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CONFLCTS OF INTEREST
Some of the authors (CCL and CCH) are employed by the company that sells products containing the fermented herbal preparation. This manuscript was written as part of their normal employment.

Effect of an Herbal Preparation Fermented by *Lactobacillus reuteri* LR107 in Preventing Periodontal Inflammation in an Experimental Gingivitis Model

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ABSTRACT
Many oral pathogens are implicated in the periodontal diseases. Strategies such as eliminating local microbial load are effective in preventing the progression of the periodontal disease. Many herbal/medicinal plants are known for their antibacterial properties. Fermentation of herbal plants with lactic acid bacteria has known to enhance various bio-active properties, thus the study aimed to investigate the effect of a herbal preparation fermented with *Lactobacillus reuteri* LR107 in *in vitro* antibacterial assays and in the experimental periodontal disease. The antibacterial activities were first compared in fermented and non-fermented herbal preparation. Fermented herbal preparation demonstrated a wide-spectrum anti-microbial activity to seven oral pathogens. HPLC and NMR studies further identified the major bioactive markers in the fermented herbal preparation to be berberine and palmatine. Lastly, ligation-induced periodontitis model is used to evaluate the in vivo effect of the fermented herbal preparation. Alveolar bone loss by the preparation was inhibited to a degree similar to that by the doxycycline control. The fermented herbal preparation demonstrated antibacterial activities and preserved alveolar bone resorption in experimental periodontitis.

Keywords: Periodontal diseases, *Lactobacillus reuteri*, fermentation.

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INTRODUCTION

Periodontal diseases are major public health issues[1]. Periodontitis is a chronic inflammatory condition. An overload of bacterial presence results in a bacterial plaque, which continues to elicit a local inflammatory reaction. This is followed by leukocyte infiltration and the release of inflammatory mediators such as cytokines[2,3]. The classic feature of chronic inflammation is tissue destruction, which results in bone resorption in the case of periodontitis[4]. One of the outcome of periodontitis is tooth loss, which affects up to 20% of adult population worldwide [5,6]. Tooth loss often leads to malnutrition, which causes morbidity and premature death. It is estimated that the oral cavity is colonized by over 750 species of bacteria[7]. Some of these bacteria are recognized as periodontogenicspecies. Bacteria like Streptococcus mutans, a Gram-positive, facultative anaerobic bacteria metabolize carbohydrates to organic acids (mainly lactic acids), which cause decalcification hence dental carries[8]. Secondary infections can be further caused by *S. sanguis, S. gordanii* and *S. ovalis*[9]. Gram negative bacteria such as *Porphyromonas gingivalis* and *Prevotella intermediaare* implicated more in the course of periodontitis[10–12]. The persistent infection results in connective tissue destruction and alveolar bone resorption.

The presence of oral pathogenic bacteria is not the sole etiologic agent for periodontal diseases. Other environmental factors such as age, smoking, oral hygiene and stress in a susceptible individual also play a role in the pathogenesis[13]. Maintaining a good oral hygiene practice and limiting sugar consumption can reduce bacterial load can further prevent the formation of dental biofilms. Antibiotics or anti-bacterial agents are commonly used for treating dental carries and periodontal diseases. Amoxicillin, tetracycline, penicillin and metronidazole are most commonly used antibiotics[14]. However, antibiotics resistance is a major issue as the prolonged uses may select bacteria with acquired antibiotic resistance. Anothercommonly used anti-bacterial reagent in oral care is chlorhexidine. Owing to its broad-spectrum antibacterial effects, chlorhexidine is considered the "gold standard" of anti-bacterial oral rinses[15]. However, chlorhexidine causes side effects including tooth staining, loss of sensation, dry and sore mucosa, which is not recommended for long term use[15]. Chemicals like chlorhexidine are edible and may cause some toxic effects when ingested. In the light of needs for natural and less-toxic anti-bacterial agents, herbal medicine is an attractive option. There are approximately over 500,000 plant species discovered worldwide, but only 1% has been explored for their bioactive properties and have been phytochemically analyzed[13]. However, of those phytochemically analyzed, many demonstrated remarkable antibacterial properties. Over the years, increasing research has indicated that herbal products demonstrated anti-microbial effects to oral pathogens. For example, the boiled extract of *Coptidis rhizome* (Ranunculacea), which is commonly used in concoctions in the traditional Chinese medicine (TCM) is bactericidal against periodontogenic bacteria[16]. Many herbal products contain phytochemicals such as alkaloids, flavonoids and terpenes, many of which are known for anti-bacterial activity[13]. The application of herbs and TCM in oral care has gained great interest as many of them are edible hence the reduced toxicity.

Fermentation process is believed to reduce toxic effects and enhance bio-active components in medicinal herbs according to traditional Chinese medicine knowledge*. Echinacea purpurea*, a purple coneflower fermented with *Lactobacillus plantarum* enhanced anti-microbial and anti-oxidant properties of the plant[17]. Enhanced anti-oxidant effects of AnoectochilusformosanusHayata fermented by Lactobacillus acidophilus were also reported[18]. Lactobacillus fermentation has not only been applied in a herbal preparation constituted by single plant species, similar process has also been used for multi-species herbal formulations, many of which are the traditional "recipe" that have been used for centuries. Hwangryun-haedok-tang (HRT), a traditional herbal medicine has been widely used in Asian countries for treating hepatitis, gastritis and dermatitis owing to its anti-inflammatory function [19]. After fermentation by Lactobacillus curvatus, HRT can inhibitosteoclastogenesis and bone loss.Fermentation with lactic acid bacteria (LAB) has also been used in enhancing bioactivities of red ginseng *Panax ginseng* C. *A. Meyer* (Araliaceae) and *Codonopsis lanceolata* extracts[20,21].

The aim of this study is to investigate whether lactic acid fermentation of herbal preparation can be utilized in development of natural oral care with a focus on periodontal disease. We first examined the antibacterial effects of the fermented herbal preparation against bacteria implicated in periodontal disease as a starting point. HPLC analysis accompanied with *in vitro* anti-microbial tests were performed to identify the major phytochemical components responsible for anti-bacterial effects. Lastly, we

investigated the potential of fermented herbal preparation in the animal model of periodontitis.

METHODS

Preparation of fermented herbal preparation:

Angelica sinensis, Rehmanniaglutinosa, Coptischinensis, Lonicera japonica, Echinacea purpurea, Salvia nipponica and Chamomile were first extracted with water at 121℃ for 20 minutes. The extract was cooled and centrifuged. The supernatant was then fermented with *Lactobacillus reuteri* LR107 (New Bellus Enterprises Co., Ltd).

Glycerol stocks of *Lactobacillus reuteri* LR107 were inoculated into 10 mL MRS broth (DIFCO) at 37℃ for 24 hours. The activated cultures were then inoculated into 100mL of MRS broth 37℃ for 24 hours. 10L of herbal extract was inoculated with 280 mL of *Lactobacillus reuteri* LR107 (1.3 x 109) inoculum at 37℃ for 24 hours. The fermented herbal extract was then concentrated and lyophilized. Water, and methanol extracts were prepared by refluxing 1g of dry powder in 10 mL of each solution. The solution after cooling to room temperature was evaporated completely to yield extracts. Dry powder (100mg) was extracted with 1mL DMSO for 24h at room temperature, and the mixture was centrifugation for 1h at 16,000g. The clear supernatant was then evaluated in in vitro antibacterial assay.

Disc diffusion method:

Test materials for disc agar diffusion test were dissolved in DMSO to yield a concentration of 100 mg/mL. Each disc (5mm) contained 4 mg of the test sample.

The test strains including *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis*, *Streptococcus mutans, Streptococcus sangui*, *Actinomyces viscosus*, *Prevotella intermedia,* and *Fusobacterium nucleatum* were first seeded in to the Muller Hinton Broth and incubated at 37℃for 20 hours. The turbidity was compared with the standard 0.5 McFarland solution and 100 mL of culture of test strains were plated on the Mueller Hinton agar. The 5 mm disc was placed on the plate coated with test organism and incubated at 37℃ for 24 hours. Zones of inhibition were measured from the edge of each disc after 24 hours of incubation.

Determination of minimum inhibitory concentration (MIC):

Mueller Hinton Broth, which contained logarithmic, serially twofold-diluted amounts of the extracts (12 concentrations tested), were inoculated with 5x105 cfu of actively dividing bacterial culture. The cultures were incubated for 24 hours at 37℃. the growth was monitored visually and spectrophotometrically (at 595 nm). The MIC was defined as the lowest concentration required to arrest the growth of the bacteria at the end of 24 h of incubation

Preparation of the extracts for HPLC analysis:

Lyophilized mycelia were extracted with methanol in a 1:10 (w/w) ratio for 1 hour under pulsed sonication. The extract was recovered by filtration and the solvent was removed by rotary evaporation under reduced pressure at temperatures below 45°C.

Twenty grams of the dried extract were re-suspended in 100 mL of distilled water and sequentially extracted three times with 100 mL of ethyl acetate and n-butanol. The solvent of each extraction was removed on a rotary evaporator under vacuum.

For the isolation of the active ingredients, the n-butanol fraction was further separated by semi-preparative reverse phase HPLC on an COSMOSIL MS Ⅱ column (20.0 mm I.D. × 250 mm, 5 μm). The column was run in a gradient mobile phase generated by mixing an aqueous solution (A) and acetonitrile (B). The samples were eluted by

a series of linear gradients of B into A as follows:, 5% B isocratic (0-10 min), 5–20% B (10–40 min), 20–28% B (40–90 min), and 28–60% B (90-120 min). The flow rate was set at 10 mL/min and column temperature was maintained at 30°C. The UV absorbance was monitored at the 210 nm and 254 nm wavelengths. A total of 120 fractions were recovered and pooled for the further assays.

The HPLC fraction containing the active compound was analyzed by HPLC-MS on a COSMOSIL 5C18-MS-II column (250 mm × 4.6 mm i.d.) at the flow rate of 0.8 mL/min under the control of a Thermo Accela HPLC system equipped with LCQ Fleet MS system. Mobile phase compositions were (A) water with 10mM formic acid and (B) acetonitrile. Gradient was set as follows: 0–5 min, 5% B; 5–40 min, 95% B; Flow rate was constant at 0.8 mL/min. Injection volume was 20 μL. Mass spectrometry was operated under the conditions as follows: drying gas N2, 10 L/min; capillary voltage, 20 v; pressure of nebulizer, 30 psi; ion spray voltage, 4 kV; and capillary temperature, 325°C.

Animal model of periodontal disease:

Sprague Dawley (SD) males rats were purchased from National Laboratory Animal Center with a weight between 280 to 310g. The animals were housed in the animal center of National Defense Medical Center at 22 ±3°C with 12 hour light/dark cycles. The animals were fed *ad libitum*. The study was approved by the Institutional Animal Care and Use Committee (IACUC). The animals were randomly assigned to four groups: the un-ligated control group; the ligated group; the ligated animals treated with 100mg/kg doxycycline; 300 mg/kg fHP. Either doxycycline or fHP treatment was orally administered to the animals for 13 days. The animals were anaesthetised by peritoneal injection. Silk ligatures were applied to the necks of the first molars of the lower jaw and the second molars of

the upper jaw. The knots were located on the buccal and mesial surfaces. Animals were sacrificed on Day 14. Micro-CT was used to examine changes in the height of alveolar bones. Soft tissue were first removed to expose cemento-enamel junction (CEJ) The change in the height of the alveolar bone was determined by measuring the distance between the CEJ and alveolar bone crest.

RESULTS

Comparison of fermented and non-fermented herbal preparation for antibacterial activities against common oral pathogens:

The disc diffusion test was performed to compare antibacterial activity of the herbal preparations. Frozen powder of the herbal preparations including fermented herbal preparation (fHP), non-fermented herbal preparation (HP) and bacterial mass (BM) were extracted using DMSO, methanol and water. The mixture was subjected to antibacterial testing against seven common oral pathogens. As shown in Table 1, regardless of methods used for extraction, the HP demonstrated no antibacterial effects to the oral pathogens except for the water-extracted HP against Streptococcus sangui. Bacterial mass from the fermentation extracted by water and methanol showed a broad range of anti-bacterial activities. Overall, for fHP, methanol extraction is more superior to other two methods in producing an inhibition on the growth of oral pathogen. Thus, this is the preferred method of extraction for the rest of the experiments.

	DMSO			Methanol			Water		
	NB 7	NB 8	NB 9	NB 7	NB 8	NB 9	NB 7	NB 8	NB 9
A.a	ND	ND	ND	ND	ND	1.2	ND	ND	ND
P.g	ND	ND	ND	ND	ND	1.5	ND	ND	ND
S. m	ND	1.8	ND	ND	1.5	2.0	ND	1.2	1.2
S.s	ND	ND	ND	ND	2.4	2.4	1.1	1.6	1.8
A.v	ND	ND	ND	ND	ND	1.8	ND	ND	ND
P.i	ND	ND	ND	ND	2.0	ND	ND	2.0	2.4
F.n	ND	1.1	ND	ND	2.0	ND	ND	1.4	2.0

Table 1. Antimicrobial activity of HP, fHP and BM extracted by either DMSO, methanol and water

A.a, *Actinobacillus actinomycetemcomitans;* P. g, *Porphyromonas gingivalis*; S.m, *Streptococcus mutans*; S.s, *Streptococcus sangui*; A.v, *Actinomyces viscosus*; P. i, *Prevotella intermedia*; F. n, *Fusobacteriumnucleatum*.

HPLC analysis of the herbal preparations:

To distinguish the difference of major components contributing to the antibacterial effects in the herbal preparation, HPLC analysis was performed. By comparing HPLC fingerprints of HP, fHP and BM, the bioactive compounds were likely to appear between retention time of 20-25 minutes (Figure 1).

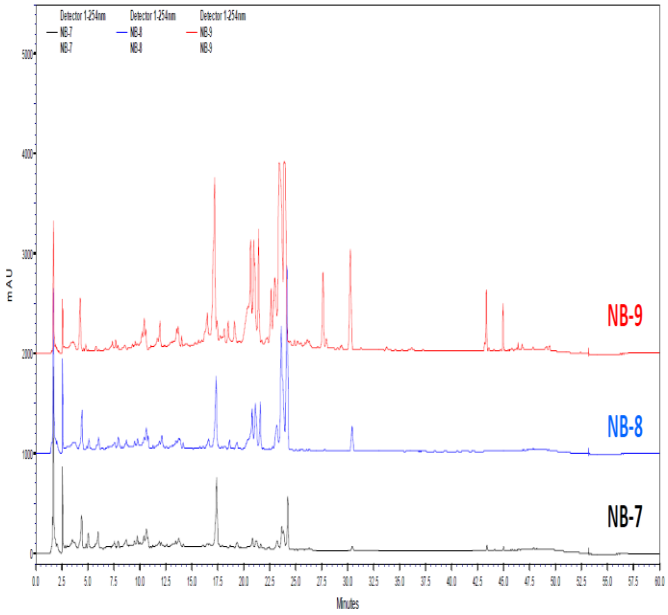

Figure 1. HPLC analysis of HP, fHP and BM. The bioactive compounds for antibacterial activities were predicted to be the peaks between retention time 20-25 minutes.
Abbreviations: NB-7, HP; NB-8, fHP; NB-9, BM.

Anti-bacterial activities of the HPLC fractions from the herbal preparation

200 mg of fHP were further fractionated by preparative HPLC using COSMOSIL MSⅡ C18 250×20mm column at 10mL/minute. Elution was performed with a linear gradient of acetonitrile in the presence of 0.1% trifluoroacetic acid at 1.5 min/tube. In total, four fractions were performed. In total, 115 crude extracts (CR) were obtained, which were then combined to 15 fractions. Each combined fraction was tested for the minimum inhibitory concentration (MIC) (data not shown). The oral pathogen Streptococcus mutanswas used as the first screen for the effective fractions. The final concentration of S. mutanswas 3-5 x 105CFU/mL. The fHP had a MIC greater than 1024 μg/mL, however the fractions H10, H11 and H12 had a MIC of 128-64μg/mL, 64-16μg/mL and 256 μg/mL. The fractions H8 to H14 were subjected to MIC analysis against seven periodontal disease pathogen including *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Prevotella intermedia, Fusobacterium nucleatum, Actinomyces viscosus, Streptococcus mutans* and *Streptococcuss anguis*. Among all the fractions, H11 displayed the highest anti-bacterial activity, especially for *Fusobacterium nucleatum, Prevotella intermedia, Actinomyces viscosus, Streptococcus mutans*, hence further analysis of the fraction H11 was performed (Table 2).

Fractions	*P. g.*	*A. a.*	*P. i.*	*F. n.*	*A. v.*	*S. m.*	*S. s.*
NB8 (fHP)	>512	>512	512	512	128	512-256	>512
H08	>512	>512	>512	>512	>512	>512	>512
H09	>512	>512	256	256	128	256	256
H10	256	256	32	32	16	32	32
H11	128	128	16	8-16	8	8-16	32
H12	>512	>512	>512	>512	>512	>512	>512
H13	>512	256	>512	>512	>512	>512	>512
H14	>512	>512	256	64	128-256	>512	128

Table 2. MIC results (mg/mL) of each fractions of hHP on oral pathogens
P.g,*Porphyromonas gingivalis*; A.a, *Actinobacillusa ctinomycetemcomitans*; P. i, *Prevotella intermedia*; F. n, *Fusobacterium nucleatum*; S.m, *Streptococcus mutans*; S.s, *Streptococcus sangui*; A.v, *Actinomyces viscosus*.

Identification of phytochemical markers of the fermented herbal preparation:

For further analysis of the active components of the fHP, 15g of solid mass concentrated from 500 mL of fHP was dissolved in water and extracted using methanol. The extract was subjected to solvent-solvent partition three times using ethyl acetate and n-Butanol. HPLC analysis of each organic layer showed that the butanol layer contained the highest content of candidate bioactive compounds (H10 and H11). The butanol layer of extracted fHP was then fractionated by preparative HPLC with the same procedures described in the previous section. The candidate H11 fraction with the highest wide-range bioactive activity yielded two pure compounds H11-1 and H11-2. The molecule weight of H11-1 and H11-2 was determined by GC-MS as 352 and 336, respectively. Using NMR and the results from GC-MS, H11-1 and H11-2 was identified as palmatine and berberine, respectively.

The anti-bacterial activity of these two pure compounds palmatine and berberine was assayed by the MIC assay. The two compounds displayed comparable anti-bacterial properties. However, the anti-bacterial activities of these two compounds were not superior to the bioactive fraction.

	P. g.	*A. a.*	*P. i.*	*F. n.*	*A. v.*	*S. m.*
H11-1	128	128	256	32	32	128
H11-2	256	128	64	128	32	64

Table 3. MIC results of pure compounds H11-1 and H11-2
P.g, *Porphyromonas gingivalis*; A.a, *Actinobacillus actinomycetemcomitans*; P.i, *Prevotella intermedia*; F.n, *Fusobacterium nucleatum*; S.m, *Streptococcus mutans*; S.s, *Streptococcus sangui*; A.v, *Actinomyces viscosus*.

Animal models of periodontal diseases:

The periodontal disease in rats was induced by ligature-induced model. In these rats, the micro-CT scans were taken to generate lingual and buccal views. From both lingual and buccal views, alveolar bone loss was most severe in the untreated ligature groups. Doxycycline (100mg/kg) treated groups served as positive controls in which the boss loss was ameliorated. However, the reduction in the alveolar bone loss was observed in the groups treated with fHP (Figure 2).

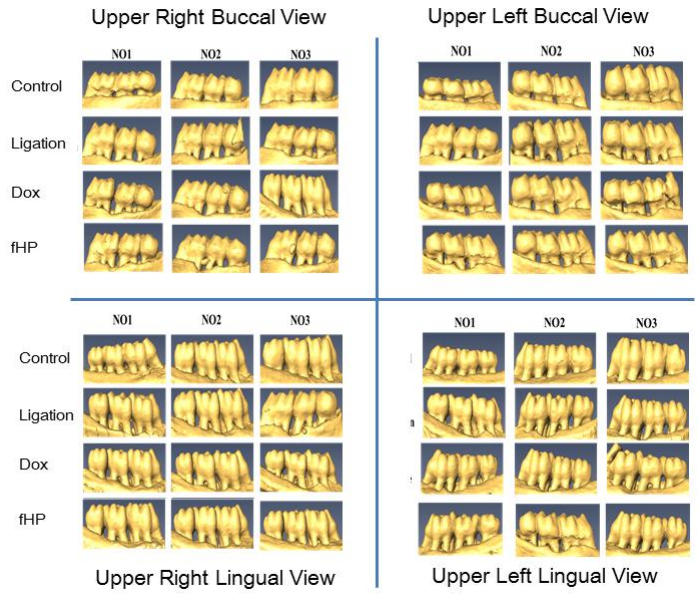

Figure 2. fHP treatment demonstrated the similar effect on preventing alveolar bone loss in the rat model of ligature-induce periodontitis. Three representative images were shown for each group. Micro-CT scans were taken from different views indicated in the figure.

DISCUSSION

Bacteria are one of the primary etiologic agents of periodontal disease. The use of antibacterial oral care is particularly important in preventing the onset and development of periodontal disease. The current therapy including antibiotics and cholorhexadine are effective, however when used long term bacterial resistance and side effects are inevitable. This warrants research on discovering novel therapeutic compounds. To this end, herbal plants may provide a good resource pool for such development. Apart from their conventional uses, fermentation can enhance the effects of the herbal plants.A fermentation process has been traditionally used in food industry for improving sensory characteristics and for preserving nutritional properties[21]. Lactic acid bacteria (LAB) such as Lactobacilli and Bifidobacteria are two common species widely used in such process. Fermentation with LAB can induce modifications of naturally occurring compounds in the plants[21]. For example, the fermentation can convert glycosylated isoflavones into aglycones in soy due to the beta-glycosidase activity of the LAB[22]. Production of secondary metabolites, removal of undesirable compounds and enhancement

of bioactive compounds are also features of LAB fermentation[21,22].

In this study, we demonstrated that fermentation of a herbal preparation by *Lactobacillus reuteri* LR107 enhanced the anti-microbial effects of a herbal preparation. We have chosen seven common oral pathogens as our target micro-organisms. First, we showed that in the via disc diffusion assay, fermentation effectively enhanced the anti-bacterial effects of HP. Interestingly, the bacterial biomass also had similar effects, which may also imply that anti-bacterial effects can be attributed to the presence of *Lactobacillus reuteri* LR107 and/or the process of fermentation. Next, fractions from the hHP were screened for anti-bacterial effects and one fraction was particular effective. The bioactive components in the fraction were identified to be palmatine and berberine. Surprisingly, MIC of palmatine or berberine alone was not superior to the fraction from which both compounds were identified. The reason for this is unclear, and there may be some synergistic actions of these two compounds.

Alkaloids berberine and palmatine have been known for their anti-cancer and anti-microbial effects. A previous study showed that the extract of *Coptidis rhizoma* exhibited a MIC of 31-250μg/mL against *Actinomyces naeslundii*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Actinobacillus actinomycetemcomitans*[16]. A less effective MIC (500-2,000μg/mL) against Streptococcus and Lactobacillus spp. Berberine was identified as the major active component of *C. rhizome* extract. In this study the fHP exhibited MIC of (8-128μg/mL) against *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Actinomyces viscosus*, *Streptococcus mutans* and *Streptococcus sanguis*. fHP demonstrated a higher anti-bacterial potency to Streptococcus spp. compared to the *C. rhizome* extract. This discrepancy was possibly attributed to multiple plant species used and the process of fermentation. Hwangryunhaedok-tang containing four herbs *Coptis japonica, Phellodendron amurense*, *Scutellaria baicalensis* and *Schisandra chinensis* was fermented with *Lactobacillus acidophilus* KFRI 128 [19]. The amounts of palmatine were reduced whereas the content of berberine was increased. The net effects were enhanced neuroprotective properties.

Results from our ligature-induced periodontal disease model indicated that the fHP effectively prevented bone loss, and such effects were comparable to doxycycline. Apart from its anti-bacterial effects, berberinehas been shown to prevent degradation of extracellular matrix by metalloproteinase (MMPs). Over the course of periodontitis, tissue destruction is a result of chronic inflammation mediated by MMPs. Tissue degradation in rats with ligature-induced periodontitis was attenuated in rats fed with berberine, which was in concordance with our data. The active fraction of the fHP was shown to be more effective than berberine or palmatine alone. It will be interesting to investigate that weather synergistic actions of these two and potential uses for treating periodontal disease.

The study is the first to demonstrate the anti-bacterial effects of LAB fermentation on herbal preparation for applications in therapeutic treatment of periodontal diseases. Though we identified berberine and palmatine as major bioactive compounds in this preparation, further studies are required to identify other phytochemical compounds which may also contribute to the antibacterial activity as berberine and palmatine alone was not as effective as the fermented herbal preparation. Clinical effect of the fermented herbal preparation on the control of periodontal diseases can also be addressed in a randomized and double blind trial.

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