of the probiotic strains

As shown in Table 1, two strains of lactobacilli out of 50 strains of lactic acid bacteria exhibited strong antibacterial activity against *P. gingivalis*: *L. plantarum* 122 (8 Units) and *L. fermentum* ALAL020 (16 Units). Other strains of the same species, showed only 2 Units. Therefore, the following analysis of the antibacterial constituents was performed with these two strains.

Fractionation of the *L. plantarum* 122 and *L. fermentum* ALAL020 culture supernatants.

The culture supernatant of *L. plantarum* 122 was separated on a Sephadex G-25 column into four fractions by absorbance at 260 nm; a substantial amount of carbohydrates was contained in fractions 3 and 4 (Figure 1A). The culture supernatant of *L. fermentum* ALAL020 was separated into five fractions, however, the carbohydrate level was very low in comparison to *L. plantarum* 122 (Figure 2A).

The antibacterial activity of the *L. plantarum* 122 fractions

An antibacterial assay was performed. At the maximum dilution, there were 4 Units each in fractions 3 and 4 (Figure 1B). The concentrations of sodium lactate in fractions 3 and 4 were 23.4% (w/w) and 41.7% (w/w), respectively (Figure 1B). When the antibacterial activity of sodium lactate against *P. gingivalis* was measured in advance, the MIC of sodium lactate was 2.0% (20 mg/ml). The sodium lactate concentrations of fractions 3 and 4 were higher than the MIC of sodium lactate.

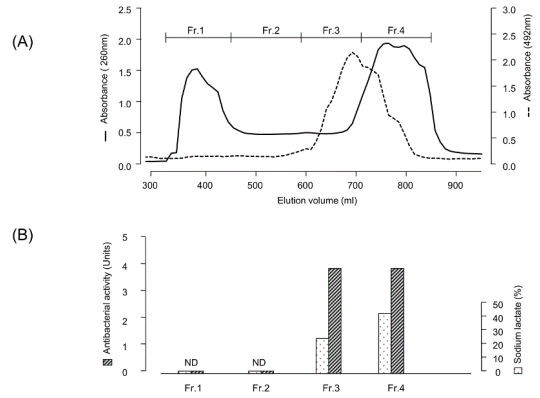

Figure 1: The fractionation of the culture supernatant of *L. plantarum* 122 using a Sephadex G-25 column:
(A) Gel filtration chromatography of the water soluble phase of the culture supernatant. The solid line shows the UV absorption at 260 nm; the dotted line shows the chromogenic reaction of carbohydrate detected at 492 nm. (B) Antibacterial activity and sodium lactate were detected in fractions 1 to 4. Each fraction was adjusted to pH 7.0 before these experiments. Antibacterial activity against *P. gingivalis* was defined as the highest dilution (Units) to exhibit growth inhibition in the measurement of absorbance at 620 nm. ND: not detected.

The antibacterial activity of the *L. fermentum* ALAL020 fractions

As shown in Figure 2B, fraction 3 showed the highest antibacterial activity of 1.82 SLU. The concentration of sodium lactate in fraction 3 was 1.3% (w/w) (Figure 2B), which was less than the MIC of sodium lactate (2.0%). This result indicated that *L. fermentum* ALAL020 might have some active substances other than sodium lactate. Therefore, the fractions were purified and the following analyses were performed.

Four volumes of acetone were added to the fraction 3 solution and the antibacterial activity of the acetone supernatant and precipitate was determined, revealing that the antibacterial activity of the acetone supernatant was as strong as 8.0 SLU (Figure 2C). The culture supernatant was then subjected to acetone precipitation for further experiments and the supernatant was separated using a Sephadex G-25 column. One peak fraction was obtained by this procedure (Figure 3A). This peak fraction was subsequently separated in the ODS (C18) column by reverse-phase HPLC and divided into eight fractions: H1-

H8 (Figure 3B). H8-1 showed the strongest antibacterial activity against *P. gingivalis* (5.0 SLU), followed by H8-2 (3.1 SLU) (Figure 3C).

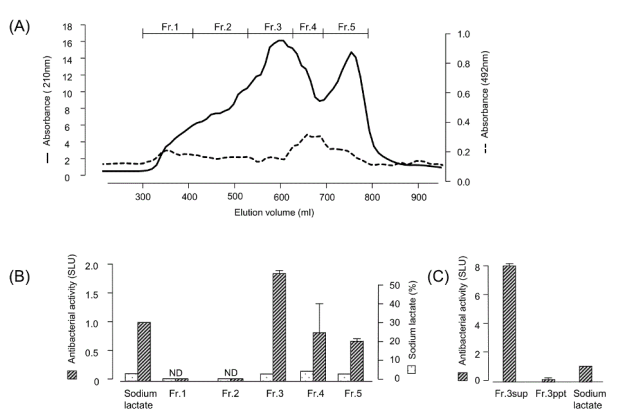

Figure 2: The fractionation of the culture supernatant of *L. fermentum* ALAL020 using a Sephadex G-25 column:
(A) Gel filtration chromatography of the water soluble phase of the culture supernatant. The solid line shows the UV absorption at 260 nm; the dotted line shows the chromogenic reaction of carbohydrate detected at 492 nm. (B) Antibacterial activity and sodium lactate were detected in fractions 1 to 5. Each fraction was adjusted to pH 7.0 before these experiments. Activity units were based on the sodium lactate equivalent (SLU) antibacterial activity. ND: not detected. SLU=(1/C)/(1/L)=L/C. C: The concentration of the fraction that inhibited *P. gingivalis* to below the detection limit. L: MIC of sodium lactate against *P. gingivalis*. (C) The antibacterial activity of the acetone supernatant and precipitate of Fr. 3 are shown as SLU.

The LC-MS analysis of the antibacterial constituents of *L. fermentum* ALAL020

An amino acid analysis of H8-1 and H8-2 was performed, however, no apparent peak was detected (data not shown). We therefore performed an LC-MS analysis. H8-1 and H8-2 were subjected to precision mass spectrometry. The molecular weights of both were estimated to be 226.131 Da (Figure 4A, 4B), with a molecular formula of $C_{11}H_{18}O_3N_2$.

Discussion

P. gingivalis is the most important periodontal pathogen [17-19] because it is frequently isolated from patients with chronic periodontal disease. Vivekananda *et al.* [13] reported that *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289, which we also used in our screening study, reduced the number of *P. gingivalis* in the oral cavity. In addition, Ishikawa *et al.* [20] reported that *L. salivarius* LS1 reduced the number of *P. gingivalis* cells *in vitro*. In the present study, *L. plantarum* 122 and *L. fermentum* ALAL020 showed stronger antibacterial activities than these other lactobacilli at a neutral pH.

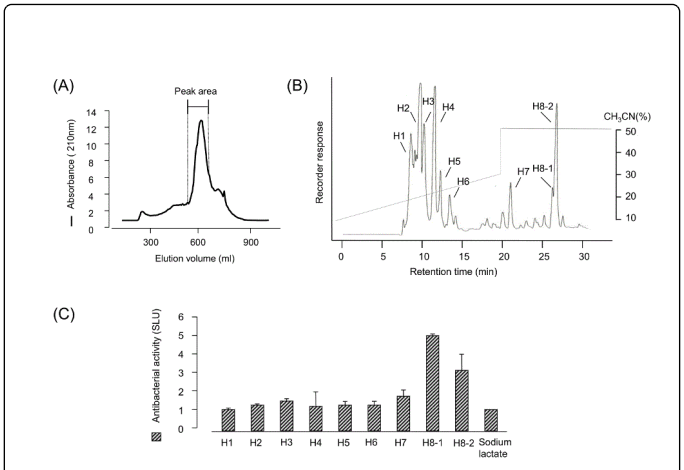

Figure 3: The purification of *L. fermentum* ALAL020:
(A) Sephadex G-25 gel filtration chromatography of the acetone supernatant: The eluted peak area was then subjected to HPLC. (B) Reverse-phase chromatography by HPLC on an ODS (C18) column. (C) Antibacterial activity was detected in H1 to H8. Activity units were based on the sodium lactate equivalent (SLU) antibacterial activity.

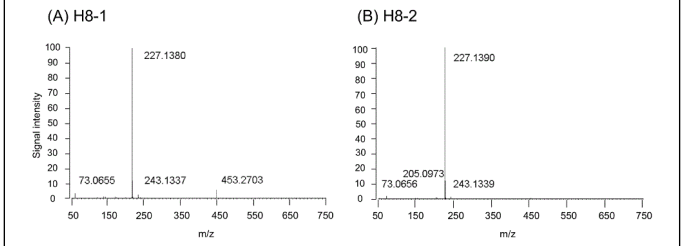

Figure 4: The LC-MS analysis of H8-1 and H8-2:
(A) The spectrum of H8-1 contained major ions of m/z 227.1380. (B) The spectrum of H8-2 contained major ions of m/z 227.1390. The molecular weights of H8-1 and H8-2 were estimated to be both 226.131 Da.

The sterilization or antibacterial substances produced by lactic acid bacteria include organic acids such as lactic and acetic acids, hydrogen peroxide and bacteriocins [21-23]. Kang *et al.* [24] reported that *L. reuteri* showed antibacterial activity against oral pathogens including *P. gingivalis*, but it was decreased when the pH was neutralized. Thus, they concluded that the antibacterial substance of *L. reuteri* was lactic acid. Furthermore, Takahashi *et al.* [25] reported that *P. gingivalis* proliferation was inhibited at a pH ≤ 6.5. However, it is possible that low pH conditions in the oral cavity may induce dental caries or hypersensitivity. In this study, the antibacterial test against *P. gingivalis* was performed after the pH neutralized. Despite the neutral pH of the *L. plantarum* 122 and *L. fermentum* ALAL020 culture supernatants, both strains strongly inhibited the growth of *P. gingivalis*. These results indicate that sodium lactate, a neutralized form of lactic acid, also has antibacterial properties. This result is in line with the findings of Matsuoka *et al.* [26]. The antibacterial fraction of *L. plantarum* 122

contained more than 2% sodium lactate (MIC of sodium lactate against *P. gingivalis*). The presence of sodium lactate strongly contributed to the antibacterial activity of *L. plantarum* 122.

As for *L. fermentum* ALAL020, strong antibacterial activity was observed in the ethyl acetate or acetone supernatant of fraction 3 (Figure 2B, 2C), but the concentration of sodium lactate was only 1.3% (Figure 2B). Therefore, fraction 3 was divided in HPLC (Figure 3B) to obtain purified active fractions and analyzed by LC-MS (Figure 4). In *L. fermentum*, bacteriocin-like substances have been reported [27-29]. However, the antibacterial substances obtained from H8-1 and H8-2 in the present study showed a different molecular weight (226.131 Da, Figure 4) from bacteriocin-like proteins and the following composition formula: $C_{11}H_{18}O_3N_2$. The substances from H8-1 and H8-2 showed different behaviors in the LC, and were divided into two proximal peaks; we speculate that the substance was a structural isomer. An amino acid analysis revealed that this substance did not contain amino acids, and it was confirmed with FT-IR (data not shown). Reuterin, an antibacterial substance in *L. reuteri* (also known as 3-hydroxypropionaldehyde; molecular weight, 74 Da; composition formula, $C_3H_6O_2$) also has a low molecular weight and does not contain amino acids [30,31]. Because the molecular weight and formula of our isolated substance were different from those of reuterin, the isolated substance may be a novel antibacterial substance. In our preliminary experiment, *L. fermentum* ALAL020 was less effective against *Prevotella intermedia* or *Fusobacterium nucleatum* (date not shown), but highly effective against *P. gingivalis*. These results indicate the possibility that it may specifically target *P. gingivalis*. According to these results, it is necessary to analyze the mechanisms that underlie its antibacterial activity.

In conclusion, an antibacterial substance which exhibited activity against the periodontal pathogen *P. gingivalis* was isolated and identified from *L. plantarum* 122 and *L. fermentum* ALAL020. It was hypothesized that the major antibacterial substance produced by *L. plantarum* 122 was lactic acid. The antibacterial substance produced by *L. fermentum* ALAL020 we purified was a novel low molecular substance active in neutral pH. This substance might be useful in dental treatment and for the prevention of periodontal diseases. A further structural analysis of this novel substance is necessary to elucidate its mechanisms before its use in the clinical setting.

Acknowledgments

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