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Original Research

Inhibitory Effects of the Heat-Killed Lactic Acid Bacterium *Enterococcus faecalis* on the Growth of *Porphyromonas gingivalis*

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ABSTRACT

Background: *Porphyromonas gingivalis*, a gram-negative obligate anaerobic bacterium, is a major pathogen involved in the onset and progression of periodontal disease, a chronic inflammatory disorder observed in approximately two-thirds of the Japanese population older than age 30 years. *P gingivalis* cells produce and secrete gingipain, a powerful proteolytic enzyme, on their surfaces and in external environments.
Objectives: The effects of heat-killed *Enterococcus faecalis* (Hk*Ef*), a lactic acid bacterium, on the growth of *P gingivalis* were evaluated in vitro by measuring the viable cell count of *P gingivalis* and gingipain activity.
Methods: Hk*Ef* solution (1.63 or 163 mg/mL) was added to 1 mL *P gingivalis* culture to generate a final Hk*Ef* concentration of 0.64 or 64 mg/mL. The cultures were incubated anaerobically. The number of viable *P gingivalis* cells and gingipain activity were measured after incubation for 0, 12, 24, 48, and 72 hours. The number of viable *P gingivalis* cells was calculated by counting the number of colonies after culture. Gingipain activity was quantified by adding a chromogenic substrate to *P gingivalis* culture medium and measuring the absorbance of the reaction solution with a plate reader. Mean (SE) was calculated for viable cell counts and gingipain activity, and Wilcoxon rank-sum test was used to test for significant differences.
Results: The counts of viable *P gingivalis* cells in the control group increased as incubation time progressed for 12, 24, 48, and 72 hours; similar results were observed in the low-concentration Hk*Ef* group. In the high-concentration Hk*Ef* group, the increase in the viable cell count was significantly inhibited compared with that of the control group. Furthermore, gingipain activity in the low- and high-concentration Hk*Ef* groups was significantly inhibited over time compared with that of the control group. Although the pH of the culture solution tended to decrease in the high-concentration Hk*Ef* group, it was not considered to have affected the growth of *P gingivalis*.
Conclusions: Hk*Ef* exhibits inhibitory effects on the growth of *P gingivalis* and gingipain activity.

Introduction

Porphyromonas gingivalis, a gram-negative obligate anaerobic bacterium (Figure 1), is a major pathogen involved in the onset and progression of periodontal disease, a chronic inflammatory disorder observed in approximately two-thirds of the Japanese population older than age 30 years.[1] *P gingivalis* is found in the subgingival lesions of adult patients with periodontitis and is known to be strongly associated with various systemic diseases[2–9] that extend beyond the oral cavity; as the bacteria spreads to the saliva, tongue, tonsils, and other organs it induces inflammation and subverts host immunity.[3] Periodontitis caused by *P gingivalis* has been identified as a secondary risk factor for serious systemic diseases, including cardiovascular diseases (endocarditis and coronary heart disease),[5,6] pneumonia,[8] and premature and low-birth-weight babies.[9] Therefore, the relationship between *P gingivalis* and systemic diseases is of great interest. Recently, virulence factors such as gingipain, a powerful proteolytic enzyme secreted by *P gingivalis*, have been detected in the brain of patients with Alzheimer disease and in animal models.[10–12] The association between chronic periodontitis caused by *P gingivalis* infection and Alzheimer disease has also attracted attention.[13,14]

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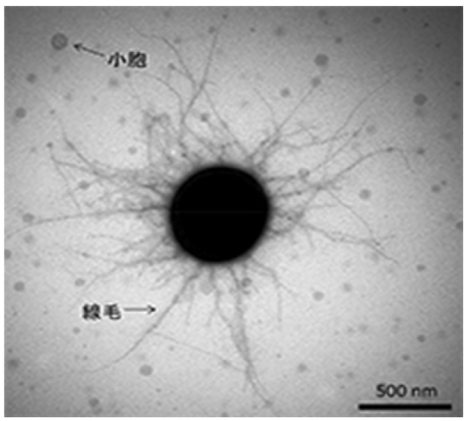


Fig 1. Electron microscope image of *Porphyromonas gingivalis.* Koji Nakayama, Japanese Society for Bacteriology. https://jsbac.org/youkoso/porphyromonas Gingivalis.html.

P gingivalis does not ferment carbohydrates; *P gingivalis* cells produce and secrete gingipain on their surfaces and in external environments. Therefore, the intake of nutrients and energy sources depends on external proteins and peptides.[15] Gingipain causes loss of adhesion in gingival fibroblasts and vascular endothelial cells, structurally and functionally destroys periodontal tissues, and causes host protein degradation, blood coagulation, vascular hyperpermeability, leukocyte dysfunction, and cell death.[16,17] Therefore, gingipain is highly pathogenic and induces the development of various pathological conditions. In addition, gingipain has 2 enzyme groups in accordance with the specificity of the peptide cleavage site: Arg-gingipain (Rgp), which cleaves the C-terminus of the arginine residue, and Lys-gingipain (Kgp), which cleaves the C-terminus of the lysine residue.[18] These enzyme groups can maintain and exert their activity without being inactivated in vivo, thus damaging the host cells; they are essential for the survival and growth of the bacterium. Gingipain is suppressed by a specific inhibitor.[19] Suppression of *P gingivalis* proliferation has been reported by knocking out the gene for gingipain.[18]

Studies using animal models have shown that oral administration of heat-killed *Enterococcus faecalis* (Hk*Ef*), a lactic acid bacterium, has protective effects against infections caused by several bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), multiple-drug-resistant *Pseudomonas aeruginosa* (MDRP), *Streptococcus pneumoniae*, and *Clostridioides difficile*, which are all responsible for nosocomial infections.[20,21] Furthermore, in a previous study, we confirmed the direct inhibitory effects of Hk*Ef* on the growth of *C difficile*, which causes antibiotic-associated colitis, in an in vitro test using a mixed culture.[22] In the present study, we conducted an in vitro co-culture study to evaluate the effect of Hk*Ef* on *P gingivalis* growth and gingipain activity.

Materials and Methods

Test microorganisms

A stock solution of 500 mg/mL Hk*Ef* in brain-heart infusion broth was prepared and serially diluted to 1.63 and 163 mg/mL before use. The concentration of the Hk*Ef* suspension was set to 12 billion cells/kg/d based on previous studies; Hk*Ef* showed a significantly higher survival rate in MRSA-, *S pneumoniae*-, MDRP-, and *C difficile*-infected animal models.[20,21] In addition, the survival rates of MDRP-infected animals in the group that received 3 times the dose of Hk*Ef* cells showed no significant difference.[21] In an in vitro mixed culture test, an Hk*Ef* dose of 600 billion cells/d showed direct inhibitory effects against the growth of *C difficile.*[22] Therefore, 12 billion Hk*Ef* cells/kg/d was set as the low-concentration group (1.63 mg/mL), and 1200 billion Hk*Ef* cells/kg/d was set as the high-concentration group (163 mg/mL).

Bacterial cultures

The stock culture of *P gingivalis* ATCC® 33277™ was thawed and spread onto *Brucella* HK agar medium, followed by culturing under anaerobic conditions at 37°C for 3 days in an incubator (ILE800, Yamato Scientific Co Ltd, Tokyo, Japan). After culturing, the colonies were inoculated into brain-heart infusion medium containing 5 μg/mL hemin and 1 μg/mL menadione, followed by incubation under anaerobic conditions at 37°C for 2 days. The cultures were incubated until the turbidity (optical density at 650 nm) reached 0.6; these cultures were used as the stock solutions.

Preparation of bacterial inoculum solution and measurement of viable cell counts

The inoculum stock solution was prepared by diluting the test microorganism culture by 10^4 times in brain-heart infusion broth. One part of the inoculated bacterial solution was collected, diluted by 10, 10^2, and 10^3 times with physiological saline, spread onto *Brucella* HK agar medium, and incubated in an incubator (ILE800, Yamato Scientific Co Ltd) under anaerobic conditions at 37°C for 4 days. After incubation, colonies were counted using a colony counter (CC-1; AS ONE Corporation, Osaka, Japan), and the number of viable bacterial cells in 1 mL inoculum stock solution was determined. The sample size was set to 5.

Measurement of viable *P gingivalis* cells

Hk*Ef* solution (0.65 mL low or high concentration) was added to a test tube containing 1 mL test microorganism culture, followed by incubation at 37°C for 72 hours in an anaerobic jar. After 0, 12, 24, 48, and 72 hours of incubation, the pH of the culture broth was measured, and an aliquot of the culture was collected to measure the counts of viable *P gingivalis* cells. The culture solution was appropriately diluted and spread onto *Brucella* HK agar medium, followed by incubation at 37°C for 4 days under anaerobic conditions. Viable cell counts were calculated by measuring the number of colonies that grew after incubation. The compositions of the test groups are listed in the Table.

Measurement of gingipain activity

The test tubes used for measuring the viable cell counts were centrifuged at 2000 rpm ($780 \times g$) for 10 minutes using a centrifuge (AX-310; Tomy Seiko Co Ltd, Tokyo, Japan), and the supernatants were collected. To the supernatant, 50 mM Tris-HCl buffer (pH 8.5), 0.2 mM chromogenic substrate (Rgp: *Nα*-benzoyl-L-arginine 4-nitroanilide hydrochloride, or Kgp: *N*-[*p*-tosyl]-Gly-Pro-Lys 4-nitroanilide), and 10 mM 1,4-dithiothreitol (DTT) were added. The reaction solution (100 μL) was incubated at 37°C for 10 minutes, and 25 μL 50% acetic acid was added to terminate the reaction. A plate reader was used to measure the absorbance of the reaction solution and the supernatant, obtaining values of 405 and 620 nm, respectively (Multiskan FC; Thermo Fisher Scientific, Waltham, Massachusetts, USA). The gingipain activity was calculated by dividing the 405 nm absorbance value by the 620 nm value.[23]

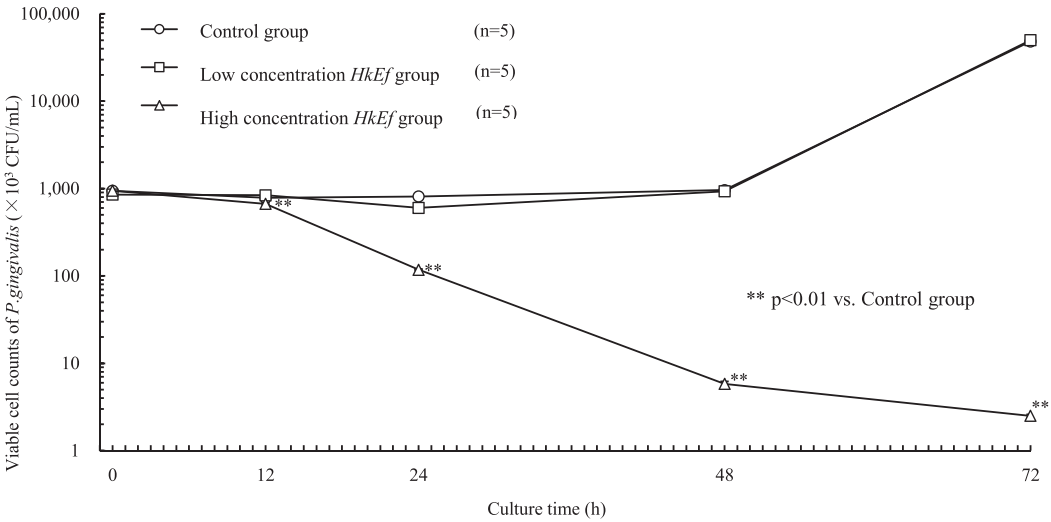
Statistical Analysis

The mean (SE) of the viable cell counts and gingipain activity were calculated, and significant differences relative to the control

Table
Compositions of the test groups in this study.*

Test group	Concentration of Hk*Ef* (mg/mL)	Concentration of *P gingivalis* (CFU/mL)
Control group	0	1.2×10^6
Low-concentration Hk*Ef* group	0.64	1.2×10^6
High-concentration Hk*Ef* group	64	1.2×10^6

Hk*EF* = heat-killed *Enterococcus faecalis*.
* The concentration indicates the final concentration after mixing.



Culture time	0	12	24	48	72
Test group	Mean ±S.E.	Mean ±S.E.	Mean ±S.E.	Mean ±S.E.	Mean ±S.E.
Control group	942.0 ± 23.5	784.0 ± 11.2	810.0 ± 55.9	960.0 ± 34.2	48380.0 ± 3239.4
Low concentration *HkEf* group	854.0 ± 38.8	838.0 ± 20.1	602.0 ± 52.0	928.0 ± 30.1	49960.0 ± 3498.4
High concentration *HkEf* group	944.0 ± 26.2	670.0 ± 17.3 **	117.8 ± 2.4 **	5.8 ± 0.4 **	2.5 ± 0.1 **

Fig 2. Time-courses of the viable cell counts of *P gingivalis*. Each value shows mean ± SE ($\times 10^3$ CFU/mL). **Significantly different from the control group at $P < 0.01$.

group were examined using the Wilcoxon rank-sum test. Statistical significance was set at $P < 0.05$. A commercially available statistical program (SAS system; SAS Institute Japan Ltd, Tokyo, Japan) was used for the statistical analysis.

Results

Effect of Hk*Ef* on the *P gingivalis* viable cell count

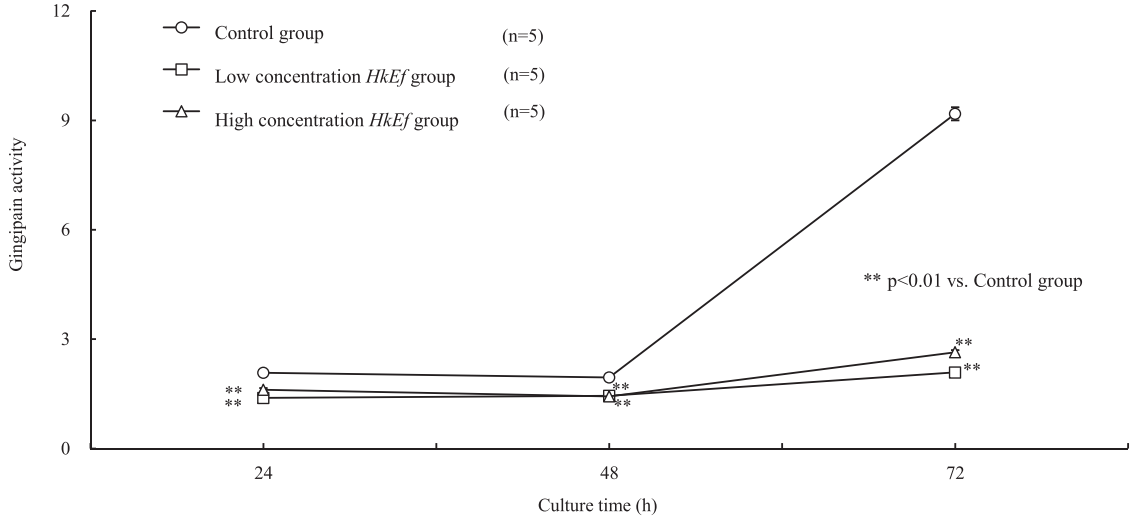
P gingivalis and Hk*Ef* were co-cultured for different time periods and the count of viable *P gingivalis* cells was calculated (Figure 2). The viable cell count of *P gingivalis* in the control group was 942.0 (23.5) $\times 10^3$ CFU/mL at the start of incubation, and 784.0 (11.2), 810.0 (55.9), 960 (34.2), and 48,380.0 (3239.4) $\times 10^3$ CFU/mL at 12, 24, 48, and 72 hours, respectively. The viable cell count of *P gingivalis* in the low-concentration Hk*Ef* group was the same as that in the control group, whereas the cell count in the high-concentration Hk*Ef* group was significantly lower than that in the control group; that is, 944.0 (26.2) $\times 10^3$ CFU/mL at the start of incubation, and 670.0 (17.3), 117.8 (2.4), 5.8 (0.4), and 2.5 (0.1) $\times 10^3$ CFU/mL at 12, 24, 48, and 72 hours, respectively.

Effect of Hk*Ef* on gingipain activity

Gingipain activity was observed after *P gingivalis* and Hk*Ef* were co-cultured for different time periods (Figures 3A and B). The solvents used in this study (culture medium, Hk*Ef*, and chromogenic substrate) showed the same absorption as that of gingipain, with a maximum optical density value of 0.98. Therefore, values < 1.0 were considered below the detection limit, and values ≥ 1.0 were used for the evaluation of gingipain activity. When either *Nα*-benzoyl-L-arginine 4-nitroanilide hydrochloride or *N*-(*p*-tosyl)-Gly-Pro-Lys 4-nitroanilide was used as the substrate, gingipain activity in the control group increased after 72 hours. When *Nα*-benzoyl-L-arginine 4-nitroanilide hydrochloride was used as the substrate, the increase in gingipain activity was suppressed in the low- and high-concentration Hk*Ef* groups; gingipain activity was significantly lower in both groups than in the control group. In contrast, when *N*-(*p*-tosyl)-Gly-Pro-Lys 4-nitroanilide was used as the substrate, gingipain activity was significantly lower in the high-concentration Hk*Ef* group than in the control group until 72 hours, whereas gingipain activity was significantly suppressed after 72 hours in the low-concentration Hk*Ef* group.

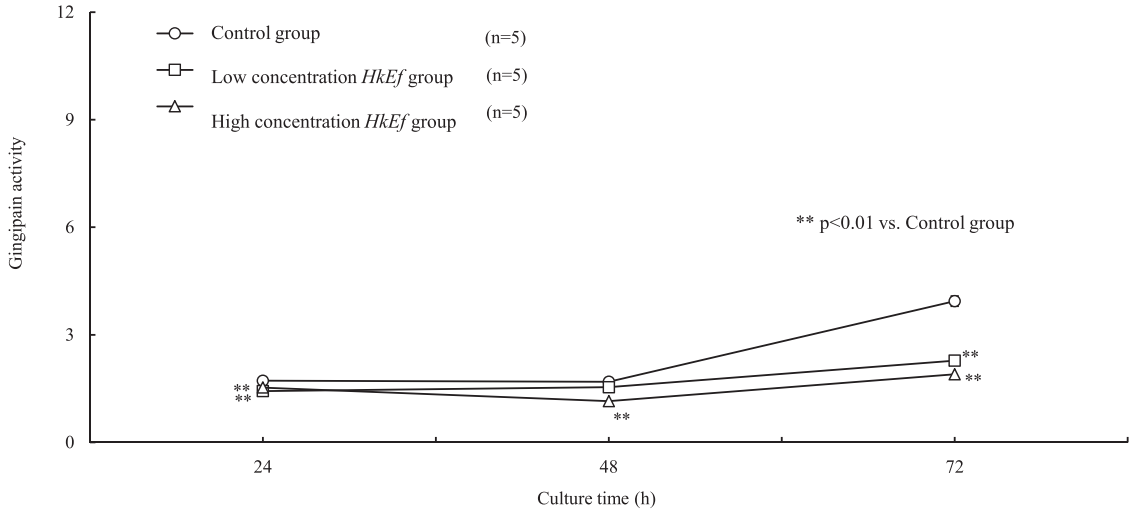
Discussion

The present study revealed that the increase in the viable cell counts of *P gingivalis* in the control group was significantly suppressed in the high-concentration Hk*Ef* group but was not affected in the low-concentration Hk*Ef* group. These results confirmed the inhibitory effects of Hk*Ef* on the growth of *P gingivalis*. The increase in the activities of both Rgp- and Kgp-gingipain (Rgp and Kgp are toxins produced by *P gingivalis*) was significantly inhibited in both the low- and high-concentration Hk*Ef* groups. Thus, the inhibitory effects of Hk*Ef* on the growth of viable *P gingivalis* cells differed between the groups. Moreover, inhibition of the increase in gingipain activity was observed in the low-concentration Hk*Ef* group, in which the viable cell count of *P gingivalis* remained unaffected. These results confirmed that Hk*Ef* has an inhibitory effect on the growth of *P gingivalis* and increases gingipain activity.



Culture time	24		48		72	
Test group	Mean ±S.E.		Mean ±S.E.		Mean ±S.E.	
Control group	2.08 ± 0.05		1.95 ± 0.03		9.18 ± 0.18	
Low concentration *HkEf* group	1.39 ± 0.03	**	1.45 ± 0.03	**	2.09 ± 0.02	**
High concentration *HkEf* group	1.61 ± 0.05	**	1.43 ± 0.01	**	2.64 ± 0.06	**

(A)



Culture time	24		48		72	
Test group	Mean ±S.E.		Mean ±S.E.		Mean ±S.E.	
Control group	1.72 ± 0.01		1.69 ± 0.06		3.94 ± 0.14	
Low concentration *HkEf* group	1.43 ± 0.03	**	1.54 ± 0.02		2.28 ± 0.02	**
High concentration *HkEf* group	1.53 ± 0.01	**	1.15 ± 0.01	**	1.90 ± 0.03	**

(B)

Fig 3. Time-courses of the gingipain activity (Arg-gingipain). (A) Chromogenic substrate; Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride. (B) Chromogenic substrate; N-(p-tosyl)-Gly-Pro-Lys 4-nitroanilide. Each value shows mean ± SE **Significantly different from the control group at $P < 0.01$.

Two strains of viable *Lactobacillus delbrueckii*, STYM1 and GVKM1, isolated from commercial yogurt products, have been reported to inhibit *P gingivalis* growth in agar overlay and spot assays.[24] In addition, antibacterial peptides[25] and natural or synthetic gingipain inhibitors[26] have been studied; however, problems regarding their specificity, safety, efficacy, and manufacturing costs have been noted. *P gingivalis* slowly forms subgingival biofilms. Moreover, gingipain contains high concentrations of numerous cysteine proteinases with trypsin-like activity,[27] which damage host cells, cause adhesion loss in gingival fibroblasts and endothelial cells of blood vessels, and induce cell death. Gingipain decomposes proteins in cooperation with Rgp and Kgp enzymes, both of which degrade human type I and IV collagen, fibronectin, and laminin in the extracellular matrix.[28–30] Gingipain also degrades the $\alpha 2$, $\beta 1$, and $\beta 3$ subunits of integrin, a fibronectin receptor protein.[18] Additionally, Rgp and Kgp have been shown to suppress neutrophil phagocytosis owing to their ability to destroy human immunoglobulins (eg, immunoglobulin G and immunoglobulin A), degrade complement systems (eg, C3 and C5), and inactivate cytokines (eg, interleukin 6, interleukin 8, and tumor necrosis fac-

tor α), thereby impairing biological defense mechanisms.[31–34] The strong damaging effect of gingipain on host cells, which occurs via protein degradation, is a risk factor for various systemic diseases, such as cardiovascular disease, pneumonia, Alzheimer disease, and chronic periodontitis. Considering these findings, the inhibitory effects of Hk*Ef* on both Rgp- and Kgp-gingipain activity observed in the present study are important for the prevention of various systemic diseases. Moreover, lactic acid bacteria produce organic acids, such as lactic acid and acetic acid, which decrease the pH and produce antibacterial substances, such as hydrogen peroxide and bacteriocin,[32–34] thereby inhibiting the growth of other bacteria. Matsuoka et al[35] determined the amount of lactic acid accumulated in a mixed culture solution of *Lactobacillus salivarius* and *P gingivalis* and the pH change in the culture solution. The authors observed a sharp decrease in *P gingivalis* abundance when the amount of lactic acid was <50 nmol/L or the pH <6.0. However, because the lactic acid bacteria used in the present study were heat-sterilized and therefore did not produce lactic acid during the culture process, changes in the pH of the culture solution of the samples from the high-concentration Hk*Ef* group were likely due to factors other than lactic acid. The pH of the medium was not considered to have a significant influence on the inhibitory effects on *P gingivalis* growth because the pH change during the incubation period ranged from 6.33 to 6.89 (data not shown). Collectively, our results showed that Hk*Ef* inhibited the growth of viable *P gingivalis* cells and gingipain activity, revealing the antibacterial effect of heat-sterilized, nonliving lactic acid bacteria on *P gingivalis*. The mechanisms underlying the inhibitory effects of Hk*Ef* on *P gingivalis* have not yet been clarified, and further studies are needed to elucidate these mechanisms.

Recent studies suggest that periodontitis caused by *P gingivalis* infection is a secondary risk factor for diabetes,[4] arteriosclerotic disease,[5,6] rheumatoid arthritis,[7] and obesity/nonalcoholic fatty liver disease.[2] The existence of a relationship between Alzheimer disease[11,12] and *P gingivalis* has also received significant attention. In this study, we showed that Hk*Ef* inhibited *P gingivalis* growth and gingipain activity, suggesting that Hk*Ef* may contribute to the prevention and treatment of systemic diseases. We believe that the application of Hk*Ef*, either orally or as an oral care application against *P gingivalis*, which is believed to act on various systemic diseases, would be of therapeutic significance owing to its inhibitory effect on gingipain activity. However, because these results were obtained in vitro, we intend to determine the gingipain activity inhibition by the 2 substrates in vivo and plan to verify the Hk*Ef* growth-inhibitory effect in clinical studies in the future. Future studies should investigate how Hk*Ef* works against *P gingivalis* and exerts its antimicrobial effect, including the mechanism by which the production of gingipain from *P gingivalis* is inhibited.

Conclusions

In this study, we evaluated the effects of Hk*Ef* on *P gingivalis* growth in vitro. The number of viable *P gingivalis* cells in the high-concentration Hk*Ef* group was significantly lower than that in the control group; however, the low-concentration Hk*Ef* group had the same cell count as the control group. Gingipain activity was significantly inhibited in both the low- and high-concentration Hk*Ef* groups compared with the control group. In conclusion, Hk*Ef* inhibited the proliferation of *P gingivalis* cells and gingipain activity.

Declaration of competing interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

Acknowledgments

T. Matsuo contributed to the data analysis and manuscript writing. K. Nakao and K. Hara contributed to the collection of the data and data analysis. All the authors read and approved the final manuscript.

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