

Effects of *Streptococcus salivarius* K12 on Experimental Periodontitis and Oral Microbiota in Mice

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How to cite this paper: Zhu, L., Li, H., Yang, X.Q., Xue, L., Li, X. and Du, J.R. (2019) Effects of *Streptococcus salivarius* K12 on Experimental Periodontitis and Oral Microbiota in Mice. *Journal of Biosciences and Medicines*, **7**, 95-111. https://doi.org/10.4236/jbm.2019.712009

Received: October 26, 2019 Accepted: December 3, 2019 Published: December 6, 2019

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Abstract

Background: Periodontal diseases comprise a wide range of inflammatory conditions that affect the supporting structures of the teeth, and may lead to severe periodontal destruction and even tooth loss. Streptococcus salivarius K12 (S. salivarius K12), one of oral probiotics, has been reported to be able to inhibit various potentially deleterious bacteria. This study was the first time to investigate the effects of S. salivarius K12 on ligature-induced periodontitis in mice. Methods: A silk ligature was tied around the second left maxillary molar to establish the model of periodontitis, and then, mice in group S. salivarius K12 were administered with S. salivarius K12 (2×10^9 CFU) twice daily for 30 d, the others were treated with isopyknic water. Results: Administration with S. salivarius K12 markedly decreased the anaerobic bacteria accumulation on the teeth, and it also considerably alleviated periodontitis-induced alveolar bone resorption and attachment loss. Moreover, S. salivarius K12 administration increased the relative species abundance of Gram-positive bacterium in oral cavity while decreased Gram-negative bacterium, as well as the diversity of the bacterial community. Conclusion: Oral administration of the probiotic S. salivarius K12 may mitigate the alveolar bone resorption and attachment loss of periodontitis by modulating the oral microbiota.

Keywords

Alveolar Bone Resorption, Oral Microbiota, Periodontitis, Probiotic, *Streptococcus salivarius* K12

1. Introduction

Periodontitis is an inflammatory disease caused by bacterial plaque, the main

clinical symptoms of which are red and swollen gums, bleeding, periodontal pocket formation, clinical attachment loss, alveolar bone resorption, and eventually tooth loosening [1]. The initiation and propagation of periodontitis is through a dysbiosis of the commensal oral microbiota (dental plaque), which, in general, will first induce gingivitis. If not treated in time, the gingiva, bone and ligament will be severely damaged and eventually develop into periodontitis [1]. In addition to the local lesions of the teeth, periodontal disease may contribute to the body's overall inflammatory burden, worsening conditions such as cardiovascular and cerebrovascular diseases, diabetes mellitus and atherosclerosis [2] [3] [4]. Periodontal diseases are prevalent and affect more than 47% of adults in the United States [5]. In Western Europe, more than 46% of people who are 35 - 44 years old suffered moderate or severe chronic periodontitis. In Eastern European countries, more people are affected by periodontitis (up to 75%) [6].

Despite more than 50 years' development of antibiotics, infectious diseases remain a primary health problem. Continuous exposure to different antibiotics during clinical application has led to the cumulative acquisition of resistant traits in major human pathogens, resulting in the generation of multidrug-resistant bacteria that are virtually impossible to treat [7]. As the antibiotic pipeline dries up, multidrug-resistant bacteria continue to emerge, with pathogenic microorganisms linked to the induction or worsening of many chronic diseases. Thus, new approaches to restore and maintain health are urgently needed. Probiotics are defined by the United States Food and Agriculture Organization and the World Health Organization Working Group (2002) as "Live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [8]. Probiotics have become a hot topic in medicinal research.

Studies have shown that *S. salivarius* as a probiotic is the first colonized bacteria in the oral cavity of infants and is a normal flora in the mouth. S. salivarius participates in the composition of the host's local biofilm barrier and maintain the ecological balance of the host oral flora [9] [10]. S. salivarius K12 is a subspecies of *Streptococcus*, which was originally isolated from the saliva of a healthy child and was shown to produce several megaplasmid-encoded bacteriocin-like inhibitory substances (BLISs), such as Lantibiotics salivaricin A and salivaricin B [11] [12]. Therefore, *S. salivarius* K12 has the antimicrobial activity towards various pathogenic bacteria, it can also compete with pathogens to inhibit its combination with the host and regulate the immune function of the organism [9]. What's more, it is known to be a pioneer colonizer of oral surfaces and its colonization can hinder the adhesion of periodontopathogens due to the competing for nutrients [13] [14]. S. salivarius K12 has been used commercially as a probiotic for more than a decade, and numerous studies have supported its safety [15] [16] [17]. It is shown to have a role in the control of consortia bacterial infections, such as otitis media and halitosis, and also possibly in the reduction of oral candidosis and infections by certain upper respiratory tract viruses. These characteristics indicate that S. salivarius K12 has potential therapeutic effects on oral health [11] [18].

In this study, ligature-induced periodontitis model in mice was adopted to investigate *S. salivarius* K12's protective ability against periodontitis. Its protective effects were reflected by reduced alveolar bone resorption, attachment loss and osteomorphic integrity. These effects may be mediated through the regulation of *S. salivarius* K12 on bacterial microenvironment in the oral cavity.

2. Materials and Methods

2.1. Animals

Male specific-pathogen-free (SPF) C57BL/6 mice, 8 weeks old, were used (Sichuan Provincial People's Hospital Institute of Experimental Animals, Chengdu, China). The mice were housed under a 12 h/12 h light/dark cycle with controlled humidity and temperature. Sterile food and water were available *ad libitum*. All of the experiments were performed in accordance with the ethical guidelines of the Sichuan University Animal Research Committee.

Fifty-four mice were randomly divided into three groups (n = 18/group): control group (Con; mice without experimental periodontitis [EP], untreated with *S. salivarius* K12), EP group (mice with EP, untreated with *S. salivarius* K12), and *S. salivarius* K12 group (mice with EP, treated with *S. salivarius* K12). On day 0 of the experiment, silk ligature was placed around the second maxillary molar in the EP group and *S. salivarius* K12 group.

2.2. Ligature-Induced Periodontitis

A 5 - 0 silk ligature was tied around the second left maxillary molar to establish the model of periodontitis. Briefly, 5 - 0 silk suture was firstly passed through interdentium between first molar and second molar. Then, the suture was passed through interdentium between second molar and third molar. Finally, the suture was tied firmly using a triple-knot. The entire ligature process was performed as gently as possible to avoid secondary injury to the periodontium. The ligatures were inspected once weekly and repositioned if necessary to maintain the ligature in place throughout the experimental period. The contralateral molar remained unligated and served as a baseline control for bone height measurements [19].

2.3. Probiotic Treatment

The probiotic solution (BLIS K12 [*S. salivarius*] oral probiotic, Nature's Trove, LLC Toms River, NJ, USA; GMP NSF-certified facility and Kosher-certified) contained only *S. salivarius* K12 and inactive ingredients, including Sorbitol, Mannitol, Croscarmellose sodium, Silicon dioxide, Vegetable stearic acid and natural cherry flavor. Next day after ligaturing, mice in group *S. salivarius* K12 were administered with *S. salivarius* K12 (2×10^9 CFU). Probiotic solution was prepared by crushing probiotic tablets and dissolving each tablet (containing 1×10^9 CFU/tablet of *S. salivarius* K12) in 200 µl purified water, and then applied directly to periodontitis lesion with a micropipette twice daily for 30 days. The *S. salivarius* K12 group were administered with 200 µl *S. salivarius* K12 solution each time (supplied in three divided doses (about 70 μ l)). After administration, food and water were unavailable within the next 30 min to keep the probiotics in mouth as long as possible. The Con group and EP group were treated with isopyknic water as placebo. The other conditions in the Con and EP groups were the same as those in the *S. salivarius* K12 group. All of the mice were sacrificed 24 h after the last administration.

2.4. Determination of Bacterial Accumulation

One day after the last treatment, the mice were anesthetized by 4% chloral hydrate (i.p.). The ligatures were removed from the second molars and gently washed with phosphate-buffered saline (PBS) to remove food residue and other debris. The ligatures were then placed in Eppendorf tubes with 1 ml of PBS and the bacteria on the ligature were extracted by vortex mixing for 2 min at 3000 rotations per minute [19]. The bacterial suspension was diluted and inoculated on a blood agar plate at 37°C. After 5 days of anaerobic culture, the living bacterium are predominantly (including facultative anaerobe) anaerobes and the colony forming units (CFU) of living bacterial in each group was calculated [19]. The colony forming unit (CFU/ml) were normalized by dividing CFU by the length (in millimeters) of the corresponding ligature [19].

2.5. Bone Loss Measurement

Mice were sacrificed by perfusing ice-cold saline after removing its ligature. The maxilla was detached and soft tissue was removed. The maxilla was fixed in 4% paraformaldehyde for 48 h and then stained with 1% methylene blue [20]. Periodontal bone heights were assessed under a Nikon SMZ1270 microscope (Tokyo, Japan) using a 40× objective. The periodontal bone height was measured from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC). The measurements were performed on the palatal and buccal surfaces of the maxillae as described previously [19]. Five sites of the first molar, three sites of the second molar, and the palatal or buccal cusp of the third molar were evaluated. To calculate the total bone loss, the six-site total CEJ-ABC distance on the ligated side was subtracted from the six-site total CEJ-ABC distance on the contralateral unligated side of the same mouse [19].

2.6. Histomorphometric Analysis

The maxillae were decalcified in 10% ethylenediaminetetraacetic acid (pH 7.2) at 4°C for approximately 4 weeks. The decalcification was accomplished when the needle of a syringe could insert unimpededly into the bone tissue around the teeth. The decalcified maxillae were dehydrated in sequentially increased concentration of ethanol. After that, the maxillae were embedded in paraffin according to standard methods and cut into 4-mm-thick mesio-distal serial sections. Hematoxylin eosin (HE) staining and tartrate resistant acid phosphatase (TRAP) staining were performed with the Hematoxylin-Eosin/HE Staining Kit (G1005, Wuhan Sercicebio Technology, Wuhan, China) and Acid Phosphatase

Leukocyte Kit (G1050, Wuhan Sercicebio Technology, Wuhan, China), respectively.

A panorama of the maxillae, sections of the furcation area of the first maxillary molar, and sections of the alveolar bone between the first molar and second molar were captured by light microscopy (Nikon Eclipse CI, Nikon DS-U3, Tokyo, Japan) [21]. Similar anatomical locations were selected for quantitative measurements in order to avoid the deviation. The distance between the first and second molar was measured of the enamel from the first molar to the second molar at 200× magnification. And the distance of CEJ-ABC, that is, the vertical distance from the midpoint of the two molar enamel to the alveolar crest, was measured at 200× magnification. Multinuclear TRAP-positive cells (*i.e.*, osteoclasts) in the interproximal bone and the furcation of the maxillary first molar were counted at 200× magnification and osteoclast density expressed as number of osteoclasts per mm² (IPP).

2.7. 16S Ribosomal RNA Gene Sequencing

Saliva, obtained from the oral cavity of all mice, were collected by sterile cotton swabs and stored in sterilized equipment at -80°C. Genomic DNA was extracted using a DNA extraction kit (Sangon, Shanghai, China) according to the manufacturer's instructions. DNA quantification was performed using a Nanodrop 2000 device (Thermo Scientific, Waltham, Massachusetts, USA). Based on the measured concentration, DNA was diluted to 1 ng/ml with sterile water. 16S rirRNA/ITS genes bosomal RNA (rRNA)/18S of distinct regions (16SV4/16SV3/16SV3-V4/16SV4-V5, 18S V4/18S V9, ITS1/ITS2, and Arc V4) were amplified using specific primers with a barcode. All of the polymerase chain reactions (PCRs) were performed with Phusion High-Fidelity PCR Master Mix (New England Biolabs). PCR products were divided by electrophoresis and samples with bright main strip between 400 - 450 bp were isolated and purified for further analysis. Sequencing libraries were generated using a TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, California, U.S.) The library was then sequenced on an Illumina HiSeq 2500 platform, and 250 bp paired-end reads were generated. Paired-end reads from the initial DNA fragments were merged using FLASH [22]. Sequence analysis was performed using UPARSE software. Sequences with \geq 97% similarity were assigned to the same operational taxonomic units (OTUs). To understand the diversity and composition of microbial community of the samples, alpha diversity (e.g. richness), beta diversity (weighted and un-weighted UniFrac, namely, principal component analysis) and relative abundance profiles of microbial community were further analyzed.

2.8. Statistical Analysis

The data were analyzed using Student's t-test for comparison of two groups, one-way analysis of variance (ANOVA) followed by LSD test and Tamhane's T2 test for multiple groups and KruskalWallis tests when the variance is uneven. The data expressed as mean \pm SEM. SPSS 20.0 software was used for the statistical analyses. Values of p < 0.05 were considered statistically significant.

For 16s rRNA sequencing, Paired-end reads were merged using FLASH [23]. Quality filtering on the raw tags was performed according to the QIIME (V1.7.0) [22]. The Effective Tags obtained by the UCHIME algorithm [24]. Sequences analysis was performed by Uparse software (Uparse v7.0.1001) [25]. For each representative sequence, the GreenGene Database [26] was used based on RDP classifier (Version 2.2) [27]. Multiple sequence alignment was conducted using the MUSCLE software (Version 3.8.31) [28]. Alpha diversity and Beta diversity were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

3. Results

3.1. S. salivarius K12 Relieves Ligature-Induced Bone Resorption

The appearance of tissue loss and deep periodontal "pocket" are the hallmark of chronic periodontitis. Exposure of the cementum (the surface layer of the root) and resorption of the lacuna were induced in the EP group (Figure 1(A)), while the administration of S. salivarius K12 could significantly alleviate those symptoms, such as the attachment loss and the alveolar bone resorption (Figure 1(A)). Similar results could be seen in the change of bone height (Figure 1(B)), as we could see, very slight changes in bone height were observed at three sites in the mesial region. The other six sites on the palatal side and three sites on the buccal side in the S. salivarius K12 group presented significant decrease in alveolar bone resorption (Palatal surface: P Cusp: p = 0.000, F = 80.715; D-P Cusp: p = 0.000, F = 55.817; P Groove: p = 0.000, F = 81.996; M-P Cusp: p = 0.000, F = 78.642; D Cusp: p = 0.000, F = 39.190; D-P Groove: p = 0.010, F = 23.144; Buccal surface: B Cusp: p = 0.038; D-B Cusp: p = 0.021; B Groove: p = 0.024; Figure **1(B)** compared with the EP group. In addition, alveolar bone loss on the palatal side was generally more severe than that on the buccal side in both the S. salivarius K12 group (0.383 mm vs. 0.135 mm) and EP group (0.664 mm vs. 0.301 mm) compared with the Con group (Figure 1(C)).

3.2. S. salivarius K12 Reduces Local Accumulation of Anaerobes

To determine the local longitudinal accumulation of anaerobically-grown bacteria in the ligature, bacteria were extracted from the sutures and counted under anaerobic conditions. After 30 d of treatment with *S. salivarius* K12, anaerobic bacteria in the *S. salivarius* K12 group decreased by 75% compared with the EP group (7×10^4 vs. 28×10^4 CFU/mm, p = 0.002, Figure 2), which means that the releasing of harmful substances by pathogens may also be controlled.

3.3. *S. salivarius* K12 Alleviates Ligature-Induced Teeth Pathological Changes

To gain further insights into the modulation effects of S. salivarius K12 on



Figure 1. The effect of *S. salivarius* K12 on bone morphology in mice. (A) Representative images of bone loss of maxillae both in the palatal and buccal surface. (B) Distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) at eighteen predetermined sites in the maxilla. Negative values of the buccal surface indicate bone loss relative to their corresponding baselines. p < 0.05, p < 0.01, p < 0.001, *vs.* EP group. (C) Total bone loss on the palatal surface and buccal surface. The data are expressed as mean \pm SEM. n = 10.

periodontitis, the pathological status of teeth were investigated through different method. The HE staining result showed dramatically different teeth morphology among the Con group (Figure 3(A), Figure 3(D), Figure 3(G)) and the EP group (Figure 3(B), Figure 3(E), Figure 3(H)), the *S. salivarius* K12 group (Figure 3(C), Figure 3(F), Figure 3(I)). Compared with the Con group, the EP group showed a greater CEJ-ABC distance (p = 0.000, F = 272.333, Figure 3(K)), while the administration of *S. salivarius* K12 narrowed the distance from CEJ to ABC and the distance between the first and second molars (p = 0.000, F = 275.350, Figure 3(J); p = 0.000, F = 272.333, Figure 3(K)). Furthermore, *S. salivarius* K12 notedly alleviated the ligature-induced atrophy and necrosis of the



Figure 2. The effect of *S. salivarius* K12 on the amount of anaerobic bacteria. Bacteria were extracted from the sutures after placement in placebo- or *S. salivarius* K12-treated mice and cultured for 5 days. To determine anaerobic growth and quantify CFU, serial dilutions of bacterial suspensions were plated on blood agar plates. Each symbol represents an individual sample. The data are expressed as mean \pm SEM. n = 6. "p < 0.01.



Figure 3. The effect of *S. salivarius* K12 on pathological state of teeth. (A-C) Representative histologic images of sections of the palatal surface. (D-F) Topical examination of the interproximal area between the first and second maxillary molars. (G-I) Topical examination of the furcation area of the first maxillary molar. (J) Distance between the first and second molar, and (K) distance of CEJ-ABC. *p < 0.05, **p < 0.01, *vs.* EP group. Blue arrows, alveolar bone crest. Turquoise arrows, distance from CEJ to ABC. $40\times$ magnification in A, B and C. $200\times$ magnification in D, E, F, G, H and I. Scale bar = 100μ m. The data are expressed as mean ± SEM. n = 3.

sulcular epithelium and resorption of alveolar bone crest and intrinsic cementum. In the furcation area of the first maxillary molar, severer bone resorption could also be seen in the EP group compared with the S. salivarius K12 group and Con group (Figures 3(G)-(I)).

Tartrate-resistant acid phosphatase (TRAP) is a specific marker enzyme of osteoclasts. Through the TRAP staining of the teeth, the alveolar bone resorption and the expression of osteoclasts were observed. As shown in Figure 4, ligature placement significantly increased bone resorption and osteoclast density, while the administration of S. salivarius K12 notably reduced bone resorption and osteoclast density (Figure 4(A) and Figure 4(B)). In addition, TRAP-positive cells were counted in the interproximal bone (Figure 4(C)) and furcation of the maxillary first molar (Figure 4(D)). The amount of osteoclast in the S. salivarius K12 group was $1.39/\text{mm}^2$ in interproximal bone (p = 0.004, F = 24.642) Figure 4(C)) and $1.85/\text{mm}^2$ in furcation of the maxillary first molar (p = 0.012, F = 27.770, Figure 4(D)), both lower than those in EP group (2.80/mm² and 2.61/mm², respectively).

3.4. S. salivarius K12 Changes the Composition of Oral Bacterial **Community**

The bacterial in saliva was analyzed using 16s rRNA gene sequencing technology



Figure 4. The effect of S. salivarius K12 on bone resorption caused by osteoclasts. Bone tissue slices were performed with TRAP staining, and representative images of (A) interproximal bone and (B) furcation of the maxillary first molar. 100× magnification in a, b, c and 400× magnification in d, e, f. Scale bar = 100 μ m. TRAP-positive cells in the interproximal bone (C) and furcation of the maxillary first molar (D) were counted at 200× magnification. Osteoclast density was defined as active osteoclast count around the interproximal bone divided by the total area of the interproximal bone, so was the furcation of the first molar. The data are expressed as mean \pm SEM. n = 4. *p < 0.05 and **p < 0.01 vs.

EP group.

to explore the possible mechanism of *S. salivarius* K12 on periodontitis. The analysis of 16s rRNA gene copy number revealed marked differences between the EP and *S. salivarius* K12 group, the copy number of the *S. salivarius* K12 group was 3.9×10^4 copies/ng DNA and the EP group was 4.5×10^4 copies/ng DNA (p = 0.025, F = 6.021, Figure 5(B)).

According to the sequencing results, the content of gram-positive bacteria, such as *Lactobacillus, Streptococcus et al.*, in the Con group and the *S. salivarius* K12 group was 30%, which was much higher than 13% in the EP group. While the content of gram-negative bacteria, such as *Pseudomonas, Paracoccus, Escherichia-Shigella et al.*, in the EP group was 75.4%, which was much higher than 45.8% in the Con group, whereas *S. salivarius* K12 dramatically decreased its content to 30% (**Figure 5(C)**).

3.5. *S. salivarius* K12 Modulates the Microbial Community Diversity

The microbial diversity within each group (*a*-diversity) was assessed by calculating the Chao 1 index, and there was a significant decrease in *S. salivarius* K12-treated periodontitis mice compared with that in EP group (280.4 vs. 369.2, **Figure 6(A)**), which means the diversity of communities in group *S. salivarius* K12 has reduced.

Principal component Analysis (PCA) was utilized to assess the dissimilarity of individual microbial communities between different groups (β -diversity). The more similar the colony composition of samples, the closer their distances on the PCA map were, thereby demonstrating the validity of the experimental grouping design. In the present study, PCA plotting of weighted β -diversity dissimilarity matrix data, revealed a distinct clustering effect on mice in each group (**Figure 6(B)**), as well as the un-weighted β -diversity PCA dissimilarity matrix data (**Figure 6(C)**).

LEfSe analysis was used to find species with significant difference between groups. When the LDA value was 4, LEfSe could identify significant differences between the EP and *S. salivarius* K12 group. It can be clearly seen from **Figure 6**(**D**) that the amount of streptococci in the *S. salivarius* K12-treated group was much higher than that of the EP group.

4. Discussion

Plenty of evidence has suggested that the colonization of probiotics in biofilms can protect against oral disease [8] [29]. The most famous probiotics, *Lactobacil-li* and *Bifidobacteria*, have shown obvious advantage in both clinical trials and preclinical studies. However, according to some studies, they may have difficulty in colonizing the mouth [30]. Facing these problems, seeking for substitutes of conventional probiotics is a necessity. *Streptococci* is known to be a pioneer co-lonizer of oral surface which would predominantly occupy the biofilm. Teughels *et al.* demonstrated that some species of *Streptococci* prominently inhibited periodontopathogens *A. actinomycetemcomitans* from the colonization of



(A)



Figure 5. The effect of *S. salivarius* K12 on the composition of oral microbiome. (A) Phylogenetic tree and (B) qPCR analysis of DNA isolated from saliva to obtain copy numbers of total 16s rRNA. (C) Diversity of microbial composition based on quality-controlled OTU reads. The colors that correspond to the genus are the same as in panel A. (D) Corresponding taxa heat map. The data are expressed as mean \pm SEM. n = 8. p < 0.05.



Figure 6. The effect of *S. salivarius* K12 on the community diversity of oral flora. (A) *a*-Diversity analysis of the sequencing data using the Chao 1 index. Principal component analysis of (B) unweighted and (C) weighted β -diversity, illustrating distinct alterations of microbial diversity in the *S. salivarius* K12 group. The percentage of data variance explained by each PC is displayed. The data are presented as X/Y scatter or mean ± SEM. (D) LDA value distribution. Red, taxa in EP group. Green, taxa in *S. salivarius* K12 group. The data are expressed as mean ± SEM. *n* = 8. *p < 0.05.

epithelial cells *in vitro* [31]. Van Hoogmoed CG and co-workers found that pre-adhering of *Streptococcus* strains successfully antagonized adhesion of *P. gingivalis* [32].

S. salivarius K12 has been marketed internationally by the New Zealand Company BLIS Technologies Ltd (Dunedin, New Zealand) and consumed as a probiotic for more than a decade [16] [17]. Importantly, its safety and tolerance have been well demonstrated in clinical studies. Furthermore, *S. salivarius* K12 has shown therapeutic effects on many pathogens-related diseases, such as oral malodor, secretory otitis media, pharyngotonsillitis and so on [17] [32] [33] [34]. Periodontitis share some common pathogens with those infective diseases [33]. All of the connections indicated that *S. salivarius* K12 might prevent or alleviate periodontitis.

Periodontitis is characterized by inflammatory destruction of alveolar bone and connective tissues that surround and support the teeth [4] [35] [36]. In this experiment, the second molar of mice ligated with silk thread exhibited obvious alveolar bone loss, ligament deficiency and tooth root exposure. Destroyed tissue integrity of teeth, in addition to the increased CEJ-ABC distance indicated the successful establishment of a periodontal model. While the *S. salivarius* K12-treated mice showed integrated periodontal tissues, complete alveolar bone morphology, and significantly reduced CEJ-ABC distance, which means that *S. salivarius* K12 could alleviate the lesion of experimental periodontitis to a large extent.

Dysbiotic microbial communities consisting predominantly of anaerobic bacteria are heavily involved in the initiation and progression of periodontitis [36] [37]. In fact, the transition from health to periodontitis in humans correlates with an increased microbial burden [38] [39]. In the present study, ligature-induced murine periodontitis can cause local accumulation of bacteria around the teeth, and the accumulation of a large number of pathogenic bacteria in turn accelerates the development of periodontitis. The microbiological analysis of recovered sutures revealed that topical treatment with *S. salivarius* K12 indeed affected the anaerobic microbiotas. Specifically, *S. salivarius* K12 treatment reduced the amount of anaerobic bacteria compared with the EP group. Although *S. salivarius* K12 itself belongs to facultative anaerobes, the total amount of anaerobes (including facultative anaerobes) in the *S. salivarius* K12 group still reduced by 75%, which indicated an obvious inhibitory effect of *S. salivarius* K12 on other anaerobes.

Additionally, the amelioration of the pathological state of mice teeth were observed in the *S. salivarius* K12 group, including sulcular epithelium repair, restoration of alveolar bone and cementum. In the course of bone resorption, active osteoclasts will decompose and absorb bone matrix, causing the bone mass in this area to decrease. The expression of TRAP-positive multinucleated osteoclasts in the teeth of mice treated with *S. salivarius* K12 was significantly reduced, suggesting that the effects of *S. salivarius* K12 on periodontitis were remarkable.

The relative abundance of bacterial species is often related to its function. In the healthy oral cavity, there is a relatively coordinated, dynamic and stable micro-ecology. Imbalance of oral flora will induce chronic inflammatory, such as periodontitis. Since periodontitis is an infective disease that is predominantly caused by gram-negative anaerobes, the proliferation of those bacteria in periodontium results in a host inflammatory response and then leads to alveolar bone loss, which is the most basic phenotypes of periodontitis [38] [40] [41]. In this study, after evaluating the therapeutic effects of S. salivarius K12 on periodontitis, we envisaged the possible mechanism of action may be achieved by the adjustment of oral microbiota and its composition. 16s rRNA sequencing technology was used to analyze salivary flora to explore the possible mechanism, and the results showed that the amount of each kind of bacteria in the three groups was different, but the relative abundance of gram-positive anaerobes in the Con and S. salivarius K12 group were markedly higher than that of the EP group, while the relative abundance of gram-negative anaerobes decreased. This explained in a certain sense that S. salivarius K12 did have a regulatory effect on the oral flora.

Combining with the analysis of microbial diversity, we found that the bacterial diversity of *S. salivarius* K12-treated mice was dramatically reduced compared with the EP group in this study, which was consistent with the results of Griffen *et al.* and GE *et al.* [38] [42]. Griffen *et al.* found that the community diversity of the periodontal health group was significantly lower than that of chronic periodontitis group. The results of β -diversity then demonstrated the validity of the grouping design in this experimental. Collectively above data highlighted that microbial diversity and total abundance were altered by treatment of *S. salivarius* K12 in ligature-induced periodontitis mice.

However, there is one thing to point out, *Lactobacillus, Streptococcus* and *Bi-fidobacterium* are generally considered as beneficial bacteria, In EP group, the relative abundance of *Lactobacillus* and *Streptococcus* decreased as expected, while *Bifidobacterium* increased a lot. Besides, in *S. salivarius* K12 group, al-though *S. salivarius* K12 treatment reduced the amount of almost all gram-negative bacteria, it inhibited some beneficial bacterial, such as *Lactoba-cillus*. That is to say, the introduction of *S. salivarius* K12 didn't recover the oral microbiota to its original homeostasis, instead, it led the oral microbiota to another constitution. In fact, the oral environment is a homeostasis including both probiotics and pathogens. The efficacy of probiotic therapy on oral disease, sometimes, cannot be illustrated by changes in a single strain, instead, it should be based on changes in the entire microbial environment. This is the charm that microbiology possesses, and it is also a difficult point in this filed.

It should be admitted that the complex interactions between bacteria have yet to be further studied. As far as the current technology is concerned, the RNA sequencing technology can only be accurate to the genus level. If one day, it can be accurate to species or even strains, the authors believe that it would be useful to analyze the role of probiotics on oral diseases.

5. Conclusion

In conclusion, *S. salivarius* K12 does have beneficial effects on preventing and alleviating periodontitis, and it may be achieved by adjusting the proportion of gram-positive and gram-negative bacteria in the oral cavity. In short, *S. saliva-rius* K12 will have good prospects as an adjuvant for nonsurgical periodontal therapy.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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