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Promotion of Bone Nodule Formation and Inhibition of Growth and Invasion of *Streptococcus mutans* by *Weissella kimchii* PL9001

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Abstract Lactic acid-producing bacteria (LABs) are known to have various beneficial properties for health. However, they are generally considered to have an adverse effect on teeth, since they produce acid. Nonetheless, milk and cheese containing specific LAB strains were recently found to have an inhibitory effect on dental caries in children, with an inhibitory activity towards the growth of *Streptococcus mutans* suggested as the responsible mechanism. Accordingly, the current study selected a probiotic candidate for oral health and studied its inhibitory mechanism against dental caries. Twenty-two LAB species belonging to eleven genuses were screened for promoting bone nodule formation using direct microscopic examination. Only one isolate, *Weissella kimchii* strain PL9001, increased the bone nodule formation significantly. The addition of *W. kimchii* strain PL9001 to bone cells prepared from mouse calvaria increased the bone nodule formation, calcium accumulation, and activity of alkaline phosphatase (the osteoblastic marker). Moreover, *W. kimchii* strain PL9001 inhibited the invasion of *Streptococcus mutans* into bone cells, and an organic extract of the culture supernatant of *W. kimchii* strain PL9001 inhibited the growth of *Strep. mutans*. Therefore, the results suggest that *W. kimchii* strain PL9001 can be used as a preventive measure against dental caries. This is the first time that a LAB has been shown to promote bone nodule formation and prevent the invasion of *Strep. mutans* into bone cells.

Key words: Bone nodule formation, LAB, invasion, probiotic, *Streptococcus mutans*, *Weissella confusa*, *Weissella kimchii*

Dental caries are caused by acids produced from the fermentation of food in the mouth dissolving the calcium component and finally resulting in teeth loss [13]. One of the complex reasons for dental caries is the formation of plaque resulting from mucus, epithelial cells, and bacteria sticking to the surface of teeth. Although various bacteria are involved in plaque, the majority is *Streptococcus mutans*, and *Actinomyces gerencseriae*, *Bifidobacterium*, *Strep. oralis*, *Strep. sanguis*, *Strep. gordonii*, *Strep. salivarius*, *Strep. constellatus*, *Strep. parasanguinis*, *Candida albicans*, *Lactobacillus* spp., *Treponema denticola*, and *Veillonell* spp. have also been reported [3, 5]. Strep. mutans and Lactobacilli have both been found to produce acids from carbohydrates. Among the *Lactobacillus* spp., *L. casei*, *L. fermentum*, and *L. rhamnosus* have been reported in plaque, and Lactobacilli generally grow well in acidic intra- and interdental spaces [2].

The colonization of *Strep. mutans* on the tooth surface is considered to be the first step in the induction of dental caries, and the risk of caries is higher if the bacterium is colonized on teeth early in life [2, 13]. *Strep. mutans* adheres to the tooth surface by sucrose-independent and sucrose-dependent mechanisms [11, 17]. *Strep. mutans* has the capability of sucrose hydrolysis and produces organic acid and water-insoluble mutan, which help the plaque stick to the dental surface, and then an unspecified cell wall component of *Strep. mutans* stimulates bone absorption. The colonized *Strep. mutans* induces dental caries and finally teeth loss [2, 13, 17]. Sometimes, *Strep. mutans* invades cells and has been isolated from blood related to cardiovascular disease [22].

Dental caries can cause serious problems in children and are known to correlate well with the number of oral lactic acid-producing bacteria (LAB) in children [11, 25]. Yet, contrary to the general concept that Lactobacilli are harmful to teeth [3, 24], children with specific Lactobacilli have been found not to develop approximal and smooth-surface caries. Recently, milk containing a specific strain of LAB showed an inhibitory effect as regards dental caries in

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children [26, 34]. Furthermore, children who consumed cheese containing probiotics showed a lower number of bacteria responsible for dental caries [1]. The suggested mechanism responsible for this preventive activity was the inhibitory activity of the probiotic towards the microflora in the mouth [7–9, 29, 35]. However, the exact preventive mechanism of LAB in relation to dental caries has yet to be found.

Accordingly, this study tested 22 LAB belonging to 11 different genuses, with regards their promotion of bone nodule formation. As a possible probiotic for oral health, the LAB with the best activity was then selected for further study, including an assay of its inhibitory activity on the growth and invasion of *Strep. mutans* and the promotion of bone nodule formation, calcium accumulation, and alkaline phosphatase, the major enzyme responsible for bone nodule formation.

MATERIALS AND METHODS

Preparation of Bacteria

A total of 22 LAB isolated from baby feces and vaginas were tested in this study, including *Enterococcus durans* (1 isolate), *E. faecalis* (1 isolate), *L. brevis* (3 isolates), *L. fermentum* (2 isolates), *L. gasseri* (2 isolates), *L. paracasei* (3 isolates), *L. plantarum* (2 isolates), *L. salivarious* (1 isolate), *Leuconostoc mesenteroides* (1 isolate), *Weissella confusa* (4 isolates), and *W. kimchii* (2 isolates). Overnight cultures of the LAB in MRS (Difco, Sparks, MD, U.S.A.) were washed with phosphate-buffered saline (PBS) and diluted in PBS to make the appropriate concentration. *Strep. mutans* ATCC25175 isolated from dental caries was grown in a brain heart infusion (BHI; Difco) solid medium under 10% CO_2 at 37°C.

ARDRA to Identify *W. kimchii* PL9001

W. kimchii strain PL9001 was originally isolated from baby feces and identified as *W. confusa*. However, since *W. kimchii* was recently branched from *W. confusa* [6], *W. confusa* strain PL9001 was reidentified as *W. kimchii* strain PL9001 based on an amplified rRNA restriction analysis (ARDRA) following the procedure reported previously [15]. The PCR reaction mixture (50 μl) for the ARDRA contained 2 μl of genomic DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1.25 U Taq polymerase, 0.5 M of each primer (5'-CGTGGGAAACCTACCTCTTA-3' and 5'-CCCTCAAA CATCTAGCAC-3'), and 5 μl of a 10× reaction buffer. The PCR reaction consisted of an initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec, and polymerization at 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were treated with MnlI (MBI Fermentas, Hanover, MD, U.S.A.) for 2 h at 37°C and electrophoresed on a 2% agarose gel.

Isolation of Bone Cells from Mice

The bone cells were obtained following the procedure reported previously with slight modification [4]. Calvaria were aseptically obtained from one-day-old mice (ICR), and bone cells retreived by treating the calvaria with α-MEM (GIBCO BRL, Grand Island, NY, U.S.A.) containing 0.2% collagenase (Wako, Osaka, Japan) and 0.1% dispase (GIBCO™, Godo Shusei Co., Ltd. Japan). After 10 min, the supernatant was discarded, then the next three washes were collected and used for the experiment. Any undigested calvaria was removed via filtration through a nylon net filter (pore size, 20 μm; Millipore, Bedford, MA, U.S.A.). The filtrate was centrifuged at 2,000 ×*g* for 5 min to collect the bone cells, which were then distributed on a 30-mm cell culture plate based on 1×10^5 cells/well in α-MEM (GIBCO BRL) containing 10% fetal bovine serum (GIBCO BRL) and 1% antibiotic-antimycotics (GIBCO BRL). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until they reached a 70–80% confluence; meanwhile, the medium was changed every other day. Thereafter, a medium containing 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid was added to the cells and changed every other day until the 20th day. The osteoblastic characteristic of the cells was confirmed with histochemical staining for alkaline phosphatase using a leukocyte alkaline phosphatase kit (Sigma, St. Louis, MO, U.S.A.) following the manufacturer's instructions. The proportion of positively stained cells in each population was estimated by counting a random sample of 100 cells. Cell populations with at least 80% positive cells were further cultured and used in the assays. All incubations were carried out at 37°C in a humidified atmosphere containing 5% CO_2.

Alkaline Phosphatase Assay

After washing the cells twice with 50 mM Tris-HCl (pH 7.2), they were broken using a sonicator for 15 sec in 50 mM Tris-HCl (pH 7.2) containing 0.1% Triton X-100 and 2 mM $MgCl_2$. The alkaline phosphatase (ALPase) activity was assayed by adding *p*-nitrophenyl phosphate to the cell lysate, and the amount of protein assayed using the BCA method (Sigma).

von Kossa Staining

After being washed with PBS, the cells were fixed in 10% neutral-buffered formalin and stained *in situ* using the von Kossa technique [5]. The bone nodule formation was assayed using a Bio-1D Image analysis system (Vilber Lourmat, La Valle Cedex 1, France) based on measuring the total volume of bone nodule formed.

Assay of Amount of Accumulated Calcium

After washing the cells twice with PBS, the mineralized bone nodules were collected by treating the cells with 0.5 M HCl, then breaking them in a sonicator for 15 sec. The amount of calcium in the supernatant was assayed using the *o*-cresolphthalein complex-one method (Calcium C. Test, Wako, Japan), as described previously [12].

Inhibitory Activity of Culture Extract of *W. kimchii* Strain PL9001 on Growth of *Strep. mutans*

The supernatant of an overnight culture of *W. kimchii* strain PL9001 was extracted with the same volume of ethyl acetate and the organic phase dried in a vacuum evaporator. The concentrate was then dissolved in a 1/100 volume of the original volume. *Strep. mutans* ATCC 25175 grown on a blood agar in a CO_2 incubator was collected and dispersed in PBS, creating an absorbance of 600 nm. The *Strep. mutans* was then inoculated into a Muller Hinton (Difco) solid medium using a swab. An aliquot of the extract (10 μl) was dropped on the *Strep. mutans*, and a clear growth inhibition zone observed after incubating overnight.

Assay of Internalized *Strep. mutans* in the Presence of *W. kimchii* Strain PL9001

The internalized *Strep. mutans* was assayed using a previously reported method [16]. The bone cells were washed with α-MEM (GIBO BRL); then, after 1 h, the cells were incubated with *W. kimchii* strain PL9001 (10^7 CFU/plate or 10^8 CFU/plate, 30 mm diameter) for 30 min under 10% CO_2-90% air at 37°C. Thereafter, *Strep. mutans* ATCC 25175 (10^6 CFU/plate or 10^7 CFU/plate) was added to the cells and the mixture incubated for another 1.5 h. Any unbound bacterial cells were removed by three washes with PBS. The cells were then treated with gentamicin (100 μg/ml) for 1.5 h to kill the bound bacterial cells and washed three times with PBS. Finally, the bone cells were broken by the addition of 0.1% Triton X-100, and the number of *Strep. mutans* ATCC 25175 in the cell lysate assayed using a brain heart infusion (Difco) medium and the most probable number method (MPN method).

Statistical Analysis

The results were analyzed with Turkey's Multiple Range Test (SPSS ver. 10.0, SPSS Inc., Chicago, IL, U.S.A.) and values of $p<0.05$ were considered as significant.

RESULTS

Selection of LAB that can Promote Bone Nodule Formation

Among the 22 LABs tested in this study, only one isolate of *W. kimchii* promoted bone nodule formation when the cells were observed under a microscope (Fig. 1). This isolate was originally isolated from baby feces, identified, and named *W. confusa* strain PL9001, and it showed inhibitory activity towards the adherence and growth of *Helicobacter pylori* [25]. Since *W. kimchii* was recently branched from *W. confusa* [6, 15], *W. confusa* strain PL9001 was reidentified as *W. kimchii* strain PL9001 using an ARDRA. As shown in Fig. 2, the *W. kimchii* type strain KCTC CHJ3 (lane B) and *W. confusa* strain PL9001 (lane C) produced the same bands at 282 bp and 112 bp, whereas the *W. confusa* type strain KCTC 3499^T (lane A) produced bands at 203 bp and 94 bp, but not at 112 bp.

Bone Nodule Formation in the Presence of *W. kimchii* Strain PL9001

The bone nodule formation in the cells treated with various concentrations of *W. kimchii* strain PL9001 was quantified by measuring the stained area after von Kossa staining for the accumulated calcium (Fig. 3). In the case of the cells treated with 10^4 CFU *W. kimchii* strain PL9001/plate, the bone nodule formation increased up to 114% ($p<0.01$) and 113% ($p<0.05$) compared with the control on the 14^{th} and

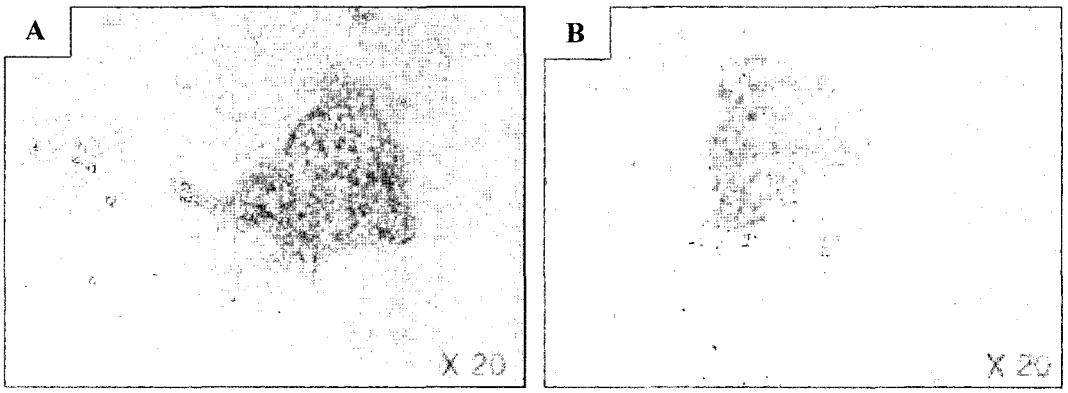

Fig. 1. Bone nodule formation observed under microscope.
The bone cells were grown for 21 days in the absence (**A**) and presence (**B**) of *W. kimchii* strain PL9001, and observed under a microscope.

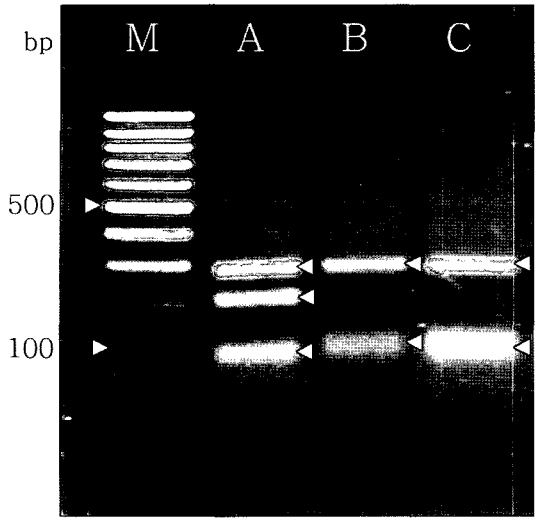

Fig. 2. ARDRA of *W. kimchii* strain PL9001.
(M) mol. wt. marker, (A) *W. confusa* type strain KCTC 3499^T, (B) *W. kimchii* type strain KCTC CHJ3, (C) PL9001.

20th day, respectively (Fig. 4). There was no significant difference in the bone nodule formation among the control cells and the cells treated with 10^5 CFU and 10^6 CFU.

Assay of Amount of Accumulated Calcium

As expected, the amount of accumulated calcium increased as the bone nodule formation increased (Fig. 5).

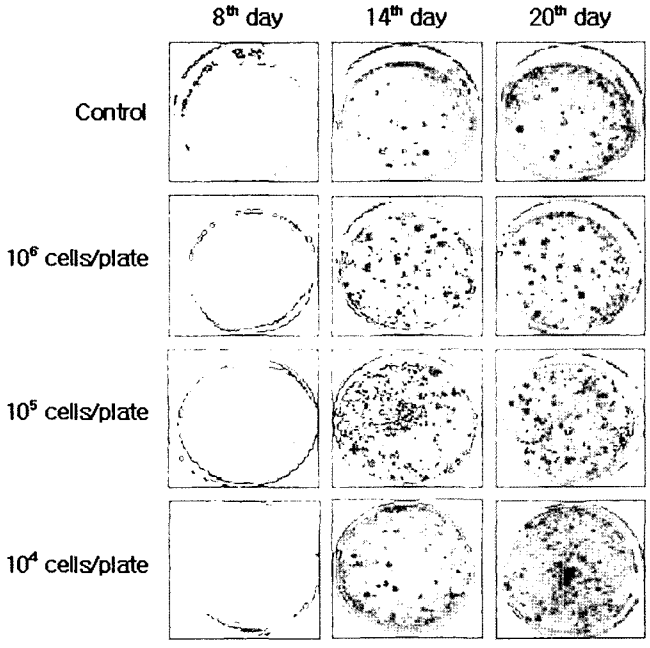

Fig. 3. von Kossa staining of bone cells.
Cells isolated from the calvaria of mice were cultured with different amounts of *W. kimchii* strain PL9001, as described in Materials and Methods. The accumulated calcium was stained with von Kossa staining.

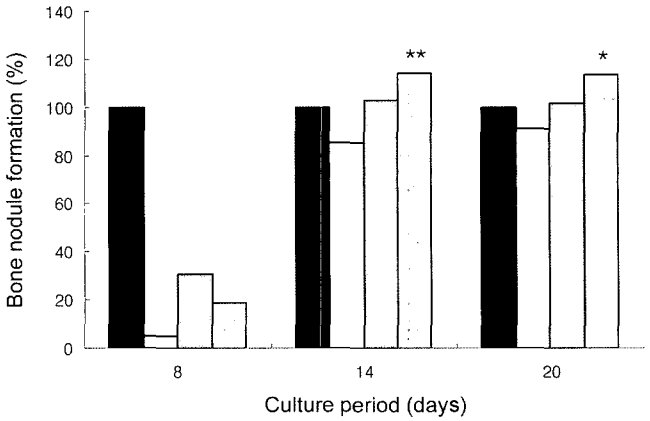

Fig. 4. Effect of *W. kimchii* strain PL9001 on bone nodule formation.
Cells isolated from the calvaria of mice were cultured in the presence of different amounts of *W. kimchii* strain PL9001, as described in Materials and Methods. The number of bone nodules formed was counted under a microscope. The number of bone nodules formed in the absence of *W. kimchii* strain PL9001 was considered as 100% (*, $p<0.05$; **, $p<0.01$; ■, control; □, 10^6; □, 10^5; □, 10^4).

Alkaline Phosphatase Activity Determined by Histochemical and Biochemical Assays

The ALPase activity increased significantly in both the control cells and the cells treated with *W. kimchii* strain PL9001 until the 14th day, and then decreased except for in the cells treated with 10^4 CFU/plate. The cells treated with 10^4 CFU/plate showed the highest ALPase activity on the 21st day, indicating active bone formation even on the 21st day (Fig. 6).

Inhibitory Activity of Culture Extract of *W. kimchii* Strain PL9001 on Growth of *Strep. mutans*

The culture supernatant of *W. kimchii* PL9001 was extracted with ethyl acetate to avoid any effect from acids

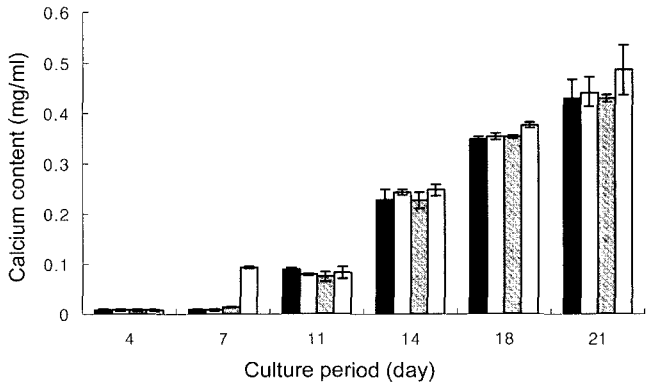

Fig. 5. Assay of calcium accumulation.
The calcium accumula-tion in the presence of various amounts of *W. kimchii* strain PL9001 was assayed using the o-cresolphthalein complexone method, as described in Materials and Methods. (■, control; □, 10^6; □, 10^5; □, 10^4).

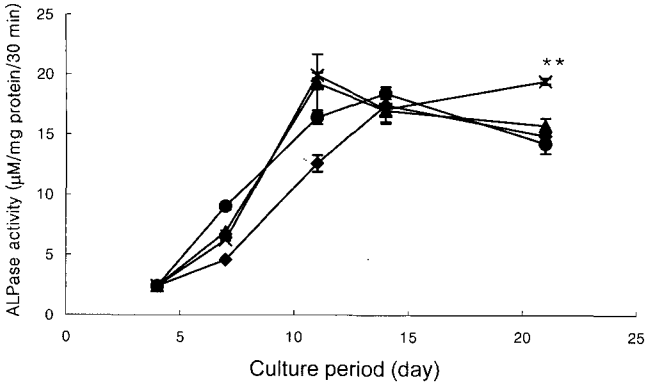

Fig. 6. Effect of *W. kimchii* strain PL9001 on alkaline phosphatase in bone cells.
The alkaline phosphatase (ALPase) activity in the bone cells in the presence of *W. kimchii* strain PL9001 was assayed by adding *p*-nitrophenyl phosphate to the cell lysate and the amount of protein assayed using the BCA method. (**, $p<0.01$; ●, control; ◆, 10^6; ▲, 10^5; ×, 10^4).

and hydrogen peroxide, and the extract inhibited the growth of *Strep. mutans*, producing a clear growth inhibition zone after culturing overnight (Fig. 7).

Inhibitory Activity of *W. kimchii* Strain PL9001 on Internalization of *Strep. mutans*

In the presence of a ten-fold concentration of *W. kimchii* strain PL9001 (10^8 CFU/plate), the internalization of *Strep. mutans* (10^7 CFU/plate) into the bone cells significantly decreased to 14.9% of that in the control cells (Fig. 8). Meanwhile, in the case of a low concentration of *Strep. mutans* (10^6 CFU/plate), the internalization of *Strep. mutans* decreased to 62.7% of the control with a ten-fold concentration of *W. kimchii* strain PL9001.

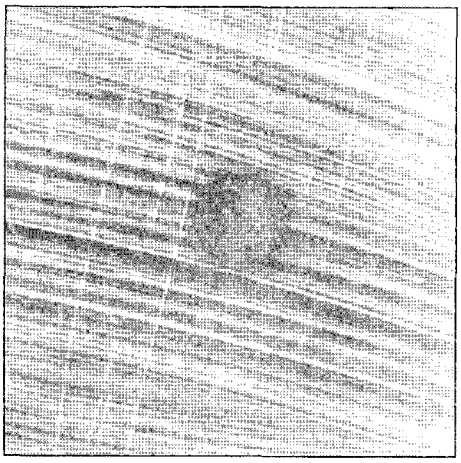

Fig. 7. Inhibitory activity of *W. kimchii* strain PL9001 on growth of *Strep. mutans*.
An ethyl acetate extract of the spent culture supernatant of *W. kimchii* strain PL9001 was dropped on *Strep. mutans* and a growth inhibition zone observed after culturing overnight.

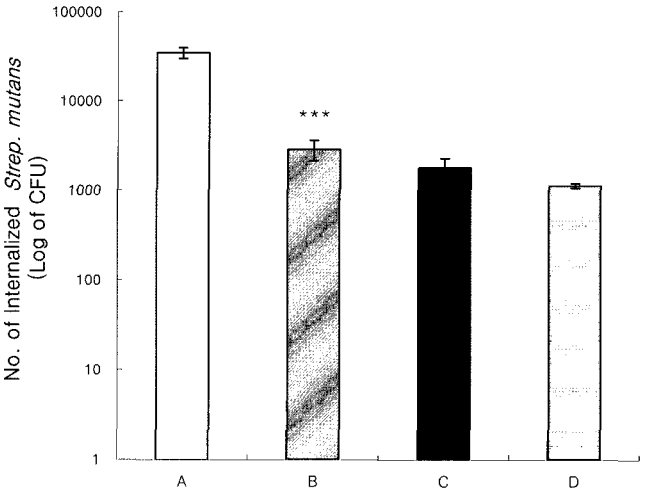

Fig. 8. Inhibitory activity of *W. kimchii* strain PL9001 on internalization of *Strep. mutans* in bone cells.
The inhibitory activity of *W. kimchii* strain PL9001 on the internalization of *Strep. mutans* was assayed as described in Materials and Methods. (***, $p<0.001$; A, *Strep. mutans* (10^7 CFU); B, *Strep. mutans* (10^7 CFU) and PL9001 (10^8); C, *Strep. mutans* (10^6 CFU); D, *Strep. mutans* (10^6 CFU) and PL9001 (10^7).

DISCUSSION

The concept of using Lactobacilli to treat and prevent disease, along with restoring and maintaining health, is not new. Moreover, there has been a renewed interest in the use of probiotics [31], which have been used therapeutically to modulate immunity, lower cholesterol, treat rheumatoid arthritis, prevent cancer, improve lactose intolerance, and prevent or reduce the effects of atopic dermatitis, Crohn's disease, diarrhea, and constipation, as well as candidiasis and urinary tract infections [10, 14, 21, 22, 32]. Since LAB have generally been thought to exert a harmful effect on tooth health by producing acids, the use of probiotics to reduce dental caries is quite a new idea.

Various methods of fighting dental caries have already been tried, including the use of antibodies formed in milk from cows vaccinated with *Strep. mutans* [33], milk containing specific probiotics [30, 37], essential oils [34], xylitol [19], and antimicrobial decapeptide [9]. Since probiotic treatment increases the number of LAB present in the saliva, thereby contributing to dental caries [26], a probiotic working against dental caries requires special attributes, such as a higher pH production and inhibitory activity against *Strep. mutans*.

In contrast to oral pathogens, *W. kimchii* strain PL9001 did not inhibit osteoblastic cell differentiation, but instead induced it [18]. The results of every assay, including calcium accumulation, bone nodule formation, and ALPase, showed that *W. kimchii* strain PL9001 at a low concentration (10^4 CFU/plate) had a beneficial effect on teeth. In

particular, a low concentration of *W. kimchii* strain PL9001 activated ALPase activity, which is expressed during bone nodule formation, even on the 21st day of culture. The decrease in ALPase activity with a higher concentration after the 7th day was assumed to be the result of harmful metabolites, such as acid.

Accordingly, the results of this study confirmed the inhibitory effect of *W. kimchii* PL9001 on dental caries via several mechanisms: promotion of bone nodule formation, plus inhibition of growth and internalization of *Strep. mutans*. Since LAB secrete metabolites with anti-inflamatory properties after intestinal transport [23], it is possible that *W. kimchii* strain PL9001 produces metabolites that inhibit inflammation in the mouth. In addition, *W. kimchii* produces less acid (pH=4.85) than other LAB.

An additional advantage of *W. kimchii* strain PL9001 for oral health is its inhibitory effect on *H. pylori* [25]. It has been reported that the majority of children infected with *H. pylori* have dental caries [20]. Thus, the anti-*H. pylori* activity and lower acid production of *W. kimchii* strain PL9001 are supplementary benefits of this strain as a preventive measure for dental caries.

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