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Original Article

Effects of systemic *Bifidobacterium longum* and *Lactobacillus rhamnosus* probiotics on the ligature-induced periodontitis in rat

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KEYWORDS
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Abstract *Background/purpose:* Probiotics might be beneficial in preventing periodontitis. Effects of Bifidobacterium and Lactobacillus on periodontitis were examined using the ligature-induced rat model.

Materials and methods: Thirty-five male Sprague-Dawley rats were divided into control, ligation, *Bifidobacterium longum* (BL986), *Lactobacillus rhamnosus* (LRH09), and combination groups. Periodontitis was induced in maxillary second molars. From the day before ligation, phosphate-buffered saline (for control and ligation groups) or probiotics (2×10^9 CFU/g for probiotic groups) were fed daily. On day 8, gingival mRNA expressions for interleukin (IL)-1β, IL-6, tissue necrosis factor (TNF)-α, IL-10, and NF-κB were determined *via* qPCR. Micro-computed tomography (μCT) and histomorphometry were employed to examine periodontal destruction.

Results: Compared to the ligation group, mRNA of IL-1β, TNF-α, IL-6, and NF-κB in probiotic groups were significantly decreased, but IL-10 was increased. Besides, the IL-10 was more significant in the combination group than in single-use group. Through μCT, the cementoenamel junction (CEJ)-to-bone distance and trabecular separation in combination group were less than that in ligation group, although the bone volume fraction and trabecular number/thickness showed an increase in three probiotic groups. Histopathologically, the combination group had significantly smaller gingival inflammatory cell-infiltrated area and CEJ-to-epithelium

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distance than the ligation group and the group with BL986 or LRH09. Additionally, the CEJ-to-bone distance was significantly smaller in the combination group than in the ligation and BL986 groups.
Conclusion: Systemic combination of BL986 and LRH09 had a synergistic effect on enhancing IL-10 and ameliorating the induced experimental periodontitis, although the single-use still presented partially alleviative effects.

Introduction

Periodontitis, the destruction of tooth-supporting tissue, is a complex and multifactorial infectious inflammatory disease involving interactions between bacteria, inflammatory responses, host immunity, genetics, and environmental factors.[1,2] Therefore, in addition to anti-infection control, host response modulation might be the other alternative approach. For instance, to control multidrug-resistant tuberculosis, host-directed therapy, aimed at improving innate or adaptive protective immune response to control pathogens and/or limit immunopathology, was proposed.[3] Probiotics, another example of host-directed therapy, can replace pathogens with good bacteria by stimulating the host's innate and acquired immunity and directly modifying the resident oral microbiome.[4,5]

The most commonly used probiotics are *Bifidobacterium* and *Lactobacillus* species.[6,7] *Bifidobacterium longum* (*B. longum*), existing in the human gastrointestinal tract, is recognized as a probiotic for immune regulation. The effects of immune regulation were observed for *B. longum* owing to its ability to accelerate recovery from allergen-induced pneumonia through the production of IL-10 in mice.[8] In the animal model, a mixture of probiotic strains of *B. longum* and *B. bifidum* was the most effective in preventing death and necrotizing enterocolitis.[9] Another most studied probiotics is *Lactobacillus rhamnosus* (*L. rhamnosus*), which exist in the gastrointestinal tract. It is capable of reducing inflammatory cytokines.[7,10] Clinical reviews showed the benefits of *L. rhamnosus*, such as relief of various types of diarrhea and treatment of relapsing *Clostridium difficile* colitis.[7,11]

Probiotics have been advocated as useful adjuvants for reducing dental caries,[12] inhibiting oral *Candida* infections,[12] eliminating halitosis,[13] and improving children's oral health by significantly reducing plaque and gingival scores using mouthwash containing probiotics.[4] Recently, the systemic administration of *L. rhamnosus* was found to reduce the severity of apical periodontitis in animal experiments.[14] Furthermore, the efficacy of *Lactobacillus*- and *Bifidobacterium*-based probiotics as adjuncts to periodontal treatment has been investigated in human clinical trials. For example, the *L. reuteri* probiotic application was shown to reduce clinical periodontal parameters, including plaque and gingival indices, bleeding on probing, and probing depth.[15] *B. lactis* and *L. kefiri* could reduce periodontal destruction and regulate proinflammatory/anti-inflammatory cytokines.[16,17] The clinical benefits of probiotics can be obtained mainly by suppressing the NF-κB pathways and promoting T regulatory cell accumulation as do nutritional supplements, such as n-3 fatty acids.[18] However, some of the randomized controlled trials did not exhibit the clinical benefits of probiotics as adjuncts to scaling and root planing with a follow up of 6 months.[19] It was observed in an *in vitro* experiment that a mixture of *Streptococcus* probiotics inhibited the growth of periodontal disease pathogens better than a single strain.[20] Investigators also believed that mixtures of different strains may be more effective than a single strain due to the different metabolic characteristics and immunomodulatory activities of various probiotics[21] and a potential synergistic effect on host modulation.[22] Therefore, we aimed to investigate the effects of the systemic delivery of *B. longum* (strain No. BL986) and/or *L. rhamnosus* (strain No. LRH09) on the gingival pro-/anti-inflammatory cytokine expressions and periodontal destruction in a ligation-induced periodontitis rat model.

Materials and methods

Experimental design and induction of experimental periodontitis

A total of 35 8-week-old male Sprague—Dawley rats (BioLASCO, Taipei, Taiwan) weighing 290 ± 20 g were observed for at least 1 week before the study. They received water and standard rat chow pellets *ad libitum* under a 12-h light/dark cycle. The management and experimental procedures were approved by the Laboratory Animal Center of National Defense Medical Center (IACUC-21-084). The rats were randomly allocated to five groups (each with 7 rats): control (phosphate-buffered saline, PBS), ligation, BL986, LRH09, and BL986 plus LRH09 (BL + LRH) (SYNBIO TECH INC., Kaohsiung, Taiwan) (Fig. 1). In the ligation, BL986, LRH09, and BL + LRH groups, periodontitis was induced after placing a 3-O braided silk ligation around the cervical region of the bilateral maxillary second molars. BL986 (2×10^9 CFU/g in 1-mL PBS), LRH09 (2×10^9 CFU/g), and BL + LRH (1×10^9 CFU/g for each probiotic) were administered *via* oral gavage one day before ligation (day 0), once a day for 8 consecutive days. The rats in the control and ligation groups were administered PBS alone. On day 8, the animals were sacrificed *via* excessive carbon dioxide inhalation. The gingivae on the palatal surfaces of the secondary molars were harvested for messenger (m)RNA analysis

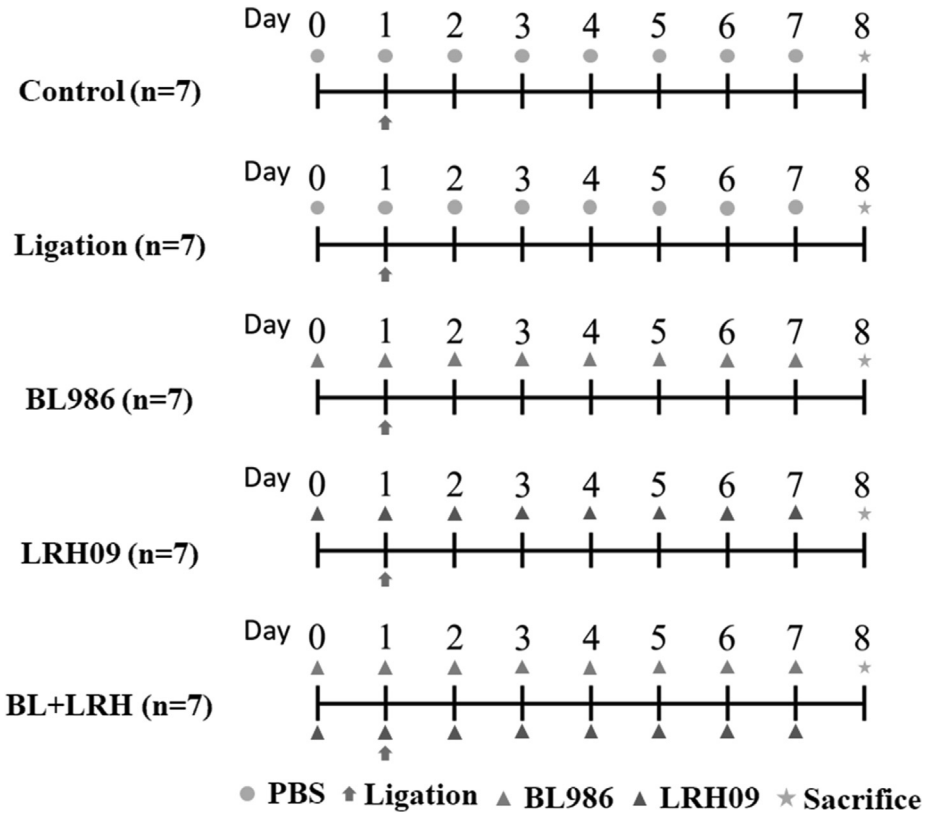

Figure 1 Experimental design. Thirty-five rats were divided into five groups: control, ligation, BL986, LRH09, and combined (BL + LRH) groups. After placing a 3-O ligation around the tooth neck of the maxillary molars, experimental periodontitis was induced in the ligation, BL986, LRH09, and BL + LRH rats. Normal saline (PBS) or the probiotic(s) was administered *via* oral gavage for 8 days 1 day before ligation. All animals were sacrificed on day 8.

via quantitative real-time polymerase chain reaction (qPCR). The remaining maxillary block, including the interdental papillary soft tissues, were fixed in 10% neutral buffered formalin for micro-computed tomography (μCT) and histological analysis.

Quantitative real-time PCR

To investigate the impacts of probiotics on pro-/anti-inflammatory cytokines and transcription factors (including TNF-α, IL-1β, IL-6, IL-10, and NF-κB) in the ligature-induced experimental periodontitis in rats, silk-surrounded gingival tissues were harvested and stored at −80 °C in a refrigerator. Total RNA was extracted using a commercially available reagent (MACHEREY-NAGEL, Düren, Germany). After defrosting, the gingivae were placed into a homogenizing tube with 3 (2.8 mm) and 24 (1.4 mm) bulk beads with lysis buffer and then ground into high-throughput tissues using Precellys homogenizers (Template: 5000-1*20-005) (Genetech Biotech Co., Taipei, Taiwan). The homogenized solution was centrifuged at 12,000 rpm, 4 °C, for 15 min. RNA extraction was performed according to the NucleoSpin® RNA Kit protocol (MACHEREY-NAGEL). mRNA quantification was determined by calibrating with RNase-free H_2O, 2 μL of mRNA was taken and placed in the spectrophotometer for measurement, and the mRNA concentration was measured. Total RNA (1 μg) was reverse-transcribed into cDNA using the RevertAid RT Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to its protocols and then used as the template for PCR reaction and analysis.

In the real-time qPCR, the Rotor-Gene® SYBR® Green PCR Kit (QIAGEN, Hilden, Germany) was used according to its protocol. About 7.5-μL SYBR® Green, 1-μL forward primer, 1-μL reverse primer, 3.5-μL H_2O, and 2-μL cDNA were added to each four-row PCR reaction tube and involved in an initial denaturation at 95 °C for 12 min; following by 45 cycles of denaturing at 95 °C for 20 s and combined annealing at 60 °C for 60 s, as per the manufacturer's instructions. The PCR primer sequences were as follows: IL-1β forward 5′-CACCTCTCAAGCAGAGCACAG-3′ and reverse 5′GGGTTCCATGGTGAAGTCAAC-3′; TNF-α forward 5′-AAATGGGCTCCCTCTCATCAGTTC-3′ and reverse 5′-TCTGCTTGGTGGTTTGCTACGAC-3′; IL-6 forward 5′-AGAAAAGAGTTGTGCAATGGCA-3′ and reverse 5′-GGCAAATTTCCTGGTTATATCC-3′; IL-10 forward 5′-GCAGGACTTTAAGGGTTACTTGG-3′ and reverse 5′-GGGGAGAAATCGATGACAGC-3′; and NF-κB forward 5′-GTGCAGAAAGAAGACATTGAGGTG-3′ and reverse 5′-AGGCTAGGGTCAGCGTATGG -3′. Relative gene expression (normalized to reference gene, β-actin forward 5′-GATATCGCTGCGCTCGTC-3′ and reverse 5′-TGGGGTACTTCAGGGTCAGG-3′ was calculated using the $2^{-\Delta\Delta CT}$ method.

Micro-computed tomography analysis

All maxillary block biopsies were scanned using a μCT system (Skyscan1076 micro-CT SYSTEM; SkyScan, Aartselaar, Belgium). The μCT parameters were set as follows: 18-μm image pixel size; 60-kV voltage, and 200-μA beam current. Three-dimensional (3D) images were generated for each specimen using CTAn (Bruker Micro-CT Software, USA) according to the manufacturer's protocol. Alveolar bone level was recorded from the cementoenamel junction (CEJ) to the alveolar bone crest (CEJ-to-bone distance) with the average of the mesial and distal sides of the second molar. Furthermore, bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, /mm), and trabecular separation (Tb.Sp, mm) in the coronal area between the first and third molars, selected as the region of interest ($3 \times 3 \times 1.5$ mm^3 apically down to CEJ), were measured as previously described.[23]

Histology and histometry

The specimens (including interdental gingivae, teeth, and alveolar bones) around the molars were dissected, fixed in 10% buffered neutral formalin for 48 h, and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 4 weeks. Each sample was paraffin-embedded and sliced into 5-μm-thick sections in mesio-distal directions. The sections were mounted on glass slides and stained with hematoxylin and eosin (H&E). Under the microscope at 40 × and 200 × magnifications, the CEJ-to-bone distance and that of the CEJ to the coronal level of junctional epithelium (CEJ-to-epithelium distance) were recorded to represent alveolar bone and attachment losses, respectively. In the subepithelial gingiva, the region of inflammatory cell-infiltrated connective tissue (ICT, %) was further measured as previously described to represent the inflammatory status of the local tissue.[24]

Statistical analysis

Data were expressed as mean and standard error and analyzed using SPSS ver.22.0 (SPSS, Chicago, IL, USA). The differences in mRNA expressions and bone parameters between the groups were analyzed *via* ANOVA with Scheffe's *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

The gene expressions for pro-/anti-inflammatory cytokines and NF-κB

Compared with the controls, the gingival mRNA expressions for proinflammatory cytokines (IL-1β, TNF-α, and IL-6) and NF-κB in the ligation group were significantly increased but significantly decreased for anti-inflammatory IL-10 (Fig. 2A–E). Compared with the ligation group, the probiotic groups (e.g., BL986, LRH09, and BL + LRH) had significantly lower pro-inflammatory cytokine and NF-κB expressions but greater IL-10. Furthermore, the IL-10 expression in the BL + LRH group was significantly greater than that in the group with single use of BL986 or LRH09 (Fig. 2D).

Dental alveolar bone destruction examined *via* micro-computed tomography

The dental alveolar bone morphology around three maxillary molars could be clearly observed *via* μCT (Fig. 3A). The CEJ-to-bone distance in the ligation group was significantly longer than that in the control group (Fig. 3B). Compared with the ligation group, the mean distances in the three probiotic groups were less; however, statistical significance was observed only for the BL + LRH group. Furthermore, the BL + LRH group had a significantly smaller CEJ-to-bone distance than the individual probiotic group of BL986.

BV/TV, Tb.Th, and Tb.N significantly decreased in the ligation group (vs the control group) (Fig. 4). However, in the probiotic groups (BL986, LRH09, and BL + LRH), these bone characteristics exhibited significantly greater than that in the ligation group. Regarding Tb.Sp, it increased in the ligation group compared with the control group. However, the BL + LRH group had a significantly lower Tb.Sp than the ligation group but also the group with single use of BL986 or LRH09 (Fig. 4B).

Periodontal tissue destruction examined *via* histology

Fig. 5A presents the histological pictures of the gingivae taken from five animal groups. Through histometry, the ICT areas were found to significantly increase in four animal groups that underwent ligation compared with the non-ligated controls (Fig. 5A). However, the increased ICT areas were significantly alleviated in the three probiotics groups compared with the ligation group. Furthermore, the BL + LRH group had a significantly smaller area than the group with single use of BL986 or LRH09 (Fig. 5B). Similar results were replicated in the CEJ-to-epithelium distance (representing periodontal attachment loss). Nevertheless, the histological bone level of the CEJ-to-bone distance was significantly lower in the BL + LRH group than the ligation and BL986 groups but not the LRH09 group (Fig. 5B).

Discussion

Probiotics are live microorganisms administered in adequate amounts and confer a health benefit to the host. Owing to their ability to safely suppress proinflammatory cytokines and exert anti-inflammatory effects,[25] the use of probiotics has been considered an alternative approach to controlling microbial dysbiotic conditions, including periodontitis.[26] Despite the use of probiotics, which seems to promote healthy subgingival microbiome and control periodontitis,[27] systemic probiotics combined with subgingival instrumentation did not provide additional benefits in randomized controlled trials.[26] Although the exact reason is still unknown, different probiotic strains have different mechanisms of action against pathogens. The efficacy of

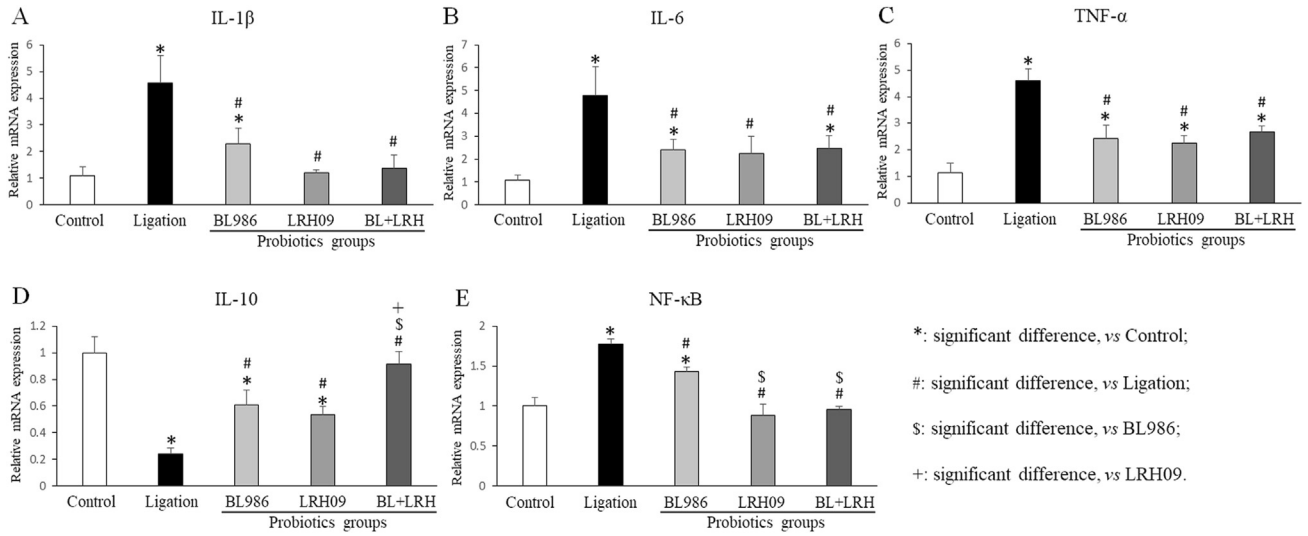

Figure 2 Pro-inflammatory and anti-inflammatory gene expressions in different rat groups. The mRNA expressions of IL-1β, TNF-α, IL-6, and NF-κB are shown in A, B, C, and E, respectively, whereas that of IL-10 is presented in D (data were analyzed *via* one-way ANOVA with *post hoc* Scheffe test when $P < 0.05$ was obtained and were expressed as mean ± SEM).

probiotics was also found to be both strain- and disease-specific.[28]

Bifidobacterium and *Lactobacillus* were selected in this study as they are the most effective species for improving immune response.[7,8] Our results showed the systemic administration of each of the two probiotics could reduce proinflammatory cytokines and NF-κB but enhance anti-inflammatory IL-10 in gingiva. Besides, the administration could also reduce connective tissue inflammation and attachment loss, but improve bone quality. However, compared with single usage, the combination of the two probiotics further enhanced anti-inflammatory IL-10. Furthermore, without an increase in probiotic contents (2×10^9 CFU/g in 1-mL PBS for the BL986 or LRH09 group and 1×10^9 CFU/g of BL986 and 1×10^9 CFU/g of LRH09 for the BL + LRH group), the induced tissue inflammation, attachment loss, and bone loss (both in μCT and histological observations) could be further reduced, whereas the width of the attached connective tissue was maintained as that in control animals. Synergic effect of *B. longum* BL986 and *L. rhamnosus* LRH09 on the induced experimental periodontitis in rats was therefore suggested.

Currently, similar finding that probiotic of *L. helveticus* SBT2171 attenuated gingival TNF-α, IL-1β, and IL-6 expressions in *Aggregatibacter actinomycetemcomitans* induced periodontitis model.[29] Studies have also shown that *Lactobacillus* is a specific and effective probiotic capable of inhibiting immune inflammatory responses by producing

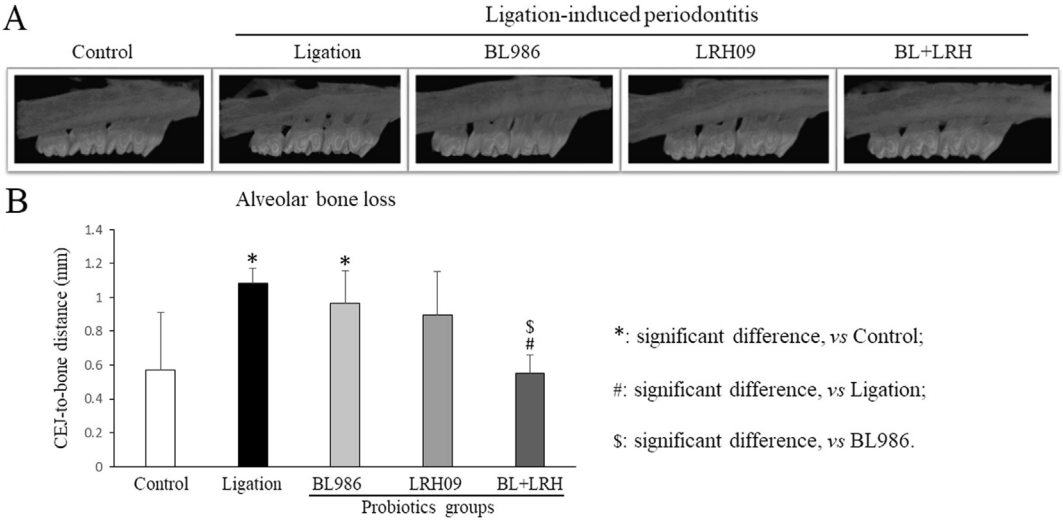

Figure 3 Losses of dental alveolar bone crest among the animal groups examined *via* μCT. Images show the dental alveolar bone morphology around the molars on the right maxillae, the palatal views (A). Comparison of the CEJ-to-bone distances among the five animal groups (B) (data were analyzed *via* one-way ANOVA with *post hoc* Scheffe test when $P < 0.05$ was obtained and were expressed as mean ± SEM).

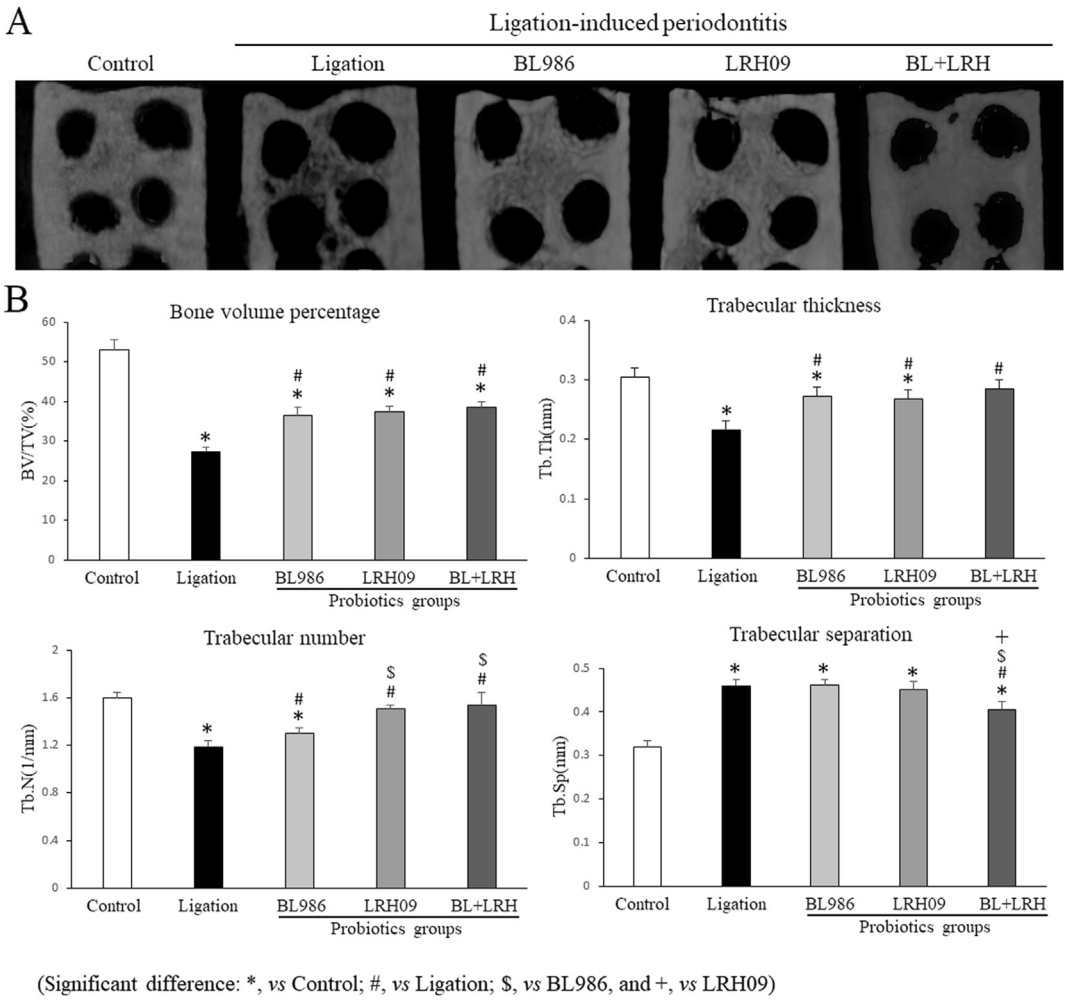

Figure 4 Dental alveolar bone qualities in five animal groups examined *via* μCT. The μCT images show the bone morphology around the maxillary second molars in the five animal groups (A). The plots present the comparisons of the bone qualities among the five animal groups (B) (bone volume fraction: BV/TV, %; trabecular thickness: Tb.Th, mm; trabecular number: Tb.N, 1/mm; and trabecular separation: Tb.Sp, mm) (data were analyzed *via* one-way ANOVA with *post hoc* Scheffe test when $P < 0.05$ was obtained and were expressed as mean ± SEM).

bacteriocins,[30] upregulating the β-defensin expression in the epithelial cells,[31] and increasing the TIMP-1 levels and reducing the MMP-8 levels in the gingival crevicular fluid.[32,33] In addition, the synergic effect of the use of *Bacteroides uniformis* FGDLZ48B1 and *B. adolescentis* FHNFQ48M5 in combination was observed in alleviating the pathological features of the antibiotic-associated diarrhea,[34] mainly through IL-6 concentration downregulation but occludin expression upregulation. Moreover, *L. plantarum* NK3 and *B. longum* NK49 may simultaneously alleviate osteoporosis by suppressing NF-κB-linked TNF-α expression through the regulation of the gut microbiota population in ovariectomy-induced osteoporosis study.[35] Therefore, the combination of different probiotics may be a valuable protocol to increase the efficacy of alleviating the periodontal inflammation and destruction.

The study by Yinhua Ni et al. demonstrated that *L. casei* LC122 and *B. longum* BL986 have anti-aging potentials, which improve learning and memory, enhance muscle function, and reduce inflammation and oxidative stress in the peripheral tissues. These anti-aging effects might be related to the alteration of the composition and function of the gut microbiota.[36] Another study of immobilization stress-induced anxiety-like and depressive behaviors demonstrated that oral administration of L. mucosae NK41 and B. longum NK46, or both mixtures, significantly alleviated the induced anxiety/depression, NF-κB activation, and gut dysbiosis.[37] Also, the surface of intestinal epithelial cells has many villi and receptors, such as Toll-like and NOD-like receptors.[38] Probiotics generally compete with pathogens and colonize and multiply by adhering to the intestinal wall *via* oral administration.[39] For example, *L. plantarum* prevents pathogens from adhering to epithelial surfaces and lipopolysaccharides from triggering Toll-like and NOD-like receptors to reduce mucosal innate immunity hyperactivation caused by inflammation.[40] The effects of probiotics on systemic immune modulation and results of periodontal improvement obtained in our study indicated that the interaction between the gut microbiota and intestinal systems could be an essentially scientific inquiry.[36]

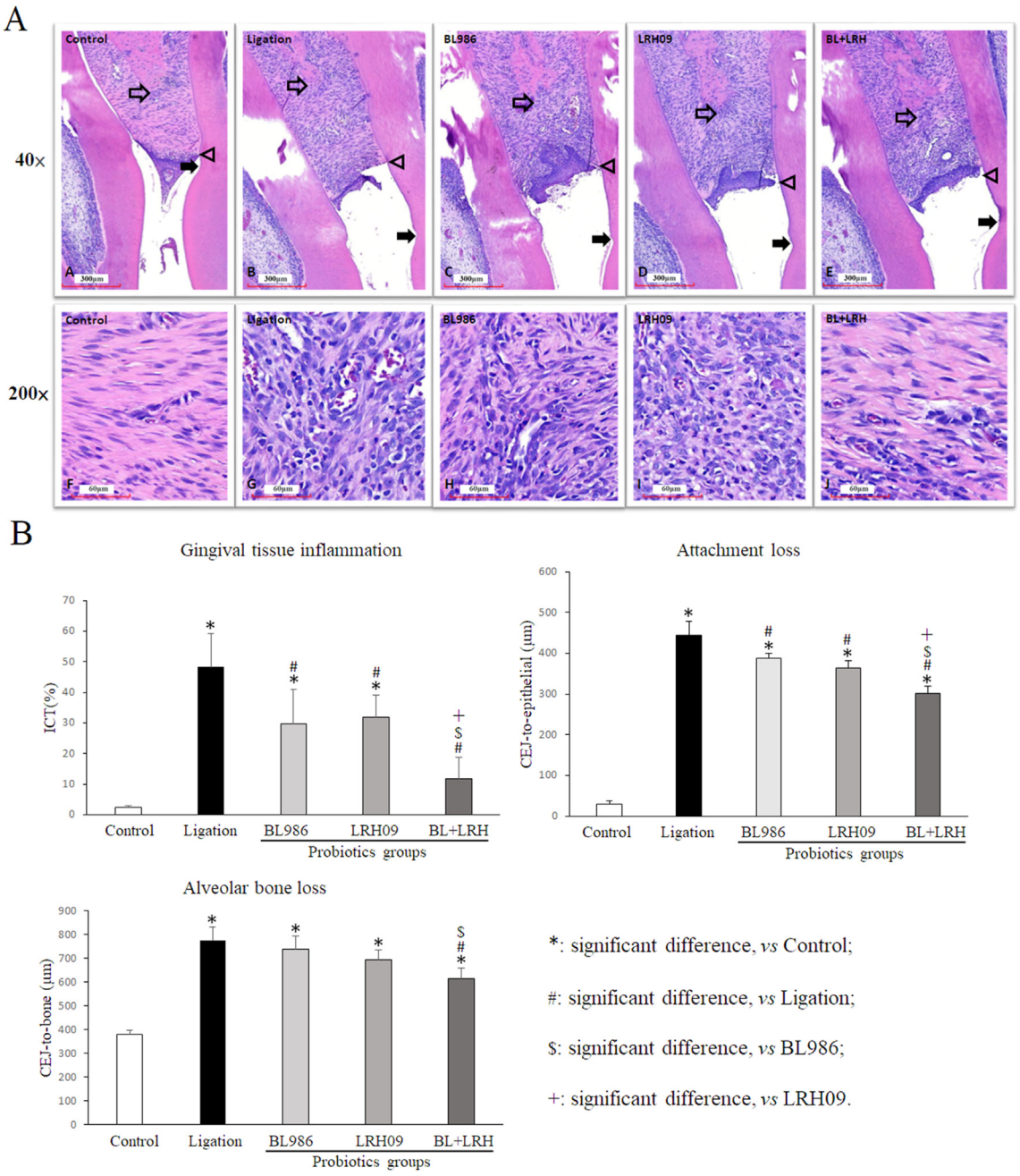

Figure 5 Periodontal tissue destructions among the five animal groups examined *via* histology. The histological images present the interdental tissues between the first and second maxillary molars of the 5 animal groups, H&E stain (A). The plots present the comparisons of the histometric measurements among the animal groups, including gingival tissue inflammation (ICT area, %), alveolar bone loss (CEJ-to-bone distance), and periodontal attachment loss (CEJ-to-epithelium distance) (B) (CEJ, black arrow; alveolar bone crest, empty arrow; junctional epithelium, arrow head) (data were analyzed *via* one-way ANOVA with *post hoc* Scheffe test when $P < 0.05$ was obtained and were expressed as mean ± SEM).

Nevertheless, further detailed study on the molecular biomechanisms related to prevention of periodontal inflammation and destruction is still needed to evaluate the long-term efficacy, safety, and modulation of probiotics on intestinal systems.

In conclusion, we found that the individual and combined use of BL986 and LRH09 attenuated the ligation-enhanced IL-1β, IL-6, TNF-α, and NF-κB expressions but enhanced anti-inflammatory IL-10 inhibition. Among the three probiotic groups, IL-10 enhancement was greater in the group with combined use than that with single use of probiotics. Through μCT and histology, the combined use was found to further reduce the inflammatory cell-infiltrated area in the gingiva and the CEJ-to-bone and CEJ-to-epithelium distances compared with the single use; however, a statically significant difference in the CEJ-to-

bone distances between the BL + LRH and LRH09 groups was observed. Therefore, a synergic effect of the systemically combined use of the two probiotics of BL986 and LRH09 enhances the anti-inflammatory IL-10 and reduces periodontal inflammation, attachment loss, and alveolar bone destruction was suggested.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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