Probiotic effect of *Bacillus subtilis* tablets on periodontopathic oral bacteria

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Abstract

Bacillus subtilis is an effective probiotic material for the prevention of enteric infections in both humans and animals. However, few products containing probiotic materials are available for the treatment of patients with periodontitis. Here, we evaluated the therapeutic effects of a tablet [VITALREX™ (VL)] containing *B. subtilis* DB9011 for patients with periodontitis. We performed a randomized, double-blind, placebo-controlled study with two parallel treatment groups made up of patients with chronic periodontitis. The subjects were randomized into two groups to receive either test (VL group, n=27) or placebo [dexstrine (DX) group, n=27] treatments for 30 days. The VL group displayed a significant change in mean BANA-test scores (mean score±standard deviation) between baseline (day 0) and day 30 (1.52±0.51 and 0.22±0.51; P<0.01), whereas no marked changes were observed for the DX group. VL treatment also resulted in a significant reduction in the Gingival Index, whereas Probing Pocket Depth and Bleeding on Probing only showed small improvements. Polymerase chain reaction analyses also demonstrated that oral VL tablets significantly reduced periodontopathic oral bacteria compared with DX tablets. Taken together, these results suggest that *B. subtilis* DB9011 tablets are an effective oral probiotic material for patients with periodontitis.

Introduction

Periodontitis is a chronic inflammatory disease of the tissues supporting the teeth and can be caused by multiple factors, including oral bacteria and the host immune system.[1] Currently, there is no definitive treatment for periodontitis. The most important initiation and progression of periodontal treatment is elimination of periodontopathic bacteria from the subgingival area. In general, bacteria are identified as periodontopathic are *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythis, Prevotella intermedia, Prevotella nigrescens, Campylobacter rectus, Eikenella corrodens, Aggrigatibacter actinomycetemcomitans, Capnocytophaga* species, and more than 300 different bacterial types. General treatments for this disease consist of surgical methods, such as scaling, root planning, and periodontal surgery, and combined surgical and chemical methods that include the use of antibacterial drugs, particularly amoxicillin, tetracycline, and azithromycin.[2,3] However, the use of antibacterial drugs as a conventional treatment method is associated with a number of risks, including the production of drug-resistant bacteria and allergic reactions in elderly individuals.[4] There is, therefore, a need for alternative materials for the treatment of periodontitis.

A few studies have shown that the use of oral probiotic tablets is effective against periodontopathic bacteria and plays an important role in the management of periodontitis patients.[5-7] However, a number of issues remain to be clarified, including whether probiotic tablets, commonly used for targeting the gastrointestinal tract, require modification or additions when used as an oral probiotic.[8]

Bacillus subtilis is an effective probiotic bacterium for the prevention of enteric infections in both humans and animals.[9,10] In a previous study, we demonstrated that rinsing with Extraction 300-E™ (E300; the supernatant of cultured medium of *B. subtilis* DB9011, a strain isolated from Japanese soil) significantly reduced periodontal pathogens compared with conventional mouth rinse (containing with benzethonium chloride), resulted in marked changes in BANA scores.[11] However, as E300 is the supernatant fraction of culture medium, it is difficult to preserve for long periods after its initial preparation. The commercial product VITALREX™ (AHC Co., Gunma, Japan) is a stable oral tablet prepared from lyophilized *B. subtilis* DB9011,[12] and has been used for the prevention of enteric infections in livestock and the control of oral health in humans.[13] VITALREX™ (VL) contains 5 ´ 10^9 colony forming units (CFU) of *B. subtilis* DB9011 per tablet, a level that purportedly improves human immunological activity, as *B. subtilis* has been shown to activate macrophages and natural killer cells, stimulate the production of immature leukocytes, and induce interferon.[14,15]

This is the first study to evaluate whether the oral administration of *B. subtilis* DB9011 tablets could reduce the levels of periodontopathic bacteria. As this bacterial strain may provide protection against infections caused by oral pathogens, the effect of VL on periodontal inflammation was examined in chronic periodontitis patients.

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Key words: *Bacillus subtilis*, periodontitis, probiotics, tablet.

Acknowledgements: the authors thank Drs. Nobuyuki Tomii, Toshimune Iizuka, and Hiromi Shimomura for helpful and critical discussions.

Contributions: the authors contributed equally to this work.

Conflict of interests: the authors report no conflict of interests.

Received for publication: 31 October 2011.
Revision received: 28 May 2012.
Accepted for publication: 11 June 2012.

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Microbiology Research 2012; 3:e23
doi:10.4081/mr.2012.e23

Materials and Methods

Patients and study design

From January 2010 to February 2011, 54 subjects (26 male and 28 female; non-smokers; age 44-62 years; mean age 52.7 years) with chronic periodontitis and no other severe general diseases were selected to participate in this study. Inclusion criteria for applicants were: i) having at least 20 natural teeth; and ii) at least eight sites with probing periodontal pocket depth (PD) over 4 mm on molar teeth. Exclusion criteria included the presence of teeth with excess mobility and/or abscess formation. All participants were selected from patients referred to the authors' clinics. The study protocol was approved by the committee of Ethics Affairs of The Nippon Dental University and was conducted according to the principles outlined in the Declaration of Helsinki for experiments involving human subjects. The subjects provided written informed consent before study start and met the following criteria: i) had received initial periodontal treatment, such as dental cleaning; ii) had taken no probiotic supplements within the previous month; and iii) had taken no antibiotic drugs within the previous month. Dental cleaning was used as dental prophylaxis to remove all supragingival plaque, stain,

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and calculus before the previous study. After the start of this study, none of the participants have had professional prophylaxis or additional brushing, such as use of an ultrasonic or power toothbrush.

A randomized, double-blind, placebo-controlled study design with two parallel groups was adopted. The subjects were randomized into two groups to receive either test (VL n=27) or placebo [dexstrine (DX) n=27] treatments according to gender, age, and clinical features at baseline (Table 1).

B. Subtillis DB9011 (5×10^9 CFU) in VL tablet are an immobilized eternity as the spore condition is freeze-dried, and these spores are stable for a long time until the tablet dissolves.

B. subtilis DB9011 is not active in the tablets until they dissolve (*i.e.* dissolved in saliva), after which *B. subtilis* DB9011 proliferation and metabolism is stimulated.

VL tablet consists of dexstrine made up of 80% maltitol, 20% microcrystalline, and an effective ingredient such as *B. subtilis* DB9011. Therefore, we used DX as placebo. Subjects were instructed to self-administer tablets by placing one in the mouth and allowing it to dissolve without chewing. The two groups took the respective VL or DX tablets twice daily (once in the morning and once in the evening after meals) for 30 days. Participants were asked to follow their usual dietary habits and brush their teeth three times a day without using toothpaste. None of the participants received any dental treatment during the study. Furthermore, participants were prohibited from using any other commercial probiotic products during the study period. Professional prophylaxis and additional brushing instructions were not given until completion of the experiment.

Four anterior or premolar teeth with a PD over 4 mm, and similar probing depths and gingival inflammation levels were selected for assessment of clinical parameters and subjected to microbial tests. All participants were asked to perform 3-minute brushing after meals without using any toothpastes or mouth rinses and probiotic supplements during the study period. We distributed standardized tooth brushes to all participants.

Clinical parameters

Probing Pocket Depth (PPD), Bleeding on Probing (BOP), and the Gingival Index (GI) were assessed for all participants at baseline (day 0), two weeks (day 14) , and one month (day 30) after subgingival scaling and root planing (SRP). Each examiner performed all measurements on six sites per tooth, excluding third molars. PPD and BOP were assessed using a manual periodontal probe (CP10SE; HuFriedy, Chicago, IL, USA). For assessing PPD, a periodontal probe equipped with a 0.5-mm diameter tip was inserted into the gingival crevice and swept from the distal to mesial aspect of the tooth at a depth of approximately 1 mm and an angle of approximately 60° while maintaining contact with the sulcular epithelium. BOP was then recorded and scored as present/absent by running the probe 1-2 mm into the gingival crevice. Gingivitis of the buccal and lingual marginal gingival, and interdental papillae of all scorable teeth was scored using the Loe-Silness GI1 on a 4-point scale from 0 (absence of inflammation) to 3 (severe inflammation).[16]

Bacterial assay

The BANA test (Knowell Therapeutic Technologies Inc., Toronto, ON, Canada) is a chair-side diagnostic system that is highly sensitive and specific for determining the presence of *red-complex* periodontal pathogens (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythis*).[17] These bacteria have been implicated in periodontal disease indication and progression. The products of this reaction can be demonstrated by the color reaction of a blue-black product on a reagent strip. Plaque was collected for BANA analysis from the same tooth sites that were used to assess the clinical parameters at days 0, 14 and 30. After incubation of plaques samples for 5 min at 35°C with Evan's black dye solution, naphthylamine, which is released as a result of the presence of any BANA-hydrolyzing bacterial species, diffuses to form a permanent blue-black color. The relative intensity of the formed blue color (strong positive, positive, or negative) was assessed.

Saliva and dental plaque sampling

After measuring clinical parameters, saliva and dental plaque samples were obtained. Unstimulated whole saliva was collected into sterile 10 mL plastic tubes. Supragingival plaque samples were collected with sterile curette scalers and suspended in 1 mL sterile distilled water. After thorough removal of supragingival plaque with sterile cotton pellets, three sterile paper points were inserted into the gingival sulcus until resistance was felt. After 30 s, all paper points were removed and pooled in 1 mL sterile distilled water. Supra/subgingival plaque samples were suspended by vortexing and then stored at -20°C until use.

Polymerase chain reaction

Saliva and dental plaque samples were subjected to polymerase chain reaction (PCR) using a mixture of the primers set (0.25 M each) listed in Table 2. Bacterial primer sequences and the predicted sizes of PCR products were tested against the seven strains listed in Table 2, cultured under appropriate con-

Table 1. Distribution of study participants.

Group	No.	Sex	Age, years (mean ± SD)
VL	13	Male	52.0±5.16
	14	Female	51.9±5.87
DX	13	Male	53.3±4.33
	14	Female	53.5±5.77

VL, VITALREX™; DX, Dexstrine; SD, standard deviation.

Table 2. Bacterial primer sequences used for polymerase chain reaction and predicted polymerase chain reaction product sizes.

Bacterium	Genbank No.	Primer sequence (5'-3')	Size (bp)
T. denticola	AJ272339	Forward AAATAATGCCGATTACGGGCTTT Reverse GCCTTCGTTACCCATCGCAA	653
P. gingivalis	D26470	Forward CGAAGTCTTCATCGGTCGTT Reverse GTACCTGTCGGCTTACCATCTT	498
A. actinomycetemcomitans	X16829	Forward GAAGGCGACGACCACTTAGC Reverse GTGCACGATCCTTTTCAGGT	400
P. intermedia	AB017537	Forward CAAAGACGCACGTACCAATC Reverse CTCTGGTGTTGTTTCCTTGCT	262
T. forsythia	AY546489	Forward CTGGAGCAGTCTTGGAATCTG Reverse GCAGCCTGAGTCAGGCTTTTT	168
S. sanguinis	AB056712	Forward GGCGCCTGTTAATACTGAGC Reverse GTTTTTCCATCCTTGAGGATAGC	330
S. salivarius	D29644	Forward CGGTCAAGATAACGTTGACCT Reverse CTGCTACGATACCGTAACGTG	212

ditions. We performed 30 cycles of touch-down PCR (94°C for 45 s, annealing for 45 s, and 72°C for 1 min) followed by a final extension at 72°C for 5 min. The annealing temperature was started from 64°C, touch-downed 1°C per cycle, and set constant once the temperature reached (56°C). PCR samples were loaded onto 2.0% agarose gels (15 L/lane) and visualized by ethidium bromide staining.[18]

Statistical analysis

The VL and DX groups were compared with the mean baseline PD, BOP, GI, and BANA using an analysis of variance and Fisher's protected least significant difference test. All data analysis was performed using Microsoft Office Excel (Windows 7 Professional, Microsoft, Redmond, W, USA). P<0.05 was considered significant.

Results

Fifty-four participants with chronic periodontitis were examined according to the study design. All participants completed the study. The distribution of study participants is shown in Table 1. The mean PPD (mm ± SD) of the study on days 0, 14 and 30 are shown in Table 3. VL showed no demonstrable change on PPD at days 14 and 30 compared with DX, respectively. The BOP scores (score ± SD) at days 0, 14 and 30 are shown in Table 4. VL showed a remarkable reduction in BOP scores on both days 14 and 30 compared with DX. VL tablets were significantly more effective (P<0.05) in lowering BOP scores from baseline (day 0) compared with DX. The mean GI scores (score ± SD) on days 0, 14 and 30 are summarized in Table 5. VL showed a remarkable reduction on day 30 and DX showed a small reduction on day 30, respectively. VL and DX were both significantly more effective (P<0.05) in lowing GI scores from baseline (day 0). The mean BANA scores (score ± SD) on days 0, 14 and 30 are shown in Table 6. VL was significantly more effective on BANA scores between day 0 and 14 (P<0.05), and day 30 (P<0.01), respectively.

The typical results of PCR for 3 participants in the VL group (VL-#1~#3) and DX group (DL-#1~#3) are shown in Figure 1A and B. Amplicons of VL-#1~#3 plaque (P) sample on the baseline (day 0) were Td, Pg and Pi for VL-#1 and Td, Pg, Sang and Pi for VL-#3, respectively. These bands had disappeared by day 30. Amplicons of VL-#1~#3 saliva (S) sample at baseline (day 0) were Td, Pg and Sal for VL#1, Sang and Sal for VL-#2 and Sang and Sal for VL-#3, respectively. Those bands based on cariogenic bacteria had not disappeared by day 30 and were rather clear. The results indicated that VL treatment reduced periodontopathic

Table 3. Mean probing pocket depth of the VITALREX™ and Dexstrine treatment groups.

Group	No.	PPD (mm ± SD) Baseline (Day 0)	Day 14	Day 30
VL	27	4.96±0.71	4.26±0.45	4.37±0.69
DX	27	4.70±0.72	4.26±0.86	4.37±0.79

PPD, probing pocket depth; SD, standard deviation; VL, VITALREX™; DX, Dexstrine. The data were the average of three experiments at each site.

Table 4. Mean bleeding on probing of the VITALREXTM and Dexstrine.

Group	No.	BOP (score ± SD) Baseline (Day 0)	Day 14	Day 30
VL	27	1.56±0.51	0.93±0.62*	0.41±0.57*
DX	27	1.59±0.50	1.52±0.51	1.11±0.51

BOP, bleeding on probing; SD, standard deviation; VL, VITALREX™; DX, Dexstrine. BOP 0: Non-bleeding-slight bleeding: % of sites 0-30; 1: Moderate bleeding: % of sites 31-60; 2: Severe bleeding: % of sites >60. *P<0.05.

Table 5. Mean gingival index scores of the VITALREX™ and Dexstrine

Group	No.	GI (score ± SD) Baseline (Day 0)	Day 14	Day 30
VL	27	2.00±0.62	1.19±0.56	0.59±0.64*
DX	27	1.88±0.75	1.44±0.80	1.33±0.73*

GI, gingival index; SD, standard deviation; VL, VITALREX™; DX, Dexstrine. GI 0: Normal; 1: Slight gingival inflammation (non-bleeding using instrument); 2: Moderate gingival inflammation (bleeding using instrument); 3: Gingival abscess, bleeding. *P<0.05. The data were the average of three-times probing at each site.

Table 6. Mean BANA score (score ± SD) of the VITALREX™ and Dexstrine.

Group	No.	GI (score ± SD) Baseline (Day 0)	Day 14	Day 30
VL	27	1.52±0.51	0.67±0.55 *	0.22±0.51°
DX	27	1.56±0.51	1.44±0.80	1.33±0.62

SD, standard deviation; VL, VITALREX™; DX, Dexstrine. BANA score: 0, Negative; 1, Positive; 2, Strong positive. *P<0.05, °P<0.01.

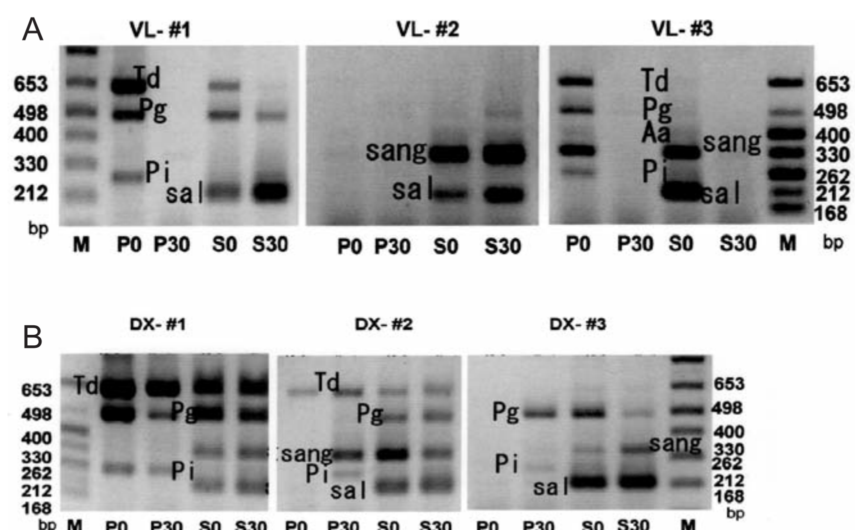

Figure 1. Typical results of polymerase chain reaction for three participants in the VITALREXTM groups (A) and the Dexstrine groups (B). Stained gel images for both saliva and plaque samples on days 0 (S0 and P0) and 30 (S30 and P30) (before and after treatment, respectively) are shown. M, markers; Td, *T. denticola*; Pg, *P. gingivalis*; Aa, *A. actinomycetemcomitans*; Pi, *P. intermedia*; Sang, *S. sanguinis*; Sal, *S. salivarius*.

bacteria rather than cariogenic bacteria. On the other hand, no remarkable change in either P or S was seen in DX-#1~#3 from baseline (day 0) to day 30. Overall, PCR data suggested that VL showed a remarkable reduction in target bacteria after the treatment period.

Discussion

In the last few decades, numerous applications have been identified for *B. subtilis* strains, including the industrial production of proteases, preparation of alkaline-fermented food, and as probiotic material for the prevention of enteric infections in both humans and animals.[19,20] Probiotics are often promoted as dietary supplements and marketed for their ability to improve or maintain health.[21] According to the newest super-clear review in 2011, Cutting reported that *Bacillus* species are useful for probiotic dietary supplements and also for medical use.[22] The future prospects for Bacillus subtills as probiotics are expanding rapidly with an increasing number of studies providing more information. However, it is less than a decade that probiotics have been extensively investigated from the perspective of oral health. During this period, reports on the beneficial effects of probiotics on oral health has been limited.

The recent literature has shown that probiotic administration effectively reduces the number of *Streptococcus mutans* in the oral cavity, suggesting a role for probiotics in caries prophylaxis.[23] Furthermore, in 2007 Hatakka *et al*.[24] reported that probiotics also reduce oral *Candida* counts in the elderly and might represent a new strategy for controlling oral yeast infections. However, few studies have examined the effectiveness of probiotics for periodontal diseases.

In 2006, Krasse *et al.*[25] showed that gingival bleeding and gingivitis decreased after administration of the probiotic *Lactobacilus reuteri*. It is well known that the normal microbiota in healthy humans displays remarkable quantitative and qualitative stability that limits the growth and persistence of interacting microbes. Thus, longer contact between probiotic bacteria and plaque is expected to increase probiotic activity.[26]

Tsubura *et al.*[11] reported that patients with chronic periodontitis who were treated with E300, a mouth rinse containing *B. subtilis* DB9011, showed not only remarkable changes in BANA scores, but also significant decreases in periodontal pathogens compared with patients using a commercially available mouth rinse containing benzethonium chloride. However, it is difficult to store E300 for long periods due to its lack of stability. It is also difficult to use for individuals who require nursing care or who have difficulty rinsing. To overcome these limitations, the VL tablet was developed as a new material with improved stability and a simple administration procedure. In the present study, the effects of VL tablets containing *B. subtilis* DB9011 as a probiotic product for patients with periodontitis were evaluated following placement of the tablets in the mouth for a few minutes to allow direct contact with the oral microbiota. VL produced marked changes in the BOP, GI, and BANA test scores compared with DX (Tables 4-6). The number of target bacteria were significantly decreased after 30 days in both the saliva and plaque of subjects in the VL group (Figure 1A), but this decrease was negligible in subjects receiving DX (Figure 1B). It is likely that VL could play a greater role in improving periodontal tissue metabolic activity and local immunocompetence in the oral mucous epithelium. Our results suggest that VL could improve conditions in the periodontal pocket and oral cavity of patients with chronic periodontitis.

In the present study, VL administration successfully decreased the numbers of periodontopathic bacteria in subgingival plaque and saliva after 30 days. As the intake of *B. subtilis* tablets is relatively simple, probiotics may be provided as homecare supplements for preventing periodontal diseases. It is also well known that the re-emergence of periodontal pathogens is correlated with a lack of clinical improvement and an increased risk of disease relapse.[27] Therefore, administration of *B. subtilis* DB9011 probiotic tablets as an adjunct to mechanical debridement might represent an effective approach for the treatment of periodontitis.

Our present results suggest that VL can control chronic or acute periodontitis, may be useful for implant treatment, and has beneficial effects on oral mucosal disease. Thus, probiotics seem to improve the oral immune response. Furthermore, in food technology, dairy products containing both probiotics and prebiotics are currently being developed and may be useful as symbiotic functional food. In the field of oncology, serious systemic infectious may occur during cancer chemotherapy because of disturbances in the oropharyngeal and gastrointestinal microflora, impaired mucosal barrier functions, and immunosupression. Bacteriotherapy in the form of probiotics provides an alternative for conventional oral health treatment and represents a promising new research field of dental science.

Many factors, including attention bias, contribute to perceived placebo effects in clinical trials. In order to justify the present study, further essential microbial experiments have to be performed on isolated periodontal pathogens, and especially the growth of *red-complex* in the presence of VL and DX should be monitored in culture. Therefore, more research is needed to identify appropriate effector strains for oral probiotics specifically designed to prevent and treat periodontal disease. The present experimental protocol did not include any oral hygiene instruction before treatment or at baseline; however, subjects in both groups might have systematically altered their oral hygiene regimens due to the observance of routine dental work.

Conclusions

B. subtilis DB9011 tablet is an effective oral probiotic material for patients with periodontitis. Probiotic tablet intervention could be a useful tool for the treatment of inflammation and clinical symptoms of periodontitis. The mechanism underlying the antibacterial effect of the VL tablets is proposed to involve the inhibition of proteases that originate from oral bacteria.

Furthermore, VL tablets may contribute to human health care with respect to not only oral diseases, but also in the control of host immunological responses.

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