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Original article

The bacteriostatic effect of erythritol on canine periodontal disease–related bacteria

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Abstract

Erythritol helps both prevent and improve periodontal disease and is therefore widely used for dental care in humans. However, only a few studies have investigated the effects of erythritol on periodontal disease in animals. We hypothesized that erythritol could be used to prevent and improve periodontal disease also in canines and investigated the effects of erythritol on canine periodontal disease–related pathogenic bacteria using both *in vitro* and *in vivo* methods.

The effect of erythritol on the proliferation of *Porphyromonas gulae*, which is reportedly associated with canine periodontal disease, was investigated *in vitro*. In addition, a 4-week intervention trial using an external gel preparation containing 5% erythritol was performed in canines with mild periodontal disease; changes in the microbiota around periodontal lesions were investigated using next-generation sequencing and bioinformatics analysis.

The growth of *P. gulae* was significantly suppressed by erythritol *in vitro*. In the intervention study, the Shannon index, an indicator of the species distribution α-diversity, and the occupancy of several canine periodontal disease – related bacteria (*P. gulae*, *P. cangingivalis*) were significantly decreased in periodontal lesions.

Based on the results of *in vitro* and *in vivo* studies, we conclude that, as in humans, erythritol has bacteriostatic effects against periodontal disease – related bacteria in canines.

Key words: erythritol, canine, periodontal disease, *Porphyromonas gulae*, *Porphyromonas cangingivalis*, *Fusobacterium*, oral microbiota

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Introduction

Periodontal disease, a chronic infection involving inflammation of the periodontal tissue, is commonly diagnosed in daily canine veterinary practice. Periodontal disease occurs due to an imbalance between plaque bacteria and the host immune response, and it involves repeated active phases in which the condition rapidly worsens and resting phases in which inflammation subsides. The condition causes redness, swelling, pain, and oral cavities in the gums, eventually leading to systemic manifestations such as loss of appetite and fever (Allaker et al. 1997, Shoukry et al. 2007, Eubanks 2010). Periodontal disease in canines occurs in the age range of approximately 3 to 5 years, and its incidence increases with age; as many as 84% of canines over 3 years old reportedly have periodontal disease (Kortegaard et al. 2008).

In recent years, studies have linked periodontal disease with systemic issues such as lifestyle-related conditions and cardiovascular disease (Santos et al. 2019). Therefore, ameliorating the effects or even preventing canine periodontal disease is an extremely important issue from the viewpoint of improving the quality of life of companion animals. Currently, the primary approach for treating canine periodontal disease includes surgical interventions such as scaling, polishing, or tooth extraction, combined with the use of antibiotics (Sarkiala et al. 1993, Nomura et al. 2020). However, as these treatment methods involve surgical therapy, they place a heavy burden on companion animals. In addition, antibiotic use is associated with problems such as the induction of drug-resistant bacteria, which is a global public health issue (Gu et al. 2021). Thus, there is an urgent need to establish new simple and safe treatment methods that can prevent or improve canine periodontal disease.

Erythritol is a type of sugar alcohol produced by yeast via the fermentation of glucose as a raw material, and as it reportedly helps to improve/prevent oral diseases such as dental caries and periodontal disease, it is widely used in oral care products (Nakakuki 1998, Cock et al. 2016, Cock 2018). Xylitol, a type of sugar alcohol like erythritol that has preventive/improving effects in oral diseases, reportedly causes acute liver failure and hypoglycemia in canines (Kuzuya et al. 1969, Murphy and Coleman 2012). In contrast, erythritol is reportedly safe for use in canines (Dean et al. 1996, Noda et al. 1996, Munro et al. 1998). However, few studies have investigated the effects of erythritol on oral diseases such as periodontal disease in canines. We hypothesized that, as in humans, oral care using erythritol may be effective in preventing or improving periodontal disease in canines. As the first step in examining this hypothesis, we investigated the effects of erythritol on canine periodontal disease – related bacteria both *in vivo* and *in vitro*.

Materials and Methods

Ethics

This study followed the Japanese national guidelines for the humane treatment of animals. All the owners provided written, informed consent for the applied procedures.

Clinical design

Eight canines with mild periodontal disease (mild inflammation, obvious color changes, mild changes in gingival surface, no bleeding) classified as described by Yumoto et al. (2004) and Allaker et al. (1997) (age, 8.20±3.33 years; sex, spayed female 6, female 2) were used in the study. The external preparation used in this study was prepared by dissolving 5% erythritol (w/w) (B Food Science Co., Ltd., Aichi, Japan) and 2% cellulose (w/w) in purified water to make a gel. The pump foam bottle was then filled, and 1.5 mL was applied to the gingiva twice a day with a finger and polished for 60 s. The oral microbiota was collected by swabbing the gingiva of the periodontal disease–affected upper jaw of canines using an oral microbiota collection kit (Mizuki Biotech Co., Ltd.).

DNA extraction

A QIAamp DNA Mini kit (Qiagen, Cat. no. 51306) was used for DNA extraction. Glass beads (SIGMA Glass beads, acid-washed, 710-1180 µm) and swab samples of oral bacteria attached were placed in a 2-mL screw cap tube (purchased separately), and 400 µL of PBS (−) was added. After shaking and crushing using a Tissue Lyser II (Qiagen) for 10 min, the supernatant was collected in a new 2-mL tube, and 20 µL of Proteinase K (included with the kit) and 400 µL of Buffer AL (included with the kit) were added and mixed. After keeping warm at 56 °C for 10 min, 400 µL of 100% EtOH was added and mixed. Next, 650 µL of the sample solution was added to the spin column (included with the kit), centrifuged at 6,000 *g* for 1 min, after which the flow-through solution was discarded, and the remaining sample solution was re-added to the spin column. After centrifuging at 6,000 *g* for 1 min, the flow-through solution was discarded, and 500 µL of Buffer AW1 (included with the kit) was added to the spin column. After centrifuging at 6,000 *g* for 1 min, the flow-through solution was discarded, and 500 µL of Buffer AW2 (included with the kit) was added

to the spin column and centrifuged at approximately 14,000 g for 3 min, after which the flow-through solution was discarded. The spin column was placed in a 1.5-mL tube (included with the kit), and 100 µL of Buffer AE was added to the spin column. After centrifuging at 6,000 *g* for 1 min, the flow-through solution was collected and stored at -30 °C.

Next-generation sequencing analysis

Six hypervariable regions of the bacteria-specific 16S rRNA gene in the extracted bacterial DNA were amplified by multiplex PCR using an Ion 16S™ Metagenomics kit (Thermo Fisher Scientific, Cat. no. A26216). The multiplex amplicon was purified using an Axygen AxyPrep MAG PCR Clean-Up kit (magnetic beads, Axygen, Cat. no. MAG-PCR-CL-50). A sample identification adapter and a sequencing adapter were combined with the amplicon of each sample to prepare a library for DNA sequencing using an Ion Plus Fragment Library kit (Thermo Fisher Scientific, Cat. no. 4471252) and purified using an Axygen AxyPrep MAG PCR Clean-Up kit. The sequencing library was prepared to a final concentration of 7 pM and amplified by emulsion PCR and fixed to beads for sequencing (Ion PGM Hi-Q View OT2 kit, Thermo Fisher Scientific). Bacterial 16S rRNA gene sequence decoding was performed using an Ion PGM Hi-QView Sequencing kit (Cat. no. A30044), Ion 316 Chip kit v2 BC (Thermo Fisher Scientific, Cat. no. 4488149), and Ion PGM Sequencer (Thermo Fisher Scientific). Bacterial information retrieval and bacterial flora analysis using MicroSEQ/GreenGenes were carried out using the cloud-based Ion Reporter ver. 5.18 software (Thermo Fisher Scientific). Finally, a table comparing the abundance ratios for bacterial species in the sample was prepared using Excel (Microsoft, Tokyo, Japan).

Measurement of the growth of periodontal disease – associated microorganisms

Porphyromonas gulae JCM 13865 was purchased from the Japan Collection of Microorganisms (JCM) and used for experiments in the present study. *In vitro* culture studies were carried out as follows. Bacteria were streaked on sheep blood agar medium for modified CDC anaerobic bacteria (Becton Dickinson, Tokyo, Japan) and cultured anaerobically at 30°C for 4 days using an Anaeropack Kenki (Mitsubishi Gas Chemical, Tokyo, Japan). Colonies were scooped with chips and suspended in 0.5 mL of modified GAM bouillon "Nissui" (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) to prepare as the seed mother suspension. Next, 20 µL of the seed mother suspension was dispensed into 600 µL of medium (modified GAM bouillon "Nissui" containing 0.4% [w/w] of glucose and 0%, 5%, 10%, and 15% erythritol) in a 96–deep well plate (AxyGen Scientific, Inc., Union, CA, USA) and cultured anaerobically at 30 °C for 5 days. A 20-µL aliquot of the primary culture solution was then suspended in 180 µL of water, and the OD_{660} was measured using a SpectraMax M2 microplate reader (Molecular Device Japan, Tokyo, Japan) to determine turbidity (n=4).

Bioinformatics and statistical analyses

The Shannon index, an indicator of the species distribution α-diversity, was calculated using Excel (Microsoft). Principal coordinate analysis (PCoA) plots, which enable visualization of the β-diversity, were prepared using SPSS® Statistics, version 26.0 (IBM). Significance testing of the occupancy of microbiota and the Shannon index in the *in vivo* study and bacterial growth analyses in the *in vivo* study was performed using the Wilcoxon signed-rank test and Tukey's methods test, respectively, with SPSS® Statistics, version 26.0 (IBM), and $p<0.05$ was considered significant. Pairwise correlation analysis of the occupancy of species was performed based on Spearman's rank correlation coefficient using SPSS® Statistics, version 26.0 (IBM).

Results

The effect of erythritol on the growth of *Porphyromonas gulae*

The growth of *P. gulae* was significantly inhibited by erythritol in a concentration-dependent (1-15%) manner compared to the control. In particular, growth was obviously inhibited with 15% erythritol (Fig. 1).

Analysis of microbiota in canine periodontal disease lesions

A heat map of the occupancy of microbiota in periodontal disease lesions before and after 4 weeks of 5% erythritol gel treatment was produced at the species level. The results showed that, before the intervention, Z-score values for each bacteria were high (Red) in most of the individuals, but Z-score values were low (Blue) after erythritol treatment (Fig. 2). The Shannon index value of microbiota in periodontal disease lesions was significantly decreased after erythritol treatment compared to before. On the other hand, PCoA plots of the microbiota showed that there was no obvious difference between before and after (Fig. 3a and b). The results of a comparison of the occupancy rate (1% or more in either before or after) of each bacterium between before and after erythritol treatment indicated

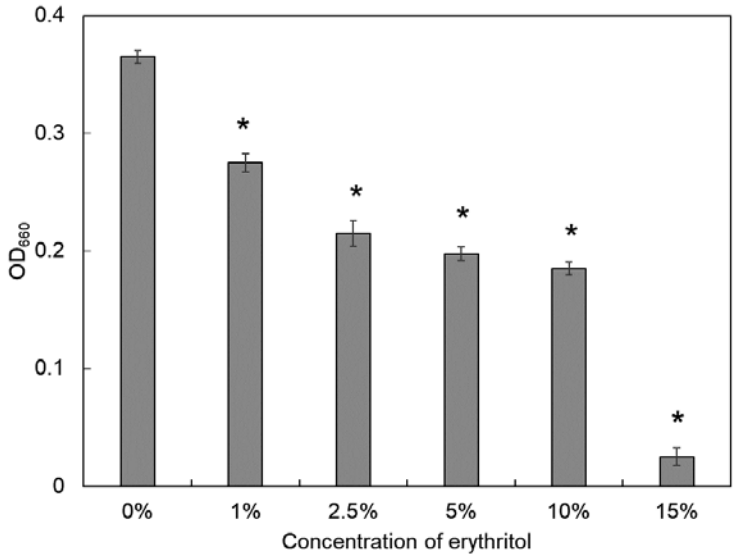

Fig. 1. Effect of erythritol on the growth of *Porphyromonas gulae*.
Graph shows the turbidity (OD_{660}) of *Porphyromonas gulae* JCM 13865 culture medium containing 0%, 1%, 2.5%, 5%, 10%, or 15% erythritol (n=4). Asterisks (*) indicate a significant difference ($p<0.05$) between the control (0%) group and the test group.

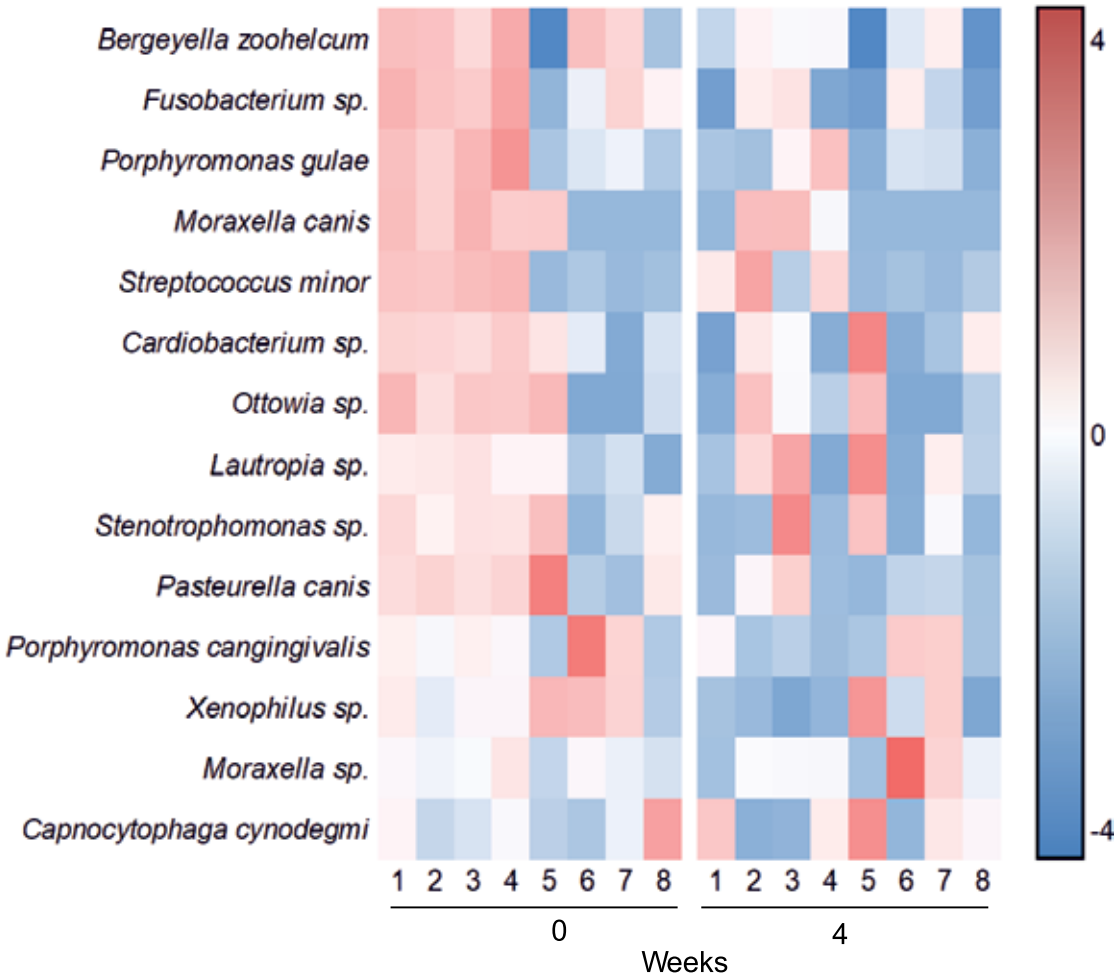

Fig. 2. Heat map visualization of the microbiota of canine periodontal disease lesions at the species level before (week 0) and after (week 4) daily erythritol treatment. Heat map cluster analysis based on the relative abundance of bacterial species level was performed using Excel. The average Z-score of the microbial relative abundance in each canine was calculated and depicted visually from blue to red; red represents the highest score, whereas blue represents the lowest score; n=8 animals per group.

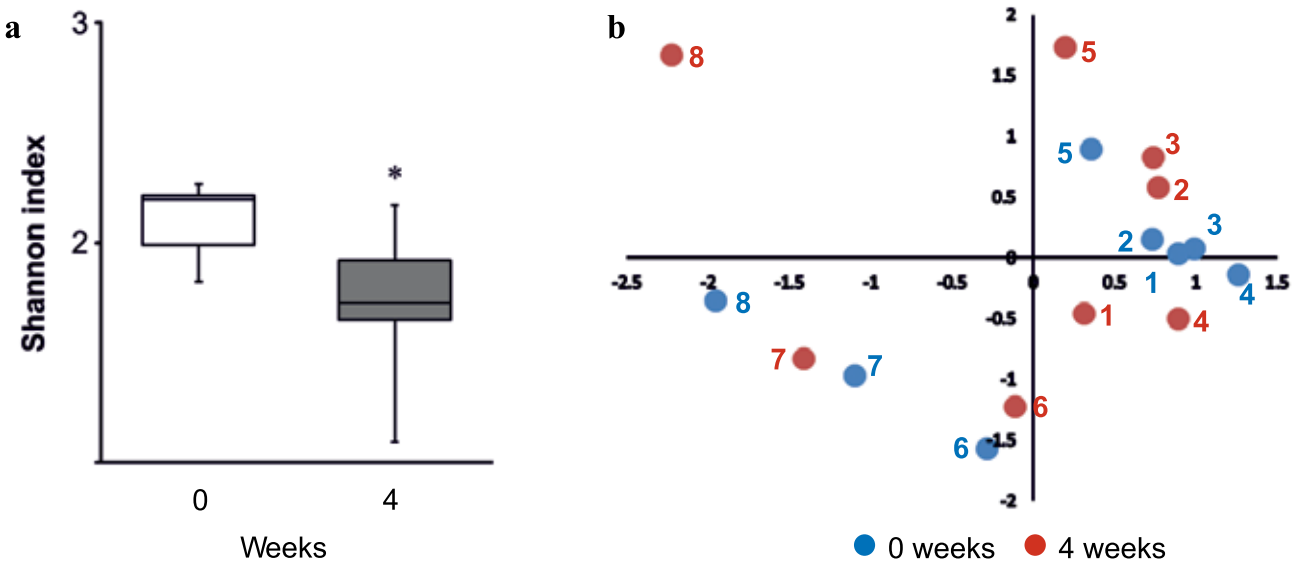

Fig. 3. The α-diversity and β-diversity data in microbiota of canine periodontal disease lesions at the species level.
(a) Shannon index.
(b) Two-dimensional PCoA analysis. The first primary component (PC1) is plotted on the horizontal axis, and the second primary component (PC2) is plotted on the vertical axis. Each point represents the microbiota structure of an individual sample. Plot colors are defined as follows: 0 weeks, blue; 4 weeks, red. Numbers 1-8 beside symbols indicate the ID of each canine.

Table 1. Metagenomic analysis of microbiota of periodontal disease lesions identified by 16S rRNA sequencing before (week 0) and after (week 4) daily erythritol treatment. Values indicate the median (IQR) of the occupancy of each bacteria among total bacteria. Bacteria with an occupancy rate of ≥1% either before or after treatment are shown. Asterisks (*) indicate a statistically significant difference ($p<0.05$) between week 0 and week 4; n=8 animals per group.

Species	Weeks	
	0	4
Lautropia sp.	5.07 (2.63-5.94)	3.84 (1.12-9.24)
Porphyromonas cangingivalis	4.57 (3.14-5.66)	1.62 (1.32-5.44)*
Porphyromonas gulae	5.34 (2.29-9.62)	1.71 (0.62-3.01)*
Cardiobacterium sp.	2.56 (1.31-2.97)	1.12 (0.23-2.17)
Moraxella sp.	1.36 (1.13-1.66)	1.43 (0.90-2.02)
Bergeyella zoohelcum	2.27 (1.66-2.54)	1.20 (0.72-1.37)*
Pasteurella canis	3.00 (2.02-3.50)	0.50 (0.13-1.08)
Fusobacterium sp.	1.90 (1.12-2.27)	0.36 (0.00-1.29)*
Capnocytophaga cynodegmi	0.91 (0.71-1.15)	1.32 (0.37-1.68)
Xenophilus sp.	1.26 (1.06-2.05)	0.29 (0.14-1.00)
Moraxella canis	4.54 (0.00-4.98)	0.00 (0.00-2.79)
Stenotrophomonas sp.	1.09 (0.82-1.27)	0.14 (0.10-1.05)
Streptococcus minor	3.62 (0.20-7.01)	0.76 (0.26-4.23)
Ottowia sp.	1.13 (0.29-1.37)	0.28 (0.02-0.80)

that the bacteria with a high occupancy rate, such as *P. gulae*, *P. cangingivalis*, *Fusobacterium*, and *Bergeyella zoohelcum* were significantly decreased after erythritol treatment at the species level (Table 1). To investigate the relationship between these bacteria significantly decreased by erythritol and the increase or decrease of other bacteria, Spearman's correlation analysis was performed. The results showed that *P. gulae* had a significant positive correlation with *Cardiobacterium sp.*, *Moraxella sp.*, and *Streptococcus*

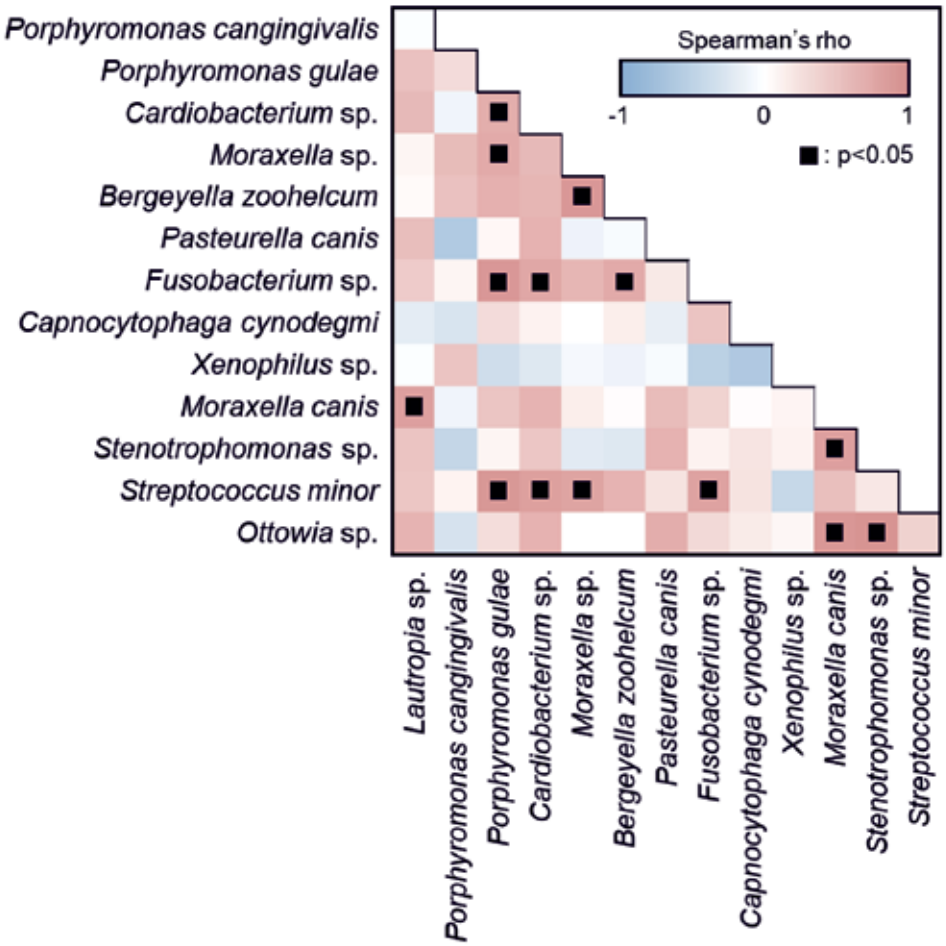

Fig. 4. Pairwise Spearman's correlation coefficient of occupancy of species before and after erythritol treatment. Species with a mean abundance >1% were subjected to the pairwise Spearman's rank test. Correlation coefficients of species are presented on a color scale. Black squares indicate significant correlations ($p<0.05$).

minor. On the other hand, there were no bacteria showing significant correlations with *P. cangingivalis* and *Fusobacterium* (Fig. 4).

Discussion

Canine periodontal disease is a chronic bacterial infection that causes inflammation of the periodontal tissue. There is no radical treatment, and surgical treatment with antibiotics such as clindamycin is primarily performed (Sarkiala and Harvey 1993, Nomura et al. 2020). The importance of dental care to prevent or improve periodontal disease in canines has been reported (Bellows et al. 2019). However, few studies have investigated dental care materials to identify those that are clearly effective in preventing or improving canine periodontal disease. In this study, it was shown that erythritol may be a useful material for the treatment or prevention of canine periodontal disease.

The effect of erythritol on the growth of *Porphyromonas gulae*, a canine periodontal disease – related species of bacteria (Senhorinho et al. 2011, Nomura et al. 2012, Nomura et al. 2020) was initially examined *in vitro*. The results showed that erythritol significantly suppressed the growth of *P. gulae* (Fig. 1). Hashino et al. reported that erythritol inhibits the formation of biofilm by *Porphyromonas gingivalis*, a bacterium commonly associated with human periodontal disease (Hashino et al. 2013). The results of our *in vitro* study were in agreement with that report and suggest that erythritol has a bacteriostatic effect on canine periodontal disease – related bacteria.

A 4-week *in vivo* intervention study using 5% erythritol gel was subsequently performed in canines with periodontal disease. A comparison of the microbiota in canine periodontal disease lesions before and after erythritol treatment showed an obvious decrease of occupancy of each of the bacteria in general (Fig. 2). In the examination of changes in species richness and evenness of distribution and comparison of species intergroup similarities following erythritol treatment, the Shannon index, an indicator of the species distribution α-diversity (Shannon 1949), was significantly

decreased. In contrast, PCoA plots, which enable visualization of β-diversity (Lozupone et al. 2007), did not show obvious changes after the treatment (Fig. 3a and b). Cock et al. (2016) reported that oral care with erythritol has a bacteriostatic effect on pathogenic bacteria associated with periodontal disease and caries. From the above results and reports, it appears that erythritol exerted a bacteriostatic effect on several bacterial species in periodontal disease lesions, resulting in no change in the composition of the microbiota, but a decrease in species evenness.

An examination of erythritol treatment – associated changes in microbiota occupancy at the species level showed significant decreases in the number of canine periodontal disease – related bacteria such as *P. gulae*, *P. cangingivalis*, *Fusobacterium*, and *Bergeyella zoohelcum* (Chen et al. 2017, Muramatsu et al. 2019) (Table 1). This result was similar to the reported effect of erythritol on periodontal disease–related bacteria in humans (Hashino et al. 2013, Cock et al. 2016) and shows that erythritol exerts the same effect on periodontal disease – related bacteria in both canines and humans. Moreover, the results of pairwise Spearman's correlation coefficient analyses of occupancy of each of the bacteria before and after erythritol treatment showed that *P. gulae* was positively correlated with most bacteria compared to other bacteria decreased by erythritol treatment (Fig. 4). From these results, it was assumed that *P. gulae* coexists with various bacteria in periodontal lesions. Future research should be conducted using co-culture and other approaches.

Based on the above results, we conclude that erythritol exerts a bacteriostatic effect on periodontal disease – related bacteria such as *P. gulae* in canines, similar to its effect in humans. On the other hand, our intervention study has some limitations, such as short duration, lack of double-blind design, and inability to observe any improvement in periodontal disease. Annual intervention studies are usually required to observe improvements in periodontal disease using erythritol treatment. Larger and longer-term clinical trials will therefore be needed in the future. Nevertheless, this study suggests that erythritol is an effective dental care material for preventing or improving canine periodontal disease.

References

Allaker RP, Young KA, Langlois T, Rosayro RD, Hardie JM (**1997**) Dental plaque flora of the dog with reference to fastidious and anaerobic bacteria associated with bites. J Vet Dent 14: 127-130.

Bellows J, Berg ML, Dennis S, Harvey R, Lobprise HB, Snyder CJ, Stone AES, Van de Wetering AG (**2019**) AAHA Dental Care Guidelines for Dogs and Cats. J Am Anim Hosp Assoc 55: 49-69.

Chen Y, Liao K, Ai L, Guo P, Huang H, Wu Z, Liu M (**2017**) Bacteremia caused by Bergeyella zoohelcum in an infective endocarditis patient: case report and review of literature. BMC Infect Dis 17: 271.

Dean I, Jackson F, Greenough RJ (**1996**) Chronic (1-year) oral toxicity study of erythritol in dogs. Regul Toxicol Pharmacol 24: S254-S260.

de Cock P (**2018**) Erythritol functional roles in oral-systemic health. Adv Dent Res 29: 104-109.

de Cock P, Mäkinen K, Honkala E, Saag M, Kennepohl E, Eapen A (**2016**) Erythritol is more effective than xylitol and sorbitol in managing oral health endpoints. Int J Dent 2016: 9868421.

Dos Santos JD, Cunha E, Nunes T, Tavares L, Oliveira M (**2019**) Relation between periodontal disease and systemic diseases in dogs. Res Vet Sci 125: 136-140.

Eubanks DL (**2010**) Periodontal disease assessment. J Vet Dent 27: 58-60.

Gu Y, Fujitomo Y, Ohmagari N (**2021**) Outcomes and Future Prospect of Japan's National Action Plan on Antimicrobial Resistance (2016-2020). Antibiotics 10: 1293.

Hashino E, Kuboniwa M, Alghamdi SA, Yamaguchi M, Yamamoto R, Cho H, Amano A (**2013**) Erythritol alters microstructure and metabolomic profiles of biofilm composed of Streptococcus gordonii and Porphyromonas gingivalis. Mol Oral Microbiol 28: 435-451.

Kortegaard HE, Eriksen T, Baelum V (**2008**) Periodontal disease in research beagle dogs-an epidemiological study. J Small Anim Pract 49: 610-616.

Kuzuya T, Kanazawa Y, Kosaka K (**1969**) Stimulation of insulin secretion by xylitol in dogs. Endocrinology 84: 200-207.

Lozupone CA, Hamady M, Kelley ST, Knight R (**2007**) Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Appl Environ Microbiol 73: 1576-1585.

Munro IC, Berndt WO, Borzelleca JF, Flamm G, Lynch BS, Kennepohl E, Bär E A, Modderman J (**1998**) Erythritol: an interpretive summary of biochemical, metabolic, toxicological and clinical data. Food Chem Toxicol 36: 1139-1174.

Muramatsu Y, Haraya N, Horie K, Uchida L, Kooriyama T, Suzuki A, Horiuchi M (**2019**) Bergeyella zoohelcum isolated from oral cavities of therapy dogs. Zoonoses Public Health 66: 936-942.

Murphy LA, Coleman AE (**2012**) Xylitol toxicosis in dogs. Vet Clin North Am Small Anim Pract 42: 307-312.

Nakakuki T (**1998**) Development of functional oligosaccharides in Japan. Trends Glycosci. Glycotechnol. 178: 19-28.

Noda K, Nakayama K, Modderman J (**1996**) Fate of erythritol after single oral administration to rats and dogs. Regul Toxicol Pharmacol 24: S206-S213.

Nomura R, Inaba H, Yasuda H, Shirai M, Kato Y, Murakami M, Iwashita N, Shirahata S, Yoshida S, Matayoshi S, Yasuda J, Arai N, Asai F, Matsumoto-Nakano M, Nakano K (**2020**) Inhibition of Porphyromonas gulae and periodontal disease in dogs by a combination of clindamycin and interferon alpha. Sci Rep 10: 3113.

Nomura R, Shirai M, Kato Y, Murakami M, Nakano K, Hirai N, Mizusawa T, Naka S, Yamasaki Y, Matsumoto-Nakano M, Ooshima T, Asai F (**2012**) Diversity of

fimbrillin among Porphyromonas gulae clinical isolates from Japanese dogs. J Vet Med Sci 74: 885-891.

Sarkiala E, Harvey C (**1993**) Systemic antimicrobials in the treatment of periodontitis in dogs. Semin Vet Med Surg Small Anim 8: 197-203.

Senhorinho GN, Nakano V, Liu C, Song Y, Finegold SM, Avila-Campos MJ (**2011**) Detection of Porphyromonas gulae from subgingival biofilms of dogs with and without periodontitis. Anaerobe 17: 257-258.

Shannon CE (**1949**) A mathematical theory of communication. Bell Syst Tech J 27: 379-423.

Shoukry M, Ben AL, Abdel NM, Soliman A. (**2007**) Repair of experimental plaque-induced periodontal disease in dogs. J Vet Dent 24: 152-165.

Yumoto T, Kowada T, Sumiya F, Matsumoto T, Fujita K, Yamamura H, Sakai T (**2004**) Surgery and bacteria isolation in treating severe periodontal disease in old dogs. J Jpn Vet Med Assoc 57: 41-45.