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Amixicile as a novel antimicrobial treatment for periodontitis: A pilot study in the non-human primate, Macaca mulatta

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Amixicile as a novel antimicrobial treatment for periodontitis: A pilot study in the non-human primate, *Macaca mulatta*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

by

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AMIXICILE AS A NOVEL ANTIMICROBIAL TREATMENT FOR PERIODONTITIS: A PILOT STUDY IN THE NON-HUMAN PRIMATE, *MACACA MULATTA*

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Abstract: Periodontitis is an inflammatory disease with a bacterial etiology in a susceptible host. Given the bacterial etiology, a selective antimicrobial agent with minimal side effects could be a useful adjunct to traditional therapy. Amixicile is a novel antimicrobial that targets the pyruvate: ferredoxin oxidoreductase (PFOR) which is an enzyme that is critical for anaerobic bacterial metabolism. It has been found to have no effect on commensal, aerobic microbes and has little to no side-effects thus far in animal models. In this study, two nonhuman primates of the *Macaca mulatta* species with naturally occurring mild chronic periodontitis were studied before and after a two-week course of systemic administration of amixicile and at 3- and 6-months post-treatment. Periodontal charting including probing depths, clinical attachment levels, presence of bleeding on probing, and presence of plaque was recorded at each visit in addition to collecting saliva and subgingival plaque samples. The microbial composition of the plaque and saliva was evaluated based on 16s rDNA analysis. Both animals' clinical conditions saw a reduction in probing depths and clinical inflammation. In the saliva samples a reduction in *Porphyromonas, Fusobacterium,* and *Alloprevotella*, all

anaerobes, was seen with a concomitant increase in *Streptococcus, Haemophilus, Gemella*, and *Escherichia*, all aerobes, was observed. Subgingival plaque samples showed similar alterations in microbial composition. Reduction of *Porphyromonas, Fusobacterium, Prevotella, Veillonella,* and *Alloprevotella*, all anaerobes, was observed with concomitant increase of known aerobes. These changes generally take place immediately post-treatment but return to baseline levels by 6-months. Thus, it was concluded that due to its selectivity for anaerobic periodontal pathogens and lack of side effects, amixicile is a strong candidate as a viable antimicrobial option for the treatment of periodontal disease.

Key words: amixicile, antimicrobial, periodontal therapy, periodontitis, PFOR

Introduction

Periodontitis is an inflammatory disease with a bacterial etiology in a susceptible host[1]. Given the bacterial etiology, antibiotics have been used regularly but judiciously in the treatment of aggressive and severe chronic periodontitis cases. Antibiotics such as amoxicillin, metronidazole, tetracycline, azithromycin, and clindamycin have been studied and found to be effective[2]. These antibiotics, however, are broad-spectrum, exerting their effects on both aerobic and anaerobic bacteria i.e. both commensal and pathogenic flora, and, if used, serve as an adjunctive therapy[3]. It has been well established that in health the microflora is dominated by aerobic species, especially *Streptococcus sp.*, while periodontitis is characterized by a shift to pathogenic, anaerobic microorganisms[4,5] (Figure 1). Thus, when a broad-spectrum antibiotic is used, it will eliminate the aerobic bacteria allowing for reinfection by the pathogenic anaerobic species, which enjoy the protection of biofilms in the forms of dental plaque and calculus. Without the competition of the commensal aerobic species the anaerobes can multiply in periodontal pockets which favor their proliferation. It is well established that these pathogenic, anaerobic bacteria are found in periodontal pockets and include such species as *Porphyromonas gingivalis (P. gingivalis), Tannerella forsythia (T. forsythia),* and *Treponema denticola (T. denticola)* which make up the so-called "red complex" while *Campylobacter rectus (C. rectus), Fusobacterium nucleatum (F. nucleatum), Parvimonas micra (P. micra)*, and *Prevotella intermedia (P. intermedia)* comprise the “orange complex”[6]. In periodontally healthy sulci species such as *Actinomyces naeslundii (A. naeslundii), Streptococcus oralis (S. oralis), Streptococcus gordonii (S. gordonii), Streptococcus cristatus (S. cristatus), Gemella haemolysans (G.*

Haemolysans), and *Neisseria spp.* are more abundant[4]. Finally, currently used antibiotics do not eradicate bacteria that are internalized by host cells and serve as a reservoir for re-infection.

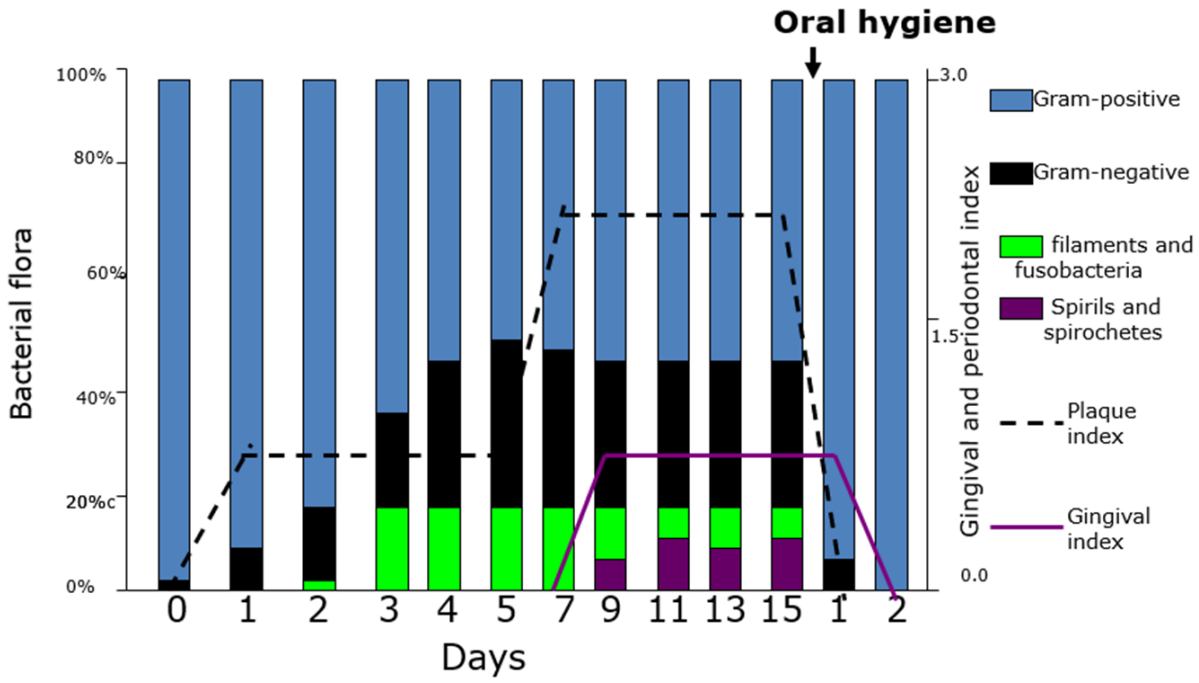


Figure 1. This figure is adapted from the findings of Löe, 1965 showing the shift in the oral microflora from health through gingivitis with the cessation of oral hygiene[5].

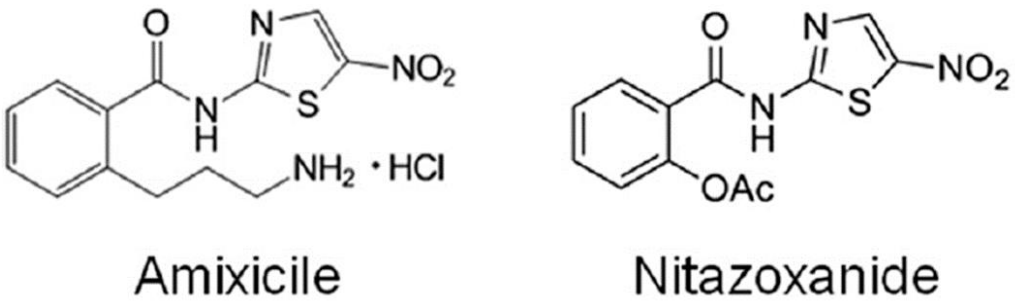
Metronidazole is a semi-selective antibiotic that targets anaerobic species and has been used as an adjunct to mechanical therapy in the treatment of aggressive and refractory periodontitis[7]. It acts as a prodrug that is partially reduced inside of anaerobic bacteria and some protozoans, which makes it selective for these populations. Once it has been reduced into its active form, it disrupts bacterial nucleic acid synthesis[8]. Metronidazole is generally used in combination with Amoxicillin as a very broad-spectrum approach based on reducing the overall bacterial load. When used in combination with Amoxicillin, significantly greater probing depth reduction and clinical attachment gain was found compared to mechanical therapy alone[9]. When used alone

Metronidazole was found to be effective in reducing the need for surgical therapy when given in conjunction with traditional mechanical therapy[7]. Its use has been limited, however, due to side effects including yeast infection, neuropathy, neurotoxicity, pancreatitis, encephalopathy, and sometimes-severe gastrointestinal effects which can lead to colitis if used repeatedly[10]. Thus, the need for a new antimicrobial agent that will specifically target periodontal anaerobes with minimal to no side effects is apparent.

Criticism of the use of antibiotics in addition to mechanical therapy for the treatment of periodontitis comes primarily due to unwanted side effects and contribution to bacterial resistance[11]. Metronidazole has a very low occurrence of acquired resistance due to its requirement to be taken into the cell and degraded into its active form inside of anaerobic cells[7]. Similar selectivity in a substitute drug would be an optimal characteristic.

Recently, the Hoffman laboratory at the University of Virginia, Department of Medicine, has developed amixicile as an anaerobe-specific antimicrobial with a novel mechanism to be used for the treatment of *Clostridium difficile* infections[11]. Amixicile is a derivative of nitazoxanide in which a 2-acetoxy group has been replaced with an aliphatic amine making the drug much more soluble as well as avoiding glucuronidation in the liver. Using a murine model, the Hoffman lab was able to show a high degree of specificity toward anaerobic gastrointestinal pathogens leaving commensal flora intact with no notable side effects. This drug's novel mechanism targets a vitamin B1 (thiamine pyrophosphate) cofactor involved in the pyruvate: ferredoxin oxidoreductase (PFOR) metabolism essential to anaerobic bacteria. This highly specific target allows both a high degree of anaerobe specificity as well as eliminating the risk of mutagenic

bacterial resistance. Aerobic bacteria rely on pyruvate dehydrogenase (PDH) for metabolism and thus are unaffected by treatment with amixicile. Even when given at very high dosages that were well beyond the minimum inhibitory concentration (MIC) no adverse effects were observed in the mice[11].



Amixicile Nitazoxanide

Figure 2. Chemical structures of amixicile and its precursor nitazoxanide.

In addition to its effect on anaerobic bacteria, amixicile would seem to be a candidate for treating periodontal infection due to its ability to localize at sites of inflammation. This property was identified during studies on amixicile's effect on *C. difficile* infections and the mechanism has to do with mucosal inflammation which causes local tissue destruction and serum leakage[12]. It is known that periodontal disease causes localized tissue breakdown as evidenced by pocket formation which is accompanied by an increase in gingival crevicular fluid which is an inflammatory exudate derived from serum[13]. It was shown that amixicile is highly soluble reaching high levels in serum thus it would be plausible that amixicile may localize to sites of periodontal inflammation and be delivered to the sulcus via gingival crevicular fluid[11].

The next step in evaluating amixicile for potential periodontal therapy was to verify that it would be effective on oral pathogens and in the oral environment. As stated previously, certain periodontal pathogens are more virulent and contribute to greater

inflammation and dysbiosis than others. Given this fact, *P. gingivalis, P. intermedia, F. nucleatum, and T. forsythia* were evaluated via the Kyoto Encyclopedia of Genes and Genomes (KEGG) and all were found to have genes encoding for the PFOR enzyme that has shown to be the target of amixicile. Known anaerobes, *S. gordonii, and A. actinomycetemcomitans*, were found only to have genes encoding for PDH and thus should be unaffected by amixicile. This was tested and confirmed using an *in vitro* model in which it was found that amixicile inhibited the growth of *P. gingivalis, P. intermedia, F. nucleatum, and T. forsythia* while it did not affect the growth of *S. gordonii* nor *A. actinomycetemcomitans* in a monoculture environment. These microbes were then combined into a multispecies culture that would represent the way they may live and interact within a biofilm and again it was found that amixicile inhibited the growth of *P. gingivalis, P. intermedia, F. nucleatum, and T. forsythia*, while it did not affect the growth of *S. gordonii* nor *A. actinomycetemcomitans* in a multi-species environment. The experiment was carried one step further with the addition of 10% saliva and 10% serum to the multi-species culture to continue to simulate an oral environment and the results continued to show that amixicile successfully and selectively targeted anaerobes in a simulated oral environment[14].

Continuing in the pre-clinical investigation of amixicile's potential application in treating periodontal disease, its efficacy in inhibiting *Treponema denticola* (*T. denticola*) was evaluated. It has been found that in a healthy periodontium oral treponemes only comprise about 1% of the sulcular microflora but in disease this proportion can reach 40-50%[15]. Again, it was shown in vitro that amixicile was a potent inhibitor of *T. denticola* and its effect on some known virulence factors was elucidated. Amixicile was

shown to inhibit both bacterial motility as well as reducing the production of hydrogen sulfides which have been shown to induce apoptosis of cells of the human periodontium[16]. Progressing to an *ex vivo* model using a well-developed oral salivary microbiome derived from human subjects it was again shown that amixicile selectively inhibited anaerobic bacteria containing the PFOR enzyme and that aerobic bacteria were unaffected in a simulated oral environment representing a plaque biofilm[17].

Finally, an *ex vivo* periodontal microbiome derived from gingival pockets of patients with periodontal disease was used to determine amixicile's efficacy in a naturally derived biofilm. The importance of this is underscored by the fact that there is a large diversity of bacterial strains in the periodontal microbiome, so it is essential to test strains derived from clinical samples from diseased sites. Again, it was found that amixicile was able to selectively inhibit anaerobes while sparing commensal aerobic flora which can help prevent reinfection[18].

Table 1. Genus and species of oral bacteria that rely on PFOR versus PDH for their metabolism of glucose. All microbes relying on PFOR should be susceptible to amixicile, while those utilizing PDH should not.

PFOR Metabolism (Sensitive to Amixicile)	**PDH Metabolism**
P. gingivalis, P. intermedia, F. nucleatum, T. denticola, P. micra, and *T. forsythia*. *Porphyromonas spp., Veillonella spp., Prevotella spp., Alloprevotella spp.,* and *Fusobacterium spp.*	*S. gordonii, S. oralis, C. rectus,* and *A. actinomycetemcomitans*. *Actinomyces spp. Streptococcus spp., Klebsiella spp., Neisseria spp., Lactobacillus spp., Haemophilus spp., Gemella spp., Escherichia spp.,* and *Leptotrichia spp.*

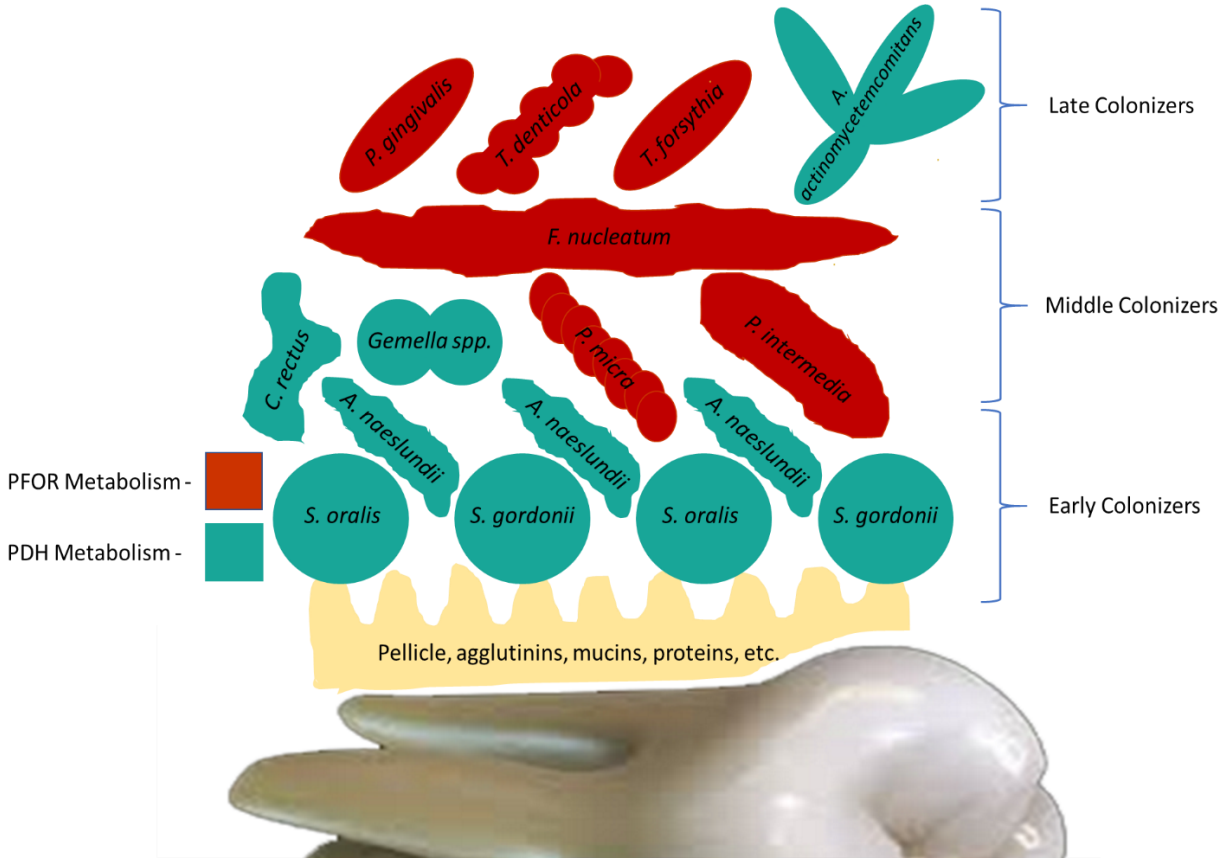


Figure 3. Representation of plaque biofilm showing early, middle, and late colonizers. Microbes are color-coded according to either PDH or PFOR metabolism.

The present study will move the examination of amixicile into an *in vivo*, preclinical animal model using the nonhuman primate (NHP), *Macaca mulatta (M. mulatta)*. In choosing an appropriate model for the study the anatomical, clinical, and microbiological features of the animal should resemble the same features of a human subject as closely as possible. In a review of animal models for the study of periodontitis, the NHP model was found to be superior to other animals including porcine, canine, and rodent models (Table 1). The anatomy of oral structures and teeth of the NHP is found to be very similar to that of the human as well as the natural occurrence and formation of dental plaque and calculus. The clinical progression of periodontitis has also been observed to be similar to the human form of the disease.

The only drawbacks of a simian model that were noted were the expensive of acquiring and maintaining the animals as well as animal husbandry issues i.e. socialization and mental stimulation, etc.[19]. A review of non-human primate species found the *M. mulatta* to be similar to the human anatomy and disease progression with some differences. There are histologic similarities in the periodontal tissues as well as the changes in those tissues from health to disease including widened intercellular spaces, an increase in PMNs, an increase of inflammatory cells in the connective tissue, destruction of collagen and formation of deepened periodontal pocketing, and finally bone resorption, all of which are similarly observed in the human disease progression. The primary difference noted was a higher proportion of *Actinomyces sp.* in NHP in both healthy and diseased sites. Microbiologically, *M. mulatta* shows an increase in motile rods and spirochetes with increased inflammation. In established gingivitis lesions the proportion of anaerobes continues to increase as well as the overall bacterial counts. In experimentally-induced periodontitis an increase in *P. gingivalis* and *P. intermedia* was observed[20].

Table 2. Comparison of animal models available for periodontal research including their anatomical features, microbiological characteristics, and logistical considerations.

Model	Anatomy	Microbiome	Logistical
Rodent	Only one incisor and three molars per quadrant. Naturally occurring periodontal disease is limited.	Microbiota differs from that of the human. *P. gingivalis* and *A. actinomycetemcomitans* do not naturally occur.	Small size makes procedures difficult and the amount of tissue for analysis is limited.

Dog	Oral structures and teeth, as well as periodontal disease progression, differ from that of the human.	Subgingival plaque consists of gram (-) anaerobic cocci and rods similar to humans including *P. gingivalis* and *F. nucleatum*.	Animal regulations issues. Need for companionship, exercise, and space can be prohibitive.
Pig	Oral structures and teeth similar to humans. Naturally occurring dental plaque, calculus, and periodontal disease.	Naturally occurring *P. gingivalis, S. mutans,* and *A. actinomycetemcomitans*	Relatively expensive. Animal care and maintenance can be an issue.
Non-Human Primate	Oral structures and teeth similar to humans. Naturally occurring dental plaque, calculus, and periodontal disease.	Very similar to human. Naturally occurring *P. gingivalis, A. actinomycetemcomitans, Haemophilus spp., Actinomyces spp., P. micra, F. nucleatum*, and *E. corrodens*, etc.	Expensive. Extensive animal care and enrichment requirements.

A greater understanding of the subgingival microflora of the *M. mulatta* species was gained and correlated to health versus clinically apparent inflammation. Mild inflammation showed an increase in *Haemophilus species (spp.)*, *Actinomyces spp.*, *P. micra*, *F. nucleatum*, *Eikenella corrodens (E. corrodens)*, and *A. actinomycetemcomitans*. The microflora was evaluated 7-days post-scaling and root

planing and a considerable increase in aerobes and simultaneous decrease in anaerobes was observed. The key difference in the NHP was again notably high *A. actinomycetemcomitans* in both healthy and diseased sites[21]. As sequencing technology improved the microflora of *M. mulatta* were more thoroughly evaluated and the findings were correlated to clinical parameters. From a global look at the microbes present it was found that 56% of the bacteria were identical to or had closely related human counterparts. Forty-eight species were unique to the macaque but all of these also had clearly and closely related human counterparts. Health associated microbes were found to be *Streptococcus spp., Lactobacillus spp., and Gemella spp*. Periodontal pathogens associated with clinical inflammation and bone loss were found to be *P. gingivalis, T. forsythia, Filifactor alocis, P. micra, Treponema spp., Fusobacterium spp.,* and *A. actinomycetemcomitans.*

It was determined that in the *M. mulatta* a 4mm probing depth (PD) along with clinical inflammation and bleeding on probing (BOP) is mild periodontitis. A PD of 5mm or greater with inflammation and BOP is considered moderate to severe periodontitis. All clinical features found in human periodontitis are present in the *M. mulatta* including increased probing depths and bone loss[22]. Bleeding on Probing (BOP) and its relevance to disease activity has been studied extensively. It has been reported that the presence of BOP can have a high false-positive for predicting periodontal breakdown but the negative predictive value was found to be 98%, meaning that an absence of bleeding is a reliable predictor of periodontal health[23]. Both visible inflammation (redness and edema) of the gingiva and BOP have been correlated histologically to an increase in inflammatory cell counts as well as collagen breakdown in the periodontal connective

tissue[24,25]. Given the value of these clinical findings, a modified Gingival Index (GI)[26] and BOP will be evaluated and correlated to the microbiological findings.

This study will aim to expand upon the *in vitro* research previously completed by testing the hypothesis that amixicile will selectively inhibit anaerobic periodontal microbes that code for the PFOR enzyme in *in vivo* conditions in the *Macaca mulatta*. Microflora will be collected, and data recorded for probing depth (PD), clinical attachment level (CAL), gingival index (GI), plaque index (PI), presence or absence of calculus (C), and bleeding on probing (BOP) at baseline, immediately post-treatment and then at 3- and 6- months following a two-week treatment with amixicile. No other treatment will be rendered.

Methods

Animals

All animal procedures were performed according to the protocol approved by the Virginia Commonwealth University (VCU) Institutional Animal Care and Use Committees (IACUC) (Approval # AD10001255). Two male non-human rhesus primates, *M. mulatta*, were used in our study. Animal G's dentition was healthy and free of caries or endodontic pathology with tooth #6 having previously treated non-surgical root canal therapy with an MF amalgam restoration. Animal T's dentition was healthy and free of caries or endodontic pathology. The animals were housed at the VCU's animal facility in extra-large enclosures. Both subjects were fed a diet of kibble (Monkey Chow, Purina) and fresh fruits and vegetables as well as foraging for dried seeds, dried fruits, and nuts daily. They were provided social and environmental enrichment through daily handling by animal technicians, visual contact with other animals, and other enrichment items (toys, videos, etc.). Animals enrolled in this study were systemically healthy.

Clinical Examination and Sample Collection

Clinical periodontal examination was performed by a graduate resident specializing in periodontics (DL) under the supervision of a faculty periodontist (JGD). The animals were placed under general anesthesia by way of an injection of ketamine (10 mg/kg) followed by intubation and administration of 2% isoflurane at 2 L/min and 100% oxygen at 1 L/min. The comprehensive periodontal examination was performed at baseline, immediately post-treatment, and then at 3- and 6- months post-treatment.

Baseline exams were performed to determine the clinical and microbiological status of the animals.

A complete set of clinical photographs was taken at each examination. The clinical photographs were used to assign a modified Gingival Index (GI)[26] score to each sextant. The scoring was based on the following classification: GI 0 = pale pink to pink, knife-edge margin, positive architecture; GI 1 = slightly more reddish, slight marginal edema, clear exudate, no BOP; GI 2 = red to bluish-red, glazy, marginal edema, BOP apparent in the photograph; GI 3 = markedly red to bluish, edematous, BOP/spontaneous bleeding apparent in the photograph. Photographs were randomized and then each sextant was scored with a single value by three independent examiners (JGD, DL, EB). Scores of each examiner were averaged to come up with the GI of each sextant for the initial exam, immediately post-amixicile, 3 months post-treatment, and 6 months post-treatment.

Saliva samples were collected using five cotton swabs equally representing all areas of the mouth by swabbing the entire oral cavity including buccal and sublingual spaces. The cotton swabs with the sample were placed into microcentrifuge tubes containing 500 µl of phosphate-buffered saline (PBS, pH 7.2) and RNAlater solution (Invitrogen, Waltham, Massachusetts, USA).

Gingival crevicular fluid (GCF) and microbiology samples were collected from the same sites to correlate the findings. Samples of GCF and subgingival plaque were taken from the following sites at baseline: #1D, 2D, 3D, 14D, 15D, 16D, 20D, 21D, 22D, 27D, 28D, and 29D. Since no mechanical therapy was to be performed as part of the study, GCF and subgingival plaque samples were taken from #1M, 2M, 3M, 14M, 15M,

16M, 20M, 21M, 22M, 27M, 28M, 29M for all follow-up exams. For subgingival plaque collection a Nevi 2 periodontal scaler (SCNEVI29E2, Hu-Friedy, Chicago, Illinois, USA) was inserted to the base of the attachment and plaque was collected from the subgingival tooth structure and placed into microcentrifuge tubes containing 500 µl of phosphate-buffered saline (PBS, pH 7.2) and stored at -80ºC. For GCF collection, a Periostrip paper (Periopaper Gingival Crevicular Fluid Collection Strip, Fisher Scientific International, Pittsburgh, Pennsylvania, USA) was inserted into the periodontal sulci of interest and left for 30 seconds or until completely visibly saturated. The samples were collected into microcentrifuge tubes containing 500 µl of phosphate-buffered saline (PBS, pH 7.2) and stored at -80ºC.

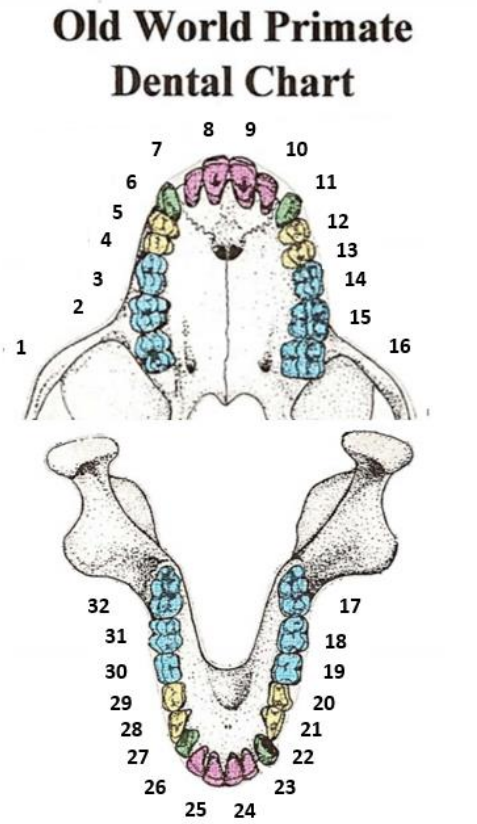


Figure 4. Nonhuman Primate Odontogram showing the position of the teeth in the arch which are very similar to the human dentition.

The clinical exam consisted of determination of 1. Probing depth (PD, distance in millimeters between the gingival margin and the base of the sulcus or pocket) and free gingival margin (FGM, distance in millimeters from the CEJ to the margin of the unattached gingiva) measured at four sites per tooth: mesiobuccal, buccal, distobuccal, and straight palatal or lingual; 2. Clinical attachment level (CAL) was calculated at each of the aforementioned sites using the formula CAL = PPD – FGM; 3. Presence or absence bleeding on probing (BOP, Bleeding on Probing); 4. Presence or absence of plaque (PI, Plaque Index); 5. Presence or absence of calculus.

Baseline exams were performed to determine the periodontal and microbiological status of the animals. Following the initial exam, the animals were then left for 14 days without any intervention prior to the administration of amixicile. The animals were then treated with 40 mg/kg/day of amixicile divided into two doses, encased in marshmallow for 14 days. No changes were made to the animals' diet and no oral hygiene measures were performed during the study period. Periodontal and microbiological exams as described above were performed immediately post-treatment, 3 months post-treatment, and 6 months post-treatment (Figure 5).

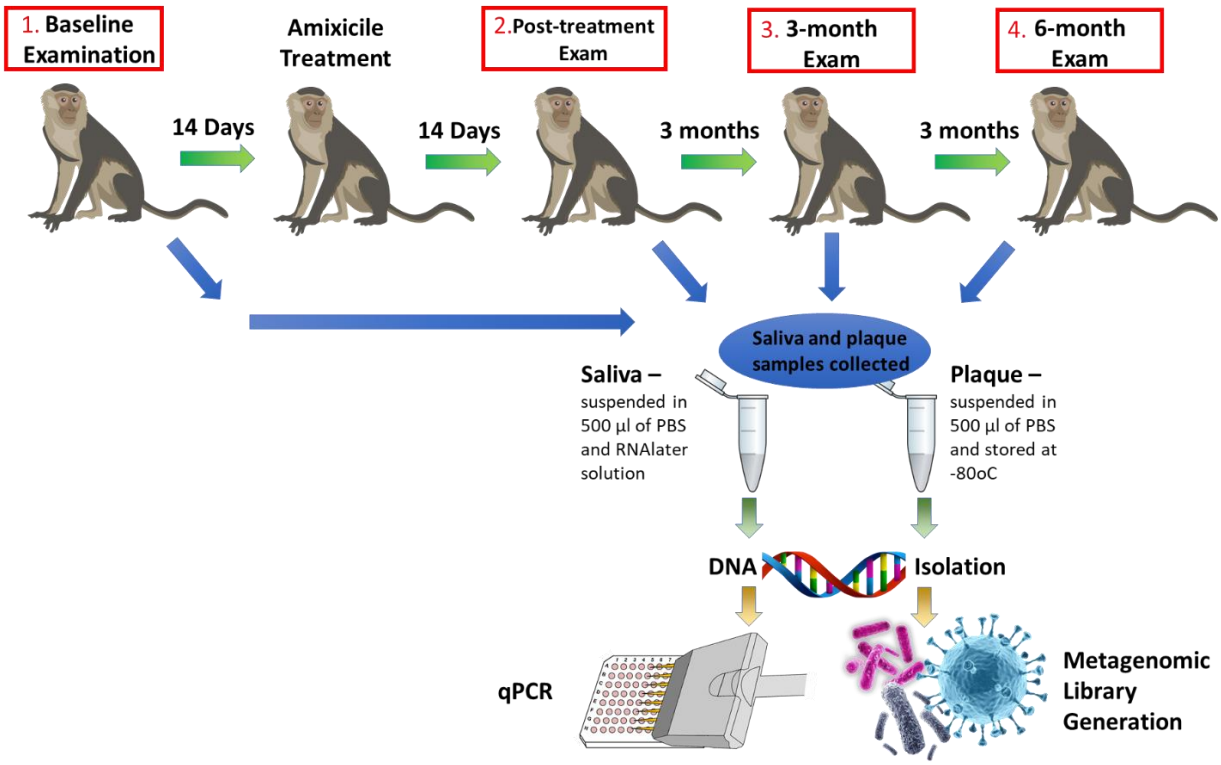


Figure 5. Experimental design showing the time frame of the exams and the sequence of events from plaque and saliva sample collection through analysis.

Microbiological Analysis

For assessment of the microbial content we analyzed both the saliva and subgingival plaque samples. Each sample for analysis was vigorously vortexed for 5 min to break down any larger plaque complexes. DNA from each sample was extracted as described below. Plaque samples were processed individually while aliquots of saliva samples were pooled together prior to analysis.

DNA Isolation. Collected plaque samples were suspended in 500 µl of RNA*later* buffer (Fisher Scientific International, Pittsburgh, Pennsylvania, USA) and stored at -80ºC. Genomic DNA (gDNA) was isolated using 200 µl of the mixture with the PureLink™ Microbiome DNA purification kit (Fisher Scientific International, Pittsburgh, Pennsylvania, USA) according to the manufacturer's instructions. Similarly, 200 µl of pooled saliva was used for DNA isolation.

Quantitative PCR (qPCR). The gDNA derived from the above microbiomes was used to quantify the presence of bacterial species in the various samples using a 7500 Fast Real-time PCR machine (Fisher Scientific International, Pittsburgh, Pennsylvania, USA). Purified DNA (1 µL) and species-specific primers were added to Fast SYBR Green Mastermix (Fisher Scientific International, Pittsburgh, Pennsylvania, USA). Reactions were run using standard cycle conditions: 95°C for 20 sec (1 cycle); 95°C for 3 sec, 60°C for 30 sec (40 cycles). The cycle threshold (Ct) data were collected and then converted to absolute fold change.

Metagenomic library generation and 16S rDNA Sequencing. Bacterial 16S ribosomal DNA (rDNA) amplification and library construction were done using the Zymo Research Quick-16S^{TM} NGS Library Prep Kit (Zymo Research, Irvine, California, USA). Low DNA input protocol was used in our study. Briefly, reactions were set up in 96 well "Targeted Plate" and the V3-V4 region of rRNA genes were amplified with the V3-V4 primers and the Quick-16STM qPCR Premix. 25 cycles (and more, if required) at the profile: 95^{o}C for 10 min, 95^{o}C for 30 sec, 55^{o}C for 30 sec, and 72^{o}C for 3 min was used for amplification. Sufficient amplification was verified using the recommended final fluorescence (that was higher than the threshold fluorescence). Following cooling at 4^{o}C the samples were transferred to collection plate and (PCR primers, dNTPs) were degraded with the enzymatic cleanup solution. Finally, the samples were transferred to a "barcoded plate" where index primers for multiplexing of the samples were added. The barcodes were added using 5 PCR cycles consisting of: 95^{o}C for 10 min, 95^{o}C for 30 sec, 55^{o}C for 30 sec, and 72^{o}C for 3 min. Sufficient amplification during barcode addition was verified through examination of the amplification curve. The library was

then pooled in equimolar amounts and purified using the MagBead kit components (Zymo Research, Irvine, California, USA). The final 16S rDNA library was sequenced with the MiSeq Reagent Kit v3 (600-cycle) with pair end-setting and 2 x 250 bp on the Illumina MiSeq platform (Illumina, San Diego, California, USA). Sequencing was performed at the VCU Genomics and Microbiome Core, Richmond, Virginia, USA. Following sequencing, the samples were deconvoluted, barcodes were trimmed, and short sequences (<100bp) were removed.

Metagenomic Data Processing. The raw read sequences were analyzed with CLC Workbench software (version 12; Qiagen, Venlo, Netherlands) equipped with the Microbial Genomics Module plugin (version 2.0; Qiagen, Venlo, Netherlands). The paired-end reads were merged into one high-quality representative by settings of CLC Workbench (mismatch cost = 1, minimum score = 25, gap cost = 4, maximum unaligned end mismatches = 5). The parameter settings for the quality trimming were as follows: trim using quality scores, limit = 0.05; trim ambiguous nucleotides, maximum number of ambiguities = 2. Operational taxonomic unit (OTU) clustering and taxonomic assignment were carried out with the reference sequences from the Human Oral Microbiome Database (HOMD, Cambridge, Massachusetts, USA, 16S rRNA gene reference sequence [16S rRNA refSeq] Version 15.2) at a level of similarity of 97% of OTU.

Bioinformatics Analysis. Data were analyzed using the bioinformatics workflows available through CLC Genomics Workbench with the CLC Microbial Genomics Module (Qiagen, Venlo, Netherlands).

Availability of Data. High throughput sequencing data were deposited to NCBI's Sequence Read Archive (SRA) with the accession number SUBXXXX (submission pending).

Due to the limited sample size and nature of this pilot study, statistical data analysis will be primarily descriptive in nature with the intent of providing information to power future studies rather than to determine statistical significance.

Results

Amixicile reduces clinical periodontal inflammation. The periodontal examination of both animals revealed mild periodontitis at several sites (Tables 5A, 5B)[22]. We examined twelve teeth per animal for bleeding on probing (BOP), pocket depth (PD), and microbiological samples of subgingival plaque and saliva. Three sites (MB, B, DB) were examined/tooth thus making up thirty-six sites per animal examined. At the baseline exam for Animal G there were 3 teeth with three sites that had PD of 4mm, which correspond to mild periodontitis for NHP, and seven sites with PD of 3mm [22]. For the second animal, Animal T, there were two teeth, each with one site with PD of 4mm and eleven sites with PD of 3mm. We thus concluded that the clinical characteristics point to an acceptable level of periodontitis to be used in our study.

A modified Gingival Index (GI) was evaluated in the two specimens examined at four timepoints during the study period. Agreement on the modified GI between pairs of independent raters ranged from 0.56 to 0.69 which is considered moderate to substantial agreement. This amounts to agreement in scores for 71%-79% of images viewed. Results are given in Table 3.

Table 3. Agreement in Gingival Index when Assessed from Clinical Photos by 3 Independent Raters

	Kappa	95% CI	Interpretation*	Percent of Cases Agreed
EEB-DJL	0.66	0.51-0.81	Substantial	77%
JGD-EEB	0.56	0.40-0.73	Moderate	71%
JGD-DLL	0.69	0.54-0.84	Substantial	79%

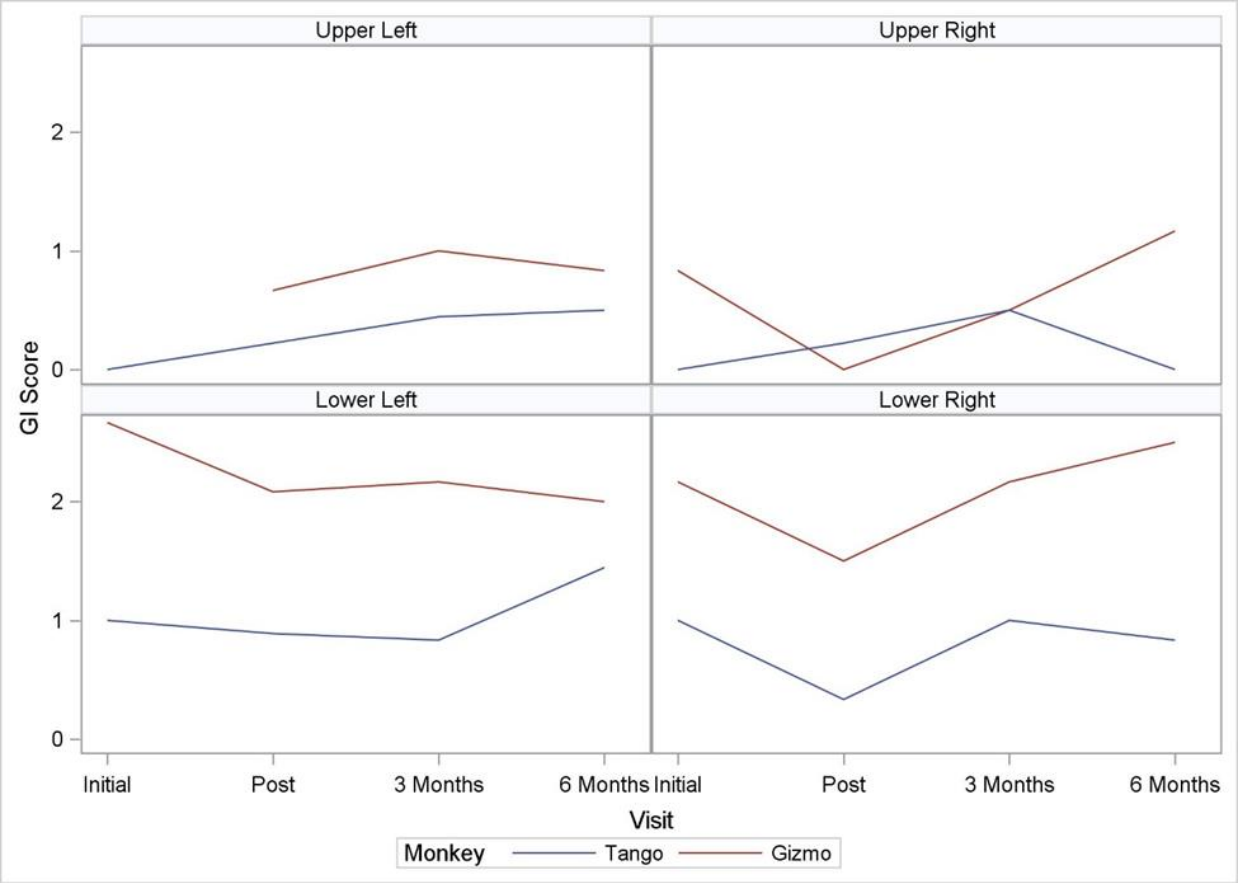


Figure 6. Gingival Index for Animal G and Animal T by Sextant Across Visits

As shown in Figure 6 above, GI was summarized by sextant (Upper Left, Upper Right, Lower Left, Lower Right). The average GI was calculated by averaging across the three raters and across all pictures as detailed in the Methods. The trends for GI by monkey and sextant are given in Figure 6. Data for the GI of Animal G for the Upper Left sextant was not available. GI was relatively steady on Upper and Lower Left. Upper Right initially decreased for Animal G and then increased steadily from 3-month and 6-month examinations. Animal T increased slightly from Initial to 3-month examinations and then decreased by the 6-month exam. The Lower Right demonstrated a decrease from the Initial exam to Post-treatment and then increased at the 3-month exam. Between the 3-month examination and the 6-month examination, Animal T remained relatively steady and Animal G had a slight increase in GI score.

Clinical findings across the study visits are summarized in Table 4. Further statistical analyses were not performed due to the limited number of animals. Average probing depth for Animal G increased from initial to both the post-treatment and 3-month examination and then decreased by the 6-month follow-up. Animal T saw an initial decrease at the post-treatment exam followed by an increase at the 3-month visit and a decrease at 6-months. Both monkeys were at or below their initial average probing depth by the 6-month examination (Animal G: 2.00 vs 1.99; Animal T: 1.93 vs 1.73; Fig. 6A). In terms of bleeding sites (Fig. 4B), both monkeys saw an initial increase from the initial examination to the post-treatment followed by a substantial improvement back to baseline at the 3-month follow-up. By the 6-month follow-up, Animal G had an additional decrease in bleeding sites, but Animal T saw a slight increase. The number of plaque sites increased for both monkeys progressively from initial examination through the 6-month follow-up (Fig. 4C). Both began with 0 plaque sites at the initial examination and finished with 96 for Animal G and 95 for Animal T.

Table 4. Summary of Clinical Findings by Visit

	Gizmo							
	Initial		Post		3 Months		6 Months	
Average Probing Depth	2.00		2.09		2.14		1.99	
Number of Bleeding Sites	12		29		17		4	
Number of Plaque Sites	0		51		80		96	
	UL	UR	UL	UR	UL	UR	UL	UR
Gingival Index	2.67	2.17	2.08	1.5	2.17	2.17	2	2.5
	LL	LR	LL	LR	LL	LR	LL	LR
	--	0.83	0.067	0	1	0.5	0.83	1.17
	Tango							
	Initial		Post		3 Months		6 Months	
Average Probing Depth	1.93		1.78		1.91		1.73	
Number of Bleeding Sites	4		23		3		9	
Number of Plaque Sites	0		47		84		95	
	UL	UR	UL	UR	UL	UR	UL	UR
Gingival Index	1	1	0.89	0.33	0.83	1	1.4	0.83
	LL	LR	LL	LR	LL	LR	LL	LR
	0	0	0.2	0.2	0.4	0.5	0.5	0

In Animal G, twelve of the thirty-six sites (33.3%) exhibited BOP at baseline. Similarly, in Animal T, there were four sites with BOP out of the thirty-six tested (11.1%). Immediately following amixicile treatment the number of sites with BOP remained similar to baseline. Three months post-treatment there were no sites with PD of 4mm in either animal. There were fourteen sites in animal G and eleven sites in animal T with PD of 3mm. Significantly, all the sites that previously were 4mm were reduced to 3mm or less at 3 months post-treatment. Bleeding on probing (BOP) remained similar to baseline at the 3-month post-treatment exam. At the final exam there were still no sites measuring 4mm and only eleven sites with PD of 3mm in each of the animals. A significant reduction in BOP in animal G was noted where we detected four sites, or 11.1%. Animal T experienced a rebound in BOP by the 6-month exam having eight sites (22.2%) exhibiting BOP. All of the above shows that a two-week treatment with amixicile

resulted in improvement in BOP in Animal G from 33.3% to 11.1% (Fig. 7) and stability of PD in Animal G and a reduction in PD in Animal T from 1.93mm at baseline to 1.73mm at the 6-month exam (Table 4, Fig. 8) all while both animals experienced a dramatic increase of detectable plaque biofilm (Animal G – 51 to 96 sites; Animal T – 47 to 95 sites)(Fig. 9).

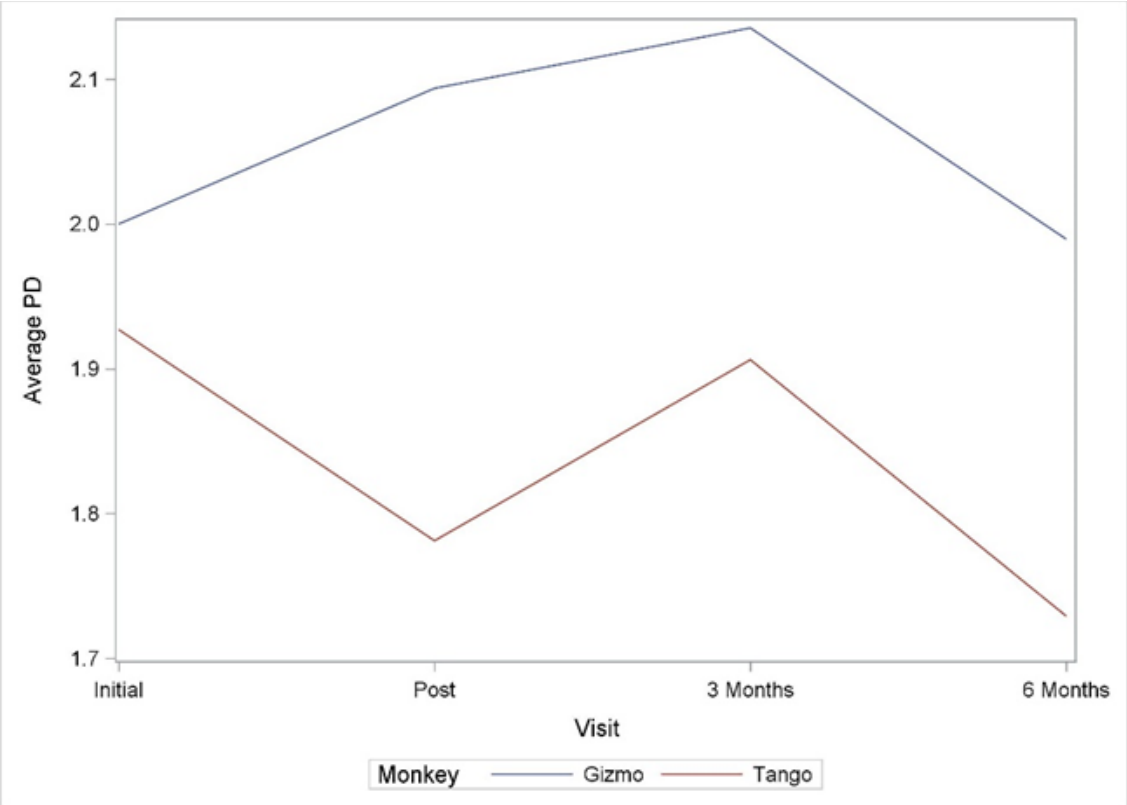


Figure 7. Average Probing Depth for Animal G and Animal T Across Visits

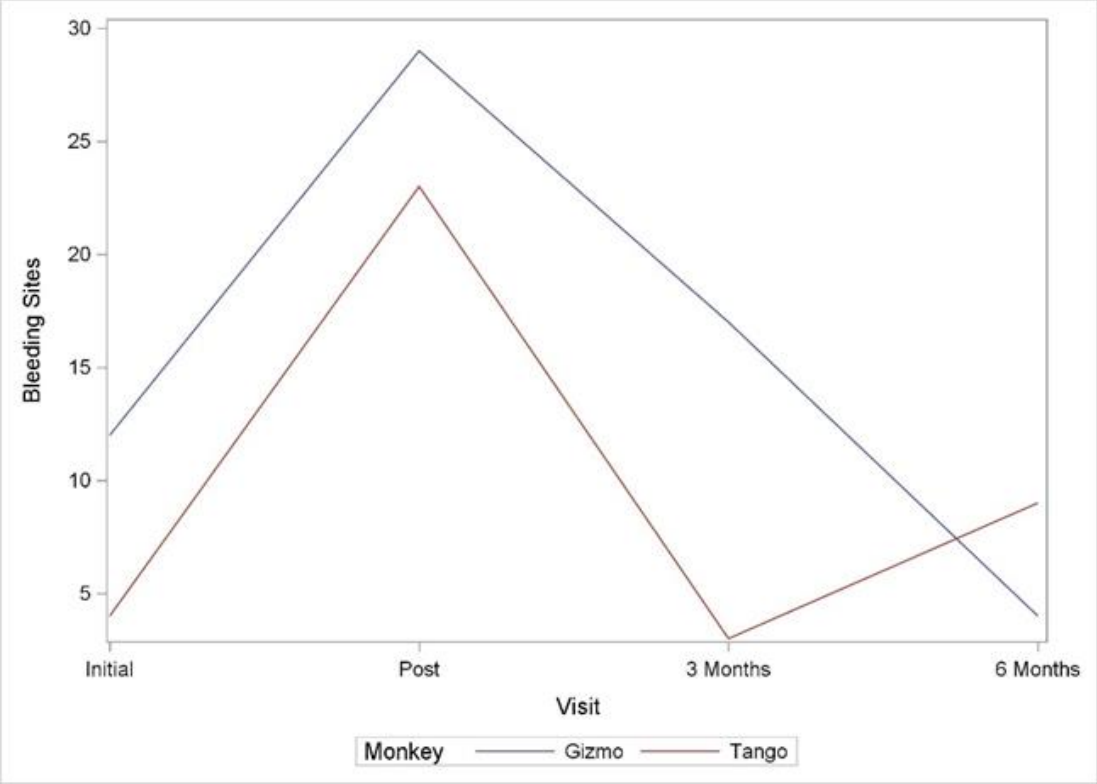


Figure 8. Average Probing Depth for Animal G and Animal T Across Visits

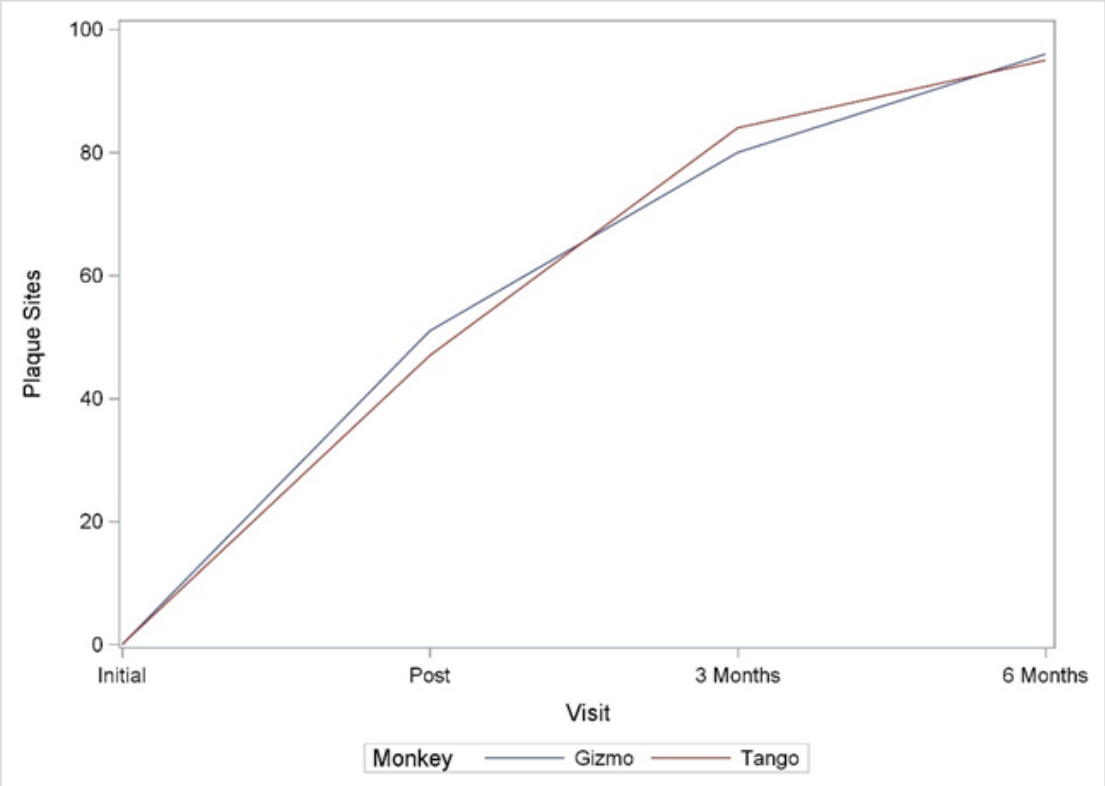


Figure 9. Plaque Sites for Animal G and Animal T Across Visits

A more focused examination of the effect of amixicile in Animal G shows teeth that had PD sites of 4mm at baseline included teeth #14, 15, and 16, all of which had PD of 3mm or less at 6 months post-treatment. At these teeth, BOP decreased from three sites to one site at three months post-treatment in animal G. That improvement was maintained through the six-month post-treatment exam. Similarly, in animal T at teeth #14, 15, and 16, sites with 4mm PD were reduced from two to zero from baseline through 6-months post-treatment. At these teeth, BOP was decreased to only one site at 3-months post-treatment, but Animal T experienced a rebound between 3 and 6 months ending with three sites with BOP.

Table 5. Clinical characteristics of Animal G at baseline (-B), Post-amixicile (-P), 3-months post-treatment (-3), and 6-months post-treatment (-6). Clinical characteristics assessed: PD – pocket depth (shown in mm), BOP – bleeding on probing (B – bleeding, N – no bleeding)

Animal G

Tooth	1	2	3	14	15	16	20	21	22	27	28	29
PD - B	323	223	223	324	324	432	212	212	221	121	222	211
BOP - B	NNN	NBN	NBN	NNN	NBN	NBN	NBN	NBN	NNN	NNN	NNN	NNN
PD – P	333	313	323	323	323	324	212	322	222	122	212	212
BOP – P	NNB	BNB	BNB	BNB	BNB	BNN	BNB	BNN	NNN	NNN	NNB	BNB
PD - 3	232	323	323	323	323	322	223	322	232	132	222	211
BOP - 3	BBN	BNB	BNN	BNN	NNN	NNN	NNN	NNN	NNN	NNN	NNB	NNN
PD - 6	322	323	323	223	313	323	222	213	221	222	222	212
BOP - 6	NNN	NNN	NNB	NNB	NNN	NNN	NNN	NNN	NNN	NNN	NNB	NNN

Table 6. Clinical characteristics of Animal T at baseline (-B), Post-amixicile (-P), 3-months post-treatment (-3), and 6-months post-treatment (-6). Clinical characteristics assessed: PD – pocket depth (shown in mm), BOP – bleeding on probing (B – bleeding, N – no bleeding)

Animal T

Tooth	1	2	3	14	15	16	20	21	22	27	28	29
PD - B	323	323	323	323	324	423	212	222	232	122	212	212
BOP- B	NNN	NBN	NBN	NNN	NNN	NNN	NNN	NNN	NNN	NNN	NNN	NBN
PD - 1	322	323	313	313	213	324	212	211	131	121	112	222
BOP- 1	NNN	NNB	BNB	BBB	NBN	NNN	NNN	NBN	BBN	NNN	NNN	BNN
PD - 3	223	323	312	313	323	323	212	222	232	122	112	212
BOP - 3	NNN	NNN	NNN	NNN	BNN	NNN	NNN	NNN	NNN	NNN	NNN	NNN
PD - 6	223	313	323	323	313	323	212	221	121	122	212	212
BOP - 6	NNN	BNB	BNB	BNB	BNN	NNN	NNN	NNN	NNB	NNN	NNN	NNN

Amixicile reduces the abundance of anaerobic bacteria within the salivary microbiome. Saliva from all sites has been collected and pooled prior to analysis. Aliquots of saliva collected during baseline and follow up periodontal exams have been used to isolate total DNA. The DNA was used for 16S rDNA sequencing and data was analyzed at the genus and species level (Fig. 10-13). Analysis of the data derived from Animal G at the genus level revealed that at baseline the most dominant were bacteria belonging to the Streptococcus genus followed by Haemophilus, Porphyromonas, Gemella, and Fusobacterium genera (Fig. 10). In the second animal, Animal T, bacteria belonging to the Neisseria genus were the most abundant while bacteria belonging to the genera of Porphyromonas, Streptococcus, and Gemella were also abundant (Fig. 12). Having large proportions of anaerobic bacteria, specifically bacteria belonging to Porphyromonas and Fusobacterium, justified the use of this model for testing of amixicile's efficacy. Following treatment with amixicile, reduction in anaerobic bacteria with a concomitant increase in aerotolerant ones was observed. Specifically, a reduction in bacteria belonging to the genera Porphyromonas, Fusobacterium, and Alloprevotella, all anaerobes, was seen in Animal G. At the same time, an increase in Haemophilus, Gemella, and Escherichia, all aerobes, was detected (Fig. 10). At the species level, Animal G showed a dramatic reduction of F. nucleatum immediately post-treatment with some rebound through 6-months but never reaching pre-treatment levels (Fig. 11). Streptococcus remained at similar levels to the pre-treatment phase. In the second animal, Animal T, we also observed a reduction in bacteria belonging to Porphyromonas (Fig. 12). However, a reduction in Neisseria, an obligate aerobe, was also observed. An increase in levels of aerotolerant bacteria belonging to the Streptococcus, Haemophilus, and Gemella genera was observed (Fig. 12). These results demonstrate that amixicile was effective in reducing the levels of anaerobic bacteria present in the salivary microbiome of an NHP model while leaving the aerobic species generally unaffected.

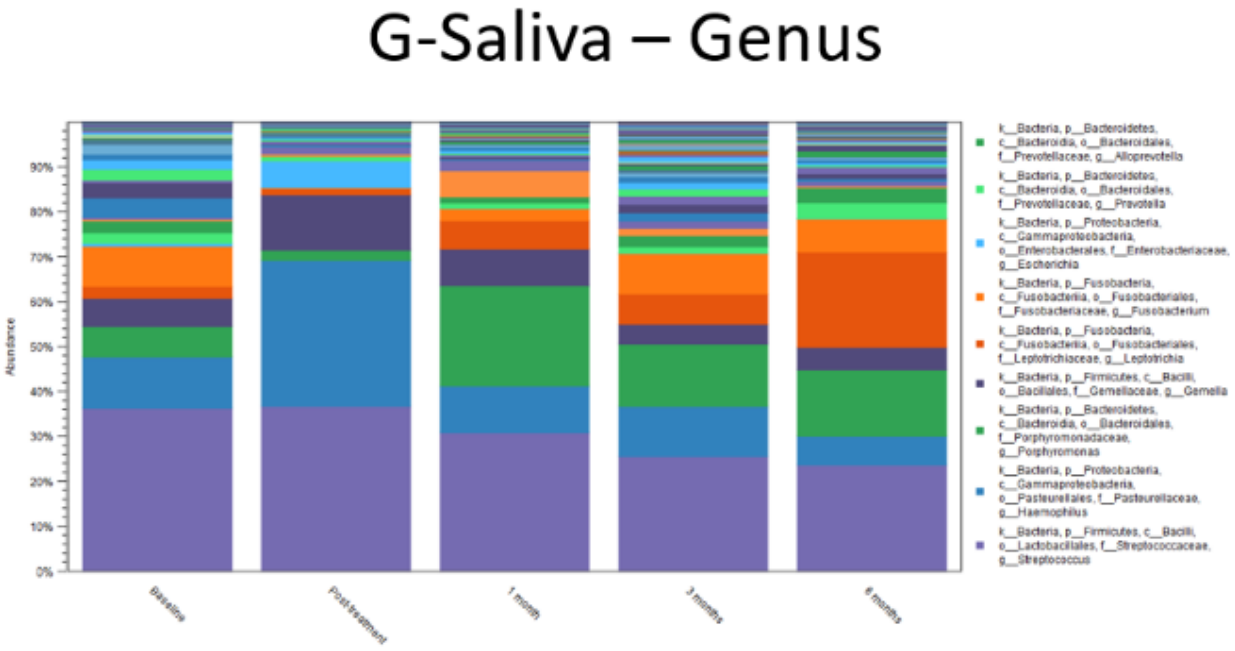


Figure 10. Amixicile reduces the abundance of anaerobic bacteria within the salivary microbiome. Animal G – Genus level analysis.

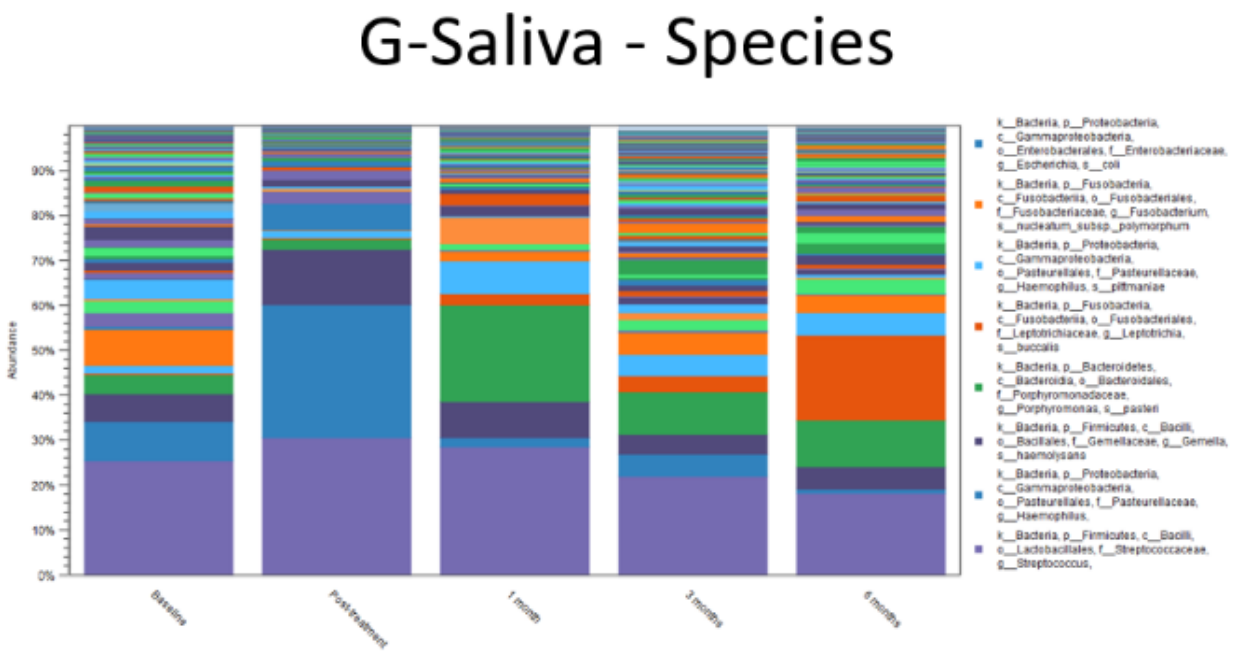


Figure 11. Amixicile reduces the abundance of anaerobic bacteria within the salivary microbiome. Animal G – Species-level analysis.

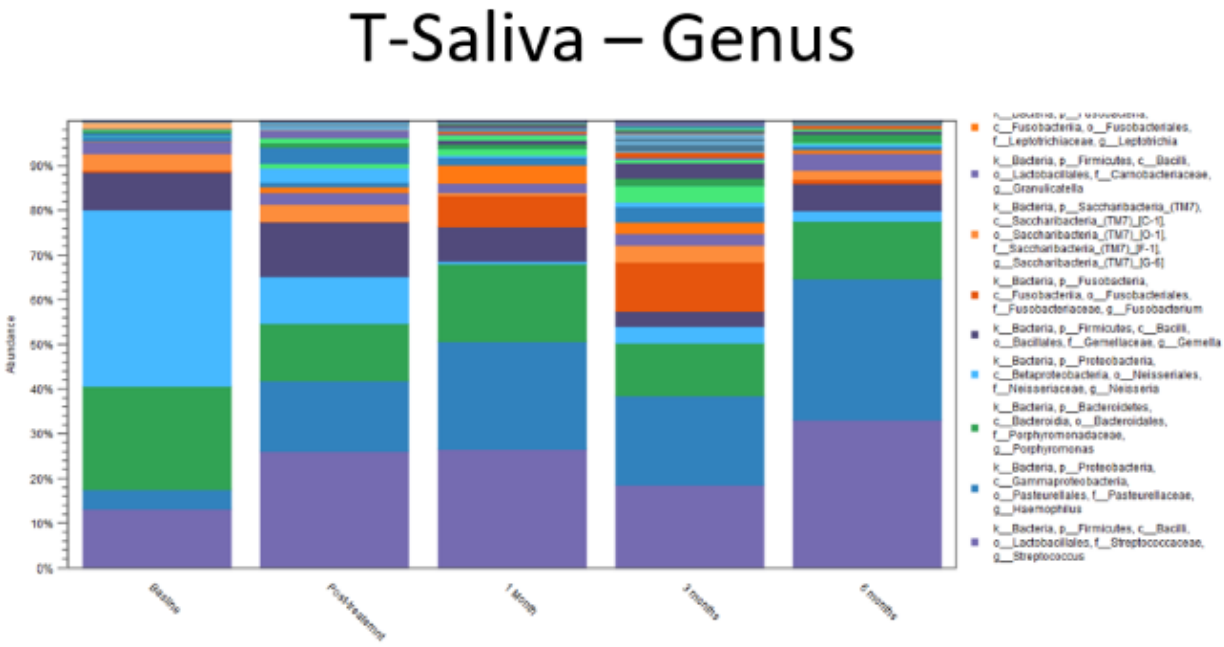


Figure 12. Amixicile reduces the abundance of anaerobic bacteria within the salivary microbiome. Animal T – Genus level analysis.

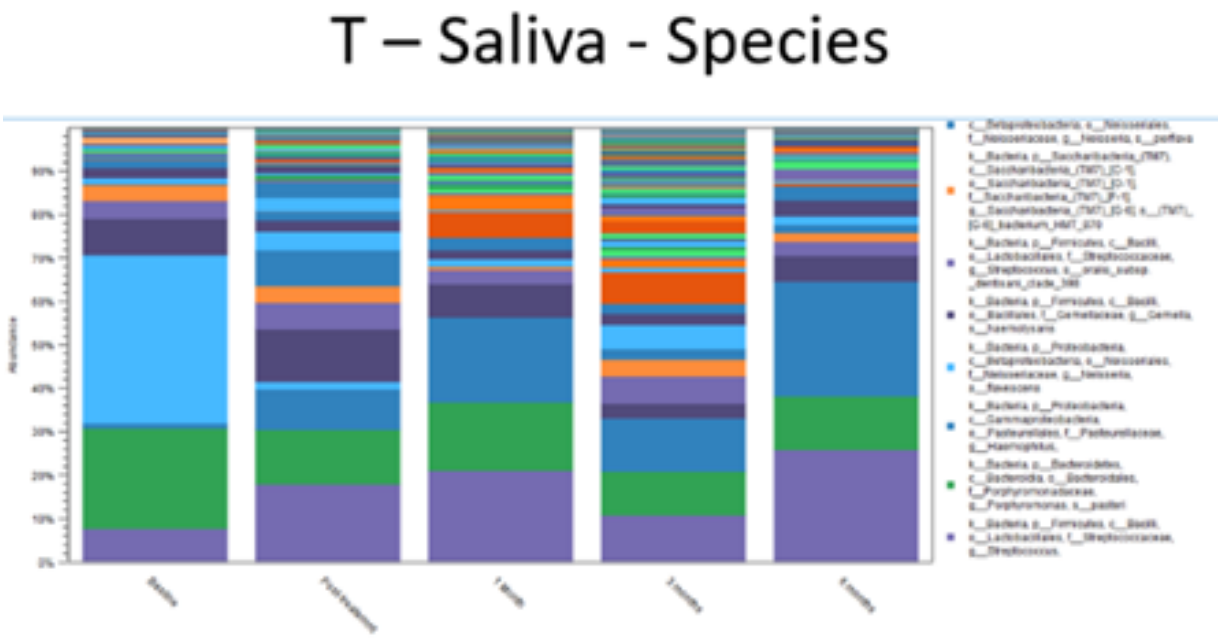


Figure 13. Amixicile reduces the abundance of anaerobic bacteria within the salivary microbiome. Animal T – Species-level analysis.

Amixicile reduces the abundance of anaerobic bacteria in gingival pockets.

Four sites of subgingival plaque biofilm from Animal G (#3,14,16,22) and four sites from Animal T (#3,14,15,16) were successfully surveyed for the composition of the oral microbiome at baseline and following amixicile treatment (Fig. 14-17). Survey of the G3 (Animal G, tooth #3) site at the genus level in Animal G indicated that at baseline *Porphyromonas* and *Fusobacterium* were highly dominant. Other abundant bacteria included *Leptotrichia, Prevotella,* and *Streptococcus.* Following amixicile treatment a significant reduction in both *Porphyromonas* and *Fusobacterium* levels was observed. This corresponded to an increase in levels of *Leptotrichia* and *Prevotella* (Fig. 14). Analysis of the G14 site has shown that at baseline *Escherichia, Prevotella* and *Streptococcus* were highly abundant genera (Fig. 15). Following amixicile treatment levels of *Escherichia* and *Prevotella* were reduced while those of *Leptotrichia* and *Fusobacterium* were increased. Baseline abundance of the G16 site has shown high levels of *Escherichia, Veillonella, Streptococcus, Leptotrichia, Prevotella,* and *Fusobacterium* (Fig. 16). Following treatment, the abundance of *Veillonella, Streptococcus, Leptotrichia, Prevotella,* and *Fusobacterium* were reduced while that of *Escherichia, Actinomyces,* and *Aggregatibacter* was elevated. The fourth site of Animal G, G22, has shown a high abundance of *Escherichia, Porphyromonas, Prevotella, Fusobacterium,* and *Alloprevotella.* Following antibiotic treatment, the levels of *Porphyromonas, Fusobacterium,* and *Alloprevotella* were reduced with an increase in *Leptotrichia, Selenomonas, Prevotella,* and *Actinomyces.* Interestingly, levels of *Streptococcus* were also reduced (Fig. 17). Overall, in Animal G we observed a reduction in levels of anaerobic bacteria and an increase in abundance of aerotolerant

ones. There was significant variation in the microbial composition of the baseline microbiome as well as post-treatment.

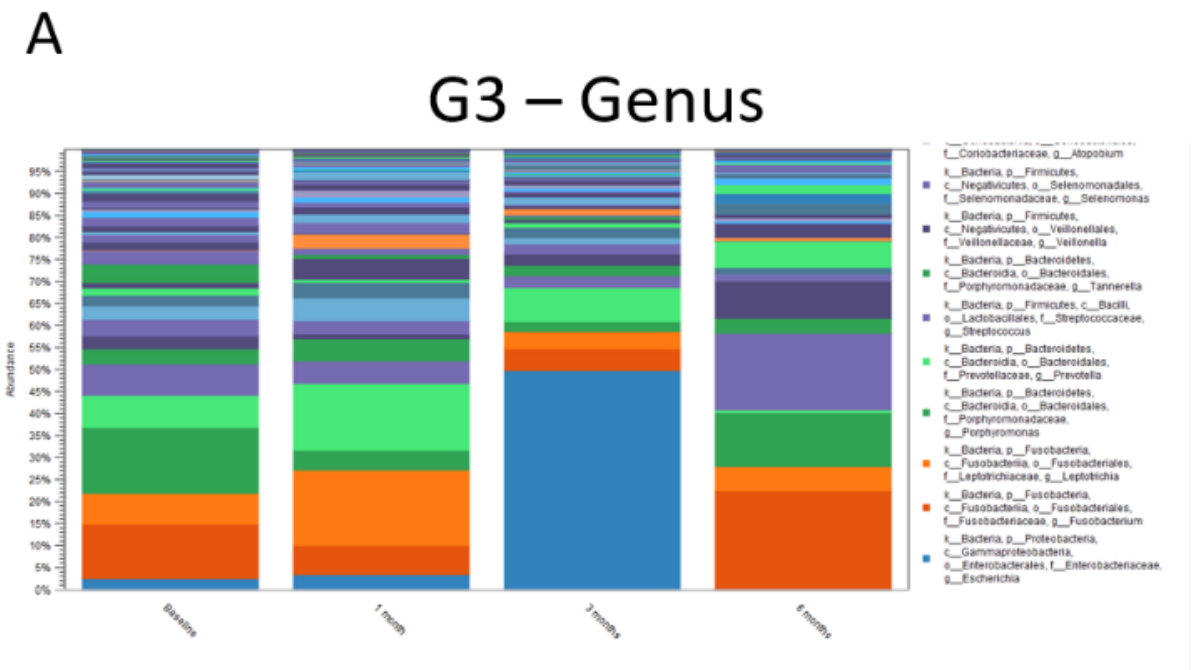


Figure 14. Amixicile reduces the abundance of anaerobic bacteria within the subgingival microbiome, site-specific analysis – Site G3.

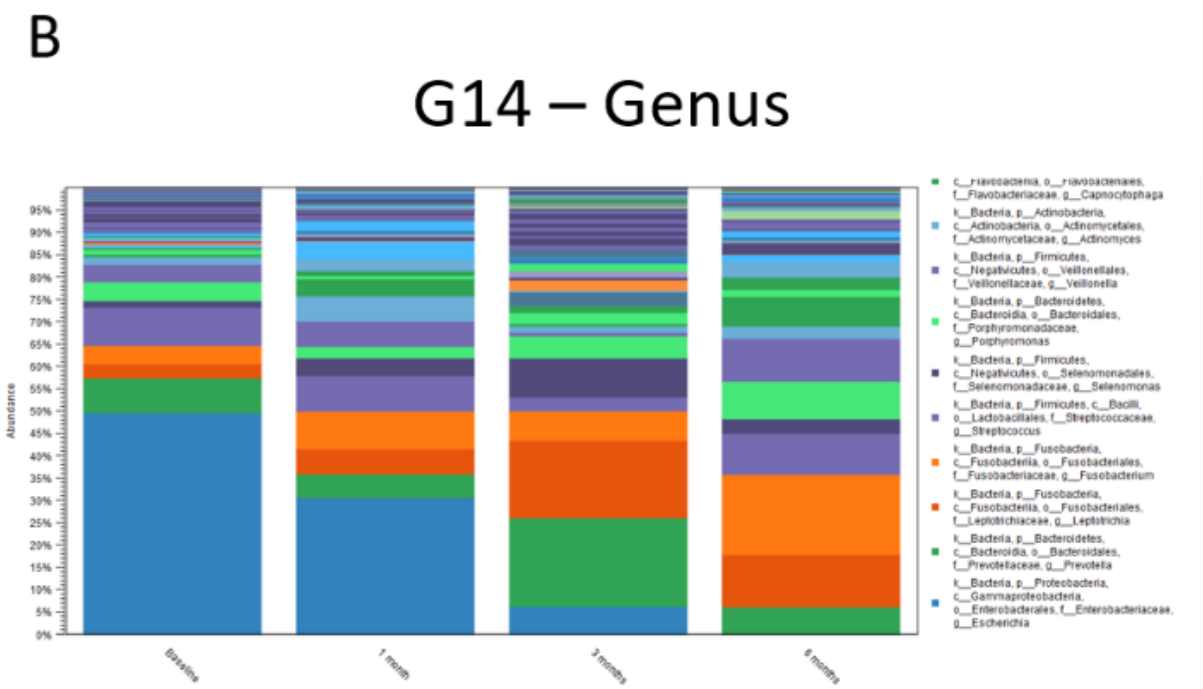


Figure 15. Amixicile reduces the abundance of anaerobic bacteria within the subgingival microbiome, site-specific analysis – Site G14.

C

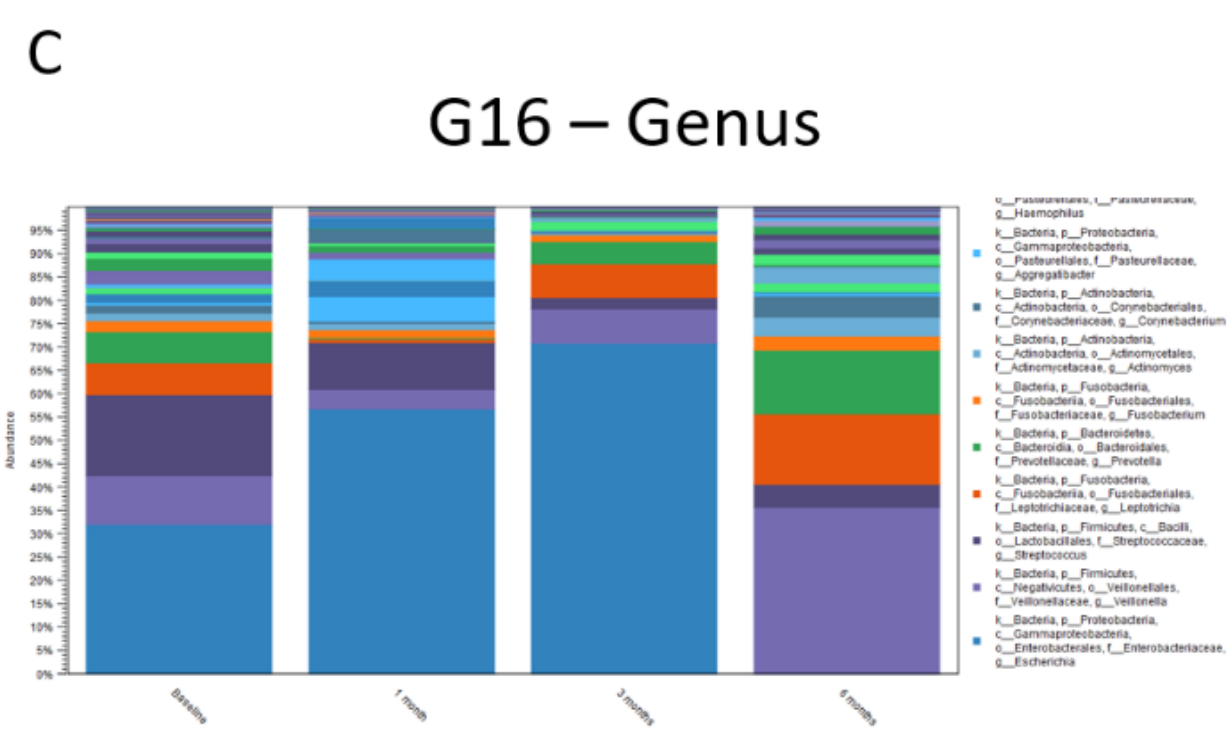


Figure 16. Amixicile reduces the abundance of anaerobic bacteria within the subgingival microbiome, site-specific analysis – Site G16.

D

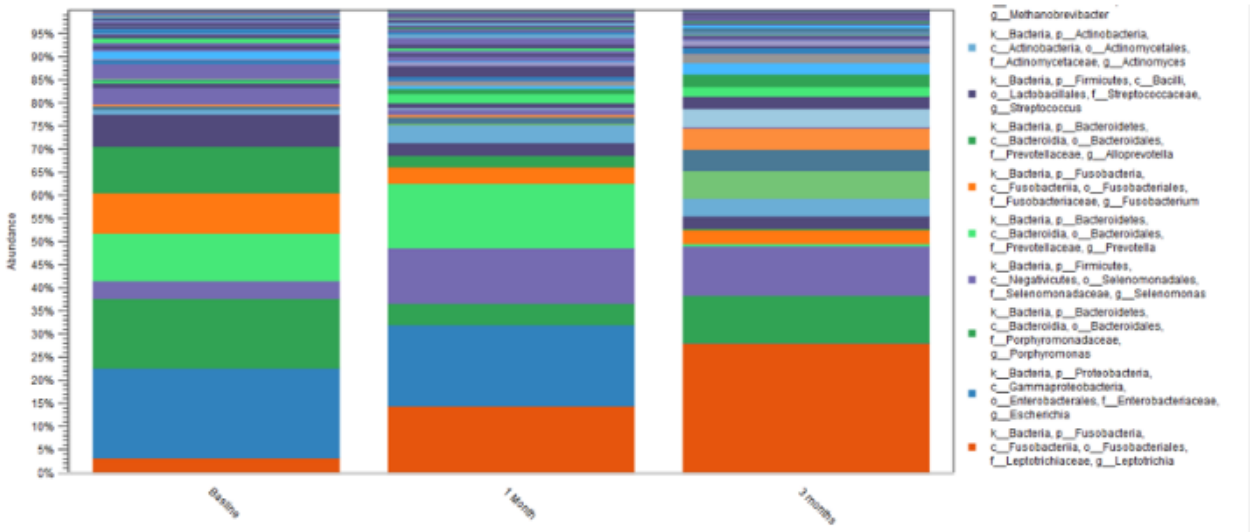


Figure 17. Amixicile reduces the abundance of anaerobic bacteria within the subgingival microbiome, site-specific analysis – Site G22.

Analysis of the subgingival microbiomes of Animal T, both at baseline and post-treatment has shown a similar trend in that levels of anaerobic bacteria were reduced in favor of the aerobic ones (Fig. 18-21). Specifically, the abundant genera of T3 (Animal T, tooth #3) were *Escherichia, Streptococcus, Porphyromonas,* and *Prevotella.* Following amixicile treatment the abundance of *Prevotella* was significantly reduced. Increased levels of *Escherichia, Streptococcus, Aggregatibacter,* and *Peptidiphaga* were observed (Fig. 18). At the second site of the Animal T, T14, *Streptococcus, Porphyromonas,* and *Fusobacterium* were the dominant bacterial genera. After amixicile treatment, the levels of *Porphyromonas, Fusobacterium,* and *Streptococcus* were significantly reduced. That corresponded to an increase in levels of *Escherichia, Aggregatibacter,* and *Peptidiphaga* (Fig. 19). The T15 site at baseline was abundant in *Escherichia, Streptococcus, Porphyromonas,* and *Haemophilus.* Following amixicile treatment the levels of *Porphyromonas, Streptococcus, Haemophilus,* and *Fusobacterium* were significantly reduced with an increase in the levels of *Leptotrichia* and *Bacteroides* (Fig. 20). The final site analyzed for microbiome composition, T16, had high levels of *Escherichia* at baseline that were reduced with a concomitant increase in levels of *Streptococcus* (Fig. 21). Thus, similar to Animal G, we observed high variability between the microbial composition of samples derived from different sites. However, the common theme from all the treated sites was a reduction in the abundance of anaerobic bacteria and an increase in levels of aerotolerant ones. In conclusion, amixicile effectively reduced levels of subgingival anaerobic bacteria in the treated samples while sparing the aerotolerant ones.

A

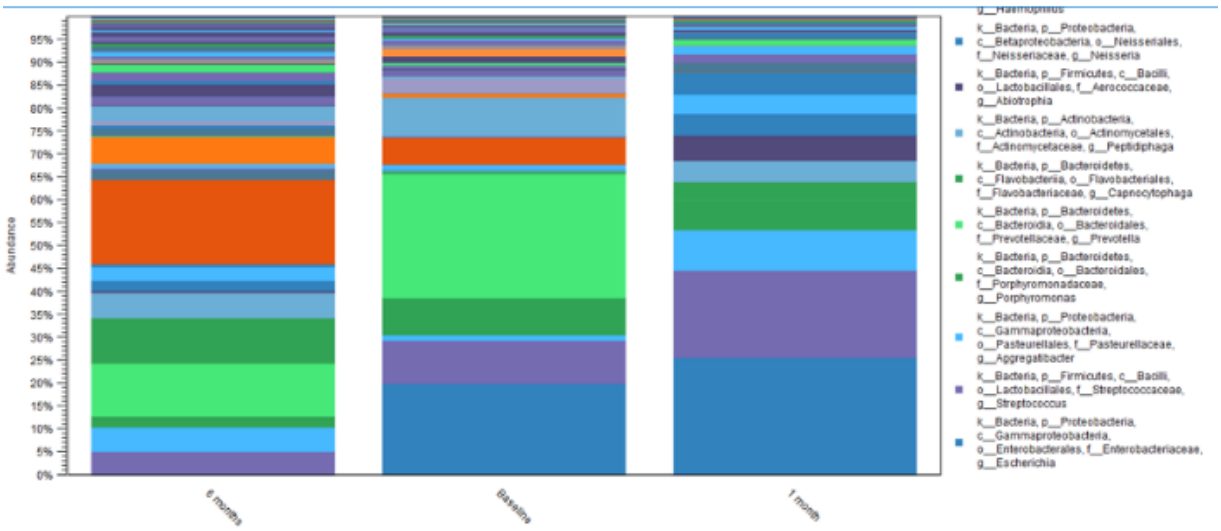


Figure 18. Amixicile reduces the abundance of anaerobic bacteria within the subgingival microbiome, site-specific analysis – Site T3.

B

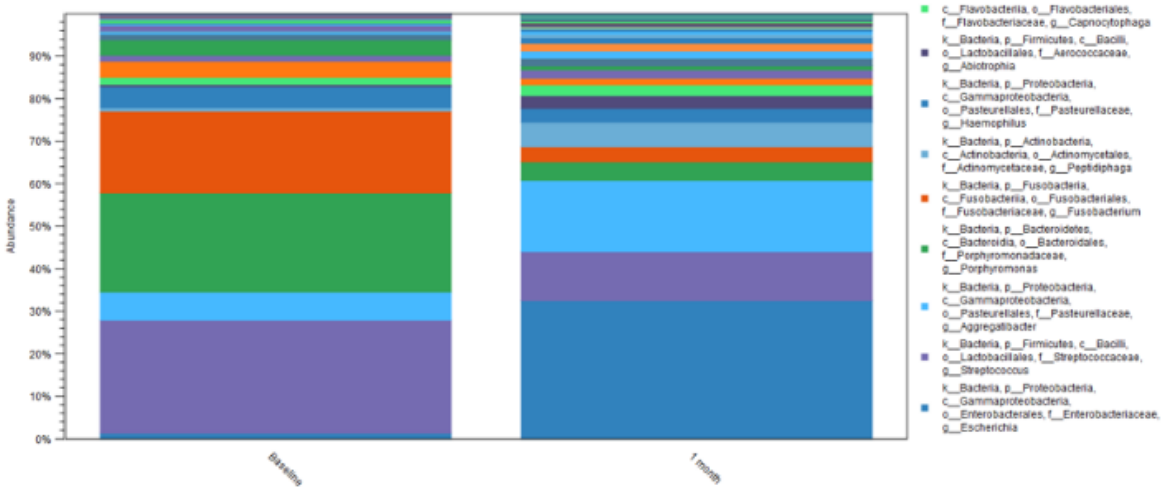


Figure 19. Amixicile reduces the abundance of anaerobic bacteria within the subgingival microbiome, site-specific analysis – Site T14.

C

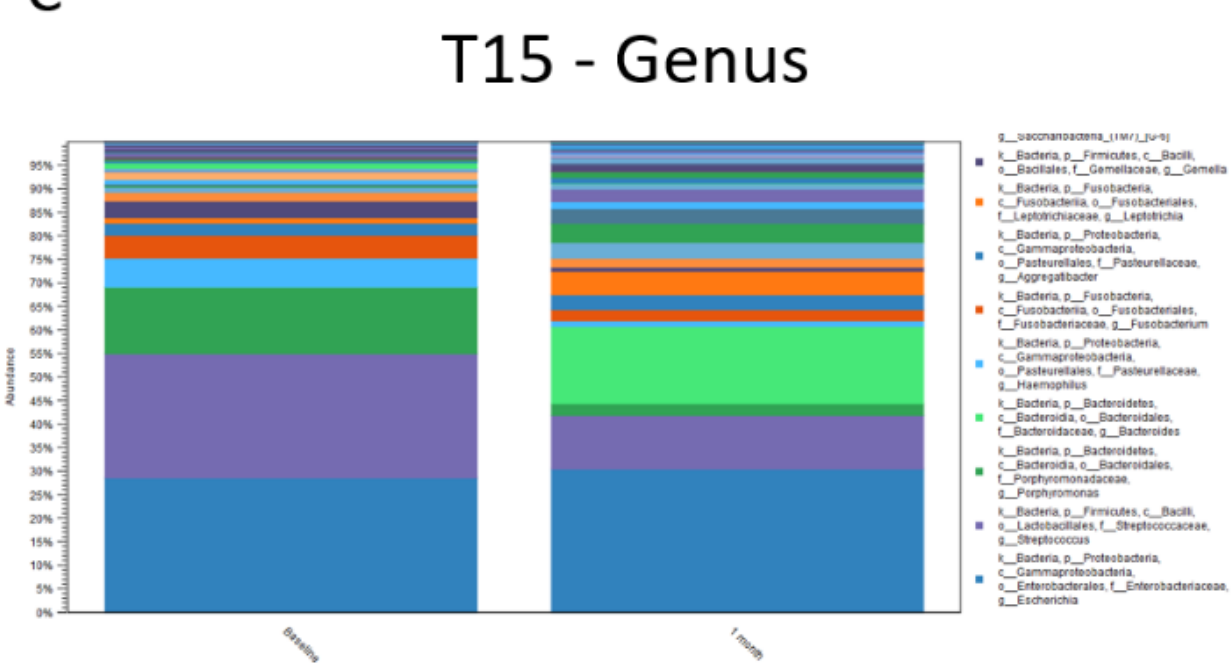


Figure 20. Amixicile reduces the abundance of anaerobic bacteria within the subgingival microbiome, site-specific analysis – Site T15.

D

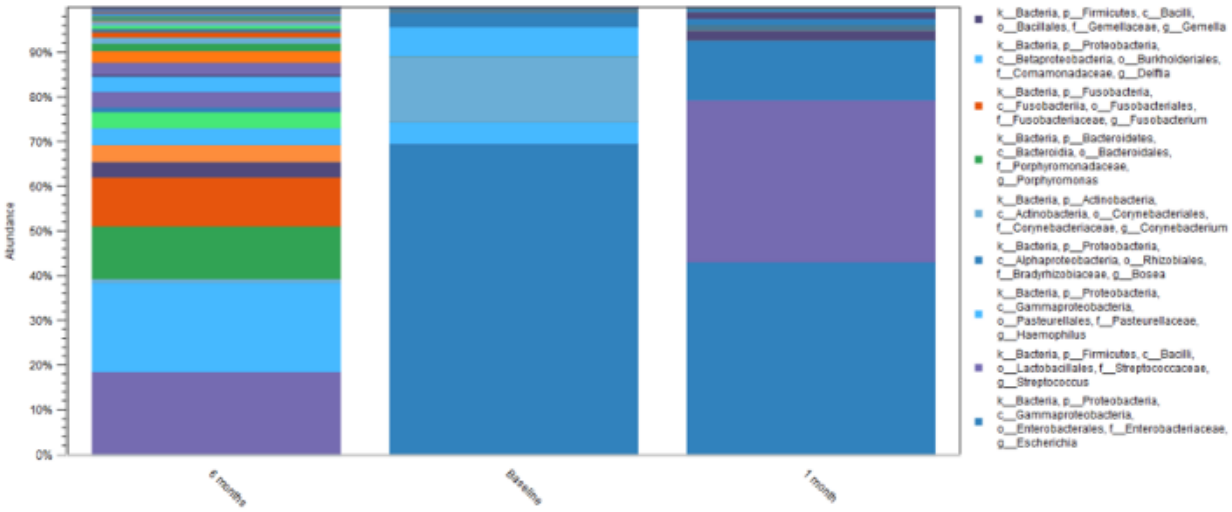


Figure 21. Amixicile reduces the abundance of anaerobic bacteria within the subgingival microbiome, site-specific analysis – Site T16.

The effect of amixicile on the oral microbiome is reversed after several months post-treatment. We followed the animals' microbiome changes up to six months post-treatment. Between 1- and 6- months post-treatment the salivary microbiome of Animal G had an increase in the abundance in *Porphyromonas, Fusobacterium,* and *Leptotrichia* while reduction in *Haemophilus, Streptococcus,* and *Gemella* was observed. The composition resembled the baseline microbiome, however, higher levels of *Leptotrichia* were seen. In Animal T, the salivary microbiome had higher levels of *Haemophilus* and *Fusobacterium* at longer post-treatment intervals. In the subgingival microbiome, an increase in the proportion of anaerobic bacteria was also observed after longer timeframes.

Correlation between microbiome and clinical characteristics. Significant clinical improvement as determined by PD, BOP, and GI was observed at three months post-treatment and continued into the sixth month. The clinical improvements were delayed with respect to the onset of the reduction of anaerobic bacteria in the microbiome. At six months, despite some reversal of the abundance of anaerobic bacteria to near baseline levels the clinical improvement persisted.

Discussion

The work presented here shows that the use of a 14-day treatment of amixicile at 40mg/kg/day is effective in reducing the symptoms of periodontal disease as manifested by probing depths (PD) and bleeding on probing (BOP). While the average PD remained relatively steady, all sites measuring 4mm (mild periodontitis in an NHP model) were reduced to 3mm or less by 3-months post-treatment, and this reduction was maintained through the endpoint of the study at 6 months. Similarly, reduction in gingival index (GI) was more notable at specific sites (Photo 1, A-C), which were not adequately captured in the average scores. Interestingly, bleeding on probing (BOP) increased immediately following the 14-day course of amixicile but then decreased dramatically at 3- and 6-months follow-up. This does not readily correlate to the GI scores given each animal at the designated timeframes. A striking observation is that while all of these clinical indices were either decreasing or remaining stable the sites with plaque were steadily and dramatically increasing (Fig. 6C). A possible explanation may be that although there is an increase in the number of microbes, the balance could be shifted to a plaque rich in commensal, aerobic bacteria due to amixicile's selective targeting of the anaerobic periodontal pathogens. Without microbes belonging to the more virulent periodontal complexes described above there may be no inflammatory response by the host and thus, no periodontal inflammation.

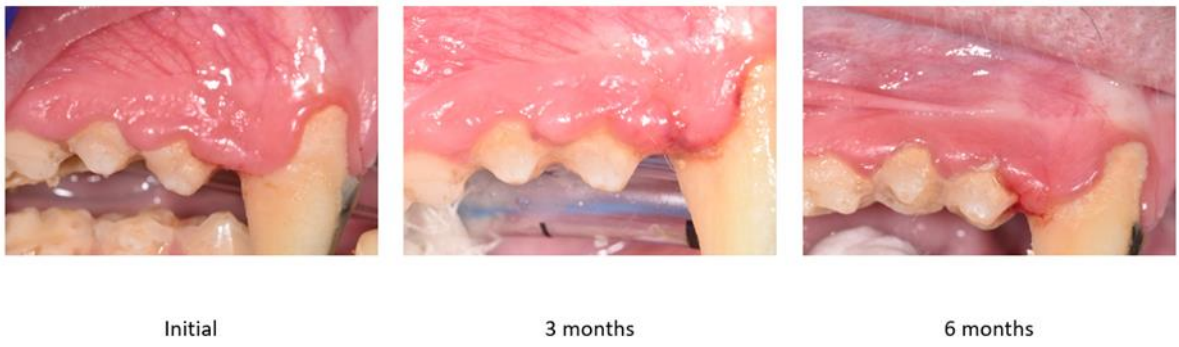


Photo 1, A-C. Clinical photos of Animal G at the Initial exam (A), 3-months post-treatment (B), and 6-months post-treatment (C). Note the marked reduction of edema of interproximal papillae.

It is noteworthy that we also see the conversion of the composition of the salivary and sulcular microbiomes from one prevalent in anaerobic bacteria to one with reduced levels of anaerobes and increased proportions of aerotolerant microorganisms (Fig. 10-21). In the saliva samples a reduction in *Porphyromonas, Fusobacterium,* and *Alloprevotella*, all anaerobes, was seen with a concomitant increase in *Streptococcus, Haemophilus, Gemella*, and *Escherichia*, all aerobes, was observed. Subgingival plaque samples showed similar alterations in microbial composition. Reduction of *Porphyromonas, Fusobacterium, Prevotella, Veillonella,* and *Alloprevotella*, all anaerobes, was observed with concomitant increase of known aerobes. These changes generally take place immediately post-treatment but return to baseline levels by 6-months.

There was a great degree of variability in microbial composition between individual sites which would be expected but makes comparison difficult. Microbial composition likely varies based on the depth of sulcus as well as environmental factors such as chewing function and self-cleansability. The lack of diversity of microflora in

Animal T, site #16 at baseline is noteworthy (Fig. 21). This site had very little biofilm and was periodontally healthy at the initial exam. With such a small amount of biomass the DNA had to amplified many times to yield any data. This means that only microbes present in high abundance would be represented in the data and that it is uncertain which others may be present at low abundance. As the biofilm accumulated a more diverse flora is noted at the 6-month exam.

When all the above data is taken together it seems that the microbiological effects of amixicile precede the clinical benefits. Although the microbiome tends to rebound between 3- and 6-months the clinical improvements persisted through the 6-month exam.

Limitations to our study include a small sample size of only two non-human primates as the expense of acquiring and maintaining these animals is very great. Given that only two subjects were available for study both animals received the treatment so there was no control in our study. The animals also only exhibited mild periodontitis at worst in a small number of sites. Another limitation is that we were unable to measure the concentration of amixicile or any inflammatory markers in the GCF or serum so while the microbiological data would support its action it can't be determined how well it was able to localize to the gingival sulcus. In our study V3-V4 primers were used which allow for broader phylogenetic coverage that V1-V2 primers. This is both an advantage and a disadvantage as these primers target 16S rDNA, but different strains of microbes cannot be distinguished using this method.

The microbiological findings support the hypothesis that amixicile reduces the proportion of anaerobic microorganisms in the oral cavity, many of which are

periodontal pathogens. These findings coupled with a reduction of clinical periodontal inflammation suggest that further study of amixicile for the treatment of periodontitis is warranted. Future studies should have a larger sample size and should include a negative control. Ideally, subjects would have more severe forms of periodontitis, and other antibiotics, possibly broad-spectrum, could be used for comparison or potentially in conjunction with amixicile.

The clinical implications of this study show great promise in amixicile as a novel antimicrobial for the treatment of periodontal disease. If amixicile continues to be found successful at selectively targeting periodontal pathogens with little to no side effects it may not only replace current antimicrobial options, such as the combination of Amoxicillin and Metronidazole, but it may make its use more routine for the treatment of periodontitis. In our study a 14-day course caused a significant reduction in pathogenic bacteria with a rebound of the microbes occurring between 3-6 months. With no side effects it may be possible to prescribe a 14-day course of amixicile once every 3-6 months as needed for patients with persistent periodontal inflammation.

Conclusion

In conclusion, amixicile seems to be a strong candidate as a viable antimicrobial option for the treatment of periodontal disease. It selectively inhibits known anaerobic periodontal pathogens including *Porphyromonas spp., Fusobacterium spp.,* and *Prevotella spp*. causing a reduction in clinical periodontal symptoms for a period of up to six months. Further research at a larger scale is needed to bring the drug closer to the possibility of a clinical trial in humans.

References

1. Eke PI, Dye BA, Wei L, et al. Update on Prevalence of Periodontitis in Adults in the United States: NHANES 2009 to 2012. *J Periodontol*. 2015;86(5):611–622.
2. Bidault P, Chandad F, Grenier D. Systemic antibiotic therapy in the treatment of periodontitis. *J Can Dent Assoc*. 2007;73(6):515–520.
3. Mombelli A, Walter C. Antibiotikarichtlinien Parodontologie. *Swiss Dent J*. 2019;129(10):835–838.
4. Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. *Clin Oral Investig*. 2003;7(4):181–188.
5. Loe H, Theilade E, Jense SB. Experimental Gingivitis in Man. *J Periodontol*. 1965;36:177–187.
6. Holt SC, Ebersole JL. Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: The “red complex”, a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000*. 2005;38:72–122.
7. Loesche WJ, Giordano JR, Soehren S, Kaciroti N. The nonsurgical treatment of patients with periodontal disease: results after 6.4 years. *Gen Dent*. 2005;53(4):298—306.
8. Soares GMS, Figueiredo LC, Faveri M, Cortelli SC, Duarte PM, Feres M. Mechanisms of action of systemic antibiotics used in periodontal treatment and mechanisms of bacterial resistance to these drugs. *J Appl Oral Sci*. 2012;20(3):295–305.
9. Zandbergen D, Slot DE, Niederman R, Van der Weijden FA. The concomitant administration of systemic amoxicillin and metronidazole compared to scaling and root planing alone in treating periodontitis: a systematic review. *BMC Oral Health*. 2016;16(1):1–11.
10. Bersani C, Berna M, Pasut G, Veronese FM. PEG-metronidazole conjugates: Synthesis, in vitro and in vivo properties. *Farmaco*. 2005;60(9):783–788.
11. Warren CA, Van Opstal E, Ballard TE, et al. Amixicile, a novel inhibitor of pyruvate: ferredoxin oxidoreductase, shows efficacy against Clostridium difficile in a mouse infection model. *Antimicrob Agents Chemother*. 2012;56(8):4103–4111.
12. Hoffman PS, Bruce AM, Olekhnovich I, et al. Preclinical studies of amixicile, a systemic therapeutic developed for treatment of Clostridium difficile infections that also shows efficacy against Helicobacter pylori. *Antimicrob Agents Chemother*. 2014;58(8):4703–4712.
13. Subbarao K, Nattuthurai G, Sundararajan S, Sujith I, Joseph J, Syedshah Y. Gingival Crevicular Fluid: An Overview. *J Pharm Bioallied Sci*. 2019;11:135–139.

14. Hutcherson JA, Sinclair KM, Belvin BR, Gui Q, Hoffman PS, Lewis JP. Amixicile, a novel strategy for targeting oral anaerobic pathogens. *Sci Rep*. 2017;7(1):1–14.

15. Lindhe J, Liljenberg B, Listgarten M. Some Microbiological and Histopathological Features of Periodontal Disease in Man. *J Periodontol*. 1980;51(5):264–269.

16. Reed LA, O'bier NS, Oliver LD, Hoffman PS, Marconi RT. Antimicrobial activity of amixicile against treponema denticola and other oral spirochetes associated with periodontal disease. *J Periodontol*. 2018;89(12):1467–1474.

17. Gui Q, Hoffman PS, Lewis JP. Amixicile targets anaerobic bacteria within the oral microbiome. *J Oral Biosci*. 2019;61(4):226–235.

18. Gui Q, Ramsey K, Hoffman PS, Lewis JP. Amixicile depletes the ex vivo periodontal microbiome of anaerobic bacteria. *J Oral Biosci*. 2020;4:1–10.

19. Oz HS, Puleo DA. Animal models for periodontal disease. *J Biomed Biotechnol*. 2011;754857. doi:10.1155/2011/754857.

20. Schou S, Holmstrup P, Kornman KS. Non-Human Primates Used in Studies of Periodontal Disease Pathogenesis: A Review of the Literature. *J Periodontol*. 1993;64(6):497–508.

21. Eke PI, Braswell L, Arnold R, Fritz M. Sub-gingival microflora in Macaca mulatta species of rhesus monkey. *J Periodontal Res*. 1993;28(1):72–80.

22. Colombo AP V., Paster BJ, Grimaldi G, et al. Clinical and microbiological parameters of naturally occurring periodontitis in the non-human primate Macaca mulatta. *J Oral Microbiol*. 2017;9(1):1403843. doi:10.1080/20002297.2017.1403843

23. Lang N, Adler R, Joss A, Nyman S. Absence of bleeding on probing: An indicator of periodontal stability. *J Clin Periodontol*. 1990;17:714–721.

24. Greenstein G, Caton J, Polson AM. Histologic Characteristics Associated With Bleeding After Probing and Visual Signs of Inflammation. *J Periodontol*. 1981;52(8):420–425.

25. Greenstein G. The Role of Bleeding upon Probing in the Diagnosis of Periodontal Disease: A Literature Review. *J Periodontol*. 1984;55(12):684–688.

26. Löe H. The Gingival Index, the Plaque Index and the Retention Index Systems. *J Periodontol*. 1967;38(6 Part II):610–616.