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Dextranase enzyme and *Enterococcus faecium* probiotic have anti-biofilm effects by reducing the count of bacteria in dental plaque in the oral cavity of dogs

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OBJECTIVE
Periodontal disease is a common clinical complication and has a negative impact on the quality of life and the welfare of companion dogs. Periodontal disease occurs when pathogenic bacteria are accumulated in the gingival sulcus, which favors biofilm formation. The oral health of dogs can be significantly compromised by dental plaque accumulation. Thus, this investigation demonstrates the effect *of Enterococcus faecium* probiotic, dextranase enzyme, and their combination on dental biofilm in the oral cavity of dogs.

ANIMALS
The 30 dogs were referred to Polyclinic with no oral ulcers, severe periodontitis, and internal diseases.

PROCEDURES
Dextranase enzyme, *E faecium* probiotic, and their combination were administered in the oral cavity of dogs. Microbiological samples were obtained from tooth surfaces and gums before and after intervention with the substances. Bacterial colonies were enumerated by using a colony counter. Also, *Porphyromonas gingivalis* hmuY gene expression was evaluated by reverse transcription quantitative real-time PCR analysis.

RESULTS
The total colony count of the bacterial culture indicated that the dextranase enzyme, *E faecium* probiotic, and their combination significantly reduced the total bacteria count in the oral cavity. Moreover, in the reverse transcription quantitative real-time PCR analysis it was observed that using the combination of *E faecium* probiotic and dextranase enzyme decreases the hmuY gene expression of *P gingivalis* bacteria.

CLINICAL RELEVANCE
The results clearly indicated that the dextranase enzyme and *E faecium* probiotic could be used as preventive agents to reduce oral biofilm in dogs. Furthermore, no side effects were observed while using these substances.

Keywords: dental biofilm, dextranase, probiotic, *Enterococcus faecium*, *Porphyromonas gingivalis*

Periodontal disease is one of the most common diseases in dogs. It is estimated that approximately 44% to 63.6% of dogs are diagnosed with periodontal disease, which compromises the overall welfare of companion dogs.[1,2] The incidence of periodontal disease results from biofilm formation and bacterial build up in the gingival sulcus. The colonized bacteria utilize adhesion and congregation mechanisms to form dental plaque on tooth surfaces, which favors the development of periodontal diseases. Dental plaque is primarily composed of bacteria, fungi, and the glycoproteins produced by salivary glands or due to bacterial decay.[3–6] Gram-positive and gram-negative bacteria are the initial colonizers on tooth surfaces.[7] A common factor in periodontal disease is the increase in the population of *Porphyromonas gingivalis*, an anaerobe, and gram-negative bacteria.[8] The up-regulation of the hmuY gene in *P gingivalis* bacteria promotes

biofilm formation in the oral cavity.[9] The calcification of plaque creates tartar, which its porous surface idealizes the further colonization and proliferation of pathogenic microorganisms. These microorganisms impair the periodontium and gingival connective tissue elements, resulting in bone resorption and tooth loss.[10–12] The usage of probiotics may be effective in the treatment of oral complications such as halitosis or periodontitis.[13] *Enterococcus faecium* is a gram-positive, non-hemolytic, or gamma-hemolytic bacteria belonging to the *Enterococcus* genus.[14] *E faecium* probiotic is used in oral care products and inhibits the growth of plaque-forming bacteria.[15] Dextran is a polysaccharide produced by dental caries pathogens and is a dental plaque or biofilm component in the oral cavity.[16,17] Dextranase is an enzyme that can be collected from diverse sources such as plants, mammalian tissues, fungi, and bacteria.[18] The primary source of dextranase enzyme is fungi, which can be efficiently collected, and has a higher activity than enzymes obtained from other origins.[19,20] The α-(1–6)-D-glycoside linkages in dextran could be catalyzed by the dextranase enzyme, destroying biofilm structure and preventing dental caries.[21,22]

Due to a significant correlation between the quality of life and oral health in dogs, this investigation examines the effect of *Enterococcus faecium* probiotic, dextranase enzyme, and their combination on dental biofilm in the oral cavity of dogs.

Materials and Methods

Study description

This investigation was conducted on 30 dogs referred to Islamic Azad University, science and research branch, Veterinary Polyclinic, Tehran, Iran, in 2021 for a dental checkup. A veterinarian evaluated all the patients to ensure no severe periodontitis or systemic diseases were present. Also, the oral cavity of dogs was thoroughly investigated by a veterinarian to ensure no oral ulcers were present. Moreover, the patients were completely conscious, and no sedative medications were utilized.

All the pet owners were provided consent letters that distinctly remarked on the investigation's procedure.

Study design

Thirty large breed male dogs aged 2 to 4 years and weighing 25 to 35 kg were divided randomly into 3 groups (10 dog models per group). Microbiological samples were taken with sterile swaps from tooth surfaces and gums of all patients before and after treatment, and both samples were placed in a laboratory sample tube containing PBS. Moreover, for follow-up, they were housed at a temperature of 22 °C to 27 °C with a 12-hour light/dark cycle in a pathogen-free isolation room and fed soft dog food for 14 days. For administration of *E faecium* probiotic, full-thickness flap surgery on the canine tooth in quadrants 2 and 3 was performed after the animals were anesthetized by combining ketamine (2 mg/kg, IV, once) and diazepam (0.5 mg/kg, IV, once). Moreover, meloxicam (0.2 mg/kg, SC, once) was administered as a painkiller. The ethics committee of the Science and Research Branch of Islamic Azad University approved the present study for dental research in dogs (Approval No. IR.IAU.SRB.REC.1400.200).

The first group of patients was treated with 1 mL of dextranase enzyme (Sigma-Aldrich) by rubbing it on the crowns of the tooth and gums with a sterile swap. After 5 minutes of administration, the second sample was taken.

The second group of patients was treated with *E faecium* probiotic (8.1 X 10^{10} CFU/mL, Sinaclon Co) while being anesthetized via performing a full-thickness flap surgery using a No. 15 scalpel blade and a periosteal elevator on the canine tooth in quadrants 2 and 3. The probiotic was combined with sterile distilled water to form a paste-like consistency and was placed 2 mm coronal to the crest of the alveolar bone. Afterward, the flap closure was performed with Vicryl 3/0 suture. The second sample was collected on day 14 of administration.

The third group of patients was treated with dextranase enzyme and *E faecium* probiotic. The patients were initially treated with *E faecium* probiotics using a full-thickness flap surgical technique. After 14 days, patients were treated with dextranase enzyme by stroking it on the crowns of the tooth and gums with a sterile swab. After 5 minutes of interaction, a secondary sample was obtained **(Figure 1).**

Inclusion criteria

The dogs were referred to Islamic Azad University Veterinary Polyclinic with no oral ulcers, severe periodontitis, and internal diseases.

Exclusion criteria

Dogs with oral disease, severe periodontitis, internal or infectious diseases, systemic complications, and dogs without consent letters were excluded from the study.

Bacterial culture

Initially, blood agar plates were prepared by adding 5% defibrinated sheep blood to a sterile plate count agar medium (PCA), prepared following the labeling instructions. The blood agar medium was distributed in 100 mm culture plates and stored at 4 °C for 24 hours. The sample tubes were vortexed for 30 seconds for uniform distribution of microorganisms. 6-fold serial dilutions were obtained with PBS, and 100 microliters of each dilution were cultured using the spread plate technique, and the plates were incubated at 38.5 °C for 3days. Afterward, the count of bacterial colonies was evaluated with a manual colony counter device (Yuchengtech).

RNA extraction and real-time PCR

The before and after oral samples containing PBS was used for (reverse transcription quantitative real-time PCR) analysis. RNA extraction and cDNA synthesis was performed by kits (Sina Colon) following the manufacturer's procedure. The reverse transcription reaction was performed at 37 °C for 1 hour, and the

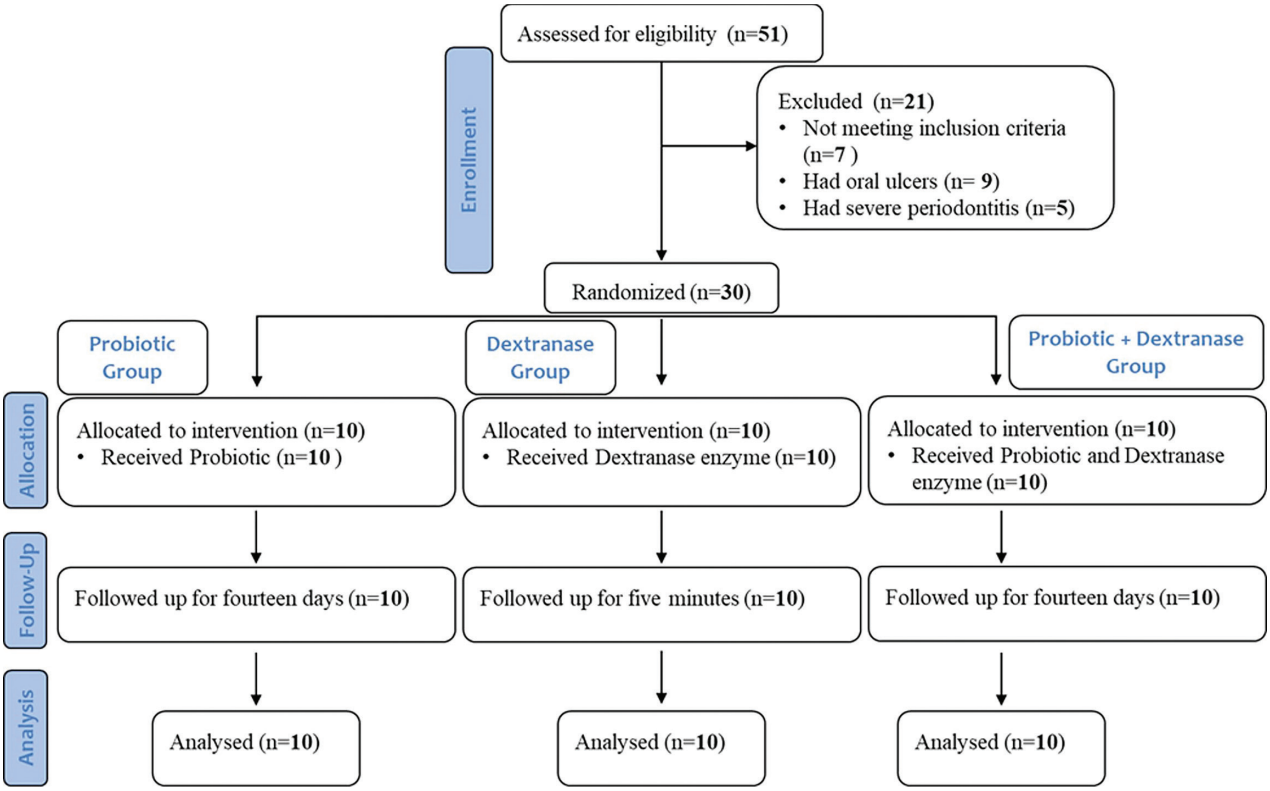


Figure 1—Consort flow diagram.

inactivation of the reverse transcriptase condition was at 70 °C for 5 minutes. The DNA polymerase activation was performed at 95 °C for 5 minutes, with 40 cycles of a 2-step PCR (95 °C for 10 seconds and 55 °C for 40 seconds).[23] A dissociation curve analysis of the hmuY gene in *P gingivalis* bacteria displayed a single peak. The SYBR Green real-time PCR primer, was reported in **(Figure 2).**

Data analysis and statistics

The data obtained from the experiment were analyzed using SPSS, version 21 (Descriptive statistics). One-way analysis of variance (one-way ANOVA) was used to determine the effect of experimental groups on the mean colony count percentage. Duncan's multiple range test compared the significant difference ($P < .05$) in means. Moreover, the delta-Ct method for non-treatment and treatment groups were subject to a simple *t*-test.

Results

Colony count analysis

The colony count was performed before and after treatment to reveal the effect of dextranase enzyme, *E faecium* probiotic, and their combination on reducing dental biofilm in the oral cavity of dogs. Dextranase enzyme in group 1 and *E faecium* probiotic in group 2 and the combination of dextranase enzyme and *E faecium* probiotic decreased the average count of bacteria by 58.5%, 83.7%, and 68.9%, respectively **(Figure 3)**. All the groups significantly decreased the mean bacteria count in the dental biofilm ($P < .05$).

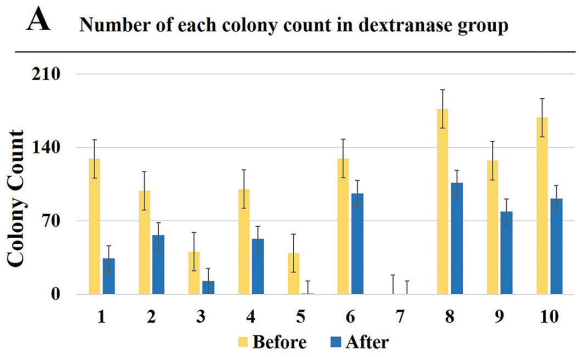
RT-qPCR analysis

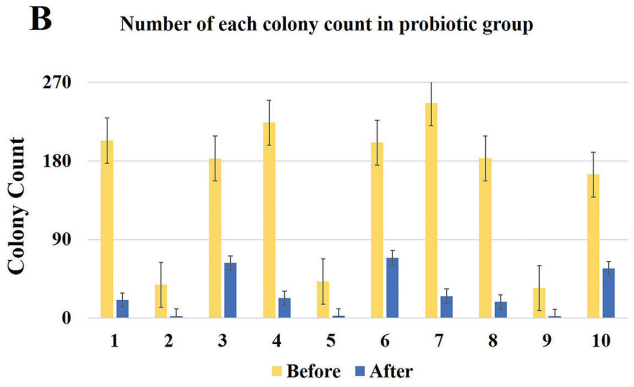
The RT-qPCR analysis revealed a significant downregulation ($P = .002$) in the hmuY gene expression in dogs treated with a combination of

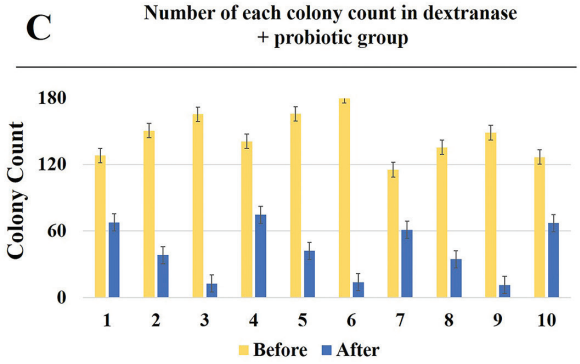
Real-time PCR primers

Gene	Forward	Reverse	OD
hmuY	GCGCTCAACGTTCAGCC	CACGAATTCCGCCTGC	2
16s RNA	AGAGTTTGATCCTGGCTCAG	AAGGAGGTGATCCAGCCGCA	9

Figure 2—Real-time PCR primers. OD = Optical density.





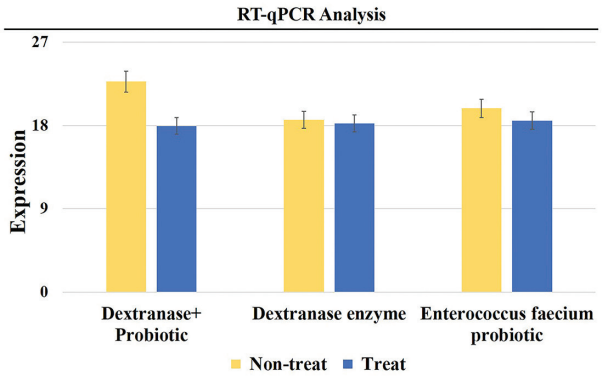


D
The mean of colony count in each group

Mean	Before treatment	After treatment	Difference(%)
Dextranase enzyme	100.6	52.1	58.5 ± 26.12
Probiotic	151.4	27.9	83.7 ± 12.28
Dextranase +Probiotic	145.3	41.9	68.9 ± 20.08

Figure 3—The colony counts indicated that the dextranase enzyme eliminates bacterial growth by 58.5% in the first group (A). The second group colony count analysis demonstrated that *Enterococcus faecium* probiotic reduces the bacteria count by 83.7% (B). The combination of dextranase enzyme and *Enterococcus probiotic* was utilized in the third group (C). The colony count examination revealed a 68.9% reduction in the total count of bacteria. Each group's mean colony count was compared using one-way ANOVA and the Duncan multiple range test (D).

dextranase enzyme and *E faecium* probiotic in group 3. Furthermore, no significant downward trend in the hmuY gene expression was detected in groups 1 and 2 **(Figure 4)**.



		Non-treat	Treat	Sig. (2-tailed)
Dextranase + Probiotic	Mean	22.74	17.94	0.002
Dextranase enzyme	Mean	18.6143	18.2143	0.119
Enterococcus faecium probiotic	Mean	19.8571	18.5286	0.123

Figure 4—The reverse transcription quantitative real-time PCR was used to detect the expression level of hmuY gene in *Porphyromonas gingivalis* bacteria. Simple *t*-test analysis revealed that the combination of dextranase enzyme and *Enterococcus faecium* probiotic significantly down-regulated the hmuY gene expression. In this study, the 16SrRNA was used as the housekeeping gene.

Discussion

Periodontal disease begins with dental plaque or biofilm forming on tooth surfaces and is a common concern in companion animals. In particular, canine dental care routines are usually time-consuming and expensive.[15,24] Many active ingredients are currently used in various formulations to prevent dental plaque and tooth decay.[25] This study investigated the dextranase enzyme's effect on preventing dental biofilm formation in dogs. Bacterial cell culture and colony count were designed to evaluate the enzyme's treatment effect on the average count of oral bacteria. It was observed that the dextranase enzyme reduces the abundance of bacteria in dental plaque. Moreover, the study designed by Juntarachot[26] found that the dextranase enzyme can improve oral health by preventing dental plaque formation. Also, Chaiyasut[27] found that mouthwash containing dextranase enzyme and nisin reduces the value of plaque index in the oral cavity.

Suzuki and Yu's studies[15,28] in 2018 demonstrated that *E faecium* probiotic decreases the growth of plaque-forming bacteria that cause periodontal diseases, and it acts as a biofilm formation inhibitor. This study also shown *E faecium* probiotic had a significant decrease in bacteria count and it prevents the formation of dental biofilm.

P gingivalis bacteria cannot synthesize protoporphyrin IX and iron by themselves. Therefore,

they are entirely dependent on hemin for growth. Moreover, *Hmu* family proteins are essential for hemin acquisition for *P gingivalis* growth.[29,30] *Hmu* family genes also stimulate mononuclear cell-mediated inflammatory responses, affecting the bacteria's virulence factor.[31,32] The Romero-Lastra study demonstrated that the upregulation of the hmuY gene in *P gingivalis* influenced the ability of this bacteria to form a biofilm.[9] In this study, the RT-qPCR assay demonstrated a downregulation in the hmuY gene in the group treated with dextranase enzyme and *E faecium*.

The novelty of this investigation was administering the combination of dextranase enzyme and *E faecium* in the oral cavity of dogs for the first time, which revealed a reduction in the count of dental plaque bacteria.

Due to the extensive period required for the probiotics to be efficacious, the dogs were kept in the hospital. Also, considering the large number of them, the before samples in each group were considered a control group since they were utterly intact without interference any interference and treatment. Furthermore, following up on the patients for more than 14 days was costly and time-consuming to estimate the long-term effects or repeated administration of the dextranase enzyme and *E faecium* probiotic.

In outline, we found that dextranase enzyme and *E faecium* probiotic can have anti-biofilm effects by reducing the count of bacteria present in dental plaque in the oral cavity of dogs. Also, hmuY gene expression in *P gingivalis* bacteria was dramatically reduced when dogs were treated with dextranase enzyme and probiotics. Therefore, combining the dextranase enzyme and *E faecium* probiotic can decrease biofilm formation.

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