

## Probiotic effect of feeding *Lactococcus lactis* and *Streptococcus thermophilus* on periodontitis induced rat model

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Dysbiosis of oral microbiome by change of the environments induces pathogen related disease. Especially, *Porphyromonas gingivalis* is associated with periodontitis. Various studies have showed probiotic efficacy for periodontitis related bacteria. The purpose of this study was to investigate the therapeutic effect of probiotics on periodontitis induced rat model. The ligature-induced periodontitis rats using *P. gingivalis* were fed the regular diet with or without probiotics for 20 days, and the periodontal status was then analyzed by micro computed tomography and levels of inflammatory cytokines. Also, the levels of *P. gingivalis* and the colonization of probiotics were analyzed by quantitative real-time polymerase chain reaction. The rat that fed on the diet containing *Streptococcus thermophilus*, *Lactococcus lactis*, and both probiotics significantly showed higher mass of the alveolar bone compared to the rat that fed on regular diet. Also, the rat that fed on the diet containing probiotics reduced the levels of inflammatory cytokines. The count of *P. gingivalis* was reduced in the rat fed containing probiotics. Furthermore, *S. thermophilus* and *L. lactis* were detected in the rat containing each probiotic bacterium. This study established that *S. thermophilus* and *L. lactis* may be suitable probiotics for therapeutic and preventive periodontitis.

**Key Words:** *Lactococcus lactis*, Periodontitis, Probiotics, *Streptococcus thermophilus*

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### INTRODUCTION

More than 700 bacterial strains may be found in oral cavity [1]. Oral microbes are composed of proportionally balanced beneficial, commensal and pathogenic bacteria and form biofilm as a micro-ecosystem [2]. Once established, the microflora in the biofilm remains relatively stable over time despite regular minor perturbations to the oral environment, and this stability is termed microbial homeostasis. Also they co-exist with communication among interspecies

or intraspecies that contribute to ecologic stability [3,4]. This microbial homeostasis can break down on occasion by substantial change of oral environment. Significant parameters regulating the homeostasis include the host defenses and the composition of the diet. In case of dental caries, the ecological biofilm was changed by the composition of the diet, and the ratio of cariogenic bacteria in the biofilm was increased [2]. Also, when the microbial homeostasis was broken by some major factors, Gram-negative anaerobes as periodontitis related microbes increase in the accu-

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mulated subgingival biofilm [1,5,6], by which periodontitis is occurred. Therefore, periodontal disease is considered to associate with multi-bacterial infection with anaerobic microbes such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* [5]. The bacterial complex interacts with host tissue and cell causing the release of broad array of inflammatory and osteoclastogenic cytokines.

Probiotics are beneficial bacteria to host and produce antimicrobial agents as bacteriocin against pathogenic bacteria [7]. Among probiotics, *Lactococcus lactis* and *Streptococcus thermophilus* are Gram-positive and facultative anaerobic bacterium [8,9]. These bacteria are widely used in the production of fermented dairy foods. Also, both probiotics produce antimicrobial agents against gut pathogenic bacteria and reduce inflammation [10-12]. Furthermore, these probiotics showed antimicrobial activity against periodontopathogens and inhibited their virulence factors in vitro study [11]. In order to provide the beneficial effects of probiotics on oral disease such as dental caries and periodontitis for the host, probiotics have to colonize on host at the site. Therefore, in this study, the beneficial effects of both probiotics on periodontitis were investigated using periodontitis rat model.

## MATERIALS AND METHODS

### Bacterial strain and cultivation

*P. gingivalis* ATCC 33277 was used for induction of periodontitis and cultured with brain heart infusion (BHI) broth (BD Biosciences, San Jose, CA, USA) supplemented with hemin (1 µg/mL) and vitamin K (0.2 µg/mL) at 37°C in an anaerobic condition (5% H<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>). Also, *L. lactis* HY 449 and *S. thermophilus* HY 9012 were gratefully donated from Yakult (Korea Yakult Co., Yongin, Korea) and cultivated with BHI broth at 37°C, anaerobically.

### Animals and induction of experimental periodontitis

Male Wistar rats (250-300 g) were purchased from ORIENT Co. (Seongnam, Korea) and used to induction of periodontitis. Also, all experiments using rat were carried out

in separate space with facilities for animal experiments. The rats were allowed food and water ad libitum and were maintained on a 12 hours light and dark cycle at 24°C with 50% to 60% humidity for 1 week before use. Also, all animals were maintained by according to the guidelines of laboratory animal ethics committee in Dankook University. The rats were anesthetized with isoflurane gas and the sterile 4-0 (diameter, 0.4 mm) braided silk (Perma-hand silk; Ethicon, Somerville, NJ, USA) was placed around the cervix of the lower second molars and knotted medially. The rats were randomly allocated into five groups with 5 rats per group. Control group was not ligated with the silk and the others were ligated with *P. gingivalis* inoculated silk. The grouping of experimental rats was described in more detail in Table 1. In order to induce periodontitis, *P. gingivalis* suspension (100 µL, 1 × 10<sup>9</sup> colony-forming unit [CFU]/mL) was inoculated into the knotted silk, and the rats were bred for 20 days. Control group (10 rats) was fed the regular diet, and the experimental groups (15 rats per group) were fed the diet containing *L. lactis*, *S. thermophilus*, or both probiotics (1 × 10<sup>7</sup> CFU/g). The diets with mixture regular food and probiotics were made by Korean Yakult. After displacing the ligatures, gingival crevicular fluid (GCF) and bacteria in subgingiva were collected with four paper points (#40, taper size 0.04 mm). Two paper points were used for enzyme-linked immunosorbent assay (ELISA) analysis to investigate inflammatory cytokines, and the remaining paper points were used for bacterial count.

### Analysis of micro-computed tomography

Mandible was subjected by micro-computed tomography (CT) using an X-ray micro-CT scanner (SkyScan 1172;

**Table 1.** Groups of experimental rats

Group	Treatment
Control	None-ligature and regular diet
Regular diet	Ligature and regular diet
<i>Streptococcus thermophilus</i> diet	Ligature and diet containing <i>S. thermophilus</i>
<i>Lactococcus lactis</i> diet	Ligature and diet containing <i>L. lactis</i>
<i>S. thermophilus</i> + <i>L. lactis</i> diet	Ligature and diet containing <i>S. thermophilus</i> + <i>L. lactis</i>

SkyScan Co., Kontich, Belgium) after collection at end time point of the experiment. The image intensifier to obtain two-dimensional images of each level, the scanning was performed under the condition of 80 keV, 100  $\mu$ A, and 16.8 magnification with the spot size of 8  $\mu$ m. The level of alveolar bone resorption was analyzed according to the method by Park et al. [13]. Briefly, linear measurements were taken from the cemento-enamel junction to the root apex line in the interdental region between the first and second molars. The ratio of remaining alveolar bone between first and second molar in the left mandible was compared among each group. The three-dimension (3D) area of alveolar bone was measured by program under development which can calculate 3D area from 3D image of micro-CT data and confocal laser microscopy data.

### Bacterial count with real-time polymerase chain reaction

For count of *P. gingivalis* or probiotics in subgingiva of the ligature site of the rats, quantitative real-time polymerase chain reaction (PCR) was performed using specific primer of each bacterium. First, the standard curve was generated using from various bacterial count ( $1 \times 10^3$  to  $1 \times 10^7$ ) and Cycle threshold (Ct) of amplified DNA of each bacterium. Each bacterial DNA was extracted from various amount of the bacteria by G-spin Genomic DNA extraction kit (iNtRON Biotech., Seongnam, Korea) after counting the bacteria with bacterial counting chamber (Marienfeld-Superior, Lauda-Königshofen, Germany). DNA was mixed with TB Green premix Ex Taq GC (Takara Bio Inc., Kyoto, Japan), 0.4  $\mu$ M of each primer pair in 20  $\mu$ L final volume. The mixture was carried out real-time PCR with ABI prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The condition of the PCR was 40 cycles at denaturing 94°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 33 seconds. The sequence of the used primers was as follows: 5'-CTC GTG GTC ACA AGC AGT AG-3' and 5'-GGA ATG ACG GTT TCA ATC GTG-3' for *L. lactis* gene; 3'-TCA CTA TGC TCA GAA TAC AAA TC-3' and 5'-ACC CAT ACA AAG ATG GAA GTA G-5' for *S. thermophilus* gene; 5'-TGC AAC TTG CCT TAC AGA GGG-3' and 5'-ACT CGT ATC GCC CGT TAT TC-3'

for *P. gingivalis* gene. The bacterial level was calculated using Ct level from the standard curve. The PCR products were investigated for each specific amplification product using a dissociation curve of amplification.

### Enzyme-linked immunosorbent assay

The collected GCF using paper point from the rat were eluted by Dulbecco's phosphate-buffered saline to investigate inflammatory cytokines, and the levels of interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$  was measured by ELISA kit (BD Biosciences) according to manufacturer's protocol.

### Statistical analysis

All results were expressed as mean  $\pm$  standard deviation and analyzed by Kruskal-Wallis non-parametric analysis and Mann-Whitney non-parametric analysis using SPSS ver. 24 (IBM Corp., Armonk, NY, USA). *p*-value less than 0.05 were considered statistically significant.

## RESULTS

### Micro-CT analysis

When the alveolar bone was investigated the periodontitis-induced rat after feeding various diets by micro-CT, the area of alveolar bone in the rat fed regular diet was 422.58  $\pm$  11.66 mm<sup>3</sup>. Compared with the control group (547.62  $\pm$  6.53 mm<sup>3</sup>), it is 125.04 mm<sup>3</sup> lower area. As shown Table 2, the rat fed the diet containing *S. thermophilus*, *L. lactis*,

**Table 2.** Alveolar bone mass of periodontitis induced rat fed various diet

Group	Alveolar bone mass (mm <sup>3</sup> )
Control	
Regular diet	547.62 $\pm$ 6.53
Periodontitis-induced rat	
Regular diet	422.58 $\pm$ 11.66
<i>Streptococcus thermophilus</i> diet	450.74 $\pm$ 11.26
<i>Lactococcus lactis</i> diet	483.47 $\pm$ 9.96
<i>S. thermophilus</i> + <i>L. lactis</i> diet	525.13 $\pm$ 6.39

Values are presented as mean $\pm$ standard deviation.

and both probiotics significantly showed higher area of the alveolar bone ( $450.74 \pm 11.26$ ,  $483.47 \pm 9.96$ , and  $525.13 \pm 6.39 \text{ mm}^3$ , respectively). When 3D image of alveolar bone was generated, the interdental space reduced in the rat fed the diet containing probiotics compared to the rat fed regular diet (Fig. 1).

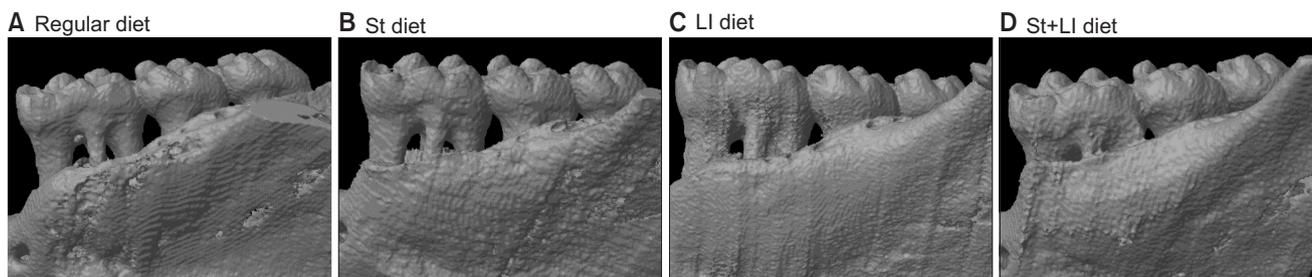
### Investigation of cytokine levels

In ligation-induced periodontitis rat model, the rat fed the regular diet showed the highest level of IL-1 $\beta$  and IL-6 expression. Also, the rat fed the diet containing *S. thermophilus* or *L. lactis* significantly exhibited higher level

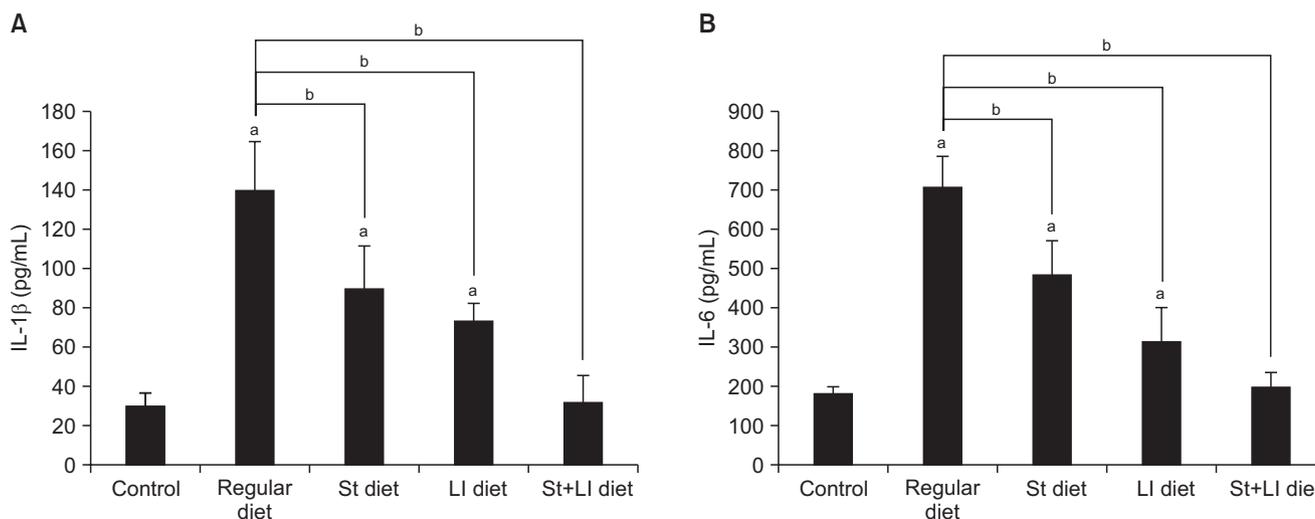
of IL-1 $\beta$  and IL-6 expression compared to control group (non-periodontitis rat) ( $p < 0.05$ ) (Fig. 2). However, comparing the rat fed the regular diet, the levels of IL-1 $\beta$  and IL-6 expression was measured less in the rat fed the diet containing *S. thermophilus* or *L. lactis*. Interestingly, the rat fed the diet containing both probiotics did not show significant difference with control group.

### Analysis of bacteria levels

Periodontitis-induced rat fed various diets in the presence or the absence of probiotics was investigated the levels of *P. gingivalis*, *S. thermophilus*, and *L. lactis* by quan-

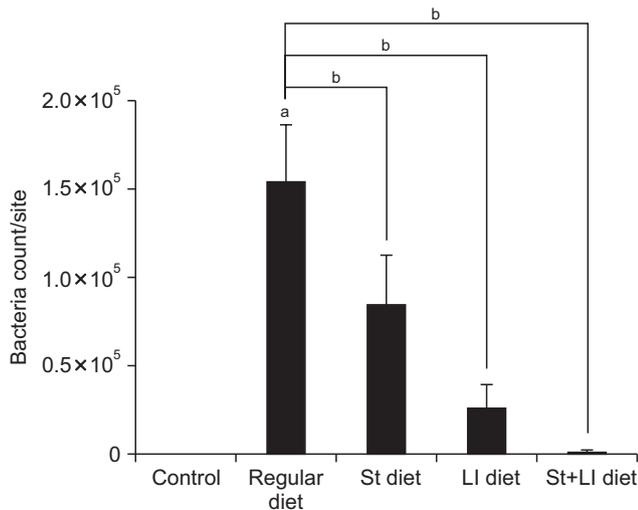


**Fig. 1.** Micro-computed tomography (CT) image of ligature-induced periodontitis rat. The ligature-induced periodontitis rats were fed the diet with *Streptococcus thermophilus* (St diet; B), *Lactococcus lactis* (LI diet; C), and *S. thermophilus*+*L. lactis* (St+LI diet; D) or without probiotics (A), and the ligature site of the rat was analyzed by micro-CT.

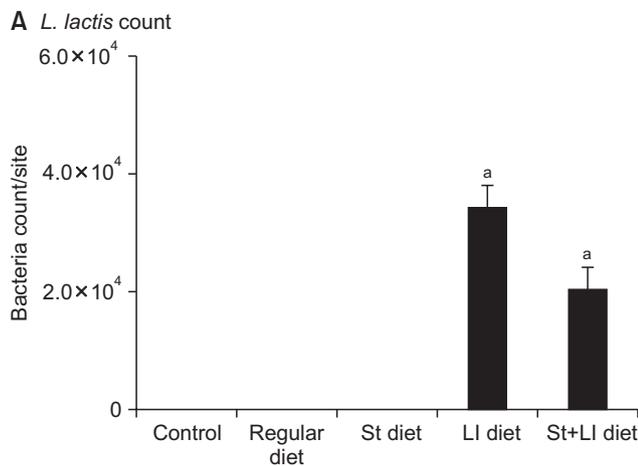


**Fig. 2.** Investigation of inflammatory cytokine production. The ligature-induced periodontitis rats were fed the diet with or without *Streptococcus thermophilus* (St diet), *Lactococcus lactis* (LI diet), and *S. thermophilus*+*L. lactis* (St+LI diet) probiotics, and the gingival crevicular fluid was collected with paper point. The levels of interleukin-1 $\beta$  (IL-1 $\beta$ ; A) and interleukin-6 (IL-6; B) were measured by enzyme-linked immunosorbent assay kit. A letter (<sup>a</sup>) represents significant difference compared to non-ligature control group ( $p < 0.05$ ), and a letter (<sup>b</sup>) expresses significant difference compared to the rat fed regular diet ( $p < 0.05$ ).

titative real-time PCR. As shown Fig. 3, control group was not detected the bacteria, and the levels of *P. gingivalis* in the rat fed diet containing each probiotics were significantly reduced ( $p < 0.05$ ). *P. gingivalis* was detected the lowest level in the rat fed the diet containing both probiotics.



**Fig. 3.** Count of *Porphyromonas gingivalis* on the periodontitis site. The ligation-induced periodontitis rats were fed the diet with or without *Streptococcus thermophilus* (St diet), *Lactococcus lactis* (LI diet), and *S. thermophilus*+*L. lactis* (St+LI diet), and the bacteria was collected with paper point. DNA from the paper point was extracted by a bacterial DNA extraction kit, and the levels of *P. gingivalis* was measured by quantitative real-time polymerase chain reaction. A letter (a) represents significant difference compared to non-ligation control group ( $p < 0.05$ ), and a letter (b) expresses significant difference compared to the rat fed regular diet ( $p < 0.05$ ).



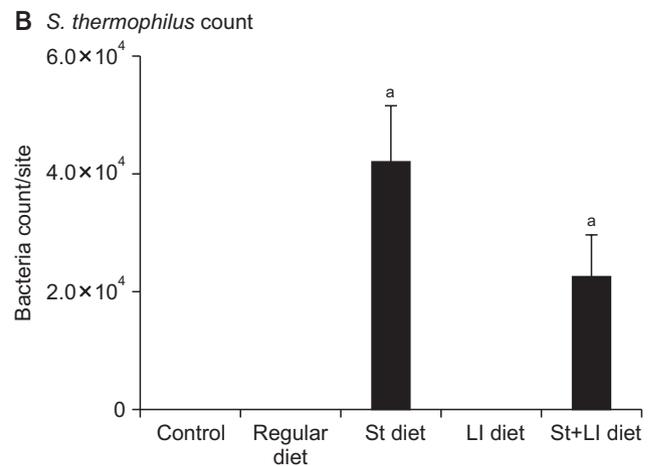
## Investigation of probiotics colonization

Probiotics colonization is important to keep beneficial effects on oral disease. Therefore, probiotics colonization in the ligation site of the rat was investigated. As shown Fig. 4, each probiotics was detected in the group of the diet contain each probiotic bacterium. Especially, *S. thermophilus* and *L. lactis* were detected together with similar levels in the rat fed the diet containing both probiotics. This data indicates that *S. thermophilus* and *L. lactis* can co-exist in oral cavity.

## DISCUSSION

Dysbiosis of oral microbiome by change of the environments induces the disease, such as dental caries and periodontitis [14,15], and epidemiological studies to dental patients suggests that *P. gingivalis*, *T. forsythia*, and *T. denticola* are related with periodontitis [1], and *Streptococcus mutans* is a cariogenic bacterium [16]. Therefore, studies have been performed to efficiently remove these bacteria.

Probiotics have been used to therapy and prevent human diseases [7]. Recently, probiotics have also been tried to apply oral diseases [17], and the antimicrobial activity of various probiotics against oral bacteria [18,19]. Previous studies showed that *S. thermophilus* and *L. lactis* have the antimicrobial activity against periodontopathogens and *S.*



**Fig. 4.** Colonization of probiotics on the periodontitis site. The rats were fed the diet with or without *Streptococcus thermophilus* (St diet), *Lactococcus lactis* (LI diet), and *S. thermophilus*+*L. lactis* (St+LI diet), and the bacteria was collected with paper point. DNA from the paper point was extracted by a bacterial DNA extraction kit, and the *L. lactis* (A) and *S. thermophilus* (B) were measured by quantitative real-time polymerase chain reaction. A letter (a) represents significant difference compared to non-ligation control group ( $p < 0.05$ ).

*mutans* and did not have aciduricity [8,11,16]. Therefore, both probiotics are considered candidate probiotics for dental caries and periodontitis. Next, efficacy of both probiotics for periodontitis was investigated using ligation-induced periodontitis animal model.

Ligature-induced periodontitis rat model by placing the wire inoculated *P. gingivalis* showed high levels of cytokine expression and reduction of alveolar bone mass. Therefore, the rat can be investigated effects of probiotics. When the ligature-induced periodontitis rats were fed the diet in the presence or the absence of *S. thermophilus*, *L. lactis*, and both probiotics, the mass of mandibular alveolar bone showed higher levels in the rat fed the diet containing *S. thermophilus*, *L. lactis*, and both probiotics compared to the rat fed the regular diet. Also, the inhibitory effect on bone resorption exhibited to be large in order of the diet containing *S. thermophilus*, *L. lactis*, and both probiotics. Next, we investigated cytokine expression of the rat in various conditions. Similar to micro-CT data, the expression of the inflammatory cytokines, which was highly induced in the rats fed regular diet compared to control group, were lower in the rats fed the diet containing probiotics. These results indicated that the diet containing probiotics showed a definite therapeutic effect on periodontitis.

Next, to investigate whether the inhibitory effects of probiotics on periodontitis was by *P. gingivalis* elimination or by reduction of inflammation, the count of *P. gingivalis* on the site was examined by real-time PCR. Various studies have suggested that *P. gingivalis* was detected greater frequency and at higher levels at the periodontitis sites, and the certain periodontitis indicators are correlated with the presence or levels of *P. gingivalis* [20–22]. *P. gingivalis* was detected lower levels in the rats fed containing probiotics compared to the rat fed the regular diet. Therefore, the inhibitory effect of *S. thermophilus* and *L. lactis* on induction of periodontitis may be caused by elimination of *P. gingivalis*.

Finally, probiotics colonization is important to keep beneficial effects on infectious disease [23]. Therefore, the rats of each group were investigated and measured probiotics levels by quantitative real-time PCR using specific primers for *S. thermophilus* and *L. lactis*. The primers did not

show non-specific reaction for any oral bacteria (data not shown). *S. thermophilus* and *L. lactis* were detected in the rats fed the diets containing each probiotic bacteria. Interestingly, *S. thermophilus* and *L. lactis* were detected in the rat fed the diet containing both probiotics. Most bacteria compete interspecies to survive and to occupy favorite place. On the basis of the results, *S. thermophilus* and *L. lactis* may not only compete with each other but coexist, and their competition, exclusion, and displacement act only against *P. gingivalis*.

Most probiotics have aciduricity (acid tolerance) which can induce dental caries [24,25]. The acid tolerance of probiotics can be an advantage for intestinal disease, but it can be a disadvantage for oral diseases. Therefore, the application of probiotics for prevention and treatment of periodontitis are careful because of dental caries. However, *S. thermophilus* and *L. lactis* did not have aciduricity [11,16] and show the antimicrobial activity against *P. gingivalis*.

*S. thermophilus* and *L. lactis* reduced cytokine expression and alveolar bone loss and showed the antimicrobial activity against *P. gingivalis* on the ligature site. Furthermore, these probiotics colonized the oral cavity of the rat model. On the basis of these results, *S. thermophilus* and *L. lactis* may be suitable probiotics for therapeutic and preventive periodontitis.

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## CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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## REFERENCES

1. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;25:134-144. doi: 10.1111/j.1600-051X.1998.tb02419.x.
2. Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res* 1994;8:263-271. doi: 10.1177/08959374940080022001.
3. Marsh PD. The commensal microbiota and the development of human disease-an introduction. *J Oral Microbiol* 2015;7:29128. doi: 10.3402/jom.v7.29128.
4. Marsh PD, Do T, Bighton D, Devine DA. Influence of saliva on the oral microbiota. *Periodontol 2000* 2016;70:80-92. doi: 10.1111/prd.12098.
5. Socransky SS, Haffajee AD. Periodontal microbial ecology. *Periodontol 2000* 2005;38:135-187. doi: 10.1111/j.1600-0757.2005.00107.x.
6. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontol 2000* 2002;28:12-55. doi: 10.1034/j.1600-0757.2002.280102.x.
7. Sanders ME. Probiotics: considerations for human health. *Nutr Rev* 2003;61:91-99. doi: 10.1301/nr.2003.marr.91-99.
8. Lee SH, Baek DH. Effects of *Streptococcus thermophilus* on volatile sulfur compounds produced by *Porphyromonas gingivalis*. *Arch Oral Biol* 2014;59:1205-1210. doi: 10.1016/j.archoralbio.2014.07.006.
9. Oh S, Kim SH, Ko Y, Sim JH, Kim KS, Lee SH, Park S, Kim YJ. Effect of bacteriocin produced by *Lactococcus* sp. HY 449 on skin-inflammatory bacteria. *Food Chem Toxicol* 2006;44:552-559. doi: 10.1016/j.fct.2005.08.030.
10. Ali D, Lacroix C, Thuault D, Bourgeois CM, Simard RE. Characterization of diacetin B, a bacteriocin from *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* UL720. *Can J Microbiol* 1995;41:832-841. doi: 10.1139/m95-114.
11. Shin HS, Baek DH, Lee SH. Inhibitory effect of *Lactococcus lactis* on the bioactivity of periodontopathogens. *J Gen Appl Microbiol* 2018;64:55-61. doi: 10.2323/jgam.2017.06.003.
12. Tong Z, Zhou L, Li J, Kuang R, Lin Y, Ni L. An in vitro investigation of *Lactococcus lactis* antagonizing cariogenic bacterium *Streptococcus mutans*. *Arch Oral Biol* 2012;57:376-382. doi: 10.1016/j.archoralbio.2011.10.003.
13. Park CH, Abramson ZR, Taba M Jr, Jin Q, Chang J, Kreider JM, Goldstein SA, Giannobile WV. Three-dimensional micro-computed tomographic imaging of alveolar bone in experimental bone loss or repair. *J Periodontol* 2007;78:273-281. doi: 10.1902/jop.2007.060252.
14. Jiao Y, Hasegawa M, Inohara N. The role of oral pathobionts in dysbiosis during periodontitis development. *J Dent Res* 2014;93:539-546. doi: 10.1177/0022034514528212.
15. Hoare A, Marsh PD, Diaz PI. Ecological therapeutic opportunities for oral diseases. *Microbiol Spectr* 2017;5. doi: 10.1128/microbiolspec.BAD-0006-2016.
16. Kim YJ, Lee SH. Inhibitory effect of *Lactococcus lactis* HY 449 on cariogenic biofilm. *J Microbiol Biotechnol* 2016;26:1829-1835. doi: 10.4014/jmb.1604.04008.
17. Han X, Zhang J, Tan Y, Zhou G. Probiotics: a non-conventional therapy for oral lichen planus. *Arch Oral Biol* 2017;81:90-96. doi: 10.1016/j.archoralbio.2017.04.026.
18. Kim SK, Lee SJ, Baek YJ, Park YH. Isolation of bacteriocin-producing *Lactococcus* sp. HY 449 and its antimicrobial characteristics. *Korean J Appl Microbiol Biotechnol* 1994;22:259-265.
19. Snel J, Marco ML, Kingma F, Noordman WM, Rademaker J, Kleerebezem M. Competitive selection of lactic acid bacteria that persist in the human oral cavity. *Appl Environ Microbiol* 2011;77:8445-8450. doi: 10.1128/AEM.06043-11.
20. Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, Norderyd OM, Genco RJ. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol* 1994;65:260-267. doi: 10.1902/jop.1994.65.3.260.
21. Haffajee AD, Socransky SS, Smith C, Dibart S. Relation of baseline microbial parameters to future periodontal attachment loss. *J Clin Periodontol* 1991;18:744-750. doi: 10.1111/j.1600-051X.1991.tb00066.x.
22. Moore WE, Moore LH, Ranney RR, Smibert RM, Burmeister JA, Schenkein HA. The microflora of periodontal sites showing active destructive progression. *J Clin Periodontol* 1991;18:729-739. doi: 10.1111/j.1600-051X.1991.tb00064.x.
23. Sanders ME. Impact of probiotics on colonizing microbiota of the gut. *J Clin Gastroenterol* 2011;45 Suppl:S115-S119. doi: 10.1097/MCG.0b013e318227414a.
24. Hassanzadazar H, Ehsani A, Mardani K, Hesari J. Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. *Vet Res Forum* 2012;3:181-185.
25. Chan ES, Lee PP, Ravindra P, Krishnaiah K, Voo WP. A standard quantitative method to measure acid tolerance of probiotic cells. *Appl Microbiol Biotechnol* 2010;86:385-391. doi: 10.1007/s00253-009-2384-y.