Integrative Diagnostic and Therapeutic Approaches to Canine Periodontal Disease: Glyconanotechnology-Based Molecular Detection of Periodontopathic Bacteria and Evaluation of Aged Garlic Extract as a Natural Gingivitis Intervention

（犬の歯周病に対する統合的診断・治療アプローチ
―糖鎖ナノテクノロジーによる病原因子の分子生物学的検出と
熟成ニンニク抽出物の歯肉炎改善効果の評価―）

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Integrative Diagnostic and Therapeutic Approaches to Canine Periodontal Disease: Glyconanotechnology-Based Molecular Detection of Periodontopathic Bacteria and Evaluation of Aged Garlic Extract as a Natural Gingivitis Intervention

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Thesis Copyright Declaration

I, Kaori Takahashi, certify that this thesis has been written by me, that it is the record of work carried out by me (unless otherwise stated), and that it has not been submitted in any previous application for a higher degree.

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Abstract

Periodontal disease is one of the most common progressive inflammatory conditions in companion animals, particularly dogs. It begins as gingivitis and gradually develops into periodontitis if not adequately managed. As the disease progresses, it leads to the destruction of periodontal tissues, tooth loss, and systemic complications, including cardiovascular, renal, and possibly cognitive disorders. The silent and chronic nature of periodontal disease, coupled with the challenges of detection in veterinary settings, often results in underdiagnosis and delayed treatment. Conventional diagnostic approaches, such as probing and radiographic assessment, are effective but invasive and typically require general anesthesia. Similarly, preventive and therapeutic interventions, including professional dental cleaning and daily tooth brushing, are hindered by compliance issues, cost, and animal temperament.

This thesis presents an integrated investigation into both the diagnostic and therapeutic aspects of canine periodontal disease through two original studies.

In the first chapter, I focused on developing a sensitive and noninvasive molecular diagnostic tool for detecting periodontopathic bacteria in dogs. Using a novel method based on sugar chain-immobilized magnetized nanoparticles (SGNP) and magnetic microparticles (MMP), combined with real-time PCR, oral swab samples from 52 dogs

were analyzed to identify eight key bacterial species implicated in periodontal disease. Among these, *Treponema denticola* exhibited the strongest positive correlation with clinical signs of gingival recession and disease severity. The SGNP/MMP-qPCR method demonstrated high sensitivity and specificity, enabling quantitative assessment of bacterial load and offering a practical solution for early-stage screening in clinical practice.

In the second Chapter, I evaluated the therapeutic potential of aged garlic extract (AGE), a natural compound known for its antioxidative, anti-inflammatory, and antimicrobial properties. While raw garlic is contraindicated in dogs due to the risk of hemolytic anemia, AGE is a processed form that eliminates toxic components and has been confirmed as safe in previous toxicological assessments. In this study, ten Beagle dogs with mild gingivitis were administered AGE at a dosage of 18 mg/kg/day for eight weeks. Clinical parameters were comprehensively evaluated, including gingival index scores, volatile sulfur compound (VSC) concentrations in exhaled air, periodontal bacterial enzyme activity, and salivary concentrations of cathelicidin, an antimicrobial peptide linked to oral innate immunity. The results revealed that AGE administration significantly improved gingival health, reduced halitosis-related compounds, and enhanced immune-related salivary markers, all without adverse effects on general health or hematological profiles.

In conclusion, these two studies offer a multifaceted approach to addressing the challenges of periodontal disease in veterinary medicine. The diagnostic study establishes a robust, noninvasive method for detecting microbial profiles associated with disease severity, enabling more accurate assessments without the need for anesthesia. Concurrently, the therapeutic study supports the clinical utility of AGE as a safe and effective natural supplement for managing gingival inflammation in dogs. The integration of molecular diagnostics and phytotherapeutic intervention represents a promising direction for improving the standard of care in veterinary oral health. Moreover, these findings contribute to the broader understanding of microbial pathogenesis, host immune responses, and the translational potential of natural compounds in veterinary practice. By bridging fundamental microbiological research with practical clinical application, this thesis provides an evidence-based foundation for the early diagnosis, targeted treatment, and holistic management of periodontal disease in dogs.

General Introduction

Periodontal disease is one of the most common oral health issues in dogs, with a reported prevalence exceeding 80% among those over two years of age [1,2,40,109]. It is a progressive inflammatory disease affecting the tissues supporting the teeth, including the gingiva, periodontal ligament, cementum, and alveolar bone. The etiology of periodontal disease is complex and multifactorial, but it is primarily initiated by bacterial plaque biofilm that accumulates on the tooth surface and gingival margin. If left untreated, the condition can lead to gingival recession, root exposure, tooth mobility, and ultimately tooth loss. In addition to local oral damage, periodontal disease is increasingly recognized as a systemic health threat in dogs, being associated with cardiac, renal, and even cognitive disorders [3-5]. As companion animals age and their roles in human families become more prominent, maintaining their oral health is essential not only for their well-being but also for their quality of life and longevity.

Despite its high prevalence, the diagnosis and monitoring of periodontal disease in veterinary medicine remain challenging. Traditional diagnostic procedures such as probing pocket depths and dental radiography often require general anesthesia, which imposes additional risks and costs [1]. Furthermore, gross visual inspection without anesthesia frequently underestimates the actual severity of periodontal disease. Thus,

there is an urgent need for simple, noninvasive, and objective methods that can be used in routine clinical settings to detect early signs of disease and monitor its progression.

My study addressed this diagnostic challenge by developing a novel molecular screening technique using sugar chain-immobilized magnetized gold nanoparticles and magnetic microparticles (SGNP/MMP) [11,25,26]. This method enables the efficient concentration and purification of bacterial DNA from oral swab samples, allowing for highly sensitive detection and semiquantitative analysis of key periodontopathic bacteria via real-time PCR (qPCR). The study focused on several bacterial species, including those belonging to the human "red complex" (*Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*) and those more commonly associated with dogs, such as *Porphyromonas gulae* and *P. cangingivalis*. Among these, *T. denticola* showed the strongest correlation with the severity of periodontal disease in dogs, suggesting its potential as a reliable biomarker. This diagnostic approach offers veterinarians a valuable tool for early identification and stratification of periodontal disease in clinical practice.

Parallel to advancements in diagnostics, there is a growing interest in developing safe and effective treatments for early periodontal inflammation. Conventional therapies such as professional dental cleaning and systemic antibiotics are often limited by practical challenges, including the need for anesthesia and concerns regarding antimicrobial

resistance. Therefore, alternative therapeutic strategies, particularly those derived from natural products, have attracted considerable attention. Aged garlic extract (AGE), a preparation derived from garlic aged in ethanol for several months, has demonstrated various pharmacological properties, including antioxidative, anti-inflammatory, and immunomodulatory effects [61-69]. My second study investigated the therapeutic potential of AGE in dogs with mild gingivitis and found that its oral administration significantly improved clinical parameters such as gingival index scores and volatile sulfur compound levels in the breath, without inducing adverse hematological effects. Notably, AGE also increased the concentration of salivary cathelicidin, an antimicrobial peptide that contributes to innate immunity in the oral cavity.

Collectively, these two studies provide a synergistic framework for managing canine periodontal disease. The SGNP/MMP-based diagnostic method enables early and precise detection of disease-associated bacteria, while AGE presents a safe and natural therapeutic option to ameliorate early gingival inflammation. Integrating these approaches has the potential to revolutionize veterinary dental care by promoting early diagnosis, guiding targeted interventions, and improving overall treatment outcomes.

Through these complementary studies, this thesis aims to explore the diagnostic and therapeutic aspects of periodontal disease in dogs. By combining innovative molecular

diagnostics with functional dietary intervention, this work contributes to developing holistic, evidence-based strategies for preventing, detecting, and managing periodontal disease in companion animals.

Chapter 1

Detection of Periodontopathic Bacteria and Their Correlation with Periodontal Disease in Dogs Using a Novel Molecular Screening Method

1-1 Abstract

This study sought to create a simple method for screening and detecting oral bacteria associated with periodontal disease as well as to identify the bacterial species that had the strongest correlation with disease severity in dogs. Fifty-two dogs from the Kagoshima University Veterinary Teaching Hospital were included. The periodontal disease status, which included gingival recession, root surface exposure, and gingival redness, was determined through visual examination. Oral swab samples were taken from each dog's gingival margin or tooth cervix using a sterile cotton swab following a standardized protocol. The collected samples were applied to our nanotechnology named sugar chain immobilized magnetized gold nanoparticle/magnetic microparticle (SGNP/MMP) method to obtain bacterial DNA, which was then subjected to qualitative and semiquantitative PCR analyses for 6 genera and 8 species of bacteria: *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*), *Treponema denticola* (*T. denticola*), *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*), *Fusobacterium nucleatum* (*F. nucleatum*), *Prevotella intermedia* (*P. intermedia*), *Porphyromonas gulae* (*P. gulae*), and *Porphyromonas cangingivalis* (*P. cangingivalis*). The severity of periodontal disease was most strongly correlated with *T. denticola* (associated with the gingival recession), followed by slight total bacteria and *P. gingivalis*+*P. gulae*, but no significant correlation was found with *P. gulae.* Within the scope of this study, the

analytical method used may represent a useful standard for screening and detecting specific bacteria, and the quantity of *T. denticola* is linked to the severity of periodontal disease in dogs.

1-2 Introduction

Periodontal disease represents the most prevalent oral condition affecting domestic dogs, with a reported prevalence exceeding 80% among animals older than two years [1, 2,40,109]. This disease is characterized by chronic inflammation of the supporting structures of the teeth, primarily driven by the accumulation of bacterial plaque and the host's immune response. If left untreated, periodontal disease progresses from reversible gingivitis to irreversible periodontitis, resulting in alveolar bone loss, tooth mobility, and eventual tooth loss. In addition to its local oral effects, periodontal disease in dogs has been increasingly recognized as a contributing factor to systemic diseases, including cardiovascular dysfunction, renal disease, and cognitive decline [3–5].

The elucidation of the microbial etiology of periodontal disease is essential for improving diagnostic and therapeutic strategies. In humans, the so-called "red complex" bacteria—*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*—are widely acknowledged as principal periodontopathic organisms [6]. These anaerobic Gram-negative bacteria possess potent virulence factors that facilitate immune evasion, tissue destruction, and biofilm formation [7, 8]. Although initially thought to be exclusive to humans, recent studies have detected these bacteria or closely related species within the canine oral microbiota, indicating the possibility of interspecies transmission [9–12]. For instance, *Porphyromonas gulae*, a close phylogenetic relative of *P. gingivalis*, has been

frequently isolated from dogs, although its specific role in disease progression remains a subject of ongoing debate [13–16].

Currently, the diagnosis of periodontal disease in veterinary practice predominantly relies on visual examination, periodontal probing, and dental radiography, procedures which are invasive and frequently necessitate general anesthesia [1, 5, 17]. These methods are prone to underestimating the true severity of disease due to inherent limitations. In contrast, molecular diagnostic methods such as real-time polymerase chain reaction (qPCR) have profoundly advanced human periodontal microbiology [6, 7, 18], but their application in veterinary practice remains limited, primarily due to technical and economic constraints.

The purpose of this study was to assess the diagnostic potential of a novel molecular approach utilizing sugar chain-immobilized magnetized gold nanoparticles (SGNP) and magnetic microparticles (MMP) to concentrate bacterial DNA obtained from canine oral swab samples. This method, in conjunction with qPCR, enables the detection and semiquantitative analysis of principal periodontopathic bacteria in dogs, including *P. gingivalis*, *T. denticola*, *T. forsythia*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, *P. gulae*, and *P. cangingivalis* [7, 8, 14, 19–22]. By integrating this novel molecular screening method with clinical periodontal assessments, the study aimed to identify specific bacterial profiles correlated with the severity of

periodontal disease. The development and validation of such a noninvasive, rapid, and objective diagnostic tool is anticipated to enhance early diagnosis and support more targeted therapeutic interventions in the field of veterinary dentistry.

1-3 Materials and Methods

Test companion animals

This study included 52 dogs who visited Kagoshima University Animal Hospital. Animals administered antimicrobials within 2 weeks of sample collection were excluded. Informed consent was provided by the owner of each dog.

Sampling

The samples were collected by wiping the buccal cervical area (gingival margin) of the target tooth(e.g., canine, premolar, or molar) 3 times in one direction with 1 cm width using a sterile cotton swab bar (SPO415-1, Osaki medical, Nagoya, Japan) without anesthesia. The target tooth was randomly chosen from an individual dog by me.

Evaluation of periodontal disease

The stages of periodontal disease in animals are generally categorized into 5 stages as 0 to 4 (PD 0~4) based on the evaluation of attachment loss with dental radiography or probing pocket depth, per the staging system standardized by the American Veterinary Dental College (AVDC) veterinary dental nomenclature [1]. However, in this research, the investigator visually studied the selected sampled teeth for gingival recession, gingival redness, and the degree of tooth root exposure without general anesthesia to assess the

status of periodontal disease using the alternative visual staging system developed for this study (Table 1). When distinguishing between 2 and 3 or 3 and 4 was difficult, the score was assigned 2.5 or 3.5, respectively. Furthermore, each tooth was divided into two groups based on a simple and more specific classification: Group 0 without gingival recession (PD 0 and 1) and Group 1 with gingival recession (PD 2, 3, and 4).

Target DNA sequences, PCR primers, and probes for each bacterium

P. gingivalis and *P. gulae* (126 bp in 16s rRNA) (common primer and probe set) [35, 37]

TACCCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACAAGCTAATGGGACGCATGCCTATCTT

ACAGCTATAAATATTTCCTTGTAATATCATGCAATAATACAAGTGTATGCGGTTTTAGTCCG;

Forward: TACCCATCGTCGCCTTGGT, Reverse: CGGACTAAAACCGCATACACTTG,

Probe: GCTAATGGGACGCATGCCTATCTTACAGCT-ROX

T. denticola (122 bp in 16s rRNA) [7,8,33]

CCGAATGTGCTCATTTACATAAAGGTAAATGAGGAAAGGAGCTACGGCTCCGCTTCAGGATGGG

CCCGCGTCCCATTAGCTAGTTGGTGAGGTAAAGGCCCACCAAGGCAACGATGGGTATC;

Forward: CCGAATGTGCTCATTTACATAAAGGT, Reverse: GATACCCATCGTTGCCTTGGT,

Probe: ATGGGCCCGCGTCCCATTAGC-Cy5

T. forsythia (127 bp in 16s rRNA) [7,8,33]

GGGTGAGTAACGCGTATGTAACCTGCCCGCAACAGAGGGATAACCCGGCGAAAGTCGGACTAAT

ACCTCATAAAACAGGGGTTCCGCATGGGGCTATTTGTTAAAGATTTATTGGTTGCGGATGGGC;

Forward: GGGTGAGTAACGCGTATGTAACCT, Reverse: ACCCATCCGCAACCAATAAA,

Probe: CCCGCAACAGAGGGATAACCCGG-FAM

F. nucleatum (101 bp in 16s rRNA) [35]

CGCAGAAGGTGAAAGTCCTGTATAAGTAAATCCTTACACATATAACTTTGCTCCCAAGTAACAT

GGAACACGAGGAATTCTGTGTGAATCAGTGAGGACCA;

Forward: CGCAGAAGGTGAAAGTCCTGTAT, Reverse: TGGTCCTCACTGATTCACACAGA

P. intermedia (103 bp in 16s rRNA) [27]

ATCCAACCTTCCCTCCACTCGGGGATACCCCGTTGAAAGACGGCCTAATACCCGATGTTGTCCA

CATATGGCATCTGACGTGGACCAAAGATTCATCGGTGGA,

Forward: ATCCAACCTTCCCTCCACTC, Reverse: TCCACCGATGAATCTTTGGTC

A. actinomycetemcomitans (77 bp in 16s rRNA) [14]

CTTACCTACTCTTGACATCCGAAGAAGAACTCAGAGATGGGTTTGTGCCTTAGGGAGCTTTGAG

ACAGGTGCTGCAT;

Forward: CTTACCTACTCTTGACATCCGAA, Reverse: ATGCAGCACCTGTCTCAAAGC

P. gingivalis (128 bp in 16s rRNA)

AACCGCATACACTTGTATTATTGCATGATATTACAAGGAAATATTTATAGCTGTAAGATAGGCAT

GCGTCCCATTAGCTGGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGGGTAGGGGAACTGA;

Forward: AACCGCATACACTTGTATTATTGC, Reverse: TCAGTTCCCCTACCCATCGT

P. gulae (116 bp in 16s rRNA)[15]

TTGCTTGGTTGCATGATCGGGCAAGGAAATATTTATAGCTGTAAGATAGGCATGCGTCCCATTA

GCTGGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGGGTAGGGGAACTGA;

Forward: TTGCTTGGTTGCATGATCG, Reverse: TCAGTTCCCCTACCCATCGT

P. cangingivalis (118 bp in 16s rRNA) [18]

ACTTTGGTTTCCGCATGGGGACTTTAGGAAAGATTTATTGCTGACAGATAGGCATGCGTTCCAT

TAGATAGTTGGTGAGGTAACGGCTCACCAAGTCAACGATGGATAGGGGAGCTGA;

Forward: ACTTTGGTTTCCGCATGGGGAC, Reverse: TCAGCTCCCCTATCCATCGTTGAC

Total bacteria using universal primers (467 bp in 16s rRNA)[16]

TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCG

CGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACATATGTGTAAGTA

ACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG

TAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTT

AAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGT

GCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACC

AGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAAC

AGGATTAGATACCCTGGTAGTCC;

Forward: TCCTACGGGAGGCAGCAGT, Reverse: GGACTACCAGGGTATCTAATCCTGTT

PCR apparatus and protocols and calibration of plasmids containing the DNA sequence of each bacterium

This study's PCR apparatus was a Thermal Cycler Dice system III (Takara-Bio Inc., Kusatsu, Shiga, Japan). DNA from all bacterial species, except *A. actinomycetemcomitans, P. gulae*, and *P. cangingivalis,* were generously donated by my colleagues from Kagoshima University and Hiroshima University. The DNA of *A. actinomycetemcomitans*, *P. gulae*, and *P. cangingivalis* was taken from the samples of the companion animals during the preliminary experiments. The PCR conditions and reaction mix were as follows:

1) *P. gingivalis* + *P. gulae* (using the same primer/probe set), *T. denticola*, and *T. forsythia*: multiplex PCR with a probe method (Takara kit RR064, Takara-Bio Inc.). The reaction mixture is illustrated in Appendix Table A1-1. The PCR protocol was 95°C for 20 s,

followed by 45 cycles at 95°C for 5 s, and 60°C for 40 sec. The cycle threshold (Ct) value, which is a quantitative evaluation based on the number of cycles required to achieve a specific amount of PCR amplification product, was calculated using the software included with the PCR apparatus.

2) *F. nucleatum*, *P. intermedia*, *P. gingivalis*, *P. gulae*, and *P. cangingivalis*: PCR was carried out using the intercalator method (Takara kit RR820, Takara-Bio Inc.) and the primer set described above. The reaction mixture is illustrated in Appendix Table A1-2. The PCR protocol was as follows: 95°C for 20 s, 45 cycles at 95°C for 5 s, and 60°C for 30 s, followed by melting analysis. Melting curves from 60°C to 95°C were obtained during increments of 1°C every 30 sec. The Ct and melting temperature (Tm) values were calculated using the software included with the PCR apparatus. The Tm value from the first melting curve was verified to be within ± 0.5°C for each assay.

3) *A. actinomycetemcomitans*: PCR was carried out using the intercalator method (Takara kit RR820) and the primer set described above. The reaction mixture is illustrated in Appendix Table A1-2. The PCR protocol was 95°C for 300 s, 40 cycles at 95°C for 15 s, and 60°C for 60 s, followed by melting analysis. The melting curves from 60°C to 95°C were obtained during increments of 1°C every 30 sec. The Ct and Tm values were obtained

using the methods described above.

4) Total bacteria (universal primers): PCR was conducted using the intercalator method (Takara kit RR070, buffer, and dNTP mixture, Takara-Bio Inc.); RR064 (EXTaq HS, Takara-Bio Inc.); and 5761A (SYBR-Green, Takara-Bio Inc.) with the primer set explained above. The reaction mixture is displayed in Appendix Table A1-3. The PCR protocol was 95°C for 300 s, 30 cycles at 95°C for 15 s, and 60°C for 60 s, followed by melting analysis. The melting curves from 60°C to 95°C were obtained during increments of 1°C every 30 sec. The Ct and Tm values were obtained using the methods described above.

Preparation of the control plasmid DNA and calibration

The obtained PCR products were examined on a 1% agarose (Nacalai Tesque, Kyoto, Japan) gel stained with 3,8-diamino-1-ethyl-6-phenylphenantridinium bromide dye (Nippon gene, Toyama, Japan). The approximate size band in the gel was cut, and the DNA of the PCR products from the cut band was extracted and purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel GmbH & Co., Gutenberg, Germany). The DNA was then ligated into the plasmid DNA using pGEM-T Easy Vector (Promega, Madison, Wisconsin, USA). The plasmid vectors were added into DH5α *E. coli* cells (DNA-903F, Toyobo Co., Osaka, Japan) and grown in the culture medium used in the kit, and plasmid

DNA was purified using a NucleoSpin Plasmid kit (Macherey-Nagel GmbH & Co.). The control plasmid DNA was quantified using the OD260 method, and a series of 10-fold dilutions ranging from 10^8 to 100 copies/μL were prepared and tested using the real-time quantitative PCR SYBR Green kit.

The Ct value for each plasmid was calibrated using PCR primer sets and either the multiplex probe for *P. gingivalis* + *P. gulae* (common primer/probe set), *T. denticola*, and *T. forsythia*; PCR primer sets for *F. nucleatum*, *P. intermedia*, *A. actinomycetemcomitans*, *P. gingivalis*, *P. gulae*, and *P. cangingivalis*; or universal primers for total bacteria (Appendix Fig.A1-1–Appendix Fig.A1-10). On each experiment day, 10,000 copies of each control plasmid were amplified using qPCR. As shown in Appendix Table 3, the maximum standard deviation values were below 1.9% for the Ct value (+/−0.5) for each bacterium (*P. intermedia*) and 3.0% (+/−0.6) for the universal primer. Based on these findings, I only considered periodontal disease severity in companion animals.

Concentration and partial purification of bacteria and DNA extraction for qPCR analysis （SGNP/MMP method）

In this study, I used the previously established sugar chain immobilized magnetized gold nanoparticle (SGNP) method for viral detection [11] to rapidly concentrate and partially purify bacteria for qPCR pretreatment. This method captures and purifies viral

particles using a virus-binding SGNP before collecting them with a magnetic microparticle (MMP) containing O, Mg, Al, Si, Ti, and Fe (atom ratio 24:2:2:2:5:64). The sugar chain immobilized on the SGNP is low molecular weight dextran sulfate. The SGNP method has been used to identify human herpes viruses, influenza viruses, dengue viruses, HIV, and SARS-CoV-2.

This study used Kit I of the commercially available kit (SUDx-SARS-CoV-2 Detection Kit, SUDx-Biotec, Kagoshima, Japan). The collected bacteria were washed with phosphate-buffered saline (PBS) buffer before being destroyed by extracting DNA with a 0.1% SDS (sodium lauryl sulfate) aqueous solution. The extracted DNA was analyzed using qPCR, as previously described. The cotton bar (SPO415-1, Osaki-Medical, Nagoya, Japan) used to collect the swab samples was soaked in 1 mL of PBS containing 1% penicillin–streptomycin (PS) solution (Nacalai Tesque) free of divalent cations and kept at 4°C. Within 4 days, the PBS-soaked sample was centrifuged at 10,000 x *g* for 1 min to remove debris. Subsequently, 0.5 mL of the supernatant was stirred well with 10 μL of SGNP and then added to a 1.5-mL tube with MMP. After thoroughly mixing with a pipette, the tube was immediately placed on a magnet stand to collect the magnetic precipitate. After 10 s, the supernatant was removed, and 0.5 mL of PBS comprising PS was added to wash the magnetic precipitate in the tube. The tube was then immediately placed back in the magnet stand. After 10 s, the supernatant was removed completely, and 20 μL of 0.1%

SDS aqueous solution was added to the precipitate. Then, the precipitate was thoroughly mixed using a tap. Finally, after returning the tube to the magnet stand, 1 μL of the supernatant was used for qPCR analysis.

Statistical analysis

The association between the Ct values of each bacterium and the status classification was investigated using Spearman's rank correlation coefficient. The results were analyzed and visualized in the R programming environment with the following packages: *dplyr, tidyr, broom, patchwork,* and *ggplot2*. As a further and more conventional clinical approach, I divided the clinical status of periodontal disease into <2 and ≥2 groups(Group0 and Group1), as the alveolar bone dissolves and aggressive treatment is required at stage 2 or above. The Ct values of these groups were compared using the Mann–Whitney U test, a nonparametric test appropriate for two independent groups. All statistical analyses were carried out using the *ggplot2* and *dplyr* packages in R programming [12,31]

1-4 Ethical statement

This study was authorized by the Ethics Review Board of Kagoshima University Veterinary Teaching Hospital (KVH240004).

1-5 Results

This study included 52 dogs ranging from 6 months to 16 years old, with a mean age of 9.9 ±4.7 years and a median age 10±3.7 years. The breeds included 13 Toy Poodles, 11 Miniature Dachshunds, 5 Jack Russell Terriers, 5 mixed breeds, 3 Golden Retrievers, 3 Shiba dogs, 2 Pomeranians, 2 Chihuahuas, and 1 of each of the following: Shih Tzu, French Bulldog, Collie, English Setter, Beagle, Boston Terrier, Afghan Hound. Groups 0 and 1 had 24 and 28 dogs with periodontal disease, respectively.

As shown in Figure 1, the qPCR for each bacterium was positive for *P. gingivalis* + *P. gulae* in 47 dogs, *P. gingivalis* in 15 dogs, *T. denticola* in 39 dogs, *T. forsythia* in 14 dogs, *A. actinomycetemcomitans* in 27 dogs, *F. nucleatum* in 51 dogs, *P. intermedia* in 14 dogs, *P. gulae* in 35 dogs, and *P. cangingivalis* in 49 dogs, for a total of 52 dogs. Many different types of oral bacteria associated with human chronic/aggressive periodontitis were detected in dogs, with detection rates for *P. gingivalis* + *P. gulae*, *F. nucleatum, and A. actinomycetemcomitans* being 90%, 98%, and 52%, respectively.

I then analyzed the correlation between visual periodontal disease status and the Ct value of each bacterial species using data from 52 dogs (Appendix Table A2). The total bacterial Ct values decreased as periodontal disease severity increased (Figure 2). Using the approximation curve slope for the total number of bacteria as a reference, bacterial species with a steeper slope (i.e., Ct value decreased as periodontal disease severity

increased) were identified as being closely related to periodontal disease. However, bacterial species with less steep slopes, such as *A. actinomycetemcomitans* and *P. cangingivalis,* were found to be insignificant in the severity of periodontal disease in dogs. Of the 52 dogs, only 15 and 14 tested positive for *T. forsythia* and *P. intermedia*, so these two bacteria were excluded from the evaluation. The bacterial species with the steepest slopes were *P. gingivalis* + *P. gulae*, *T. denticola*, and *P. gulae*. These findings for dogs are summarized in Table 2, and they indicate that *T. denticola*, a component of the red complex in humans, plays a significant role in the severity of periodontal disease in dogs.

Next, I looked at the correlation between gingival recession PD <2 (Group 0) or ≥2 (Group 1) and the respective Ct values of *P. gingivalis* + *P. gulae*, *T. denticola*, and *P. gulae.* The alveolar bone begins to dissolve and active treatment is necessary at PD 2 or above. As shown in Figure 3, identifying the periodontal disease status based on the Ct value was not feasible for *P. gulae* (P = 0.0555) but it was feasible for *P. gingivalis* + *P. gulae* (P = 0.000200), *T. denticola* (P = 0.000000233), and total bacteria (P = 0.000103).

1-6 Discussion

In this study, I sought to create a simple method for screening and detecting oral bacteria associated with periodontal disease. For this purpose, qPCR was combined with nanotechnologies such as SGNP and MMP [25, 26] to pretreatment the samples. The SGNP/MMP method allows for rapid and efficient concentration of viruses and bacteria, significantly increasing PCR sensitivity while requiring no special equipment or time-consuming procedures, making it a clinically viable alternative to traditional methods. In the preliminary study, I compared the PCR results of *P. gingivalis* using three different methods; SGNP/MMP, MMP, and Qiagen extraction (Appendix; Method A1).

Following qPCR, some human red-complex bacteria were found in most dogs. The findings are partly consistent with previous reports (3,34) demonstrating the presence of common oral bacteria that may be transmitted between dogs and humans, and they highlight the importance of precise detection of these bacteria for the optimal treatment/prevention of periodontal disease as a zoonotic disease. In particular, I discovered a statistically significant relationship between the bacteria *T.denticola, P.gingivalis* + *P. gulae,* total bacteria, gingival recession, and periodontal disease status. The severity of gingival recession increased proportionally with the abundance of these 3 types of bacteria. Based on my findings, I propose a *T. denticola* Ct value of 29 as the cutoff for PD ≥2 periodontal disease. This cutoff Ct value had a fairly good correlation with

periodontal disease status in dogs, with a sensitivity of 100% and a specificity of 63% (Appendix Table 4). This data is consistent with the reported findings [2]. *P. gulae* has been reported to be either frequently associated with or not associated with periodontal disease in dogs [10,13,23], and its pathogenicity is being investigated. In the current study, the absolute correlation value of Ct of *P. gulae* with PD 0 to 4 was lower than 0.4 (0.339). Ct values did not vary significantly between PD ≥2 and <2 periodontal disease (P = 0.0555). *P. gingivalis* was found in only 15 of the 52 specimens. However, the combined detection of *P. gulae* and *P. gingivalis* resulted in a higher absolute correlation value of Ct; *P. gingivalis* + *P. gulae* with PD 0 to 4 (0.488) and considerably distinct Ct values between PD ≥2 and <2 (P = 0.000200).

However, this study has several limitations that need to be addressed in future research. First, an expert performed a visual inspection of the status of periodontal disease on a limited number of teeth using an independently employed classification without anesthesia. Although the evaluation of each animal takes only about 30 s, the possibility of underestimating the severity of gingival recession/inflammation cannot be ruled out. Thus, a more accurate and standardized visual inspection, including intra-/inter examiner(s) calibration, should be performed under anesthesia to determine the severity and improve the assessment of periodontal disease/condition. Additionally, clinical examination (measurements of probing pocket depth, attachment level, and

bleeding on probing) as well as dental radiography to detect alveolar bone loss should be added to future studies. Second, the purpose of this study was to assess a diagnostic method capable of detecting bacterial profiles regardless of prior or concurrent systemic conditions, highlighting its potential for broader clinical application. However, the potential impact of these conditions (e.g., immune disorders or oral hygiene practices) on bacterial profiles and disease progression was not investigated, owing to the small sample size. Future research should include larger cohorts and stratify cases based on underlying conditions to better understand their effect on periodontal disease progression. The third limitation is the primer/probe cross-reaction. In this study, a comparison of the primers/probes [10, 29, 32] revealed that those used for the detection of *T. denticola* did not cross-react with those used for the five other *Treponema* species (*T. socranskii, T. vincentii, T. maltophilum, T. medium,* and *T. pectinovorum*) (Appendix Fig. A2 and Appendix Fig. A3-1 – A3-6). However, the primers/probe for *T. denticola* may cross-reactivity with those for *T. putidum* (Appendix Fig. A2 and Appendix Fig. A3-7), which was found to cross-react in only one of the six reported strains in the NCBI database. As a result, the detection of *T. denticola* had low specificity because other unknown *Treponema* species, including *T. putidum,* could have been present [17]. Additional analysis, such as protein expression patterns and antibody reactivity, of the species is required. Nevertheless, I used the term *"T. denticola,"* because the goal of this study was to identify PCR-detectable bacteria

associated with periodontitis in companion animals.

In this study, I conducted qualitative, semiquantitative, and highly sensitive qPCR using my nanotechnology as described above to determine eight bacterial species in pretreatment oral swab samples from dogs with varying levels of periodontal disease. As a result, *T.denticola* was most closely linked to the progression of periodontal disease in dogs, supporting the previous report [2]. This finding suggests that the amount of *T. denticola* can be used to assess periodontal disease severity in companion animals. Furthermore, the Ct value obtained from highly sensitive qPCR could serve as a useful new standard for assessing the severity and prognosis of periodontal disease in companion animals.

1-7 Summary

This study successfully developed and validated a novel molecular diagnostic method using sugar chain-immobilized magnetized gold nanoparticles and magnetic microparticles (SGNP/MMP) in combination with real-time PCR for the detection and semiquantitative analysis of periodontopathic bacteria in dogs. By applying this technique to oral swab samples collected from 52 dogs with varying degrees of periodontal disease, I was able to sensitively identify eight bacterial species commonly implicated in canine and human periodontitis.

Among these, *Treponema denticola* emerged as the most strongly correlated species with clinical indicators of disease severity, such as gingival recession and visual periodontal status. The total bacterial load and the combined presence of *P. gingivalis* and *P. gulae* also showed significant associations with disease progression. These findings suggest that specific bacterial profiles—particularly the presence and abundance of red complex bacteria—may serve as reliable biomarkers for periodontal disease staging in dogs.

The SGNP/MMP-qPCR method demonstrated clear advantages over conventional diagnostic approaches. It is noninvasive, requires only a simple oral swab, and provides objective, quantifiable data that can complement visual assessment. This diagnostic tool

holds great potential for use in routine veterinary practice, enabling early detection of periodontal pathogens and facilitating timely intervention before irreversible damage occurs.

Overall, this study contributes to the advancement of molecular diagnostics in veterinary dentistry and highlights the value of integrating microbiological data into clinical decision-making. Future studies involving longitudinal monitoring and larger, diverse populations will be instrumental in further validating the clinical utility of this method and establishing standardized diagnostic criteria for canine periodontal disease.

1-8 Tables and Figures

Table 1. Assessment of periodontal disease

Abbreviation	AVDC staging	Visual status in this research
PD 0	No periodontal disease	No gingival recession, no gingival redness
PD 1	Gingivitis only without attachment loss	No gingival recession with gingival redness
PD 2	Early periodontal disease with less than 25% attachment loss	Slight gingival recession with/without gingival redness
PD 3	Moderate periodontal disease with 25–50% attachment loss	Mild gingival recession (exposed root) with/without gingival redness
PD 4	Advanced periodontal disease with more than 50% attachment loss	Severe gingival recession (severe exposure of root and root furcation) with/without gingival redness

AVDC: American Veterinary Dental College

Simple and Definite classification

PD 0 and 1: Group 0 without gingival recession

PD 2, 3, and 4: Group 1 with gingival recession

Table 2. Spearman's rank correlation coefficients between the status of each bacterium and Ct values

Bacteria	**Correlation**	**p_value**
A. actinomycetemcomitans	0.263	0.06
F. nucleatum	-0.252	0.07
P. cangingivalis	-0.065	0.65
P. gingivalis	-0.085	0.55
P. gingivalis + *P. gulae* (common primer/probe set)	-0.488	<0.05
P. gulae	-0.339	<0.05
P. intermedia	-0.367	<0.05
T. denticola	-0.676	<0.05
T. forsythia	-0.168	0.23
total bacteria using universal primers	-0.475	<0.05

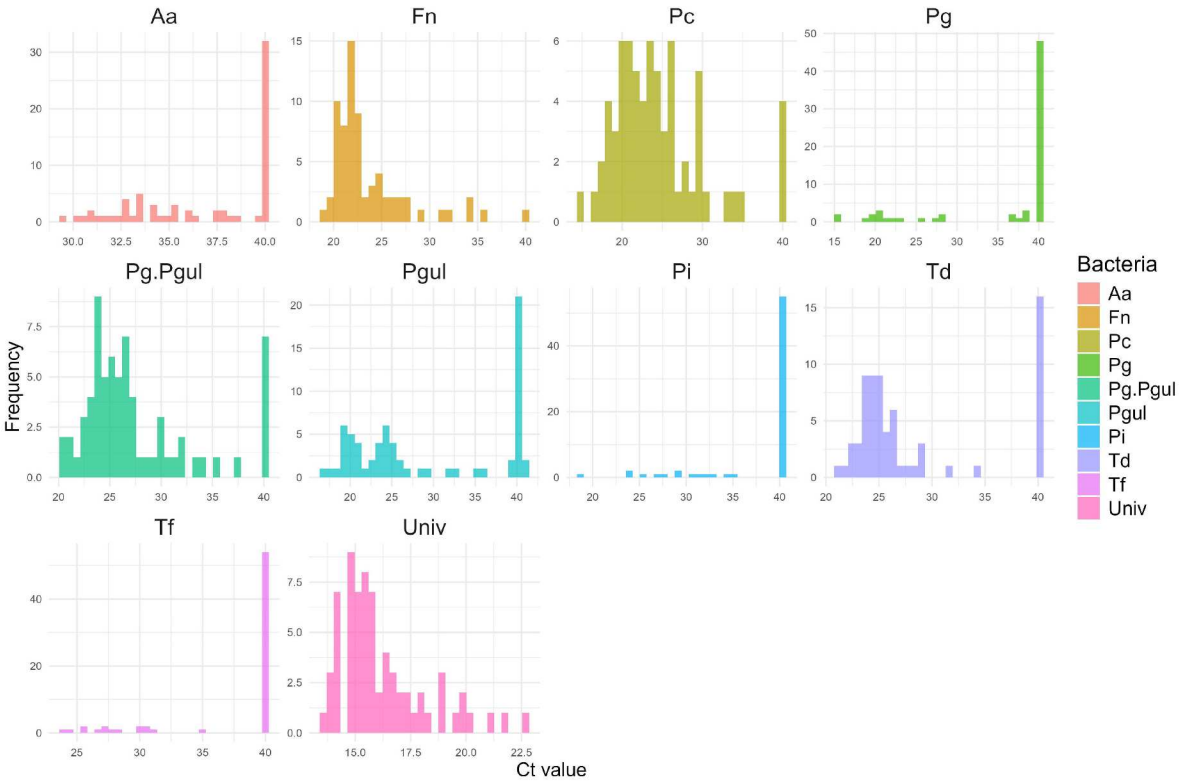

Figure 1. Histograms and Ct values of each bacterial species in the case of dog (Ct ≧ 40 means qPCR negative)

Aa: *A. actinomycetemcomitans*, Fn: *F. nucleatum*, Pc: *P. cangingivalis*, Pg: *P. gingivalis*, Pg.Pgul: *P. gingivalis* + *P. gulae* (common primer/probe set), Pgul: *P. gulae*, Pi: *P. intermedia*, Td: *T. denticola*, Tf: *T. forsythia*, Univ: total bacteria using universal primers

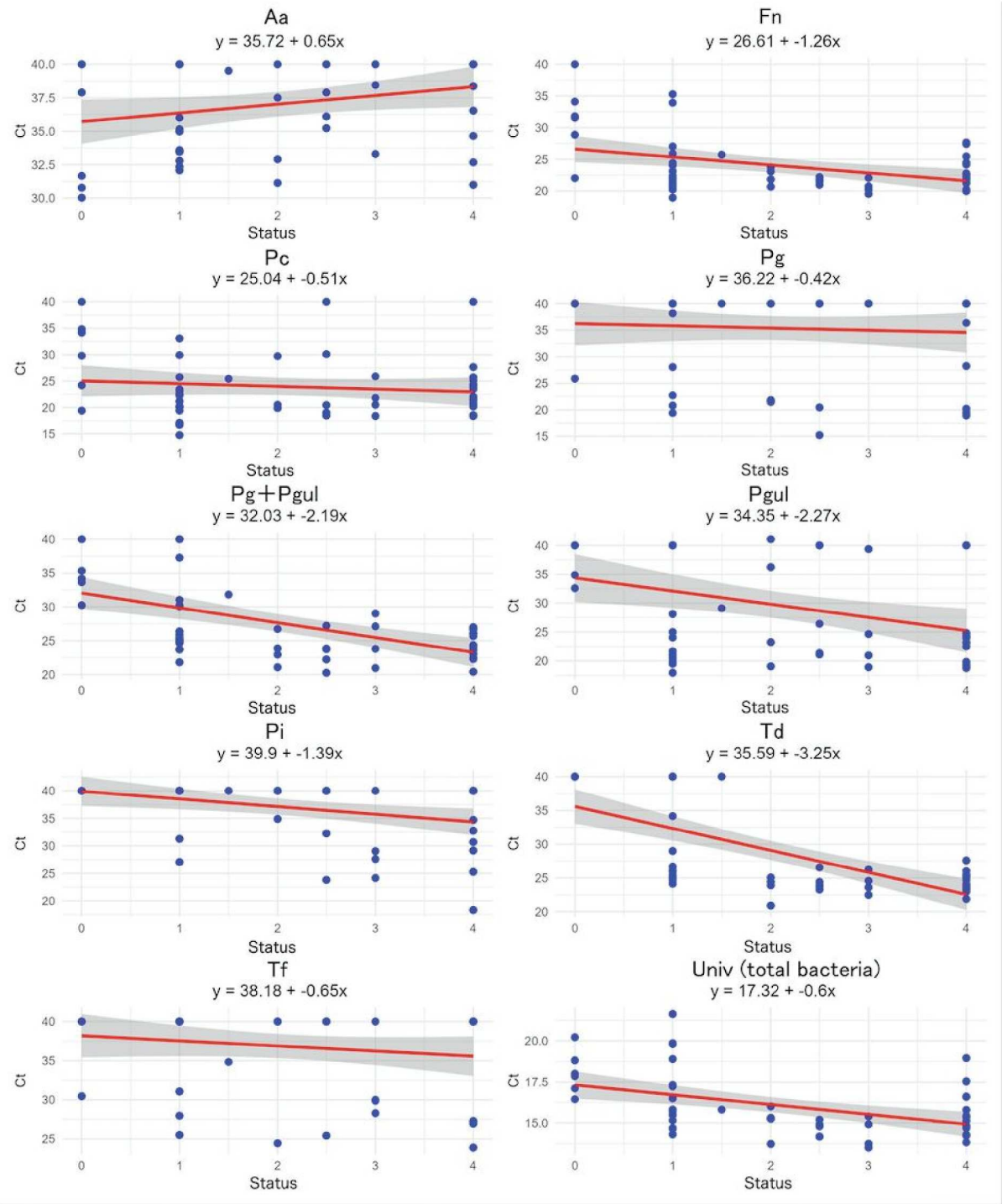

Figure 2. Scatter plot of the Ct values and status (0–4) for each bacterium

A steeper slope indicates a stronger correlation between periodontal disease status and Ct values.

Ct = 40 represents data not detected by PCR analyses. The figure was created using ggplot2 in R.

Aa: *A. actinomycetemcomitans*, Fn: *F. nucleatum*, Pc: *P. cangingivalis*, Pg: *P. gingivalis*, Pg.Pgul: *P. gingivalis* + *P. gulae* (common primer/probe set), Pgul: *P. gulae*, Pi: *P. intermedia*, Td: *T. denticola*, Tf: *T. forsythia*, Univ: total bacteria using universal primers

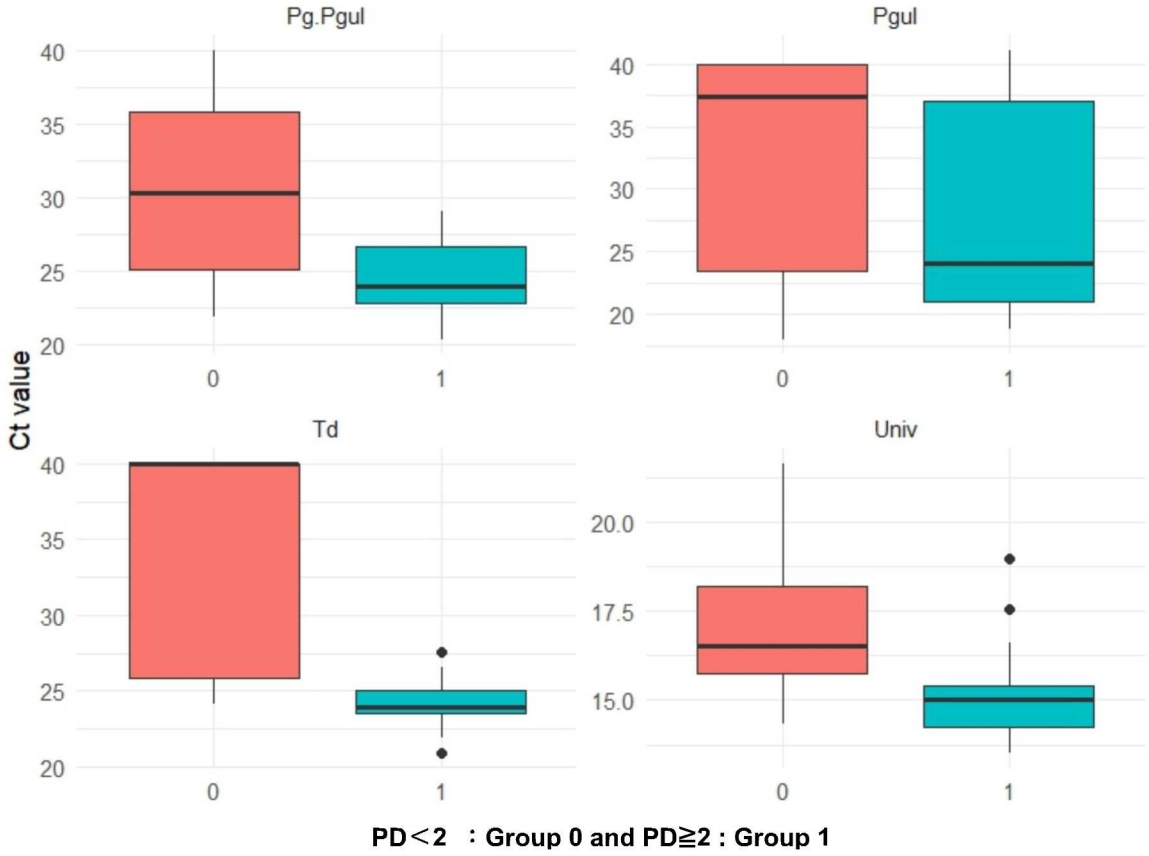

Figure 3. Differences in the Ct values of the bacteria targeted by each primer set between the two groups of gingival recession severity

PD <2 is denoted by Group 0, and PD ≥2 is denoted by Group 1.

The figure was created using ggplot2 in R.

P = 0.000200 for Pg.Pgul (*P. gingivalis* + *P. gulae* (common primers/probe)); P = 0.0555 for Pgul (*P. gulae*); P = 0.000000233 for Td (*T. denticola*); P = 0.000103 for total bacteria (Univ)

Chapter 2

Evaluation of the Therapeutic Effect of Aged Garlic Extract on Gingivitis in Dogs

2-1 Abstract

Periodontal disease is one of the most common dental health problems in dogs. Clinical studies in humans have shown that aged garlic extract (AGE), which contains stable and water-soluble sulfur-containing bioactive compounds, improves the symptoms of periodontal diseases. The previous study demonstrated that oral administration of AGE in healthy Beagle dogs at 90 mg/kg/day for 12 weeks had no adverse effects such as hemolytic anemia, which is well known to occur as a result of ingestion of *Allium* species, including onions and garlic, in dogs. However, the therapeutic potential of AGE in canine periodontal disease remains unclear. Accordingly, I investigated the therapeutic effects of AGE in Beagle dogs with mild gingivitis. Feeding 18 mg/kg/day of AGE for 8 weeks resulted in the improvement of gingival index score, level of volatile sulfur compounds in exhaled air, and enzyme activity of periodontal pathogens without any adverse effects on clinical signs and hematological and serum biochemical parameters. Moreover, AGE increased the concentration of salivary cathelicidin, an antimicrobial peptide that contributes to the oral innate immune response. These results suggest that AGE could be a potential therapeutic agent for canine gingivitis.

2-2 Introduction

As outlined in Chapter 1, periodontal disease, a set of dental inflammatory diseases initiated by oral microbiota on the tooth surface [38], is one of the most common health problems in dogs [39]. The prevalence of this disease is 44–100% across all dog breeds, based on clinical assessment and necropsy sample reports [40]. In general, periodontal disease is more prevalent in small dog breeds than in large ones [40, 41], and its incidence is strongly correlated with age [41–43]. Gingivitis is characterized by reversible inflammation and gingival redness without the loss of connective tissue attachment or alveolar bone. If this condition is left untreated, most, but not all, cases of gingivitis progress to periodontitis. Periodontitis is a severe chronic inflammation of the supporting tooth tissues that causes loss of connective tissue attachment and alveolar bone, possibly resulting in gingival recession, oronasal fistula, radicular abscess, tooth mobility, and tooth loss [44]. Periodontitis is caused by bacterial invasion and bacterial toxins, but the extent of the disease is changed by host immune reaction that depends on genetic, immunological, and environmental factors [45, 46]. Furthermore, periodontal disease is associated with systemic disorders in dogs, such as cognitive dysfunction [47], cardiac disease [41, 48], and renal disease [49, 50], ultimately leading to a poor quality of life.

Controlling the accumulation of oral microbiota on tooth surfaces and subsequent gingival inflammation is important for the prevention and treatment of periodontal diseases [51]. Treatment strategies for periodontal disease are generally based on homecare with tooth brushing and professional dental cleaning, dental gum, and antibiotics as needed. Daily tooth brushing is the most effective method for removing dental plaque. A previous study demonstrated that twice- daily tooth brushing for 18 months prevents the accumulation of plaque, debris, calculus, and the subsequent development of gingivitis and periodontitis in 10-month-old Beagle dogs [52]. Daily tooth brushing and oral administration of antibiotics are sometimes difficult to achieve because of poor compliance, lack of technique in owners, and aggressive temperament [53–55]. Therefore, more convenient oral agents for the prevention and treatment of gingivitis in pet dogs are required for owners and veterinarians.

Herbs and phytochemicals are utilized in human dentistry as antimicrobial, antiseptic, antineoplastic, antioxidant, and analgesic agents as well as for the elimination of halitosis [56, 57]. Such medicinal herbal plants include aloe (*Aloe vera*), green tea (*Camellia sinensis*), turmeric (*Curcuma longa*), kalonji (*Nigella sativa*), and neem (*Azadirachta indica*) as well as garlic (*Allium sativum*), which have been suggested as an alternate remedy for oral-dental problems in humans. Herbal-based treatments are mostly safer than synthetic drugs in humans, but some serious adverse effects may be happened [56].

Some compounds in fresh garlic have the potential to cause chemical burns to the skin and mucosa even in humans [58]. It is well- known that intake of garlic causes hemolytic anemia due to the oxidation of erythrocytes in dogs [59].

Aged garlic extract (AGE) is one of the garlic products prepared by the soaking of garlic with aqueous ethanol for more than 10 months at room temperature [60]. AGE and its bioactive sulfur-containing amino acids, *S*-allylcysteine, *S*-1-propenylcysteine, and *S*-allylmercaptocysteine, possess favorable properties such as antioxidation [61, 62], anti-inflammation [63–65], immunomodulation [66–68], and anti-cancer activities [69]. Several human clinical studies have demonstrated that AGE improves hypertension [70], atherosclerosis [71], and metabolic syndrome [72]. More recently, daily intake of AGE for 4 and 18 months was reported to improve the modified gingival index, gingival bleeding index, and probing pocket depth in human patients with mild-to-moderate periodontal disease [73, 74]. In support of these results, AGE and its bioactive sulfur-containing amino acids suppressed tumor necrosis factor-α-induced intracellular adhesion molecule-1 expression and interleukin-6 secretion in human gingival epithelial cell line Ca9-22 [75]. These results suggest that AGE suppresses gingival inflammation and the progression to periodontal disease. Furthermore, my previous study reported that oral administration of AGE at 45 and 90 mg/kg/ day for 8 and 12 weeks in Beagle dogs increased the gene expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and Nrf2- regulated anti-

oxidant enzymes NAD(P)H quinone oxidoreductase 1 and glutamate-cysteine ligase modifier subunit in whole blood without any adverse effects, including garlic-induced hemolytic anemia caused by oxidative injury of erythrocytes [76].

Although the safety and favorable bioactivity of AGE in dogs are already clarified as mentioned above, its therapeutic potential for canine periodontal disease remains unclear. The aim of this study was to evaluate the therapeutic effects of AGE supplementation on gingivitis in dogs.

2-3 Materials and Methods

Preparation of AGE and placebo powders

AGE was prepared as previously described [76]. Sliced cloves of garlic cultivated in Japan were soaked in an ethanol/water mixture for more than 10 months at room temperature. The extract was then dried using a circulation dryer (HOH-A3; Takabayashi Rika Co., Ltd., Tokyo, Japan). The AGE powder consisted of 18.0 mg of dried AGE extract, 19.0 mg of crystalline cellulose (Ceolus UF-F702; Asahi Kasei Chemicals Corporation, Tokyo, Japan), 0.9 mg of carboxymethyl cellulose calcium (E.C.G-FA; Nichirin Chemical Industries Ltd., Itami, Japan), and 5.0 mg of agar powder (Ina Food Industry Co., Ltd., Ina, Japan). The placebo powder that consists of crystalline cellulose (37.0 mg), calcium carboxymethyl cellulose (0.9 mg), and agar powder (5.0 mg) were used. The powder was stored at 4°C until further use. Produced AGE is sometimes analyzed by high-performance liquid chromatography to confirm to have several sulfur-containing compounds, *S*-methylcysteine, *S*-allylcysteine, *S*-1-propenylcysteine, and *S*-allylmercaptocysteine, which are characteristic sulfur compounds in AGE [77].

Experimental animals and treatments

The animal experiment was conducted with 10 Beagle dogs (4 males and 6 females, 2–9 years old, 9.8–11.8 kg body weight) housed at the Kitayama Labes Corporation, Narita Biocenter (Narita, Japan). Before the experiments, all dogs were confirmed to be clinically healthy based on physical examination, and hematological and serum biochemical analyzes. All dogs were housed at a temperature of 23 ± 5°C and relative humidity of 55 ± 25% under a 12 h light/dark cycle (light phase from 7:00 to 19:00). The 10 dogs were divided into two groups based on the average gingival index score: the placebo- treated control group referred as Placebo group (4.6 ± 1.2 years of age, 10.76 ± 0.41 kg body weight, and the average gingival index score 0.57 ± 0.10) and the AGE-treated group referred as AGE group (3.8 ± 0.8 years of age, 10.76 ± 0.35 kg body weight, the average gingival index score 0.53±0.13), with each group consisting of five dogs (2 males and 3 females). Each dog was fed 250 g of dry food (DS-A; Oriental Yeast Co., Ltd., Tokyo, Japan) sprinkled with either a 42.9 mg/ kg of placebo or AGE powder (18 mg/kg dried AGE extract) once daily between 11:00 and 12:00 for 8 weeks. Water was provided *ad libitum*. After feeding, I confirmed that all dogs had consumed the food completely. All animal experiments complied with the Guidelines for Animal Experiments of Kitayama Labes Corporation and were approved by the Animal Welfare Committee of Kitayama Labes Corporation (Approval Number NBC57-024).

Clinical observation and measurement of body weight

The dogs were inspected every morning for clinical manifestations, such as fecal characteristics, vomiting, coat condition, and behavior during the experimental period. The dogs were weighed at baseline (1week before treatment), and at 4 and 8 weeks after treatment. A visual inspection of the oral cavity was performed to check for teeth, oral lesions, and dental and soft issue abnormalities at baseline, and at 4 and 8 weeks after treatment.

Hematology and serum biochemistry

Hematological and serum biochemical analyzes were performed as previously described [76]. During the interdigestive period, 4 mL of blood was collected from the cephalic vein under unanesthetized conditions at baseline and 4 and 8 weeks after treatment. Approximately half of the blood sample was miscible with ethylenediaminetetraacetic acid dipotassium salt, and used for hematological analysis (FUJIFILM VET Systems, Tokyo, Japan). Serum was obtained from the remaining half of the collected blood samples, followed by biochemical analysis using a chemical analyzer (VETSCAN VS2; Zoetis, Florham Park, NJ, United States) with a rotor (Comprehensive Diagnostic Profile; Zoetis). Serum amyloid A (SAA) concentration was determined using an automated biochemical

analyzer (Pentra C200; HORIBA ABX SAS, Montpellier, France) and a particular SAA reagent for animal serum or plasma (VET-SAA "Eiken" reagent; Eiken Chemical Co. Ltd., Tokyo, Japan). The C-reactive protein (CRP) concentration was determined using a laser nephelometric immunoassay analyzer (Laser CRP-2; Arrows Co., Ltd., Osaka, Japan).

Evaluation of gingival index

The severity of gingivitis was evaluated using the gingival index at baseline and 4 and 8 weeks after treatment without sedation or anesthesia as previously described [78, 79]. The gingival index was measured on the buccal side of I3, C, P2, P3, P4, and M1 of the maxilla and C, P2, P3, P4, and M1 of the mandible. The gingival index was scored as follows: 0, no gingival inflammation; 0.5, slight gingival inflammation (slight change in color); 1, mild gingival inflammation (clear redness and edema, but no bleeding on probing); 2, moderate gingival inflammation (strong redness, edema, and bleeding on probing); and 3, severe gingival inflammation (marked redness, edema, ulceration, and a tendency to spontaneously bleed). The scores were assessed as a blinded experiment by the same experimenter at the Kitayama Labes Corporation. This experimenter was a Junior Laboratory Animal Technician certified by the Japanese Society of Laboratory

Animals, had worked for 11 years, and experienced several experiments for the evaluation of gingival index in dogs with gingivitis.

Measurement of volatile sulfur compounds (VSCs) levels

The VSCs levels in exhaled air were measured using a halimeter RH17K (TAIYO Instruments Inc., Osaka, Japan) at baseline and 8 weeks after treatment. The dogs were subcutaneously administrated 1 mg/kg maropitant (Cerenia; Zoetis) at 17:00 on the day before measurement for the prevention of vomiting. On the day of measurement, 0.03 mg/mL medetomidine (Dorbene; Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) and 0.3 mg/kg midazolam (Dormicum; Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) were administered intramuscularly to the thigh for sedation. Oral air samples were obtained by inserting a straw connected to a halimeter into the oral cavity, and VSCs levels were measured according to the manufacturer's recommendations. After measurement, 0.3 mg/kg atipamezole (Atipame; Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) was administered intramuscularly. Levels of thiol, a VSC, were measured in the gingival margin at C, P2, P3, and P4 in the bilateral maxilla and mandible using OraStrip (DS Pharma Animal Health Co., Ltd., Osaka, Japan) (80, 81) at baseline and 1, 2, 4, and 8 weeks after treatment. The OraStrip test was performed according to manufacturer's instructions. The scores were

assessed as a blinded experiment using the six chart colors on the accompanying sheet by the same experimenter at the Kitayama Labes Corporation.

Enzyme activity of periodontal pathogens

The enzyme activity of periodontal pathogens was measured using a swab in the gingival margin at C, P2, P3, and P4 in the bilateral maxilla and mandible using ADplit (Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) [82, 83] at baseline and 1, 2, 4, and 8 weeks after treatment. The score from each test in the bilateral maxilla and mandible was averaged. A score of 1–5 was assigned according to manufacturer's instructions. The cores were assessed as a blinded experiment using the five chart colors on the accompanying sheet by the same experimenter at the Kitayama Labes Corporation.

Enzyme-linked immunosorbent assay (ELISA)

Saliva was collected from outside the posterior molar using a Salivette (SARSTEDT, Nümbrecht, Germany) at baseline and 4 and 8 weeks after treatment. Quantification of immunoglobin A (IgA) and cathelicidin antimicrobial peptide (CAMP) in the saliva was performed using the Canine IgA ELISA Kit (Novus Biologicals, Centennial, CO, United

States) and Canine Cathelicidin Antimicrobial Peptide ELISA Kit (MyBioSource, San Diego, CA, United States), respectively, according to the manufacturer's protocols. The absorbance of the samples was measured at 450 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Scientific, Vantaa, Finland).

Statistical analysis

Data analyzes were performed using Kyplot 6.0 (KyensLab Inc., Tokyo, Japan). Data are expressed as mean ± standard error of the mean. Statistical significance between the Placebo and AGE groups was assessed using the Mann–Whitney U test. Statistical changes were also assessed using the Wilcoxon signed-rank test and compared with baseline. Differences at $p < 0.05$ were considered statistically significant.

2-4 Ethics statement

The animal study was approved by the Animal Welfare Committee of Kitayama Labes Corporation (Approval Number NBC57-024; approval date: 28 January 2021). The study was conducted in accordance with the local legislation and institutional requirements.

2-5 Results

All dogs consumed food without repeated vomiting during the experimental period. No significant changes in body weight were observed in the Placebo and AGE groups during the experimental period (Table 1). Furthermore, there were no changes in clinical symptoms such as soft feces, fur shedding, reddish urine color, or abnormal behaviors in either group. There were no abnormal changes including missing and fractured teeth, oral lesions, and hydration in the oral inspections during the experimental period.

During the experimental period, there were several significant changes compared with the baseline data and several significant differences between the Placebo and AGE groups, although there were no substantial changes and differences (Table 1). In the hematological data, the erythrocyte count decreased significantly ($p < 0.05$) in the AGE group at 4 and 8 weeks compared to baseline. The leukocyte count decreased significantly ($p < 0.05$) in the Placebo group at 8 weeks and in the AGE group at 4 weeks compared to baseline. The monocyte count decreased significantly ($p < 0.05$) in both groups at 8 weeks compared to baseline. In the serum biochemical data, the total protein concentration was significantly ($p<0.05$) higher in the AGE group than in the Placebo group at baseline and 4 weeks. The alanine aminotransferase activity was significantly ($p < 0.01$) lower in the AGE group than in the Placebo group at 8 weeks. Calcium

concentration was significantly ($p < 0.05$) higher in the AGE group than in the Placebo group at baseline and decreased significantly ($p < 0.001$) in the AGE group at 8 weeks compared to baseline. Sodium concentration was significantly ($p<0.05$) higher in the AGE group than in the Placebo group at baseline and at 4 and 8 weeks.

The average gingival index score obtained from 22 sites in the oral cavity decreased significantly ($p < 0.05$) in the AGE group at 4 (0.39 ± 0.12) and 8 weeks (0.25 ± 0.08) compared with that at baseline (0.53 ± 0.13), but not in the Placebo group during the experimental period (Figure 1A). There was a significant ($p < 0.05$) difference in the change from baseline between the Placebo and AGE groups at 4 weeks, but not ($p = 0.095$) at 8 weeks (Figure 1B).

The VSCs level of exhaled air measured using a halimeter increased significantly ($p < 0.05$) in the Placebo group at 8 weeks compared to that at baseline, but not in the AGE group (Figure 2A). There was a significant ($p<0.05$) difference in the change from baseline between the Placebo and AGE groups at 8 weeks (Figure 2B). The average score indicating the thiol level estimated using OraStrip decreased significantly ($p < 0.05$) in the Placebo group at 2 and 8 weeks compared to baseline, decreased significantly ($p < 0.05$) in the AGE group at 4 and 8 weeks compared to baseline (Figure 3A). However,

there was no significant difference in the change from baseline between the two groups (Figure 3B).

The average score obtained using ADplit showed no significant changes compared to baseline in both the Placebo and AGE groups (Figure 4A). However, the change from the baseline value tended to increase in the Placebo group at 2 weeks, whereas it tended to decrease in the AGE group during the experimental period (Figure 4B). There was a significant difference ($p < 0.05$) between the two groups at 2 weeks. The score was still lower in the AGE group than in the Placebo group at 8 weeks, but there was no significant difference ($p = 0.095$) between the two.

There was no significant change in the salivary IgA concentration in either the Placebo or AGE groups during the experimental period (Figure 5A). In contrast, the CAMP concentration tended to increase in the AGE group compared to the baseline value but not in the Placebo group (Figure 5B). There was a significant ($p < 0.01$) difference between the two groups at 4 weeks. The CAMP level was still higher in the AGE group than in the Placebo group at 8 weeks, but there was no significant difference ($p = 0.056$) between the two.

2-6 Discussion

Garlic intake induces the oxidation of erythrocytes and hemoglobin in dogs [84], resulting in hemolytic anemia [59]. Therefore, I first examined the safety and adverse effects of AGE in Beagle dogs using the methods described in the previous study [76]. The previous study demonstrated no adverse effects, including hemolytic anemia, when AGE was orally administered to healthy dogs at 90 mg/kg/day for 12 weeks [76]. Consistent with a previous study, the present study demonstrated that feeding of AGE at a low dose of 18 mg/kg/day for 8 weeks had no adverse effects on the general health of dogs with mild gingivitis (Table 1). Although the administration of AGE induced statistically significant differences in several hematological and serum biochemical parameters, such as erythrocyte, leukocyte, and monocyte counts, and calcium concentration compared to baseline values, the differences were quite small, and all the parameters were kept within the reference values in Beagle dogs [85, 86]. In my previous study, dogs administered AGE at 45 and 90 mg/kg/day for 12 weeks, the doses and duration of which were higher and longer than those in this study (18 mg/kg/day for 8 weeks), showed no significant changes in erythrocyte count and other hemolytic anemia-associated parameters including reticulocyte count, lactate dehydrogenase activity, total bilirubin concentration, and erythrocyte oxidation parameters such as Heinz body and eccentrocyte counts [76]. Therefore, I considered the significant decrease in erythrocyte count at 4 and 8 weeks in

the AGE group in this study to be incidental, innocuous, and unrelated to anemia. However, garlic has the potential for hemolytic anemia in dogs [59, 84], and therefore, further studies are necessary in order to confirm the complete safety of AGE for the use as a supplement.

The major objective of this study was to investigate the therapeutic effect of AGE on gingivitis in dogs using multiple evaluation indicators, including the gingival index score (Figure 1), VSCs levels in exhaled air (Figure 2), thiol levels (Figure 3), enzyme activity of periodontal pathogenic bacteria (Figure 4), and salivary IgA and CAMP concentrations (Figure 5). Based on the results obtained from these evaluation indicators, I considered that AGE has a potential therapeutic effect on canine gingivitis, as describe later.

The clinical signs of canine periodontal disease include gingival inflammation with redness, swelling, and bleeding [45]. Therefore, the severity of gingivitis was evaluated using a gingival index score between 0 and 3, as previously employed in other studies [78, 79]. The score significantly decreased in the AGE group only and was significantly lower in the AGE group than in the Placebo group at 4 weeks (Figure 1). The score in the AGE group continued to be lower than in the Placebo group at 8 weeks, although there was no significant difference. This suggests that AGE can improve the degree of gingival inflammation in dogs.

Oral malodor, also known as halitosis, is the first clinical sign of periodontal disease detected by dog owners [87]. The major sources of halitosis are VSCs, including hydrogen sulfide, methyl mercaptan, and dimethyl sulfide, which are produced by anaerobic oral bacteria [88]. Oral Gram-negative anaerobic bacteria can degrade proteinaceous components from saliva, blood cells, oral epithelial cells, and food debris into cysteine and methionine, resulting in the production of VSCs [89]. VSCs levels in exhaled air correlate positively with clinical parameters of periodontal disease, such as gingival and calculus indices in Beagle dogs [42]. In this study, the VSCs level increased significantly in the Placebo group and the OraStrip score decreased significantly in both groups regardless of the administration of AGE, possibly because the gingival inflammatory conditions of the examined dogs were still actively changing during the experimental period. In such a situation, the administration of AGE suppressed the increase in VSCs levels in the exhaled air (Figure 2). However, this study also showed that there was no significant difference in the OraStrip test score between the AGE and Placebo groups (Figure 3), which is associated with thiol levels at the gingival margin. A clinical study on human subjects indicated a relationship between halitosis and VSCs-producing microorganisms on the tongue [89]. Oral bacteria associated with halitosis, such as *Porphyromonas*, *Fusobacterium*, and *Streptococcus* species, colonize the dorsal mucosa of the canine tongue [90]. Taken together, these reports suggest that VSCs production in

exhaled air results from not only the gingival marginal microflora but also the microflora on the tongue. Therefore, AGE-induced suppression of VSCs levels in canine exhaled air may be attributed to the prevention of halitosis-associated bacterial proliferation on the tongue. Further studies are needed to determine the effects of AGE on the tongue microflora.

Human periodontal pathogens such as *Porphyromonas gingivalis*, *Treponema denticola*, *Tanerella forthysia*, and *Capnocytophaga ochracea* produce trypsin-like enzymes that hydrolyze *N*-benzoyl-DL-arginine-2-naphthylamide (BANA) [91]. Diagnostic aids that use BANA to indicate the presence of periodontopathic bacteria can be conveniently used at the chairside in human dental medicine and have been shown to correlate well with the clinical indicators used to diagnose periodontal disease [92, 93]. Oral bacteria in dental plaque play an important role in the initiation and progression of gingivitis and periodontitis [40, 42]. A cross-sectional survey reported that the prevalent pathogenic species identified in dogs with healthy gingiva, gingivitis, and mild periodontitis were *Peptostreptococcus*, *Peptostreptococcaceae*, and *Actinomyces* species, and that *Corynebacterium canis* was significantly more abundant in dogs with gingivitis and periodontitis than in healthy dogs [94]. Another study revealed that the predominant pathogens were *Bacteroides heparinolyticus*, *Pasteurella dagmatis*, *Actinomyces canis*, *Porphyromonas cangingivalis*, and *Desulfomicrobium orale* in dogs with gingivitis or

periodontitis [39]. In particular, the hydrolytic activity of BANA by *Corynebacterium* and *Actinomyces* species is positively correlated with the severity of periodontal disease in Beagle dogs [95]. In this study, the administration of AGE suppressed the hydrolytic activity of BANA compared with the placebo (Figure 4), although this is not direct evidence for periodontal pathogens. The *in vitro* studies demonstrated that ethanolic and aqueous garlic extracts inhibited the growth of human periodontal pathogens such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* [96]. Diallyl sulfide, a lipophilic constituent in AGE, induces cell death in *Aggregatibacter actinomycetemcomitans* via glutathione *S*-transferase inhibition [97]. These results suggested that garlic extract and garlic-derived phytochemicals possess direct antimicrobial activity against periodontal pathogens. However, the antimicrobial activity of AGE and its constituents, other than diallyl sulfide, against periodontal pathogens is unknown. Therefore, further studies, particularly in dogs, are required.

Saliva contains a wide variety of antimicrobial substances, and the maintenance of oral microflora by these substances plays an important role in preventing periodontal disease [98, 99]. Antimicrobial substances in whole saliva are derived from various cells, such as epithelial cells, salivary glands, and neutrophils [98]. Saliva is a rich source of oral epithelial cells that express functional toll-like receptors (TLRs) such as TLR-2 and TLR-4, which interact with most periodontal pathogens [100]. IgA is commonly known as one

of the first lines of defense against the adherence and invasion of pathogenic bacteria [101]. My findings indicated that AGE administration had no effect on salivary IgA concentrations (Figure 5A).

Antimicrobial peptides exhibit a broad spectrum of antibacterial activities [102] and play an important role in innate immune responses [103]. Furthermore, antimicrobial peptides directly regulate the balance between pro- and anti-inflammation [102]. These reports suggest that antimicrobial peptides contribute not only to the defense against oral pathogenic bacteria but also to the suppression of gingival inflammation, resulting in the prevention and treatment of periodontal disease. Cathelicidins are a family of antimicrobial peptides commonly found in numerous mammals such as humans, mice, and dogs [104]. A previous proteomics study detected CAMP in the saliva of healthy dog [97]. In this study, the administration of AGE increased the salivary CAMP concentration in dogs with mild gingivitis (Figure 5B), suggesting that the AGE-induced increase in salivary CAMP concentration might contribute partly to the suppression of gingivitis (Figure 1), VSCs levels in exhaled air (Figure 2), and enzyme activity of periodontal pathogens (Figure 4).

As mentioned, a clinical trial in humans demonstrated that the daily consumption of AGE for 4 months benefited oral health by reducing gingival inflammation and bleeding

[73]. An additional study in humans evaluated the loss of attachment caused by the formation of pockets between the tooth and gums in periodontitis following 18-month use of AGE, in which the level of periodontitis was significantly lower in the AGE group than in the placebo group [74]. These two human clinical studies indicate that AGE may become significant in the prevention of periodontal diseases [105]. However, the mechanisms through which AGE exerts its beneficial effects against periodontal diseases remain to be elucidated [105, 106]. AGE may be a promising candidate for use in the treatment of periodontal diseases in humans, but further studies are required for the clarification of the basic molecular mechanisms involved [106]. In this study, the duration (8 weeks) of use in dogs with gingivitis was shorter than those (4–18 months) in the human clinical studies, but there were significant outcomes including improved gingivitis and halitosis, suppressed hydrolytic activity of BANA, and increased salivary CAMP in dogs administered AGE. If the duration of administration is extended to a monthly or yearly level, the therapeutic effect of AGE on canine gingivitis may become clearer and the preventive effect of periodontal diseases may be exerted. An increased dosage (more than 18 mg/kg/day) may make the effect of AGE clearer. In addition, this study might suggest that the suppression of hydrolytic activity of BANA in periodontal pathogens and the increased concentration of salivary CAMP explain in part the therapeutic effects of AGE in periodontal diseases in animals and humans.

In this study, I examined the effects of AGE on gingivitis in Beagle dogs and discussed its usefulness. However, the major limitation was that the breed used was limited to Beagles and the sample size was small (five dogs in each group). A research group performed a large-scale study on the effects of *Ascophyllum nodosum* on canine oral health in 60 dogs of various breeds, including Japanese Chins, Miniature Schnauzers, Chihuahuas, Pomeranians, and West Highland White Terriers, discussing the evaluated dog breeds, body weights, and sample sizes [107]. The assessment, in comparison with their previous results [108], revealed some differences among studies. Therefore, large-scale studies are needed to investigate the therapeutic effects of AGE on periodontal disease across a wide range of breeds and body sizes. Second, this study examined the effect of AGE only on mild gingivitis with gingival index of less than 1; thus, further studies are needed on dogs with more severe gingivitis and periodontitis.

2-7 Summary

In conclusion, the present study demonstrated for the first time that feeding of AGE at 18 mg/kg/day for 8 weeks improved gingivitis and halitosis in Beagle dogs with mild gingivitis. I also suggest that the direct antibacterial property of AGE and/or an increase in salivary CAMP may be involved in the underlying mechanism. These findings may support the potential application of AGE as an oral supplement for the prevention and treatment of gingivitis in dogs. Future studies will target the role of AGE in moderate to severe periodontal disease.

2-8 Table and Figures

Table 1. Changes in body weight, and hematological and serum biochemical parameters in Beagle dogs fed aged garlic extract (AGE) and not fed AGE (Placebo) for 8 weeks.

Parameter	Group	Baseline	Week 4	Week 8
Body weight	Placebo	10.8 ± 0.4	10.5 ± 0.5	10.6 ± 0.5
(kg)	AGE	10.8 ± 0.4	10.4 ± 0.5	10.8 ± 0.5
Statistics between groups		NS	NS	NS
Erythrocyte	Placebo	7.16 ± 0.21	6.68 ± 0.27	6.83 ± 0.31
($X10^6/\mu L$)	AGE	6.82 ± 0.27	6.17 ± 0.23*	5.97 ± 0.35*
Statistics between groups		NS	NS	NS
Hemoglobin	Placebo	16.0 ± 0.4	15.1 ± 0.6	15.2 ± 0.6
(g/dL)	AGE	15.3 ± 0.4	14.2 ± 0.5	13.7 ± 0.7
Statistics between groups		NS	NS	NS
Hematocrit	Placebo	48.4 ± 1.0	46.4 ± 1.8	46.2 ± 2.0
(%)	AGE	46.6 ± 1.4	43.8 ± 1.5	41.9 ± 2.2
Statistics between groups		NS	NS	NS
MCV	Placebo	67.7 ± 0.9	69.6 ± 0.8	67.6 ± 0.9
(fL)	AGE	68.4 ± 1.0	70.9 ± 1.0	70.3 ± 1.1
Statistics between groups		NS	NS	NS
MCH	Placebo	22.3 ± 0.3	22.7 ± 0.2	22.2 ± 0.3
(pg)	AGE	22.5 ± 0.3	23.1 ± 0.4	23.0 ± 0.5
Statistics between groups		NS	NS	NS
MCHC	Placebo	33.0 ± 0.1	32.6 ± 0.2	32.9 ± 0.4
(g/dL)	AGE	32.9 ± 0.2	32.5 ± 0.2	32.8 ± 0.3
Statistics between groups		NS	NS	NS
Leukocyte	Placebo	8.86 ± 0.99	7.08 ± 0.30	6.92 ± 0.25*
($X10^3/\mu L$)	AGE	8.40 ± 0.96	6.42 ± 0.70*	6.60 ± 0.16
Statistics between groups		NS	NS	NS
Neutrophil	Placebo	6.06 ± 0.11	4.64 ± 0.19	4.56 ± 0.21
($X10^3/\mu L$)	AGE	5.46 ± 0.67	4.02 ± 0.63	4.13 ± 0.19
Statistics between groups		NS	NS	NS
Lymphocyte	Placebo	1.97 ± 0.22	1.74 ± 0.18	1.74 ± 0.14
($X10^3/\mu L$)	AGE	1.98 ± 0.25	1.65 ± 0.17	1.79 ± 0.16

Statistics between groups		NS	NS	NS
Monocyte	Placebo	436 ± 61	371 ± 28	318 ± 35*
(/μL)	AGE	520 ± 108	348 ± 43	308 ± 49*
Statistics between groups		NS	NS	NS
Eosinophil	Placebo	373 ± 62	298 ± 84	284 ± 55
(/μL)	AGE	411 ± 60	374 ± 49	348 ± 42
Statistics between groups		NS	NS	NS
Basophil	Placebo	27.7 ± 3.3	33.6 ± 1.8	20.5 ± 1.6
(/μL)	AGE	29.0 ± 6.7	34.1 ± 5.2	26.5 ± 2.3
Statistics between groups		NS	NS	NS
Total protein	Placebo	6.4 ± 0.1	6.3 ± 0.1	6.4 ± 0.2
(g/dL)	AGE	6.9 ± 0.1	6.7 ± 0.1	6.6 ± 0.2
Statistics between groups		$p < 0.05$	$p < 0.05$	NS
Albumin	Placebo	3.7 ± 0.1	3.6 ± 0.1	3.6 ± 0.1
(g/dL)	AGE	3.8 ± 0.1	3.5 ± 0.1	3.5 ± 0.1
Statistics between groups		NS	NS	NS
Globulin	Placebo	2.7 ± 0.1	2.8 ± 0.1	2.8 ± 0.1
(g/dL)	AGE	3.1 ± 0.1	3.2 ± 0.1	3.1 ± 0.2
Statistics between groups		NS	NS	NS
A/G ratio	Placebo	1.35 ± 0.08	1.31 ± 0.08	1.31 ± 0.08
	AGE	1.21 ± 0.07	1.13 ± 0.08	1.17 ± 0.08
Statistics between groups		$p < 0.05$	$p < 0.05$	$p < 0.05$
Glucose	Placebo	94.4 ± 2.3	95.0 ± 1.7	92.6 ± 2.4
(mg/dL)	AGE	99.4 ± 2.2	93.0 ± 3.1	90.0 ± 2.9
Statistics between groups		NS	NS	NS
Amylase	Placebo	430 ± 45	486 ± 63	446 ± 48
(U/L)	AGE	382 ± 34	466 ± 42	436 ± 38
Statistics between groups		NS	NS	NS
ALT	Placebo	112 ± 61	66 ± 33	43 ± 3
(U/L)	AGE	66 ± 33	30 ± 2	31 ± 3
Statistics between groups		NS	NS	$p < 0.01$
ALP	Placebo	48 ± 9	51 ± 8	52 ± 8
(U/L)	AGE	46 ± 8	46 ± 3	42 ± 3
Statistics between groups		NS	NS	NS
Total bilirubin	Placebo	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
(mg/dL)	AGE	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Statistics between groups		NS	NS	NS
BUN	Placebo	12.6 ± 0.9	11.6 ± 0.7	10.8 ± 0.2

(mg/dL)	AGE	14.0 ± 1.8	14.0 ± 1.7	12.6 ± 1.7
Statistics between groups		NS	NS	NS
Creatinine	Placebo	0.78 ± 0.11	0.70 ± 0.11	0.74 ± 0.07
(mg/dL)	AGE	0.62 ± 0.06	0.54 ± 0.05	0.64 ± 0.08
Statistics between groups		NS	NS	NS
Calcium	Placebo	9.8 ± 0.1	9.7 ± 0.1	9.8 ± 0.1
(mg/dL)	AGE	10.3 ± 0.1	9.9 ± 0.1	$10.0 \pm 0.1^{\#}$
Statistics between groups		$p < 0.05$	NS	NS
Phosphorus	Placebo	5.4 ± 0.2	5.1 ± 0.2	5.1 ± 0.2
(mg/dL)	AGE	5.4 ± 0.3	4.9 ± 0.1	5.1 ± 0.3
Statistics between groups		NS	NS	NS
Sodium	Placebo	142 ± 1	141 ± 1	141 ± 1
(mEq/L)	AGE	145 ± 1	144 ± 1	145 ± 0
Statistics between groups		$p < 0.05$	$p < 0.05$	$p < 0.01$
Potassium	Placebo	4.6 ± 0.1	4.8 ± 0.1	4.7 ± 0.1
(mEq/L)	AGE	5.0 ± 0.2	4.9 ± 0.1	4.9 ± 0.2
Statistics between groups		NS	NS	NS
SAA	Placebo	2.40 ± 1.13	1.26 ± 0.10	1.08 ± 0.05
(mg/L)	AGE	2.84 ± 0.78	1.97 ± 0.30	1.88 ± 0.33
Statistics between groups		NS	NS	NS
CRP	Placebo	1.75 ± 1.26	0.98 ± 0.52	0.36 ± 0.15
(mg/dL)	AGE	1.48 ± 1.05	0.38 ± 0.16	0.22 ± 0.15
Statistics between groups		NS	NS	NS

Data are presented as the mean ± standard error (n = 5). MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, A/G: albumin/globulin, ALT: alanine aminotransferase, ALP: alkaline phosphatase, BUN: blood urea nitrogen, SAA: serum amyloid A, CRP: C-reactive protein. $*p < 0.05$ and $^{\#}p < 0.001$ compared with the data at baseline. NS: not significant with statistics between the two groups.

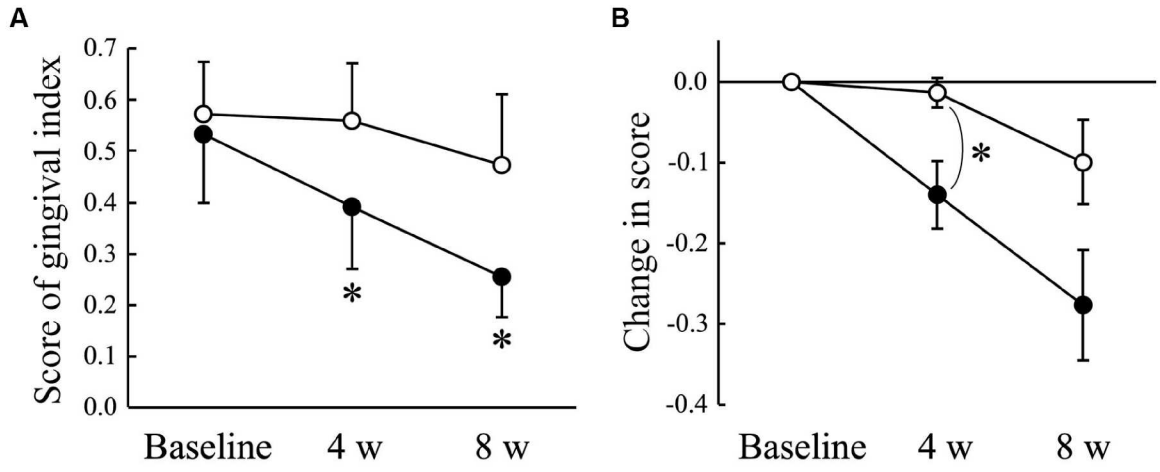

Figure 1. Gingival index score (A) and changed amount in the score (B) in Beagle dogs with mild gingivitis administered aged garlic extract (●, AGE group) and placebo power (○, Placebo group) for 8 weeks. Vertical bars indicate means ± standard error of the mean (n = 5). *$p < 0.05$, Wilcoxon signed rank test compared to baseline value, or Mann-Whitney U test compared between AGE and Placebo groups.

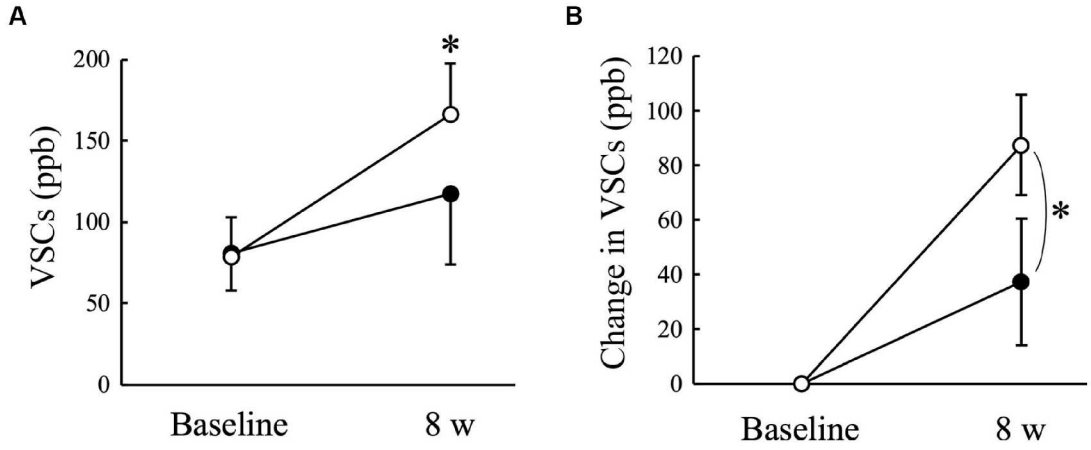

Figure 2. Level of volatile sulfur compounds (VSCs) (A) and changed amount in the level (B) in Beagle dogs with mild gingivitis administered aged garlic extract (●, AGE group) and placebo power (○, Placebo group) for 8 weeks. Vertical bars indicate means ± standard error of the mean (n = 5). *$p < 0.05$, Wilcoxon signed rank test compared to baseline value, or Mann-Whitney U test compared between AGE and Placebo groups.

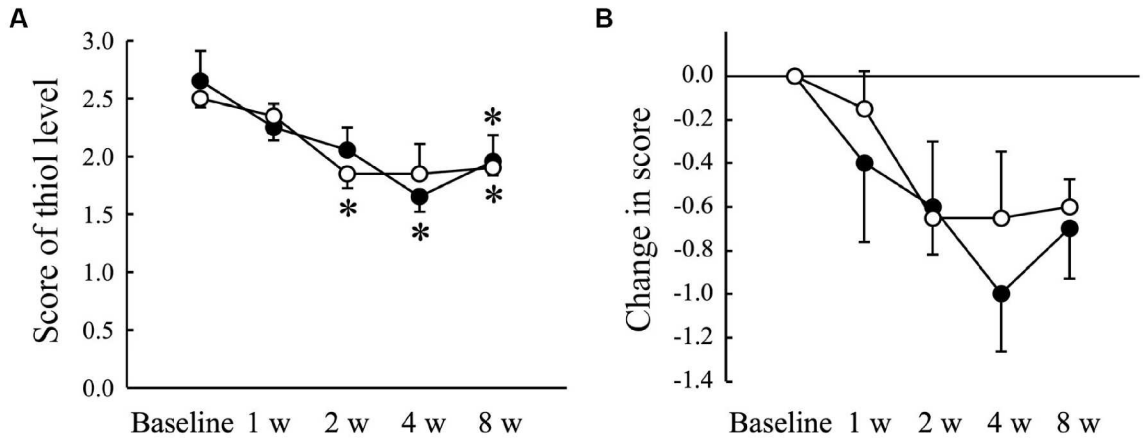

Figure 3. Score indicating the level of volatile sulfur compounds measured using OraStrip test (A) and changed amount in the score (B) in Beagle dogs with mild gingivitis administered aged garlic extract (●, AGE group) and placebo power (○, Placebo group) for 8 weeks. Vertical bars indicate means ± standard error of the mean (n = 5). *$p < 0.05$, Wilcoxon signed rank test compared to baseline value.

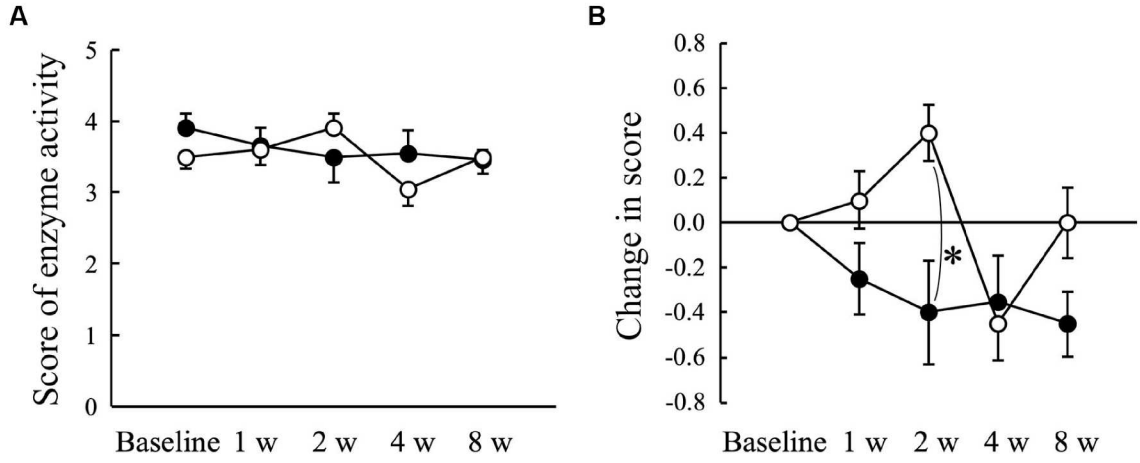

Figure 4. Score indicating the enzyme activity of periodontal pathogens measured using ADplit test (A) and changed amount in the score (B) in Beagle dogs with mild gingivitis administered aged garlic extract (●, AGE group) and placebo power (○, Placebo group) for 8 weeks. Vertical bars indicate means ± standard error of the mean (n = 5). *$p < 0.05$, Mann-Whitney U test compared between AGE and Placebo groups.

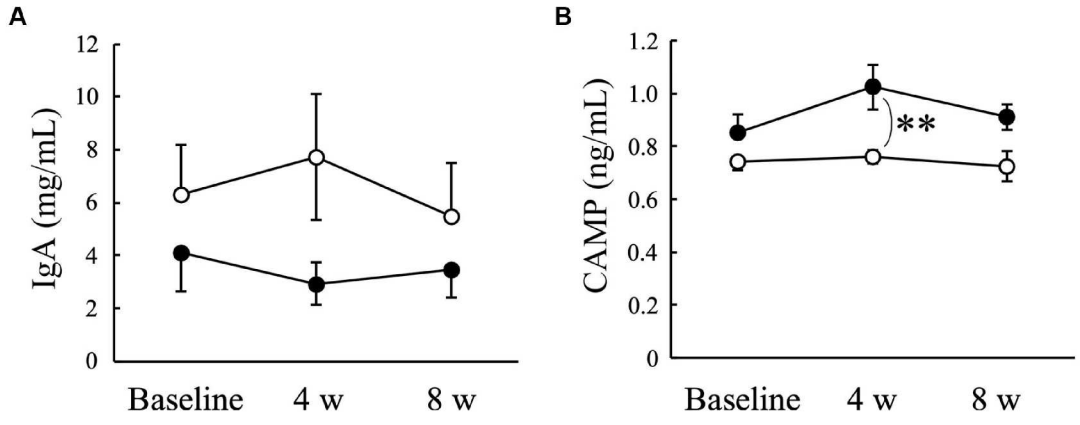

Figure 5. Concentrations of immunoglobulin A (IgA) (A) and cathelicidin antimicrobial peptide (CAMP) (B) in Beagle dogs with mild gingivitis administered aged garlic extract (●, AGE group) and placebo power (○, Placebo group) for 8 weeks. Vertical bars indicate means ± standard error of the mean (n = 5). $^{**}p < 0.01$, Mann-Whitney U test compared between AGE and Placebo groups.

Conclusion

This dissertation presents a comprehensive investigation into the diagnosis and treatment of periodontal disease in dogs, integrating two original studies that focus on microbial detection and natural therapeutic intervention. Periodontal disease is one of the most common oral pathologies in companion animals, with significant implications not only for local oral health but also for systemic conditions. Despite its prevalence, the veterinary field faces notable challenges in diagnosing and managing this disease, particularly due to the reliance on anesthesia-based examinations and the limitations of owner compliance in daily oral hygiene. Addressing this issue requires innovative yet accessible diagnostic methods and safe, effective therapeutic options suitable for routine veterinary practice.

The first study focused on the development and application of a novel glyco-nanotechnology-based diagnostic method utilizing sugar chain-immobilized magnetized gold nanoparticles and magnetic microparticles (SGNP/MMP) in conjunction with real-time PCR. This approach was designed to enable non-invasive, highly sensitive detection and semi-quantitative analysis of key periodontopathic bacteria from simple oral swab samples. When applied to 52 client-owned dogs, the method successfully identified eight periodontal pathogens, including members of the red complex. Among them, Treponema denticola exhibited the strongest correlation with clinical signs of disease severity, such

as gingival recession. The method's simplicity, rapid processing time, and lack of dependence on anesthesia make it particularly attractive for use in general veterinary clinics. Furthermore, it holds promise for early screening and risk stratification of dogs prone to periodontal disease, which could facilitate timely intervention and improved treatment outcomes.

The second study explored the therapeutic potential of aged garlic extract (AGE), a natural compound with established antioxidant, anti-inflammatory, and antimicrobial properties in human medicine. In a controlled 8-week trial involving Beagle dogs with mild gingivitis, oral administration of AGE at a dose of 18 mg/kg/day yielded several favorable outcomes. Clinical signs of gingival inflammation were significantly reduced, as reflected in improved gingival index scores and lower levels of volatile sulfur compounds (VSCs) in exhaled air. In addition, the study observed a trend toward reduced enzyme activity of periodontal bacteria and a significant increase in the concentration of salivary cathelicidin (CAMP), an antimicrobial peptide that contributes to mucosal immune defense. Importantly, no adverse effects were observed, and all hematological parameters remained within normal ranges, reinforcing the safety profile of AGE in canine use.

Taken together, the results of these two studies highlight the importance of integrating both precise diagnostics and supportive therapeutics into a unified strategy for managing canine periodontal disease. The SGNP/MMP-qPCR method provides an efficient and

objective tool for identifying bacterial risk factors and assessing disease severity, potentially transforming the current reliance on invasive diagnostic procedures. Meanwhile, AGE supplementation offers a promising, non-invasive adjunct to existing treatments, particularly for cases where daily oral care or frequent professional cleanings are difficult to maintain. The combination of these two approaches has the potential to redefine clinical protocols in veterinary dentistry by promoting early detection, individualized care, and holistic disease management.

This dissertation also opens several avenues for future research. Larger-scale clinical trials involving dogs of various breeds and disease severities are necessary to confirm the generalizability of the findings. The longitudinal application of the SGNP/MMP-based diagnostic system could help establish bacterial thresholds for disease classification and monitoring. Additionally, mechanistic studies on AGE's effects on the oral microbiome and host immune response would provide deeper insights into its mode of action. Lastly, integrating these tools into preventive healthcare models and evaluating their economic feasibility in clinical settings could enhance the overall impact of this research.

In conclusion, this work contributes significantly to the field of veterinary oral health by offering novel, evidence-based approaches to the detection and treatment of periodontal disease in dogs. Through the development of a practical bacterial screening method and the validation of a safe, natural therapeutic agent, it supports the

advancement of more accessible, preventive, and personalized veterinary care, ultimately promoting better health and quality of life for companion animals.

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Conflict of Interest

Chapter 1

This part of the study was partially supported by a research grant from SUDx-Biotec Corporation to the Laboratory of Collaborative Research on Glyconanotechnology at the Graduate School of Science and Engineering, Kagoshima University.

Chapter 2

This part of the study received funding from Wakunaga Pharmaceutical Co., Ltd., and provided the Aged Garlic Extract (AGE).

Appendix

Method A1

In the preliminary experiment, I compared the PCR results between the glycerol stock for *P. gingivalis* with or without SGNP before MMP treatment and the DNA extracted by Qiagen extraction method. For Qiagen extraction, the commercially available spin column (EconoSpin™ for DNA, EconoSpin™ column Ia/IIa; Ajinomoto Bio-Pharma, Ibaraki, Osaka, Japan) and the handmade lysis and working solution were used, according to the manufacturer's instructions. SGNP increased PCR sensitivity for *P. gingivalis* to 120% to 140%. The surface structure of *P. gingivalis* was reported to contain ethanol amine in the lipid A component of lipopolysaccharide [Kumada H, Haishima Y, Umemoto T, Tanamoto KI. 1995. Structural study on the free lipid A isolated from lipopolysaccharide of *Porphoromonas ginigivalis*. *J Bacteriol*. 177:2098-2106]; therefore, the positive charge in ethanol amine may help DS25-GNP attachment. Further analysis of the surface structure for *P. gingivalis* may be necessary. The recovered copy number ratios were 60% by the SGNP/MMP method and 107% by the Qiagen extraction method; when the copy number in the supernatant or wash solution from the magnetic precipitate of SGNP/MMP or MMP were included, the ratios were over 100% for both. These results suggested that similar to its application for viruses, the SGNP/MMP method may concentrate bacterial particles conveniently in a short time (<3 min) which is about 1/10 of time compared to Qiagen extraction. Additionally, the analysis focused on DNA derived from live bacteria rather than from lysed bacterial cells, as the former is more likely to be associated with the progression of periodontal disease.

Table A1-1 PCR reaction mixture using RR064 for one run

2 x Onestep Buffer III	7.5 µl
Water	3 µl
Ex Taq HS	0.9 µl
Primer Mixture (25 µM)	1.84 µl
Probe Mixture (10 µM)	0.92 µl
Template	1 µl

Table A1-2 PCR reaction mixture using RR820 for one run

TB Green Premix Ex TaqII	8.3 µl
Forward Primer (10 µM)	0.3 µl
Reverse Primer (10 µM)	0.3 µl
RNase free water	5.3 µl
Template	1 µl

Table A1-3 PCR reaction mixture using RR070 and part of One Step Prime Script RT-PCR Kit for one run

10 x Fast Buffer I/II (RR070)	1.6 µl
dNTP Mixture (RR070)	1.28 µl
EXTag HS (RR064)	0.5 µl
SYBR-Green I 1/2000 (5761A)	1.6 µl
Forward Primer (10 µM)	0.8 µl
Reverse Primer (10 µM)	0.8µl
RNase free water	8.52 µl
Template	1 µl

Table A2 PCR Ct values of 52 dogs

Sample #	Age (year)	stage	*Porphyromonas gingivalis (P.g.)+gulae (P. gulae) (common primers/probe)*	*Porphyromonas gingivalis (P.g.)*	*Treponema denticola (T.d.)*	*Tannerella forsythia (T.f.)*	*Actinobacillus actinomycetemcomitans (A.a)*	*Fusobacterium nucleatum (F.n.)*	*Prevotella intermedia (P.i.)*	*Porphyromonas gulae (P.gulae)*	*Porphyromonas cangingivalis (P.c.)*	Total bacteria (Universal primer)
1	13	4	26.11	28.25	27.59	-	-	24.44	29.15	-	23.95	15.44
2	3	0	34.14	-	-	-	37.9	31.79	-	-	-	17.86
3	8	1	31.02	-	34.17	-	32.09	24.42	-	28.12	25.75	19.81
4	0	0	33.62	-	-	-	30.03	34.1	-	34.88	34.82	17.11
5	10	1.5	31.81	-	-	34.84	39.52	25.71	-	29.11	25.44	15.82
6	9	2.5	23.73	-	23.8	-	35.23	22.2	23.81	21.1	18.49	14.17
7	14	4	24.26	-	25.31	27.21	-	20.23	-	24.18	21.7	15.14
8	12	4	22.45	20.20	23	-	34.65	19.95	-	19.35	18.39	14.25
9	15	4	23.91	-	21.92	-	31	22.25	18.33	24.84	20.21	14.25
10	15	2.5	27.21	-	24.42	-	37.9	21.71	-	26.42	30.1	15.21
11	9	1	25.07	20.80	-	-	33.46	22.37	-	-	22.5	16.53
12	2	0	30.24	25.87	-	-	31.67	-	-	-	24.23	18.01
13	15	4	20.4	19.47	23.61	-	-	21.87	-	-	-	13.83
14	2	1	24.9	-	24.72	-	-	20.88	-	21.01	19.4	14.69
15	11	1	25.9	-	29	-	-	27.01	-	21.66	23.2	17.33
16	8	2.5	23.83	-	23.88	-	36.1	21.55	-	21.34	19.04	14.8
17	2	1	26.36	-	25.08	-	32.79	24.05	-	24.06	20.09	14.66
18	13	2.5	22.22	20.46	23.3	25.43	-	20.97	32.24	-	20.46	14.19
19	14	2	22.93	21.81	25.07	-	37.51	23.72	-	23.24	29.72	15.25
20	14	4	23.62	18.94	23.32	23.89	-	22.61	32.75	-	24.41	16.61
21	13	2	21.07	-	20.89	-	32.91	20.67	-	19.05	20.51	13.73
22	10	4	22.28	-	23.66	-	-	27.64	-	19.09	25.72	14.7
23	14	3	20.96	-	26.26	30	38.45	20.72	-	20.94	20.52	13.74
24	5	1	23.7	38.19	-	-	32.35	22.05	-	19.79	20.23	16.51
25	1	1	-	-	-	-	-	35.29	-	-	29.92	19.86
26	8	2	26.73	-	24.45	-	31.14	23.08	-	41.07	19.93	16.01
27	14	2	23.86	21.48	23.92	24.44	-	21.81	34.89	36.25	19.91	15.32
28	13	1	21.8	-	26.68	-	35.15	20.21	-	17.95	20.07	15.82
29	14	1	30.05	28.05	24.98	-	-	20.68	-	-	16.74	15.5
30	16	3	27.11	-	22.51	29.86	33.3	20.23	27.6	39.37	25.9	15.4
31	6	1	-	-	-	27.96	33.5	25.89	-	-	17.12	15.74
32	10	1	23.67	19.4	25.98	31.05	33.59	21.95	31.27	-	23.46	16.5
33	13	1	30.25	-	26.08	-	36.01	21.6	-	-	22.47	17.23
34	7	0	35.34	-	-	-	30.79	28.87	-	32.58	29.8	18.82
35	7	1	-	-	-	25.51	-	18.91	-	-	14.77	18.9
36	13	4	26.68	36.38	24.07	-	-	22.52	-	22.52	22.07	17.55
37	12	2.5	20.26	15.22	26.57	-	-	21.85	-	-	-	14.91
38	9	3	23.79	-	23.62	-	-	19.5	29.04	18.91	18.4	13.51
39	1	0	-	-	-	-	-	31.55	-	-	34.17	20.22
40	0.5	0	—	—	—	30.46	—	22.01	—	—	19.42	16.45
41	14	4	26.9	—	25.5	—	—	21.98	—	23.96	25.17	18.95
42	9	4	26.99	—	26.06	—	—	25.46	25.28	24.11	24.28	16.6
43	8	1	25.43	—	25.39	—	—	20.66	27.067	25	21.18	15.76
44	7	1	37.28	—	—	—	—	33.94	—	—	33.07	21.63
45	9	1	24.67	22.74	25.02	—	—	21.16	—	20.43	22.64	14.32
46	13	4	24.35	—	23.87	—	—	22.79	30.7	19.82	20.74	14.3
47	13	1	25.13	—	24.16	—	34.98	23.13	—	19.49	22.27	15.16
48	13	3	29.01	—	24.59	28.29	—	22.05	24.16	24.65	21.82	14.92
49	12	4	26.06	—	24.03	—	38.37	27.41	—	23.22	27.66	15.38
50	14	4	25.68	—	25.1	27.3	36.54	21.42	—	23.15	23.52	15.8
51	16	4	26.68	—	24.69	—	—	24.17	34.71	24.53	21.36	15.38
52	12	4	23.02	—	23.45	26.94	32.68	21.21	—	18.77	18.56	14.84

—: not detected

Table A3 Average and standard deviation of control plasmid in the PCR analysis.

	P. gingivalis + P. gulae (Pg+Pgul)	*P. gingivalis (Pg)*	*T. denticola (Td)*	*T. forsythia (Tf)*	*F. nucleatum (Fn)*	*P. intermedia (Pi.)*	*P. gulae (Pgul)*	*P. cangingivalis (P c)*	*A. actinomycetemcomitans (Aa)*	*Total bacteria (Universal primers)*
average	30.91	26.41	30.44	30.57	26.36	26.63	28.11	26.50	26.48	27.32
SD	0.27	0.32	0.35	0.34	0.38	0.50	0.49	0.48	0.42	0.81
SD(%)	0.9%	1.2%	1.2%	1.1%	1.4%	1.9%	1.8%	1.8%	1.6%	3.0%

Table A4 Correlation of Cutoff Ct value of *T. denticola* wiith the periodontal disease status in dogs

	PCR +	PCR -	
Group 0 (PD <2)	9	15	24
Group 1 (PD >=2)	28	0	28
	37	15	

PCR + : Ct=< 29, PCR - : Ct>29

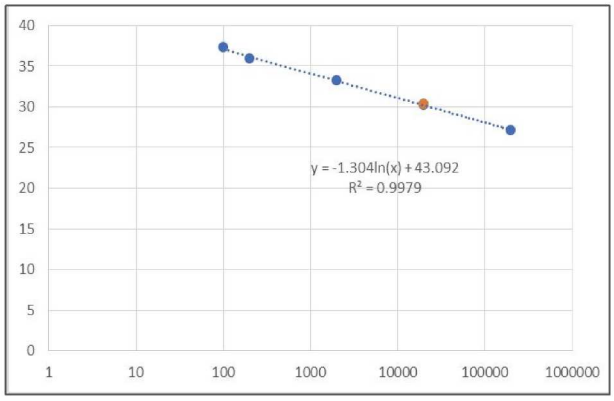

Figure A1-1 Calibration for P. gingivalis + P. gulae (Common primers/probe) (X-axis: copy number of plasmid, Y-axis: Ct value)

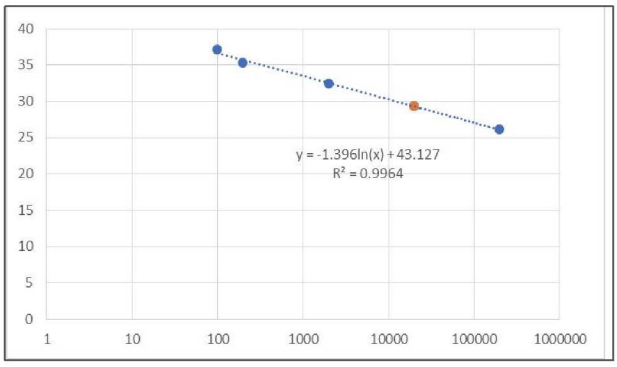

Figure A1-2 Calibration for T. denticola (X-axis: copy number of plasmid, Y-axis: Ct value)

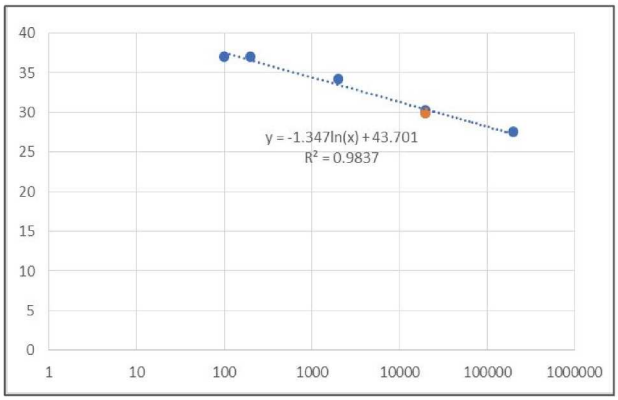

Figure A1-3 Calibration for T. forsythia (X-axis: copy number of plasmid, Y-axis: Ct value)

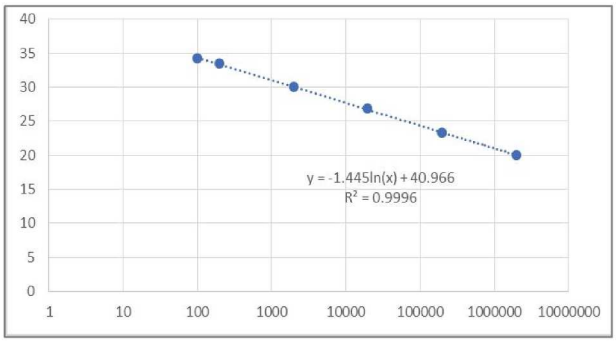

Figure A1-4 Calibration for F. nucleatum (X-axis: copy number of plasmid, Y-axis: Ct value)

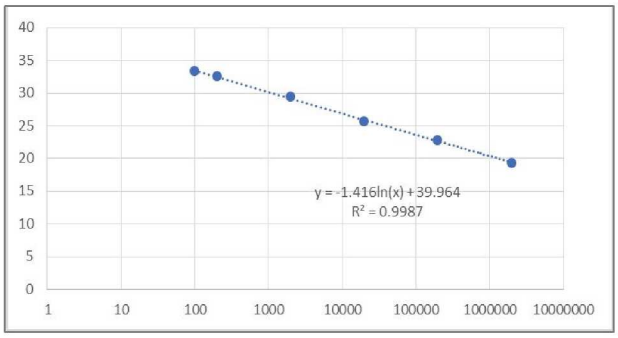

Figure A1-5 Calibration for P. intermedia (X-axis: copy number of plasmid, Y-axis: Ct value)

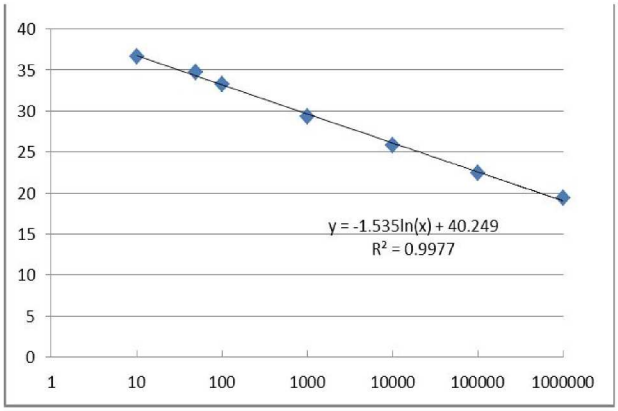

Figure A1-6 Calibration for A. actinomycetemcomitans (X-axis: copy number of plasmid, Y-axis: Ct value)

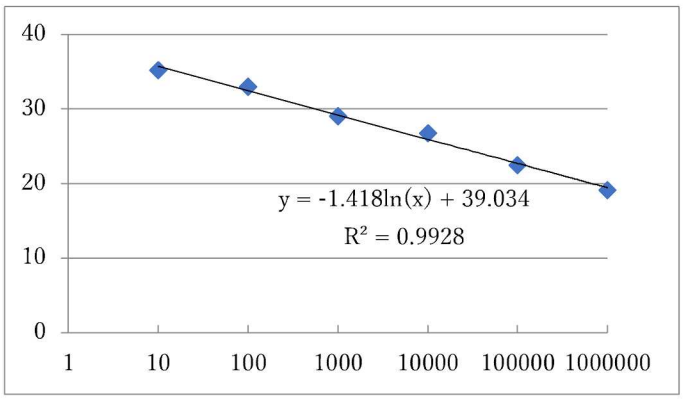

Figure A1-7 Calibration for P. gingivalis (X-axis: copy number of plasmid, Y-axis: Ct value)

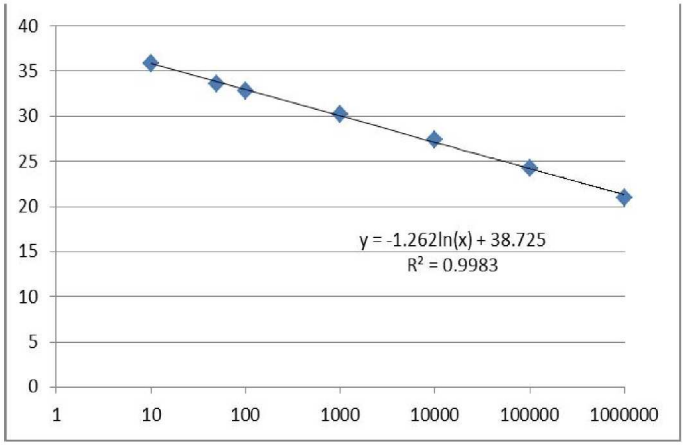

Figure A1-8 Calibration for P. gulae (X-axis: copy number of plasmid, Y-axis: Ct value)

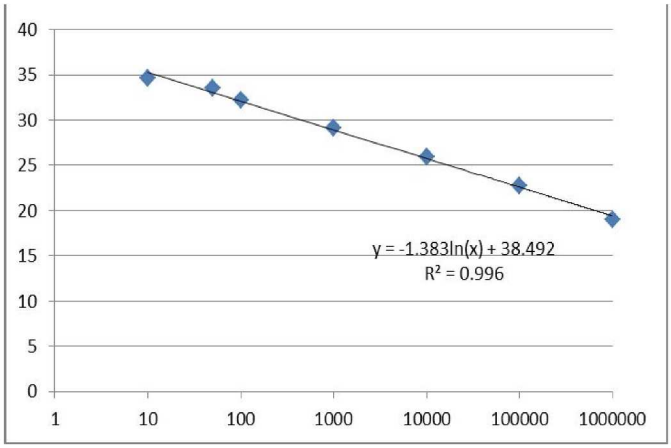

Figure A1-9 Calibration for P. cangingivalis (X-axis: copy number of plasmid, Y-axis: Ct value)

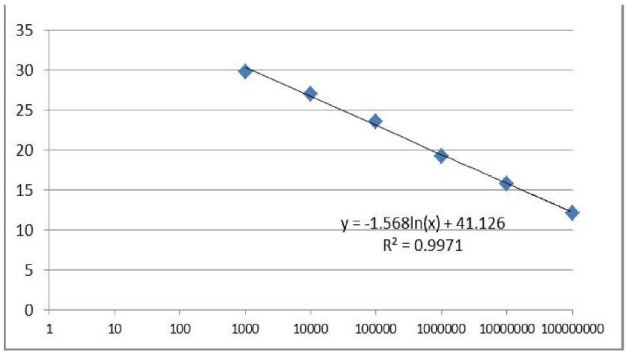

Figure A1-10 Calibration for total bacteria using universal primers (X-axis: copy number of plasmid, Y-axis: Ct value)

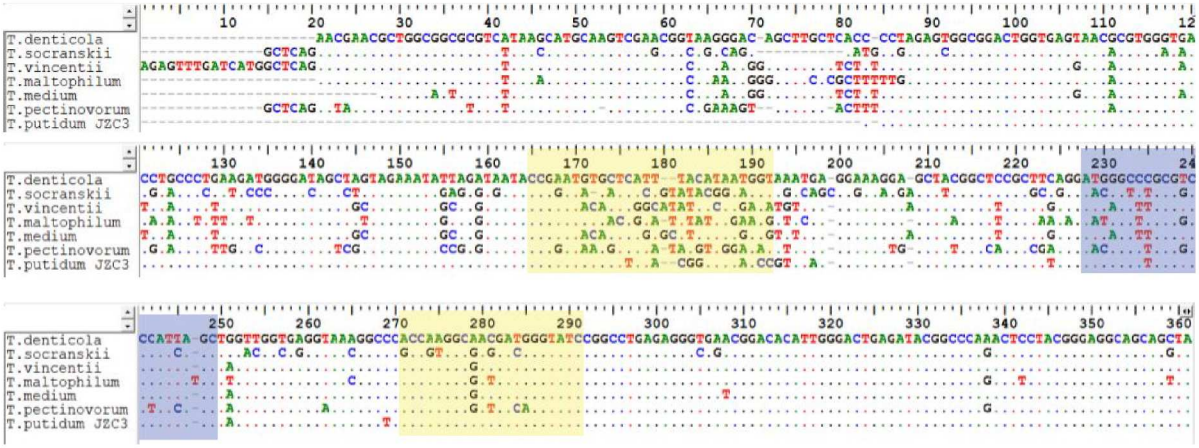

Figure A2 Comparison of primers/probe of *T.denticola* in the present study with those of 6 other Treponema species. The region of Primer was assigned with yellow back and that of probe was assigned with blue. The following information were used:

Treponema denticola strain a 16S ribosomal RNA, partial sequence - Nucleotide - NCBI (nih.gov)

Treponema socranskii subsp. buccale strain ATCC 35534 16S ribosomal RN - Nucleotide - NCBI (nih.gov)

Treponema vincentii strain OMZ 906 16S ribosomal RNA gene, complete se - Nucleotide - NCBI (nih.gov)

Treponema maltophilum strain BR 16S ribosomal RNA, partial sequence - Nucleotide - NCBI (nih.gov)

Treponema medium strain G7201 16S ribosomal RNA, partial sequence - Nucleotide - NCBI (nih.gov)

Treponema pectinovorum strain VPI D-36-DR-2 16S ribosomal RNA, partial - Nucleotide - NCBI (nih.gov)

Treponema putidum strain JZC3 16S ribosomal RNA, partial sequence - Nucleotide - NCBI (nih.gov)

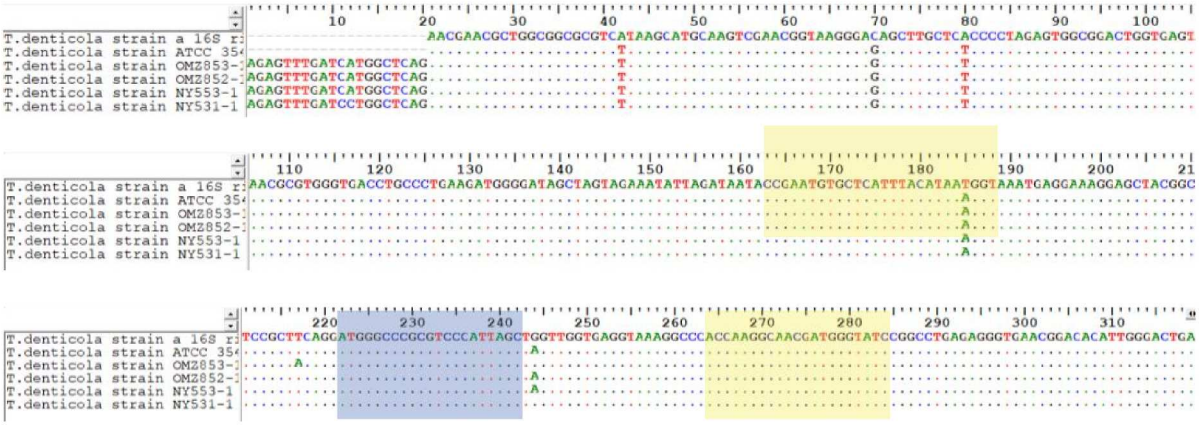

Figure A3-1 Comparison of primers/probe of *T.denticola* in the present study with those of 5 other strains. The region of primer was assigned with yellow back and that of probe was assigned with blue. The following informations were used:

Treponema denticola strain a 16S ribosomal RNA, partial sequence - Nucleotide - NCBI (nih.gov)

Treponema denticola strain ATCC 35405 16S ribosomal RNA, partial seque - Nucleotide - NCBI (nih.gov)

Treponema denticola strain OMZ853-1 16S ribosomal RNA gene, partial se - Nucleotide - NCBI (nih.gov)

Treponema denticola strain OMZ852-1 16S ribosomal RNA gene, partial se - Nucleotide - NCBI (nih.gov)

Treponema denticola strain NY553-1 16S ribosomal RNA gene, partial seq - Nucleotide - NCBI (nih.gov)

Treponema denticola strain NY531-1 16S ribosomal RNA gene, partial seq - Nucleotide - NCBI (nih.gov)

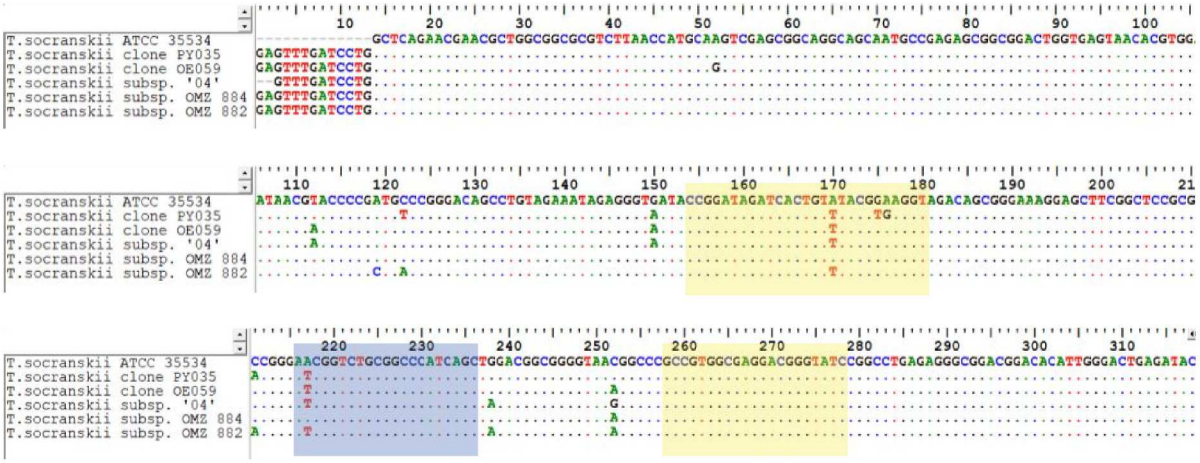

Figure A3-2 Comparison of primers/probe of *T. Socranskii* in the S-Figure 2 with those of 5 other strains. The region of primer was assigned with yellow back and that of probe was assigned with blue. The following informations were used:

Treponema socranskii subsp. buccale strain ATCC 35534 16S ribosomal RN - Nucleotide - NCBI (nih.gov)

Treponema socranskii canine oral taxon 088 clone PY035 16S ribosomal R - Nucleotide - NCBI (nih.gov)

Treponema socranskii canine oral taxon 088 clone OE059 16S ribosomal R - Nucleotide - NCBI (nih.gov)

Treponema socranskii subsp. '04' 16S ribosomal RNA gene, partial seque - Nucleotide - NCBI (nih.gov)

Treponema socranskii subsp. buccale strain OMZ 884 16S ribosomal RNA g - Nucleotide - NCBI (nih.gov)

Treponema socranskii subsp. paredis strain OMZ 882 16S ribosomal RNA g - Nucleotide - NCBI (nih.gov)

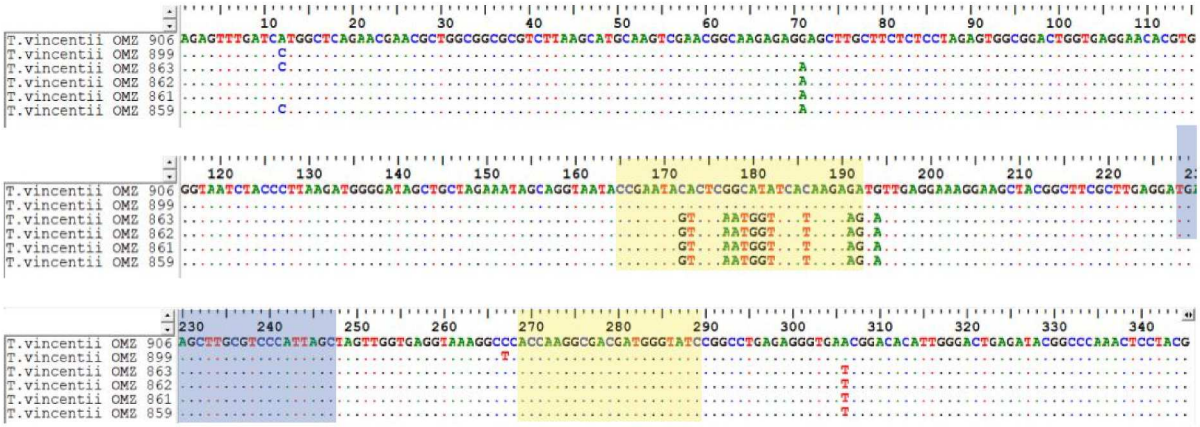

Figure A3-3 Comparison of primers/probe of *T. vincentii* in the S-Figure 2 with those of 5 other strains. The region of primer was assigned with yellow back and that of probe was assigned with blue. The following informations were used:

Treponema vincentii strain OMZ 906 16S ribosomal RNA gene, complete se - Nucleotide - NCBI (nih.gov)

Treponema vincentii strain OMZ 899 16S ribosomal RNA gene, complete se - Nucleotide - NCBI (nih.gov)

Treponema vincentii strain OMZ 863 16S ribosomal RNA gene, complete se - Nucleotide - NCBI (nih.gov)

Treponema vincentii strain OMZ 862 16S ribosomal RNA gene, complete se - Nucleotide - NCBI (nih.gov)

Treponema vincentii strain OMZ 861 16S ribosomal RNA gene, complete se - Nucleotide - NCBI (nih.gov)

Treponema vincentii strain OMZ 859 16S ribosomal RNA gene, complete se - Nucleotide - NCBI (nih.gov)

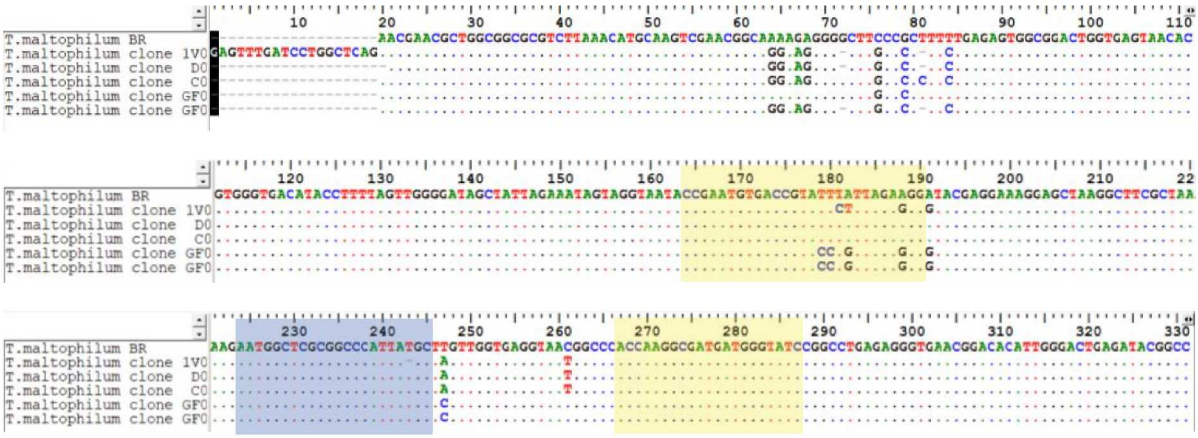

Figure A3-4 Comparison of primers/probe of *T. maltophilum* in the S-Figure 2 with those of 5 other strains. The region of primer was assigned with yellow back and that of probe was assigned with blue. The following informations were used:

Treponema maltophilum strain BR 16S ribosomal RNA, partial sequence - Nucleotide - NCBI (nih.gov)

Treponema maltophilum canine oral taxon 353 clone 1V058 16S ribosomal - Nucleotide - NCBI (nih.gov)

Treponema maltophilum clone D036 16S ribosomal RNA gene, partial sequ - Nucleotide - NCBI (nih.gov)

Treponema maltophilum clone C006 16S ribosomal RNA gene, partial sequ - Nucleotide - NCBI (nih.gov)

Treponema maltophilum clone GF038 16S ribosomal RNA gene, partial sequ - Nucleotide - NCBI (nih.gov)

Treponema maltophilum clone GF029 16S ribosomal RNA gene, partial sequ - Nucleotide - NCBI (nih.gov)

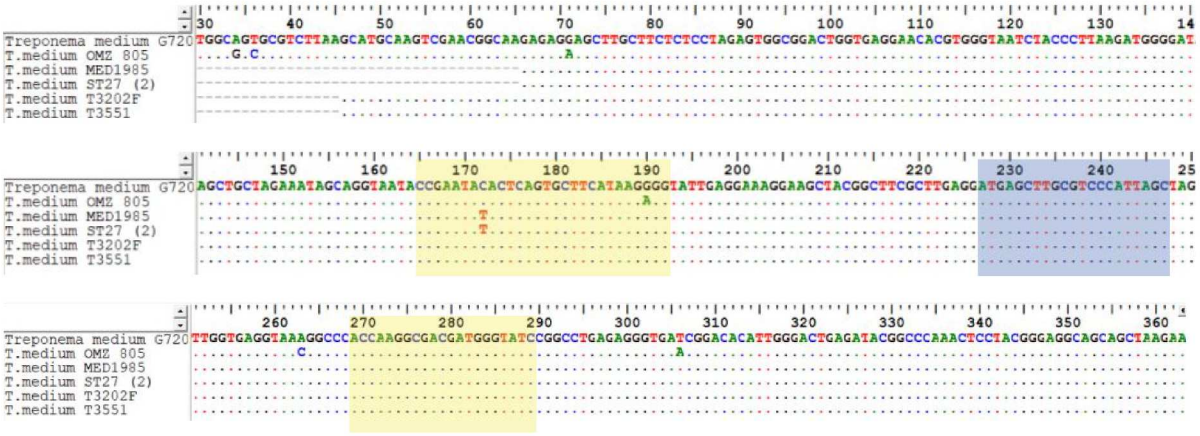

Figure A3-5 Comparison of primers/probe of *T. medium* in the S-Figure 2 with those of 5 other strains. The region of primer was assigned with yellow back and that of probe was assigned with blue. The following informations were used:

Treponema medium strain G7201 16S ribosomal RNA, partial sequence - Nucleotide - NCBI (nih.gov)

Treponema medium strain OMZ 805 16S ribosomal RNA gene, complete seque - Nucleotide - NCBI (nih.gov)

Treponema medium strain MED1985 16S ribosomal RNA gene, partial sequen - Nucleotide - NCBI (nih.gov)

Treponema medium strain ST27 (2) 16S ribosomal RNA gene, partial seque - Nucleotide - NCBI (nih.gov)

Treponema medium strain T3202F 16S ribosomal RNA gene, partial sequenc - Nucleotide - NCBI (nih.gov)

Treponema medium strain T3551 16S ribosomal RNA gene, partial sequence - Nucleotide - NCBI (nih.gov)

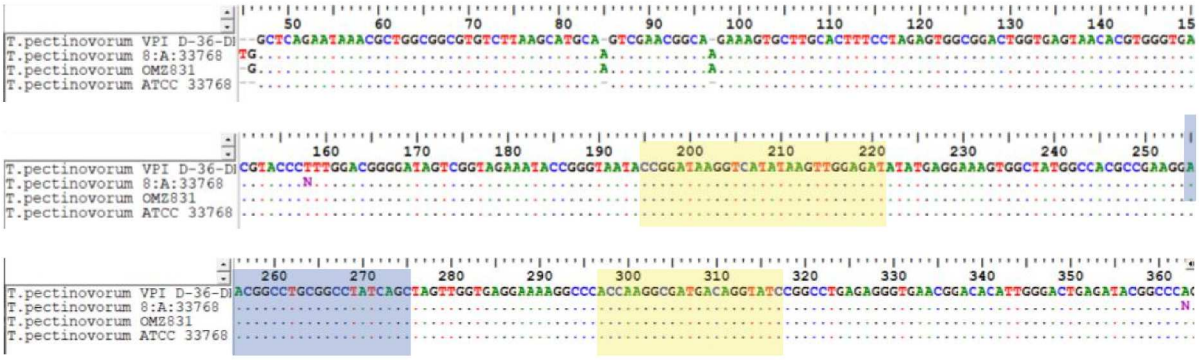

Figure A3-6 Comparison of primers/probe of *T. pectinovorum* in the S-Figure 2 with those of 3 other strains. The region of primer was assigned with yellow back and that of probe was assigned with blue. The following informations were used:

Treponema pectinovorum strain VPI D-36-DR-2 16S ribosomal RNA, partial - Nucleotide - NCBI (nih.gov)

Treponema pectinovorum strain 8:A:33768 16S ribosomal RNA, partial seq - Nucleotide - NCBI (nih.gov)

Treponema pectinovorum OMZ831 16S ribosomal RNA gene, partial sequence - Nucleotide - NCBI (nih.gov)

Treponema pectinovorum strain ATCC 33768 16S ribosomal RNA gene, parti - Nucleotide - NCBI (nih.gov)

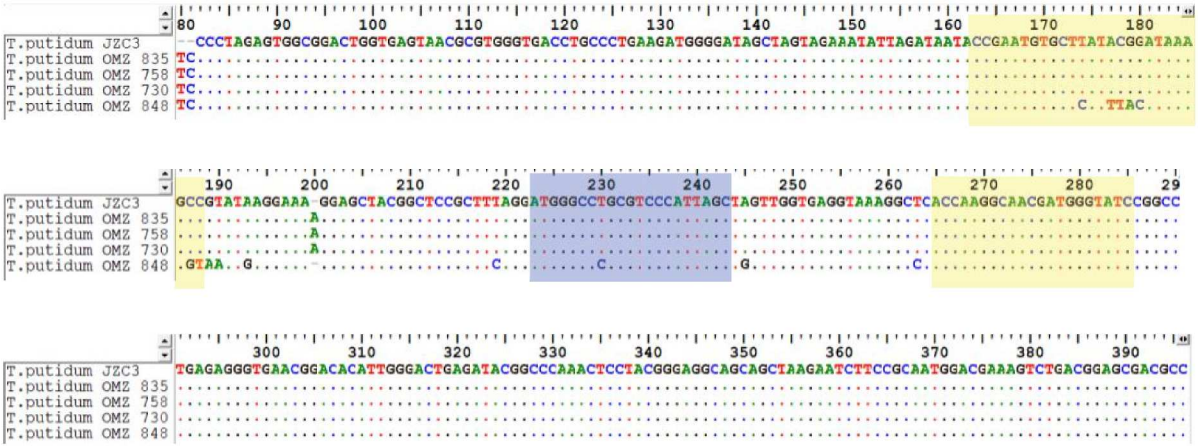

Figure A3-7 Comparison of primers/probe of *T. putidium* in the S-Figure S2 with those of 4 other strains. The region of primer was assigned with yellow back and that of probe was assigned with blue. The following informations were used:

Treponema putidum strain JZC3 16S ribosomal RNA, partial sequence - Nucleotide - NCBI (nih.gov)

Treponema putidum strain OMZ 835 16S ribosomal RNA gene, complete sequ - Nucleotide - NCBI (nih.gov)

Treponema putidum strain OMZ 758 16S ribosomal RNA gene, complete sequ - Nucleotide - NCBI (nih.gov)

Treponema putidum strain OMZ 730 16S ribosomal RNA gene, complete sequ - Nucleotide - NCBI (nih.gov)

Treponema putidum strain OMZ 848 16S ribosomal RNA gene, partial seque - Nucleotide - NCBI (nih.gov)

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