Orally Ingested *Lactobacillus crispatus* KT-11 Inhibits *Porphyromonas gingivalis*-infected Alveolar Bone Resorption

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Abstract

Lactobacillus crispatus KT-11, which was originally isolated from the feces of healthy infants, has been reported to show multiple immunoregulatory effects. However, there have been no reports about the effect of the *Lactobacillus* on periodontal disease, a chronic destructive inflammatory disease of the tissues supporting the teeth. We used a model of periodontal disease in which mice were infected with *Porphyromonas gingivalis*. As we report here, oral ingestion of KT-11 exerted inhibitory effects on alveolar bone resorption in this model, suggesting potential preventive activity of the *Lactobacillus* in periodontal disease.

Mice were given free access to feed containing dead *Lb. crispatus* KT-11 over 6 weeks, and were then orally infected with *P. gingivalis* 10 times for 2 weeks from 4 weeks later of *Lb. crispatus* KT-11 administration. The results showed a marked attenuation of alveolar bone resorption in mice that ingested the *Lb. crispatus* KT-11-containing feed. Induction of total IgG in plasma and total secretory IgA in saliva was observed. A specific plasma IgG antibody response to *P. gingivalis* also was induced.

Based on the immune response to *P. gingivalis* infection induced by ingestion of *Lb. crispatus* KT-11, this study suggests that oral administration of *Lb. crispatus* KT-11 is effective in preventing chronic periodontitis.

Keywords :

Lactobacillus crispatus KT-11;
periodontal disease;
lactobacilli; specific antibody

Introduction

In recent years, there have been numerous reports on periodontal and systemic diseases, mainly regarding the association between periodontal disease and diabetes, cardiovascular disease, and respiratory infection. Thus, prevention of periodontal disease is a focus of attention not only for oral health, but also as a means of maintaining general health(1-4). Conventionally, tooth brushing and interdental cleaning, such as the use of dental floss or interdental brushes, has been recommended for periodontal disease prevention. In Japan, the proportion of people who brush their teeth 2 or more times per day is over seventy

percent, and tooth brushing is thought to be a well-established habit(5). In contrast, the proportion of people who use interdental cleaning instruments is only about forty percent(5). Currently, there is a rising trend in the percentage of people with periodontal disease, with a gradual increase reported from the age of 20 years to more than 40% in those aged 55 years or more(5).

In addition to conventional physical plaque removal, internal medicine treatment using systemic or local administration of antimicrobial agents or antibacterial gargles also is performed in periodontics with the purpose of eliminating periodontal disease-associated bacteria(6-8). However, this approach includes problems in terms of adverse effects from antibacterial drugs and the emergence of drug-resistant bacteria. Moreover, antibacterial drugs act

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not only on harmful bacteria, but also on beneficial indigenous bacteria and are reported to disrupt the bacterial balance in the human body(9). As an alternative to antibacterial drugs, probiotics have been examined, including the use of microorganisms such as lactobacilli and bifidobacteria that act beneficially by improving the balance of the intestinal flora(10, 11). These agents have been approved as foods for specified health uses, are considered generally safe, and often are a component of fermented foods; the widespread use of such agents is a promising strategy for the maintenance and promotion of human health. In the field of dentistry, there also have been reports on the inhibition of periodontal disease bacteria in human subgingival plaque and saliva by the oral ingestion of lactobacillus pills and sugarless gum(12, 13).

Lactobacilli produce antimicrobial substances such as organic acids, hydrogen peroxide, and bacteriocin; lactic acid, the final metabolic product in fermentation, is known to serve as a major antibacterial substance. Lactobacilli are thought to produce an antibacterial effect against periodontal disease bacteria. Reports on the direct microbicidal effects of lactobacilli on periodontal disease and clinical cases are occasionally observed, but there have been few reports on periodontal disease prevention via oral immune response effects(14, 15). *Lactobacillus (Lb.) crispatus* KT-11 is a lactobacillus strain belonging to the *Lb. acidophilus* group. KT-11 has been reported to strongly induce proliferation of IFN- γ ⁺CD4⁺ cells in mouse spleen cell cultures(16). *Lb. crispatus* KT-11 also has been shown to be effective in improving atopic dermatitis (16) and reducing allergic rhinitis(17). The strength of the immunoregulatory activity of lactobacilli is reported to depend not on the species but on the strain, and the immunoregulatory activity of *Lb. crispatus* KT-11 is thought to derive from the *Lb. crispatus* KT-11 strain itself, not the *Lb. acidophilus* group or *Lb. crispatus* species. Given this background, there are also strong expectations for the immune response effects of *Lb. crispatus* KT-11 in the oral cavity.

Increases in IgA and IgG antibodies specific to the influenza virus were reportedly seen in bronchoalveolar lavage fluid of mice infected with the influenza virus who had been given *Lb. delbrueckii* ssp. *bulgaricus* OLL1073R-1; this antibody induction correlated with prolonged survival periods(18). This effect is thought to be due to the induced production of specific IgA species in antibody-secreting cells in the airway mucosa, as activated lymphocytes from

intestinal Peyer's patch cells move to and establish themselves in nasopharyngeal tissue as a result of the mice ingesting lactobacilli(18). Periodontal disease is a chronic inflammatory condition in which the acquired immune response from highly specific immune memory is thought to play a more important role than the innate immune response.

This study employed a murine model of periodontal disease to investigate the potential role of the oral immune response to *Lb. crispatus* KT-11 in preventing periodontal disease.

Materials and Methods

Lb. crispatus KT-11 preparation

The *Lb. crispatus* KT-11 strain was obtained from Kitii Co., Ltd. (Tokyo, Japan). *Lb. crispatus* KT-11 was propagated as a static (i.e., without shaking) culture for 24 hours at 37°C in MRS (De Man, Rogosa, and Sharpe) liquid culture medium, after which the bacteria were washed 3 times with phosphate-buffered saline (PBS), heat treated for 15 minutes at 110°C, and then freeze-dried for use in experiments.

General animal methods

This study was carried out with optimal concern for experimental animals, in accordance with the standard practices for feeding, maintaining, and minimizing the distress of experimental animals (Ministry of the Environment Notification No. 88, April 28, 2006) and the animal experiment guidelines of the Nihon University School of Dentistry at Matsudo. This study was approved by the Nihon University School of Dentistry at Matsudo Animal Experiment Committee (AP 12 MD 024).

Female, specific pathogen-free BALB/c mice were purchased from Sankyo Labo (Tokyo, Japan) and were 7 to 8 weeks old at study start. Mice were maintained in a specific pathogen-free environment in the animal experiment facility of the Nihon University School of Dentistry at Matsudo. Throughout the experimental period, animals were provided with free access to food (as described below) and water.

Test diet

The standard chow consisted of sterilized, powdered mouse food MF (ORIENTAL YEAST CO., LTD., Tokyo, Japan). The chow+KT-11 consisted of the same sterilized, powdered mouse food supplemented with *Lb. crispatus* KT-

11 at 5×10^7 /g.

Animal model

Animals of Groups 1-4 were initiated on chow as follows: Groups 1 (n=7) and 2 (n=3), feed+KT-11; Groups 3 (n=3) and 4 (n=3), unsupplemented feed. After 4 weeks, animals of Group 2 were terminated (hence serving as a "before infection" control group); animals of Groups 1 and 4 were infected with CMC-*P. gingivalis*; and animals of Group 3 were subjected to mock infection (CMC vehicle alone). Periodontal disease was initiated according to the method of Momoi et al. (19). Briefly, mice were orally infected with *P. gingivalis* ATCC381 (*P. gingivalis* suspended at a concentration of 1×10^9 cfu/100 μ l in 3% carboxymethyl cellulose prepared with PBS) over 2 weeks (5 times/week). Two weeks after initiation of real or mock infection (i.e., at 6 weeks total), remaining mice (Groups 1, 3, and 4) were shifted to unsupplemented feed. Blood, saliva, and fecal were collected from the remaining mice on the first and thirtieth days after completion of infection. Mice were euthanized at 30 days after completion of infection procedure (i.e., following the second specimen collection time point). At sacrifice, gingival and alveolar bone samples were collected and processed as described below.

Antibody titers

The antibody titer in plasma, saliva, and fecal was determined by enzyme-linked immunosorbent assay (ELISA). Total antibody titer was determined with anti-mouse Ig (H+L)-UNLB. In determining the antibody titer to *P. gingivalis*, a suspension of heat-killed *P. gingivalis* cells adjusted to 1×10^9 cfu/ml was diluted 500-fold. After coating each well in a 96-well ELISA plate with 100 μ l of the 1:500 dilution of the heat-killed *P. gingivalis* cells, blocking was performed with PBS containing 1% bovine serum albumin. After specimens were dispensed on each plate and were allowed to react, horseradish peroxidase (HRP) -labeled anti-mouse IgG or IgA (Southern Biotechnology Associates, Birmingham, AL) was added for further reactions. Next, a coloring reaction solution (0.1 M sodium hydrogen phosphate, 0.1 M 2-hydroxypropane-1,2,3- tricarboxylic acid, and hydrogen peroxide) was added to each well. After incubation for 15 minutes to allow the color reaction, absorbance was measured at 415 nm. In determining the antibody titer against *P. gingivalis*, the starting dilutions of the plasma and saliva samples were 1:2⁶ and 1:2³. The

antibody titer endpoint was taken to be the log₂ value that yielded an A₄₁₅ of 0.1 above background.

Alveolar bone resorption measurements, and hematoxylin and eosin double staining

Horizontal bone resorption was assessed as described by Klausen et al. (20) using specimens from the upper molar region obtained at 30 days after infection. In short, after removal of the gingival tissue, specimens were immersed overnight in 3% hydrogen peroxide; transferred to PBS and sonicated for 1 minute; and then stained with 1% methylene blue. The distance from the cement-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at a total of 7 sites on the buccal side of the specimen from each mouse, in accordance with the analytical methods reported by Momoi et al. (21). A microscope (x20) (VHX-100; Keyence, Osaka, Japan) that was part of a video image measurement system was used for measurements, which were standardized to obtain micrometer values.

Mandibles collected from the 30-day post-infection animals were fixed by 24-hour immersion at room temperature in PBS containing 1% paraformaldehyde. The fixed tissues were decalcified by submersion at 4°C for 5-7 days in PBS containing 150 mM EDTA, and then embedded in paraffin blocks. Thinly sliced sections of 4- μ m thicknesses were prepared and stained with hematoxylin and eosin (HE) by standard methodologies.

Data analysis

Data are expressed as means $\pm$ standard error of the mean (SEM). Two-group comparisons of antibody levels were performed by Mann-Whitney U-test, and 3-group comparisons of bone resorption levels were performed by non-parametric (Kruskal-Wallis) tests. Multiple comparisons were performed by Ryan's method. *p* values of <0.05 were considered statistically significant.

Results

Alveolar bone resorption and histopathology observations 30 days after infection

In the KT-11+*P. gingivalis* group, alveolar bone resorption from oral infection with *P. gingivalis* was significantly attenuated when compared with the *P. gingivalis*-only group (*p*<0.05), but bone resorption was not significantly changed compared to that in the Sham group (Fig.1). Inflammation of the gingiva was nominally increased in both

Fig.1 Assessment of alveolar bone loss using methylene blue-stained palatal bone and teeth from mice infected with *P. gingivalis*. When assessed at thirty days after the initiation of *P. gingivalis* infection, mice that had orally ingested KT-11 exhibited attenuation of the alveolar bone loss caused by *P. gingivalis* infection. Alveolar bone loss of palatal bone and teeth was visualized by methylene blue staining (A). Bone levels were determined using the VHX-100 image analysis system (B). Linear measurements were determined between the cementum-enamel junction and the alveolar bone crest of the maxillary molars at 7 landmark sites per mouse. Data are presented as means $\pm$ SEM for 3 or 7 mice per group. *: $P < 0.05$ (compared to sham-infection or *P. gingivalis*-only groups, as indicated).

the KT-11+*P. gingivalis* group and the *P. gingivalis*-only group compared to the Sham group. This inflammation included apparent lymphocyte infiltration, acanthosis, and epithelial thickening. Differences in inflammation between the KT-11+*P. gingivalis* group and the *P. gingivalis*-only group were not significant (Fig. 2).

Total IgG in plasma and total secretory IgA in saliva and fecal extracts

Total IgG levels in plasma on Days 1 and 30 after *P. gingivalis* infection were significantly higher in the KT-11+*P. gingivalis* group than in the *P. gingivalis*-only group ($p < 0.05$) (Fig. 3). Total secretory IgA (S-IgA) levels were significantly higher in the KT-11+*P. gingivalis* group than in the *P. gingivalis*-only group ($p < 0.05$) on post-infection Day 1 (Fig. 4). Total S-IgA levels in fecal extracts did not differ significantly between KT-11+*P. gingivalis* and *P. gingivalis*-only at either time point (Fig. 5). Thus, induction of total IgG was observed in plasma at Day 1 and 30; induction of total S-IgA antibodies was observed in saliva (Day 1 only) but not in fecal extracts.

Antibody titer to *P. gingivalis*

Specific antibody levels against *P. gingivalis* were tested after oral infection with *P. gingivalis*. The results showed elevation of the anti-*P. gingivalis* IgG antibody levels in plasma in the KT-11+*P. gingivalis* group ($p < 0.05$) (Fig. 6). No elevation was seen in the *P. gingivalis*-only group. *P. gingivalis*-specific IgG antibody levels were not elevated in saliva (data not shown).

Discussion

Previous work has shown that lactobacilli improve the balance of intestinal bacteria by lowering the pH of the intestinal environment (for example, through the activity of intestinal flora activity and production of short-chain fatty acids in the intestinal tract), and by favoring the retention of beneficial bacteria (22). Research also has revealed that lactobacilli produce at least one bacteriocin (an antimicrobial peptide), enhance natural killer (NK) cell activity, and increase IgA production (21, 23-25); all of these properties are thought to affect innate immunity in the intestinal immune system. Some lactobacilli strains also have been reported to be effective in reducing allergies (26). The *Lb. crispatus* KT-11 strain used in this study was isolated from the feces of healthy infants. Tobita et al. showed that heat-treated *Lb. crispatus* KT-11 markedly increased IFN- γ ⁺ CD4⁺ cell proliferation while also elevating the IFN- γ ⁺ CD4⁺ /IL-4⁺ CD4⁺ cell ratio (16, 17). KT-11 additionally has been associated with higher IgA levels and increased innate immune response in the intestine (27). Based on these data, *Lb. crispatus* KT-11 is considered promising as a foodstuff with potential anti-allergenic and immunoregulatory properties.

In the present study, we employed a murine model to investigate the efficacy of *Lb. crispatus* KT-11 in the treatment of periodontal disease. In this model, animals were infected with *P. gingivalis*, an organism that has been detected from the deepest parts of the lesions in advanced adult human periodontitis and has been shown to be associated with multiple systemic conditions, including atherosclerosis, diabetes, and premature birth. Mice that ingested *Lb. crispatus* KT-11 exhibited attenuation of alveolar bone resorption at 30 days after *P. gingivalis* infection compared to infected animals that lacked KT-11 exposure. Bone resorption in the KT-11+*P. gingivalis* mice was not significantly different from that observed in Sham mice that were not infected with *P. gingivalis*. These results

Fig.2 Hematoxylin and eosin (HE) staining of lower jaw tissues from mice infected with *P. gingivalis*. Thirty days after the initiation of *P. gingivalis* infection, decalcified mandibles were prepared and stained with HE (scale bars, 500 µm (upper row), 200 µm (lower row)). ↓, slight lymphocyte infiltration. *, acanthosis. ▲, epithelial thickening.

Fig.3 Levels of total IgG antibody in plasma. Plasma samples were collected from each group on the first and thirtieth days after the initiation of the infection procedure. Total IgG levels in plasma were determined using ELISA. Treatment groups were as follows: KT-11-supplemented chow (hatched line bar); standard chow+oral infection with *P. gingivalis* (stippled bar); or KT-11-supplemented chow+oral infection with *P. gingivalis* (black bar). Day 1 and Day 30 correspond to the 1st and the 30th days (respectively) after initiation of the *P. gingivalis* infection procedure. Data are presented as means $\pm$ SEM for 3 or 7 mice per group. *: $P<0.05$, **: $P<0.01$ (compared to *P. gingivalis*-only group).

Fig.4 Levels of total S-IgA antibody in saliva. Saliva samples were collected from each group on the first and thirtieth days after the initiation of the infection procedure. Total S-IgA levels in saliva were determined using ELISA. Treatment groups were as follows: KT-11-supplemented chow (hatched line bar); standard chow+oral infection with *P. gingivalis* (stippled bar); or KT-11-supplemented chow+oral infection with *P. gingivalis* (black bar). Day 1 and Day 30 correspond to the 1st and the 30th days (respectively) after initiation of the *P. gingivalis* infection procedure. Data are presented as means $\pm$ SEM for 3 or 7 mice per group. *: $P<0.05$ (compared to *P. gingivalis*-only group).

suggested that ingestion of *Lb. crispatus* KT-11 is effective in preventing periodontal disease. Ingestion of *Lb. crispatus* KT-11 increased antibody production, as assessed by total IgG in plasma and secretory IgA in saliva. Secretion of total IgG, a component of the systemic immune system, was elevated on Day 1 after completion of infection, and continued to rise through the following month. These results

indicated that KT-11 also has an effect within the oral cavity, since secreted antibody is expected to be present in the gingival crevicular fluid, which is derived from the adjacent capillaries. The secretory IgA in saliva is part of the local mucosal immunity produced by the salivary glands; thus, a local immune response was induced by ingestion of *Lb. crispatus* KT-11. Ingestion of *Lb. crispatus* KT-11 also

Fig.5 Levels of total S-IgA antibodies in feces. Fecal samples were collected from each group on the first and thirtieth days after the initiation of the infection procedure. Total S-IgA levels in fecal extracts were determined using ELISA. Treatment groups were as follows: KT-11-supplemented chow (hatched line bar); standard chow+oral infection with *P. gingivalis* (stippled bar); or KT-11-supplemented chow + oral infection with *P. gingivalis* (black bar). Day 1 and Day 30 correspond to the 1st and the 30th days (respectively) after initiation of the *P. gingivalis* infection procedure. Data are presented as means $\pm$ SEM for 3 or 7 mice per group. Differences were not statistically significant on a given day.

elevated the titer of *P. gingivalis*-specific IgG. The increase in IgG antibody production and induction of specific antibodies seen in *Lb. crispatus* KT-11 -exposed animals is expected to block the adhesion of *P. gingivalis* to gingival epithelial cells and therefore defend against infection(28). This mechanism is thought to be the source of inhibition of alveolar bone resorption.

Ongoing research on sublingual and nasal immunity is expected to facilitate the development of a vaccine against human periodontal disease(19, 28-31). For instance, the *P. gingivalis* recombinant 40-kDa outer membrane protein (40K-OMP) has been shown (when administered in combination with cholera toxin) to induce a significant level of 40K-OMP-specific IgG in serum(19, 28-31). Heat-treated *Lb. casei* displaying a modified version of the 40K-OMP has been shown to elicit not only IgG and IgA antibodies in serum but also a 40K-OMP-specific IgA antibody response in saliva. The antibody titers detected in the present study did not appear to be as high as those seen in nasal immunity due to oral ingestion of *Lb. crispatus* KT-11(28). Nonetheless, the induction of *P. gingivalis*-specific IgG antibodies in plasma observed in the present study suggested that feeding with chow incorporating *Lb. crispatus* KT-11 may provide a means to strengthen host sensitivity without placing an immunological burden on the

Fig.6 Levels of *P. gingivalis*-specific IgG antibodies in plasma. Plasma samples were collected from each group on the first and thirtieth days after the initiation of the infection procedure. To determine the titer of *P. gingivalis*-specific antibodies, heat-killed *P. gingivalis* cells were used to coat a 96-well ELISA plate. Treatment groups were as follows: KT-11-supplemented chow (hatched line bar); standard chow+oral infection with *P. gingivalis* (stippled bar); or KT-11-supplemented chow+oral infection with *P. gingivalis* (black bar). Day 1 and Day 30 correspond to the 1st and the 30th days (respectively) after initiation of the *P. gingivalis* infection procedure. Data are presented as means $\pm$ SEM for 3 or 7 mice per group. Differences were not statistically significant on a given day. n.d.: not detectable. *: $P < 0.05$ (compared to *P. gingivalis*-only group).

host.

Lactobacilli are deeply involved in intestinal immunity and display an effect there(21,23-25). Secretory IgA antibodies are the predominant antibodies in the intestinal tract, but in the present study, no increases in secretory IgA in fecal extracts were detected following the ingestion of *Lb. crispatus* KT-11. In contrast, Tobita et al. reported a significant increase in intestinal total IgA levels in mice given *Lb. crispatus* KT-11 when compared to mice who did not receive this supplement(27). Tobita et al. administered *Lb. crispatus* KT-11 as an aqueous suspension; their findings are consistent with their use of this route and of intestinal tissues as test specimens. In the present study, animals were provided with free access to *Lb. crispatus* KT-11 blended into chow; our findings are consistent with induction of the immune system via exposure to regional lymph nodes in the oral mucosa. Note that we also compared the effect of ingestion of *Lb. crispatus* KT-11 -supplemented chow with that of ingestion of unsupplemented chow.

A draft report by a 2002 FAO/WHO working group exploratory committee proposed defining probiotics as live bacteria that show a beneficial effect (action) when ingested in appropriate amounts(32). However, research on sterilized milk and heated lactobacilli has demonstrated that

even dead lactobacilli are effective in improving the intestinal flora, and has showed that immune stimulation from cell components of lactobacilli play the main role. Thus, even dead lactobacilli have similar health effects as living lactobacilli(33).

Cases of opportunistic infection from lactobacilli also have been reported(34-36), and there are some concerns regarding their safety(37). The *Lb. crispatus* KT-11 used in this study consisted of dead (heat-treated) cells. Application of probiotics are not limited to the intestines; probiotics also are reported to reduce the number of *Helicobacter pylori* cells in the stomach(38) and to prevent or alleviate allergies(39, 40), and their use is likely to spread in the future. Probiotics also have shown an effect in preventing or improving aging phenomena and so are promising for use in older adults who require care. In such older adults, the risk of opportunistic infections should be avoided; the use of dead cells is expected to present a significant advantage. The population of Japan is aging rapidly; in the coming years, the country is expected to experience a sharp increase in the number of older adults requiring care. The ingestion of probiotics has great potential as preventive therapy and may contribute to the extension of healthy lifespan in Japan, which is becoming a "super-aged" society. However, some aspects of the mechanisms of probiotics remain unclear. A detailed elucidation of the mechanisms of *Lb. crispatus* KT-11 remains necessary. We plan to further characterize these mechanisms, and to undertake clinical research on the preventive effects of *Lb. crispatus* KT-11 against periodontal disease in humans.

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