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Amixicile, a novel antimicrobial agent, and its effect on the salivary and subgingival microbiome in rhesus macaque monkeys

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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Acknowledgements

The author wishes to thank several people. Special thank you to family and friends for their endless love and constant support. Thank you to Drs. Janina Lewis, Janina Golob Deeb, Caroline Carrico, Thomas Waldrop, and B. Ross Belvin for their aid in designing the project, providing the resources and laboratory assistance, and being there every step of the way. This project was truly a team effort with both laboratory and veterinary support from Stacie Havens, Dr. Denver Lyons, Ellen Soehngen, and Dr. Katherine Nicholson.

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Abstract

AMIXICILE, A NOVEL ANTIMICROBIAL AGENT, AND ITS EFFECT ON THE SALIVARY AND SUBGINGIVAL MICROBIOME IN RHESUS MACAQUE MONKEYS

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Background/Purpose: Periodontitis is an inflammatory disease with a bacterial etiology in a susceptible host. A selective antibiotic with minimal systemic side effects could be a useful adjunct to traditional periodontal therapy. Amixicile is a novel antimicrobial agent that targets pyruvate: ferredoxin oxidoreductase (PFOR), an enzyme that is critical for anaerobic bacterial metabolism and has been found to have little-to-no side-effects in animal models. The aim of this study was to prospectively evaluate the effect of amixicile on clinical periodontal parameters and the composition of the oral microbiome in rhesus macaque monkeys. It was hypothesized that the amixicile would reduce growth of anaerobic bacteria and thus shift the microbiome from one of disease-promoting bacteria to that of predominately health-promoting, aerobic bacteria. By doing so, it was hypothesized that following amixicile therapy there would be a

decrease in clinical parameters of periodontal disease activity (i.e. bleeding on probing, progressive change in probing depth and attachment loss).

Methods: A total of six non-human primates of the *Macaca mulatta* species were studied. Three animals were treated with a two-week course of systemic administration of amixicile and the remaining three served as controls. Periodontal examinations were performed at baseline, immediately post-treatment, and 1-, 3-, and 6-months post-treatment. Periodontal charting, including probing depths, bleeding sites, plaque sites, calculus sites, and gingival index, was recorded at each visit. Saliva, plaque, and gingival crevicular fluid at specified sites were collected as well.

Results/Conclusion: There was no statistically significant differences amongst the clinical parameters evaluated when comparing control versus experimental animals. Microbiologically, there seemed to be a trend in which amixicile-treated animals demonstrated a dramatic reduction in anaerobic, pathogenic bacteria while having an increase or no change in aerobic bacteria. Comparatively, in the control animals an increase or at times slight decrease in anaerobic bacteria was seen while results varied for the aerobic bacteria. This demonstrates amixicile's ability to shift the oral microbiome from one associated with periodontal pathogenic bacteria to one associated with periodontal health.

Introduction

Periodontitis is an inflammatory disease with a bacterial etiology in a susceptible host[1]. The focus of periodontal therapy is antimicrobial, being traditionally achieved with mechanical therapy (i.e. scaling and root planing) and/or adjunctive antibiotic therapy. Adjunctive antibiotic therapy is regularly used in the treatment and management of both Molar/Incisor Pattern or Generalized Stage 3 Grade C Periodontitis (previously known as aggressive periodontitis) and Localized or Generalized Stage 3 Grade B Periodontitis (previously known as severe chronic periodontitis)[2]. Those antibiotics that have been studied and demonstrated as effective for periodontal therapy are broad spectrum, meaning that they target both “health-promoting” aerobic bacteria and “disease-causing” anaerobic bacteria. This leaves the treated oral environment susceptible for re-infection by periodontal pathogens, as they do not have to compete with resident, healthy microflora. Examples of antibiotics used to treat periodontitis include amoxicillin, tetracycline, metronidazole, azithromycin, and clindamycin[3]. It is well studied and established that the oral microflora in health is dominated by aerobic species whereas in a periodontal-diseased state the oral microflora has a significant shift to microorganisms that are both pathogenic and anaerobic[4]. Thus, anaerobic microorganisms are known to be the major drivers of periodontal disease[5, 6].

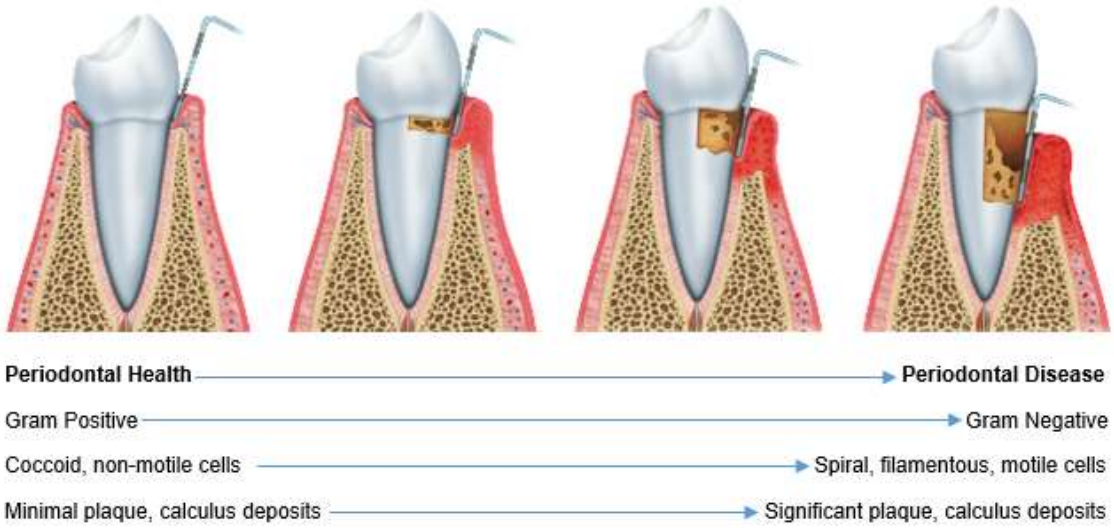

Figure 1. The shift in oral microbiome from periodontal health through periodontal disease

Therefore, with the use of the aforementioned broad spectrum antibiotics, there would be an elimination of healthy, aerobic bacteria in addition to the pathogenic, anaerobic bacteria. Metronidazole is a semi-selective therapeutic which targets anaerobic species specifically, thus making it useful in the management of periodontitis. Studies on this antibiotic have shown promising results clinically; those who received adjunctive metronidazole therapy have had a reduced need for periodontal surgical intervention[7, 8]. However, it can cause numerous adverse effects most notably severe gastrointestinal disruption leading at times to colitis if used repeatedly making its use limited for periodontal therapy[9]. Thus, there is a great need for a novel therapeutic with the ability to target periodontal pathogens specifically and have minimal to no systemic adverse effects.

The Hoffman laboratory at the University of Virginia, Department of Medicine, has recently developed a targeted antimicrobial agent, called amixicile[10]. Amixicile is a

derivative of nitazoxanide, the difference being the replacement of a 2-acetaoxy group with an aliphatic amine thus making the drug more soluble as well as avoiding glucuronidation in the liver (Figure 1).

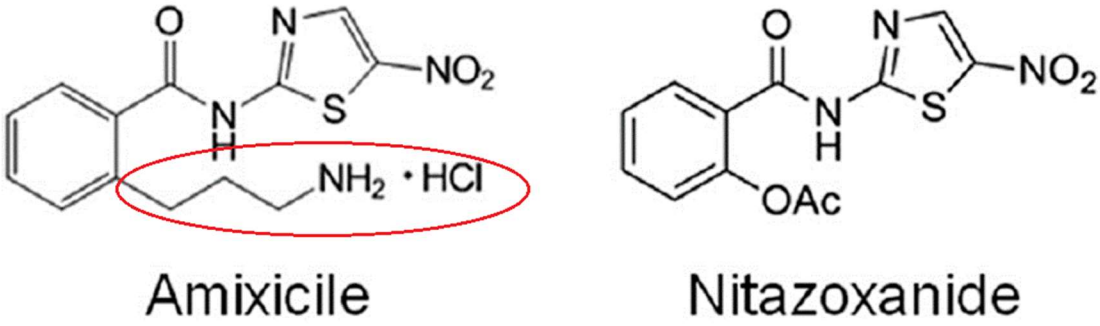

Figure 2. Chemical structures of amixicile and its precursor, nitazoxanide

Amixicile acts via targeting pyruvate:ferredoxin oxidoreductase (PFOR), a major metabolic enzyme involved in the generation of energy through oxidative decarboxylation of pyruvate and coenzyme A (CoA) to carbon dioxide and acetyl-CoA (Figure 2). PFOR functions as an important component of various metabolic pathways in anaerobic bacteria, specifically periodontal pathogenic bacteria, and is the target of amixicile. Small molecules of amixicile are able to inhibit PFOR activity by outcompeting pyruvate for binding to thiamine pyrophosphate (TPP), which is a cofactor of PFOR in the active site. Thus, this novel therapeutic only affects anaerobic pathogens, not aerobic pathogens or eukaryotic cells. Conversely, aerobic bacteria rely on pyruvate dehydrogenase (PDH) for metabolism and, therefore, are not affected by the inhibitory effects of amixicile.

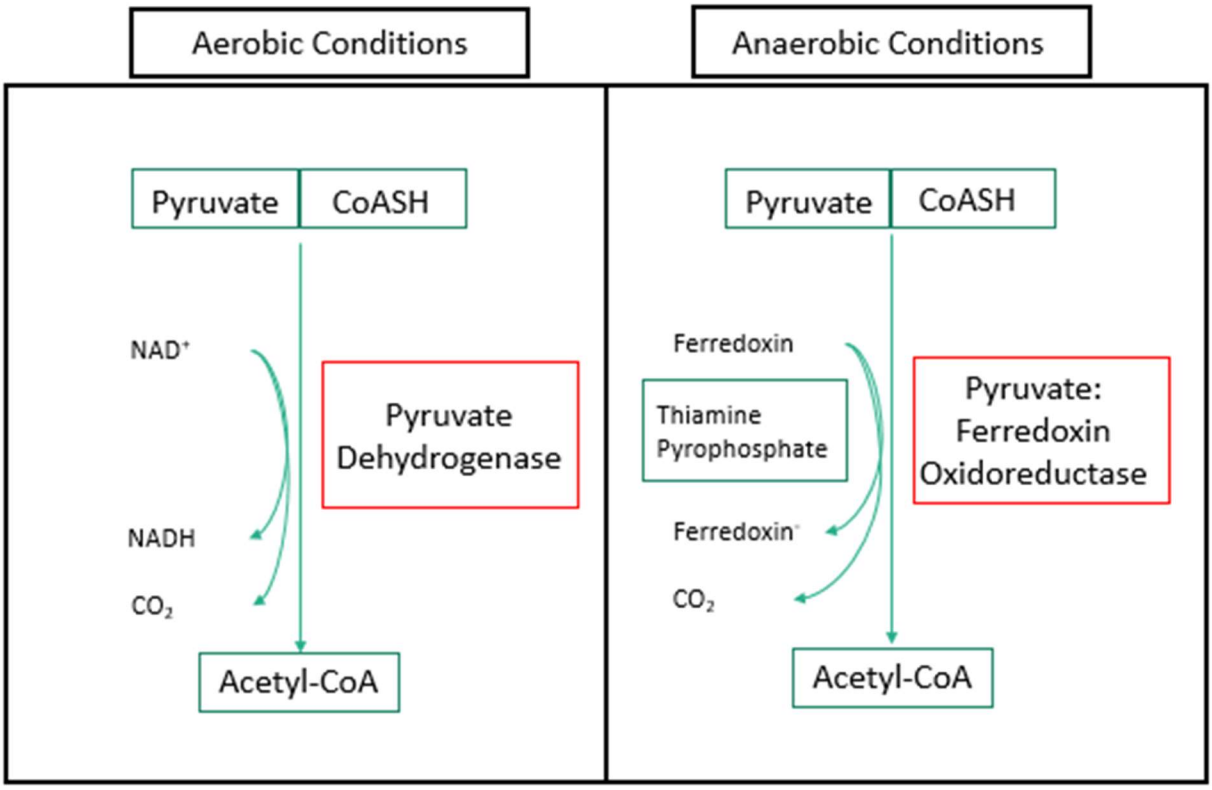

Figure 3. Pyruvate Dehydrogenase (PDH) versus Pyruvate:Ferredoxin Oxidoreductase (PFOR) metabolism. In aerobic conditions, Pyruvate and Co-enzyme A are metabolized via PDH to Acetyl-CoA. Conversely in anaerobic conditions, Pyruvate and Co-enzyme A are metabolized via PFOR to Acetyl-CoA.

PFOR Metabolism	PDH Metabolism
P. gingivalis	*S. gordonii*
P. intermedia	*A. actinomycetemcomitans*
F. nucleatum	*Streptococcus spp.*
T. denticola	*Klebsiella spp.*
T. forsythia	*Neisseria spp.*
Porphyromonas spp.	*Lactobacillus spp.*
Veillonella spp.	*Haemophilus spp.*
Prevotella spp.	*Gemella spp.*
Alloprevotella spp.	*Escherichia spp.*
Fusobacterium spp.	*Leptotrichia spp.*

Table 1. Examples of periodontal microbes according to their metabolism- PDH or PFOR

This newly developed antimicrobial agent has shown promising results thus far in research in regards to its utility in the management of periodontal disease and infection. It has been shown to be effective against periodontal-specific pathogens[11, 12, 13, 14, 15], and in previous research has been shown to have no adverse effects in mice[10] or on macaque monkeys in the pilot study completed at Virginia Commonwealth University, School of Dentistry, Department of Periodontics and the Philips Institute[16]. Also, the latter has shown reduction of the abundance of periodontal pathogens as well as a reduction in clinical symptoms of periodontal disease in treated animals, thus setting the stage for more extensive investigation.

In choosing an appropriate model for the study, the anatomical, clinical, and microbiological features of the subject should ideally resemble features of a human as closely as possible. The nonhuman primate (NHP), more specifically *Macaca mulatta (M. mulatta)* in this study, was found to be superior to other animal subjects such as canines, rodents, or porcine models[17]. The oral structure, dental anatomy, as well as the formation of dental plaque and calculus of the NHP is similar to that of humans. Periodontitis clinical progression in the NHP model has, also, been observed to be similar to that of humans. However, a major drawback of the NHP was the expense of acquiring and maintain the subjects as well as the animal husbandry issues, such as mental stimulation and socialization[17]. A review of NHP species found the *M. mulatta* to have many similarities to the human anatomy and periodontal disease progression with the exception of some differences. Some of the histologic similarities between the two groups when evaluating tissues in a diseased state includes the presence of widened intercellular spaces, an increase in inflammatory cells within connective tissue, an

increase in polymorphonuclear leukocytes, the destruction of collagen, formation of periodontal pockets, and ultimately resorption of alveolar bone. The main difference noted between the two groups was a higher proportion of *Actinomyces spp.* in the NHP in both diseased and healthy sites. In *M. mulatta* species specifically, an increase in motile rods and spirochetes has been noted microbiologically with increased inflammation[18]. The proportion of anaerobes as well as the overall bacterial counts continue to increase during established gingivitis lesions. In periodontitis lesions that were experimentally induced, an increase in both *P. gingivalis* and *P. intermedia* was observed[18]. As sequencing technology advanced and improved, the microflora of *M. mulatta* was more thoroughly evaluated and findings were then correlated to clinical parameters. It was found that 56% of bacteria were identical to or had closely related human counterparts. Forty-eight species were unique to the NHP but these also had clear and closely related human counterparts. The microbes that were found to be associated with health were *Streptococcus spp., Lactobacillus spp.,* and *Gemella spp*. Periodontal pathogens associated with disease were found to be *P. gingivalis, T. forsythia, Filifactor alocis, P. micra, Treponema spp., Fusobacterium spp.,* and *A. actinomycetemcomitans*[19]. Specifically in the NHP species *M. mulatta,* mild periodontitis is defined as a 4mm probing depth (PD) along with clinical inflammation and bleeding on probing (BOP). Moderate to severe periodontitis is defined as a PD of 5mm or greater with inflammation and BOP. As in humans, periodontitis in the *M. mulatta* includes clinical parameters such as increased probing depths and bone loss[20].

The aim of this study was to expand upon the referenced *in-vitro* research studies[10, 11, 14, 15] and the pilot study at Virginia Commonwealth University, Department

of Periodontics and Philips Institute[16], in which there were no control subjects, by testing the effects of amixicile in *in-vivo* conditions in six rhesus macaque monkeys. Three macaque monkeys served as controls while three served as experimental subjects for the effectiveness of amixicile as a possible tool for the management of periodontal disease. It was hypothesized that the clinical indices would improve and microbiological composition would shift to that of a healthier oral microbiome following systemic administration of amixicile compared to the control subjects.

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). Four male and two female non-human primates *Macacca mulatta* were used for this study (Table 2). All six animals were housed at the VCU's animal facility in extra-large enclosures. Their diet consisted of kibble (Monkey Chow, Purina), fresh fruits and vegetables, as well as foraging for dried seeds, dried fruits, and nuts daily. Enrichment was provided to the animals through daily handling by veterinary technicians, visual contact with other animals, and other enrichment items (toys, videos, etc.). All animals enrolled in this study were systemically healthy. No changes were made to the animals' diet and no oral hygiene measures were performed during the study period.

	Name	Study Group	Sex	Age (years)	Average Weight (kg)
Pair 1	Animal C	Control	M	10	13.7
	Animal S	Experimental	M	12	10.2
Pair 2	Animal L	Control	F	12	5.7
	Animal E	Experimental	F	8	5.7
Pair 3	Animal K	Control	M	14	11.9
	Animal J	Experimental	M	14	13.0

Table 2. Demographics of Study Animals

Clinical Examination and Sample Collection

Prior to examination, the animals were anesthetized by a veterinarian by means of ketamine injection (10mg/kg) followed by intubation and subsequent administration of 2% isoflurane at 2 L/min and 100% oxygen at 1 L/min. A periodontal exam was performed by a trained periodontist and resident, respectively (JGB, EEB), to determine the baseline oral and periodontal status of the animals (pocket depth [PD], bleeding on probing [BOP], presence of plaque, presence of calculus, and gingival index [GI]). This comprehensive periodontal examination was completed at baseline as stated and then subsequently immediately post-amixicile treatment, 1-month post-treatment, 3-months post-treatment, and 6-months post-treatment.

Clinical photographs were taken at each examination for each animal. The clinical photographs were used to assign a modified GI score (REF) to each sextant. The scoring was based on the following classification[21]: 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 two independent examiners (JGD, EEB). Scores of each examiner were averaged to determine the GI of each sextant for the baseline, immediate post-treatment, 1-month post-treatment, 3 months post-treatment, and 6 months post-treatment exams.

Additionally, salivary samples, GCF samples, and plaque samples were collected by trained periodontal resident (EEB) assisted by Dr. Lewis at each exam. Following the

baseline exam, the animals were left without any intervention for two weeks to allow for stabilization of the oral microbiome. The experimental animals were then treated with 20mg/kg of body weight with amixicile compounded and mixed into an edible form given daily. The antibiotic therapy was carried out for 14 days. Salivary samples were collected using five sterile cotton swabs equally representing all areas of the mouth by swabbing the entire oral cavity, including sublingual and buccal spaces. The cotton swabs were then 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 following sites: for control animal C #1 mesio-buccal (M), #2M, #5M, #11 disto-buccal (D), #13D, #15D, #18M, #19M, #22D, #26D, #27 straight buccal (B), #31M; for experimental animal S #1M, #3M, #5D, #6D, #11D, #12D, #15B, #19D, #21M, #22B, #27B, #29D, #32B; for control animals L & K and experimental animals E & J #1M, #2M, #5M, #11D, #13D, #15D, #18M, #19M, #22D, #26D, #27B, #31M. For subgingival plaque collection, a Nevi 2 periodontal scaler (SCNEVI29E2, Hu-Friedy, Chicago, Illinois, USA) was inserted to the base of the sulci of interest 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 -80^{o}C. 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 -80^{o}C.

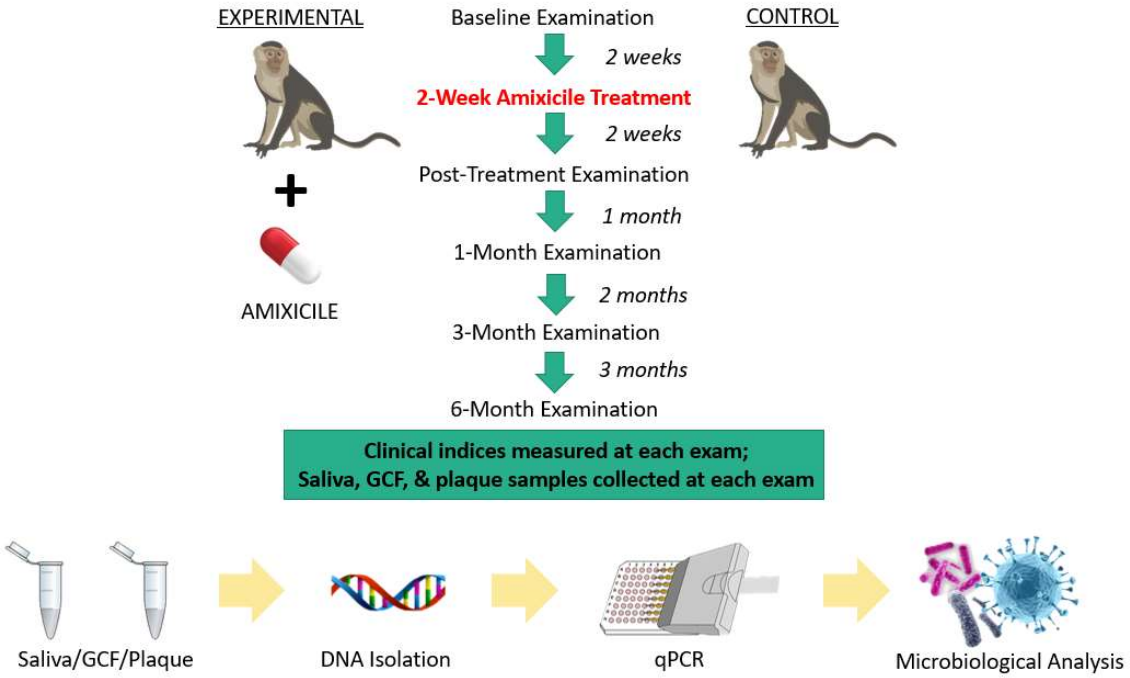

Figure 4. Experimental design demonstrating the time frame of the examinations and sequence of events from saliva/GCF/plaque sample collection through microbiological analysis. Baseline examination completed prior to amixicile therapy. All animals left for two weeks post-baseline examination to allow for stabilization of oral microbiome. Amixicile therapy administered to experimental animals after two week stabilization period. Post-treatment examination occurs immediately following completion of two week amixicile administration for experimental animals. One month examination occurs one month post-completion of amixicile and so on for three and six month examinations.

Microbiological Analysis

Both the salivary and subgingival plaque samples were analyzed to assess their microbial content. Collected plaque samples were suspended in 500 µl of RNA*later* buffer (Fisher Scientific International, Pittsburgh, Pennsylvania, USA) and stored at -80^{o}C. Each sample was vigorously vortexed for 5 minutes to break down larger plaque complexes for analysis. Plaque samples were processed individually while aliquots of salivary samples were pooled together before analysis.

DNA Isolation – Genomic DNA (gDNA) was isolated using 200 µl of the resuspended plaque mixture with the PureLink^{TM} Microbiome DNA purification kit (Fisher Scientific International, Pittsburgh, Pennsylvania, USA) according to the

instructions from the manufacturer. Similarly, 200 µl of pooled saliva was used for DNA isolation.

Quantitative PCR (qPCR) – Genus specific 16s primers and universal bacterial 16s primers used in this study are listed below (Table 3).

Primer	Sequence (5'-3')
Universal 16s-F	AGAGTTTGATCCTGGCTCAG
Universal 16s-R	GCTGCCTCCCGTAGGAGT
Prevotella 16s-F	CCAGCCAAGTAGCGTGCA
Prevotella 16s-R	TGGACCTTCCGTATTACCGC
Fusobacterium 16s–F	GATCCAGCAATTCTGTGTGC
Fusobacterium 16s–R	CGAATTTCACCTCTACACTTGT
Streptococcus 16s–F	GTACAGTTGCTTCAGGACGTATC
Streptococcus 16s–R	ACGTTCGATTTCATCACGTTG

Table 3. Genus specific 16s primers and universal bacterial 16s primers

All samples were run on a Quant Studio 3 real time qPCR thermal cycler (Fisher Scientific International, Pittsburgh, Pennsylvania, USA). For reactions, purified gDNA (1 µL) and 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 subsequently converted to absolute fold change values. All genus specific values were normalized for total bacterial load using universal 16s primers.

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 utilized in this 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°C for 10 min, 95°C for 30 sec, 55°C for 30 sec, and 72°C for 3 min was used for amplification. Amplification was verified as being sufficient by using the recommended final fluorescence (that was higher than the threshold fluorescence). Following cooling at 4°C, the samples were transferred to a collection plate and (PCR primers, dNTPs) were degraded with the enzymatic cleanup solution. Lastly, 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°C for 10 min, 95°C for 30 sec, 55°C for 30 sec, and 72°C for 3 min. Amplification during barcode addition was verified as being sufficient through amplification curve examination. The library was 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).

Statistical Methods

Periodontal health measures were averaged across the sites for each monkey at each visit. Repeated measures ANOVA models were used to determine if there were differences in average periodontal health values based on the visits, group, and if the change across visits was dependent on the treatment group. Repeated measures analysis was utilized to adjust for the repeated measures on the monkeys across visits. For GI Scores, the scores from two calibrated raters were averaged. Agreement between raters was assessed using Kappa statistic. Significance level was set at 0.05 and Tukey's HSD adjustment was used to account for multiple comparisons for post hoc tests. All analyses were performed in SAS EG v.8.2 (SAS Institute, Cary, NC).

Results

Clinical Data

A total of 6 monkeys were evaluated at 5 different time points. The average periodontal health measures are presented in Table 4. Figures 4-7 display the trend for each monkey across time for each of the four periodontal health measures. Three of the monkeys were treated with the experimental treatment (S, E, J) and the remaining three were controls (C, L, K).

Table 4. Average Periodontal Health Measures by Visit for All Animals

Periodontal Health Measure	Baseline	2 Week	1 Month	3 Months	6 Months
Average Probing Depth	2.3, 0.12	2.2, 0.13	2.3, 0.11	2.3, 0.08	2.2, 0.12
Average Bleeding Sites	56.2, 6.49	38.0 14.91	39.0, 15.63	40.5, 15.07	32, 10.28
Average Calculus Sites	75.7, 7.00	66.0, 5.29	63.8, 11.13	63.5, 13.26	47.8, 10.8
Average Plaque Sites	85.3, 6.12	88.7, 2.42	88.7, 3.33	87.5, 2.43	86.2, 2.93

Table 5. Average Periodontal Health Measures by Visit for Control Animals versus Experimental Animals

	Visit	Control	Experimental	P-value*
Plaque Sites	Baseline	83.7, 2.33	87.0, 2.33	>0.999
	2Wk	88.3, 2.33	89.0, 2.33	>0.999
	1Mo	88.3, 2.33	89.0, 2.33	>0.999
	3Mo	88.0, 2.33	87.0, 2.33	>0.999
	6Mo	86.0, 2.33	86.3, 2.33	0.9869

Bleeding Sites	Baseline	53.3, 7.81	59.0, 7.81	>0.999
	2Wk	29.0, 7.81	47.0, 7.81	0.8167
	1Mo	39.0, 7.81	39.0, 7.81	>0.999
	3Mo	39.3, 7.81	41.7, 7.81	>0.999
	6Mo	32.7, 7.81	31.3, 7.81	>0.999
Calculus Sites	Baseline	77.0, 6.30	74.3, 6.30	>0.999
	2Wk	66.0 6.30	66.0, 6.30	0.999
	1Mo	63.7, 6.30	64.0, 6.30	>0.999
	3Mo	62.0, 6.30	65.0, 6.30	>0.999
	6Mo	44.7, 6.30	51.0, 6.30	>0.999
Average Probing Depth	Baseline	2.2, 0.06	2.3, 0.06	0.992
	2Wk	2.2, 0.06	2.3, 0.06	0.962
	1Mo	2.2, 0.06	2.4, 0.06	0.801
	3Mo	2.2, 0.06	2.3, 0.06	0.969
	6Mo	2.2, 0.06	2.3, 0.06	>0.999

*Tukey's Adjusted P-value and estimated means from Repeated Measures ANOVA Models.

Plaque Sites. The change in the number of plaque sites across the study visits was not significantly associated with the treatment group (p-value=0.9181). The main effects for visit (p-value=0.5286) and treatment group (p-value=0.6155) were also not statistically significant.

Table 6. Repeated Measures ANOVA Results for Plaque Sites

	Estimate	Standard Error	P-value
Intercept	86.33	2.33	<.0001
Visit			0.5286
Initial	0.67	3.29	0.8420
2-week	2.67	3.29	0.4295
1-month	2.67	3.29	0.4295
3-month	0.67	3.29	0.8420
6-month		Reference	
Group			0.6155
Control	-0.33	3.29	0.9242
Experimental		Reference	
Visit*Group			0.9181

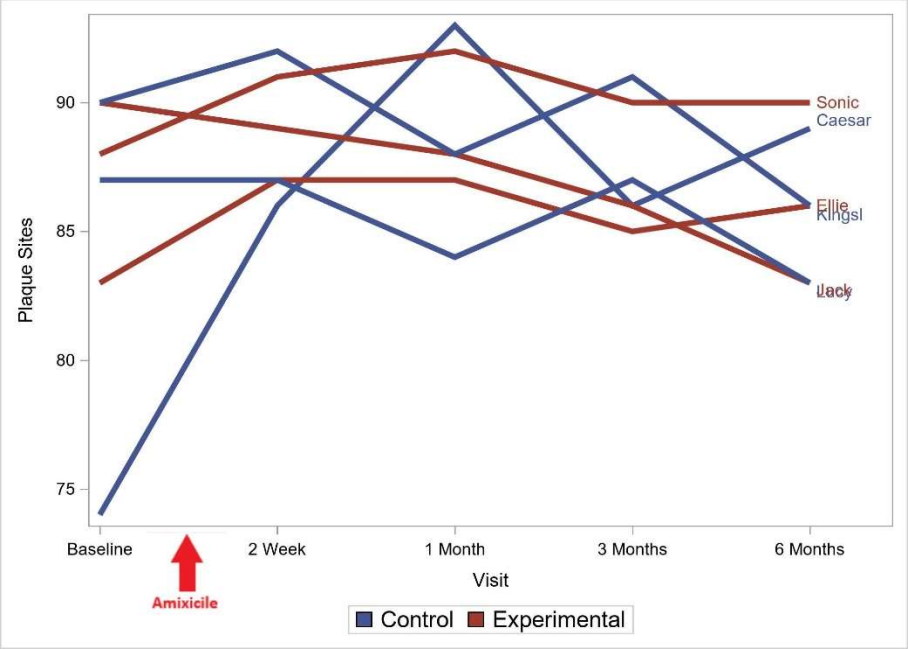

Figure 5. Average Plaque Sites across Visits for 6 Monkeys

Bleeding Sites. The changes in bleeding sites across the study visits were also not significantly dependent on the treatment group (p-value=0.7398). The main effects for visit (p-value=0.0714) and group (p-value=0.3746) were also not statistically significant. After removing group and the group by time interaction terms from the model, there were significant differences in the bleeding sites across study visits for the 6 monkeys (p-value=0.0488). Upon further investigation, the only significant difference was between the baseline value and the 6-month follow-up (Tukey's adjusted p-value=0.0307). By the 6-month follow-up, the average number of bleeding sites had reduced by an average of 24.2 (95% CI: 1.75-46.58). When limiting to the period defined as the "therapeutic interval" (2 weeks until 1 month), there was a significant difference in the bleeding sites between the experimental and control groups (p-value=0.0141). During this period, the control group sees an average increase of 10 sites and the experimental group sees a decrease of 8 (total difference in groups: 18,

95% CI: 6.00-30.0). When adjusting for the number of sites each monkey had at the two week mark, the difference is more dramatic: control sees an average increase of 24.96 more than experimental group (95% CI: 16.37-33.55; p-value=0.0027). Figure 7 demonstrates the trend for the 6 monkeys during this time period.

Table 7. Repeated Measures ANOVA Results for Bleeding Sites

	Estimate	Standard Error	P-value
Intercept	31.33	7.81	0.0160
Visit			0.0714
Initial	27.67	11.05	0.0235
2-week	15.67	11.05	0.1754
1-month	7.67	11.05	0.4978
3-month	10.33	11.05	0.3636
6-month		Reference	
Group			0.3746
Control	1.33	11.05	0.9098
Experimental		Reference	
Visit*Group			0.7398

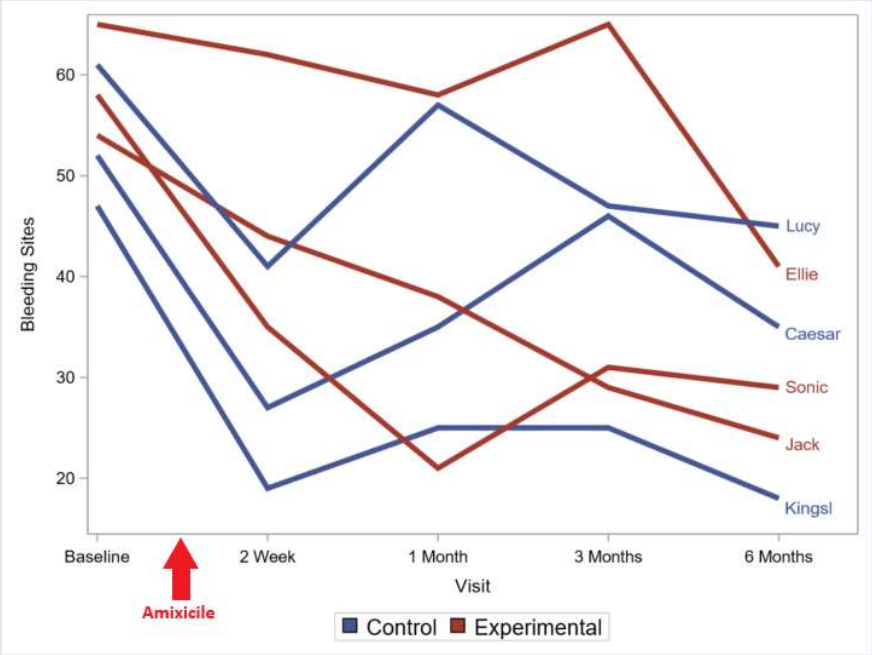

Figure 6. Average Bleeding Sites across Visits for 6 Monkeys

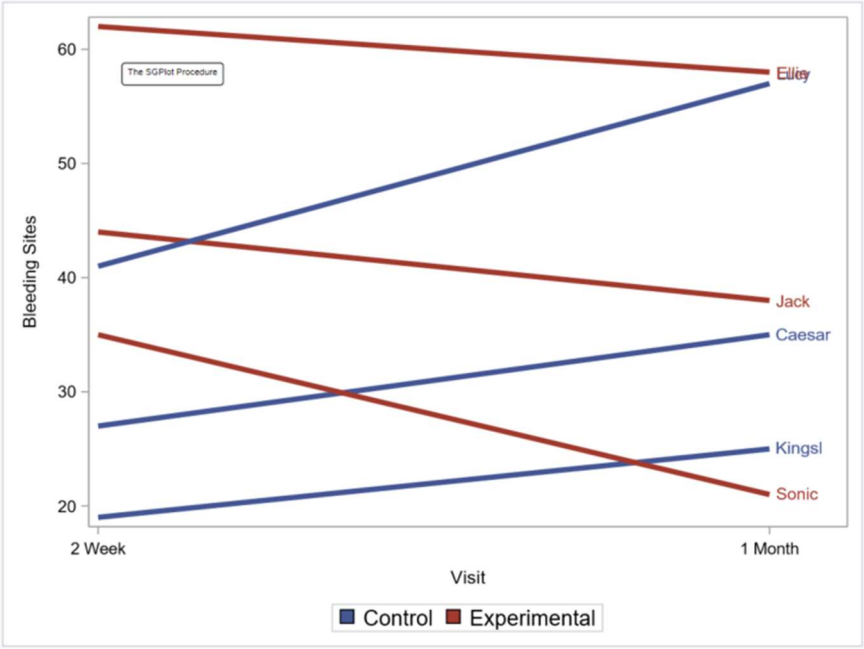

Figure 7. Average Bleeding Sites across "Therapeutic Interval." This begins at the 2 week examination, or the immediate post-amixicile treatment examination, and ends at the 1 month exam, which extends 1 month following completion of the amixicile therapy. Note the trend in which the control animals are all increasing in bleeding sites whereas all experimental animals are decreasing.

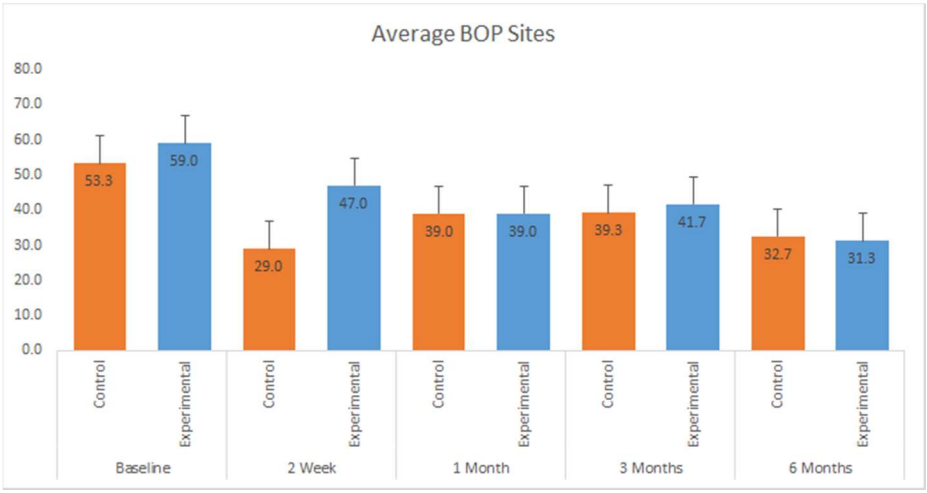

Figure 8. Average Bleeding Sites across Visits for Control versus Experimental Animals. Note the changes in average bleeding sites for control versus experimental animals from baseline to 2 weeks (or immediate post-amixicile treatment) to 1 month. Control animals saw a decrease from baseline to 2 weeks followed by an increase from 2 weeks to 1 month. Experimental animals saw a steady decrease from baseline to 2 weeks to 1 month.

Calculus Sites. The changes in calculus sites across the study visits were not significantly dependent on the treatment group (p-value=0.9619). The main effect for group was also not statistically significant (p-value=0.7431). There was a significant difference in calculus sites across the study visits for all monkeys combined (p-value=0.0081). The 6-month follow-up average calculus values were on average 27.8 less than baseline (95% CI: 10.67-44.99) and 11.8 less than the two-week visit (95% CI: 1.00-35.33).

Table 8. Repeated Measures ANOVA Results for Calculus Sites

	Estimate	Standard Error	P-value
Intercept	51.00	6.30	0.0013
Visit			0.0081
Initial	23.33	8.91	0.0186
2-week	15.00	8.91	0.1117
1-month	13.00	8.91	0.1639
3-month	14.00	8.91	0.1357
6-month		Reference	
Group			0.7431
Control	-6.33	8.91	0.5165
Experimental		Reference	
Visit*Group			0.9619

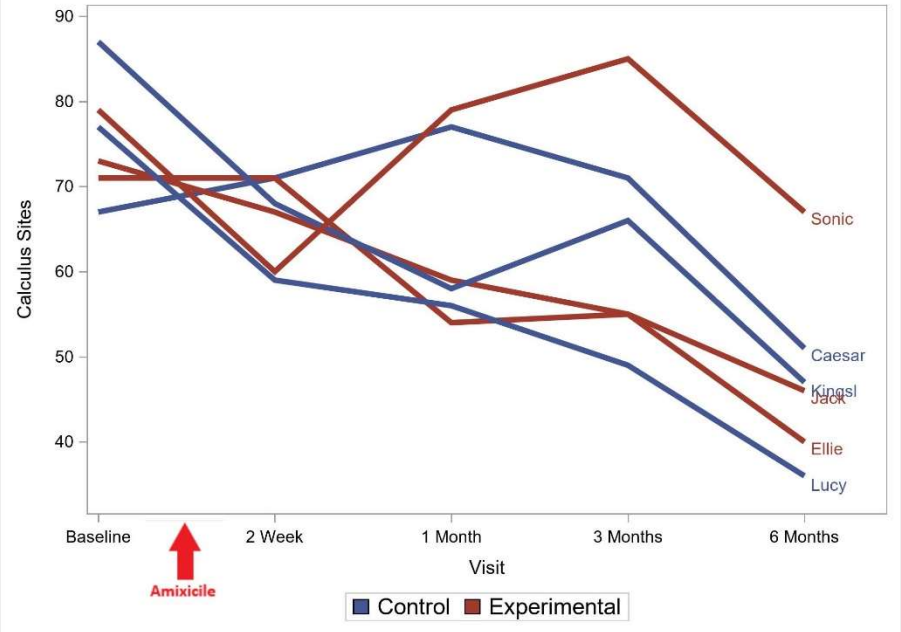

Figure 9. Average Calculus Sites across Visits for 6 Monkeys

Probing Depth. Changes in average probing depth were not significantly associated with treatment group (p-value=0.9261). The main effects for group (p-value=0.0757) and visit (p-value=0.9833) were also not statistically significant.

Table 9. Repeated Measures ANOVA Results for Probing Depth

		Estimate	Standard Error	P-value
Intercept		2.26	0.06	<.0001
Visit				0.9833
	Initial	0.05	0.09	0.6224
	2-week	0.05	0.09	0.6224
	1-month	0.09	0.09	0.3125
	3-month	0.05	0.09	0.5704
	6-month	Reference		
Group				0.0757
	Control	-0.03	0.09	0.7190
	Experimental	Reference		
Visit*Group				0.9261

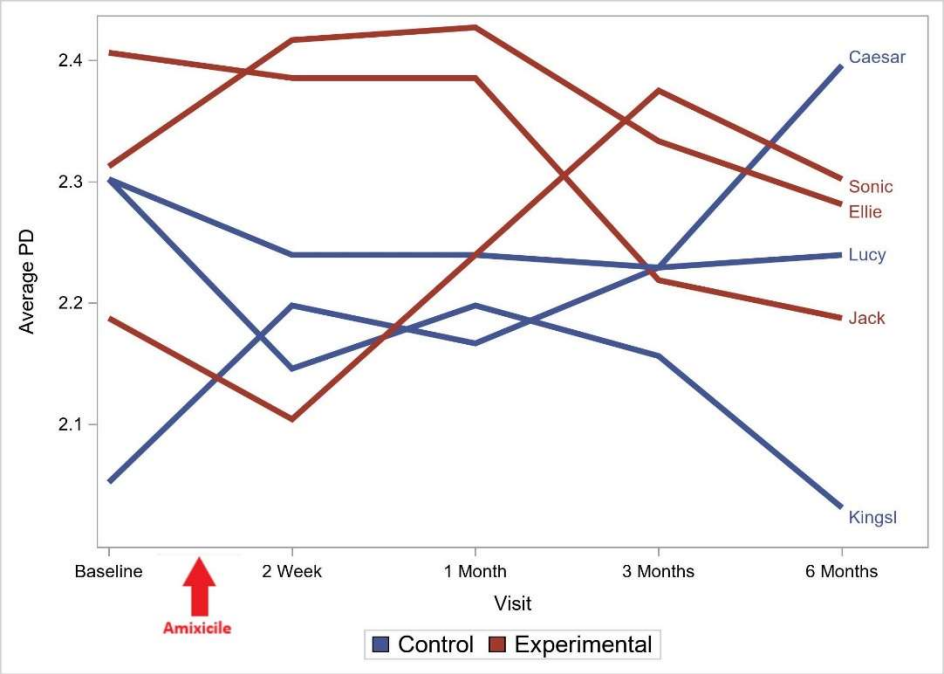

Figure 10. Average PD across Visits for 6 Monkeys

GI Scores. The agreement of GI scores between the two independent, calibrated raters was k=0.48 (95% CI: 0.36-0.61). GI scores from the two raters were averaged for subsequent analysis. Average GI scores were predominantly scored as a 1 or less (83% of observed GI scores across all study visits). The effect of treatment group on changes in average GI score across the study visits did not significantly depend on the sextants (p-value=0.8631). Further, the changes in average GI score across the study visits were not significantly dependent on the treatment group (p-value=0.2417). This indicates, for all 6 sextants, the treatment did not have a significant effect on the changes in GI scores. The only factor significantly associated with average GI score was the sextant (p-value=0.0035). Pairwise comparisons found significant differences between Upper Left and Lower Left (1.20 vs 0.72, adjusted p-value=0.0032), Lower Left and Upper Right (0.72 vs 1.13, adjusted p-value=0.0332), and Lower Right and Upper Left (0.72 vs 1.17, adjusted p-value=0.0438). None of the other pairwise comparisons were significantly different. Figure 8 displays the GI scores by sextant for each of the monkeys across the study visits. Experimental monkeys are labeled with Red and controls with Blue. Table 9 lists the average GI score across the monkeys in each group at each study visit by sextant.

Table 10. Estimated Average GI Score by Visit, Sextant and Treatment Group

	Sextant	Control	Experimental
Baseline			
	Lower Anterior	0.50	1.00
	Lower Left	1.00	0.83
	Lower Right	0.83	0.33
	Upper Anterior	1.00	0.83
	Upper Left	1.17	1.33
	Upper Right	1.17	0.83
2 Week			
	Lower Anterior	0.67	1.00

	Lower Left	0.67	0.67
	Lower Right	1.00	1.00
	Upper Anterior	1.00	1.00
	Upper Left	1.33	1.50
	Upper Right	1.00	1.50
1 Month			
	Lower Anterior	1.33	0.83
	Lower Left	1.00	0.67
	Lower Right	1.33	1.00
	Upper Anterior	1.00	0.83
	Upper Left	1.50	1.67
	Upper Right	1.50	0.83
3 Months			
	Lower Anterior	1.00	0.83
	Lower Left	0.33	1.00
	Lower Right	0.83	0.33
	Upper Anterior	0.67	1.00
	Upper Left	1.00	1.00
	Upper Right	0.83	1.00
6 Months			
	Lower Anterior	1.17	0.67
	Lower Left	0.67	0.33
	Lower Right	1.00	1.00
	Upper Anterior	1.00	0.67
	Upper Left	1.17	1.00
	Upper Right	1.33	1.33

**Average GI score estimated from Repeated Measures ANOVA model, SE was 0.28 for all means.*

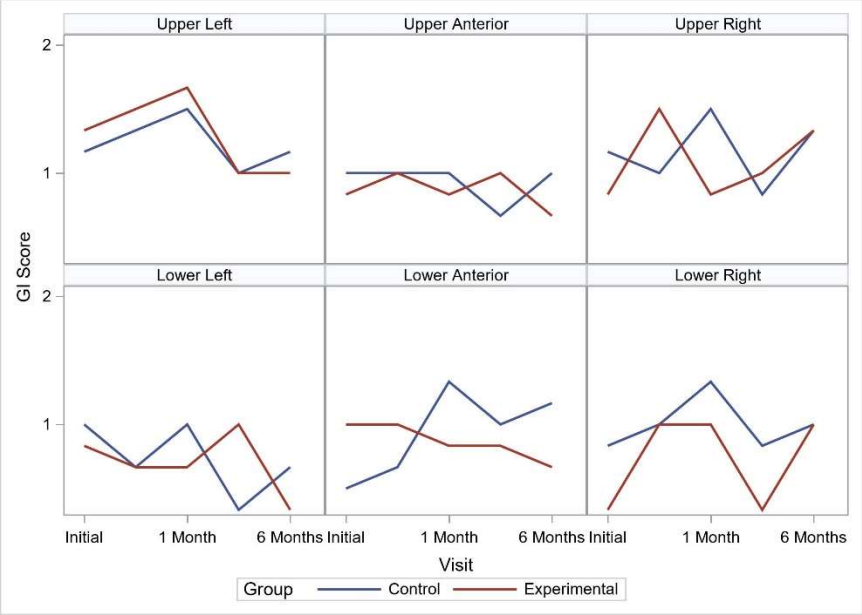

Figure 11. Average GI Scores across Study Visits by Sextant

Microbiological Data

Metagenomic 16S rDNA Sequencing — *Control Animal C, Experimental Animal S*

Salivary Microbiome. Saliva from all sites were collected and pooled prior to analysis. Aliquots of saliva collected during baseline and the immediate post-treatment evaluation were used to isolate total DNA. The DNA was then used for 16S rDNA sequencing and data was analyzed at the genus level (Figure 9). Analysis of the data derived from control animal C reveals that at baseline the most dominant bacteria were belonging to the *Haemophilus* genus followed by *Streptococcus*, *Fusobacterium*, *Leptotrichia*, and *Gemella* genera. In experimental animal S, the most abundant bacteria were belonging to the *Porphyromonas* genus followed by *Streptococcus*, *Haemophilus*, *Fusobacterium*, and *Leptotrichia* genera. Following treatment with amixicile, reduction in anaerobic bacteria with a concomitant increase in aerotolerant bacteria was observed. Specifically in experimental animal S, a reduction in bacteria belonging to the anaerobic genera *Porphyromonas* was seen. An increase in the aerobic genera *Haemophilus* was seen at the same time. Comparatively in control animal C, an increase in anaerobic genera *Porphyromonas* and *Veillonella* was observed at the follow up evaluation. Additionally, no change was seen in genera *Streptococcus* and a decrease in aerobic genera *Haemophilus* was observed. These results demonstrate amixicile's ability to effectively reduce the levels of anaerobic bacteria present in the salivary microbiome of an NHP model while leaving the aerobic bacteria unaffected for the most part.

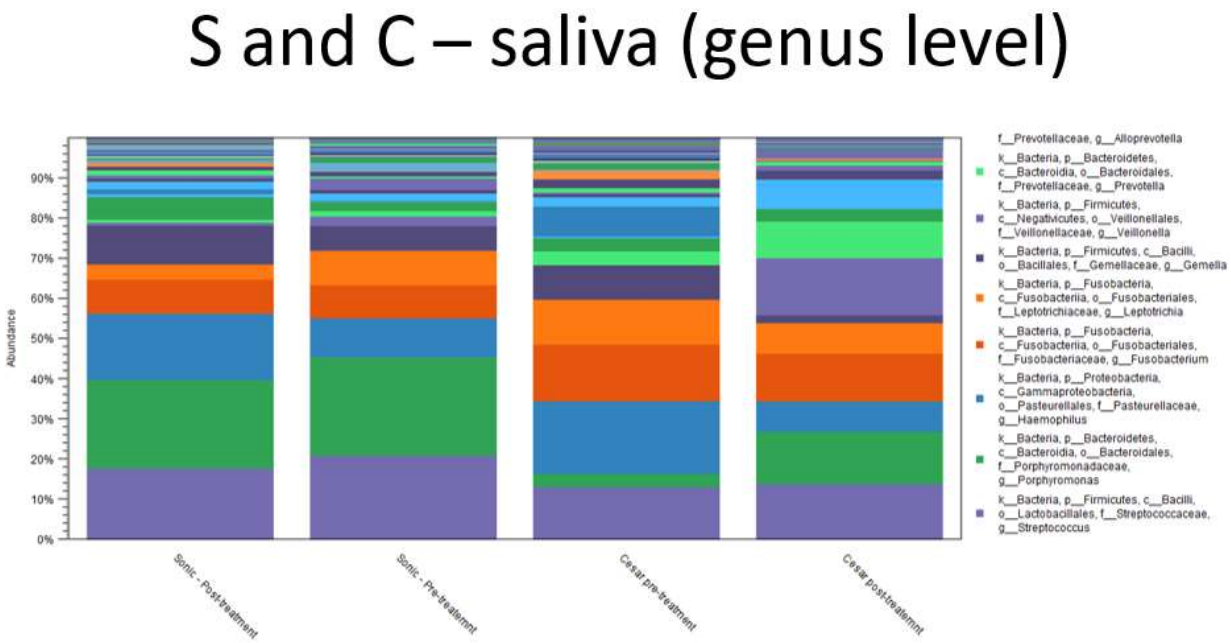

Figure 12. Salivary microbiome composition at a genus level for control animal C and experimental animal S. Microbial composition at baseline (pre-treatment) and immediately post-treatment.

Subgingival Plaque Microbiome. Three sites of subgingival plaque biofilm from control animal C (#5 mesial, #13 distal, #19 mesial) and five sites from experimental animal S (#1 mesial, #3 mesial, #5 distal, #29 distal, #32 buccal) were successfully surveyed for the composition of the oral microbiome at baseline and following amixicile treatment (Figures 10-13).

For control animal C (Figures 10-11), survey of the #5 mesial site at the genus level revealed minimal-to-no change in *Fusobacterium*, increase in *Leptotrichia* and *Prevotella*, and decrease in *Porphyromonas* and *Streptococcus* comparing baseline to the follow up evaluation. Survey of the #13 distal site at the genus level revealed increase in *Fusobacterium*, *Prevotella*, and *Streptococcus* as well as a decrease in *Leptotrichia* and *Porphyromonas*. Survey of the #19 mesial site at the genus level

revealed increase in *Fusobacterium*, *Streptococcus*, and *Leptotrichia* as well as minimal-to-no change in *Prevotella* and *Porphyromonas*. Overall, there was a tendency towards an increase in *Fusobacterium*, *Prevotella*, *Leptotrichia*, and *Streptococcus* with a subsequent decrease in *Porphyromonas*.

For experimental animal S (Figures 12-13), survey of the #1 mesial and #3 mesial sites at the genus level both revealed an increase in *Fusobacterium* and *Capnocytophaga* with a subsequent decrease in *Streptococcus* and *Porphyromonas* comparing baseline to post-treatment. Survey of the #5 distal site at the genus level revealed minimal-to-no change in *Fusobacterium*, *Streptococcus*, or *Porphyromonas* as well as a decrease in *Leptotrichia* and *Capnocytophaga* comparing baseline to post-treatment. Survey of the #29 distal site at the genus level revealed minimal-to-no change in *Fusobacterium*, an increase in *Prevotella*, and a decrease in *Streptococcus*, *Capnocytophaga*, *Porphyromonas*, and *Leptotrichia* comparing baseline to post-treatment. Survey of the #32 buccal site at the genus level revealed minimal-to-no change in subgingival plaque composition comparing baseline to post-treatment. Overall, there was a tendency towards an increase in *Fusobacterium* and a decrease in both *Streptococcus* and *Porphyromonas*.

C – subgingival plaque (genus level)

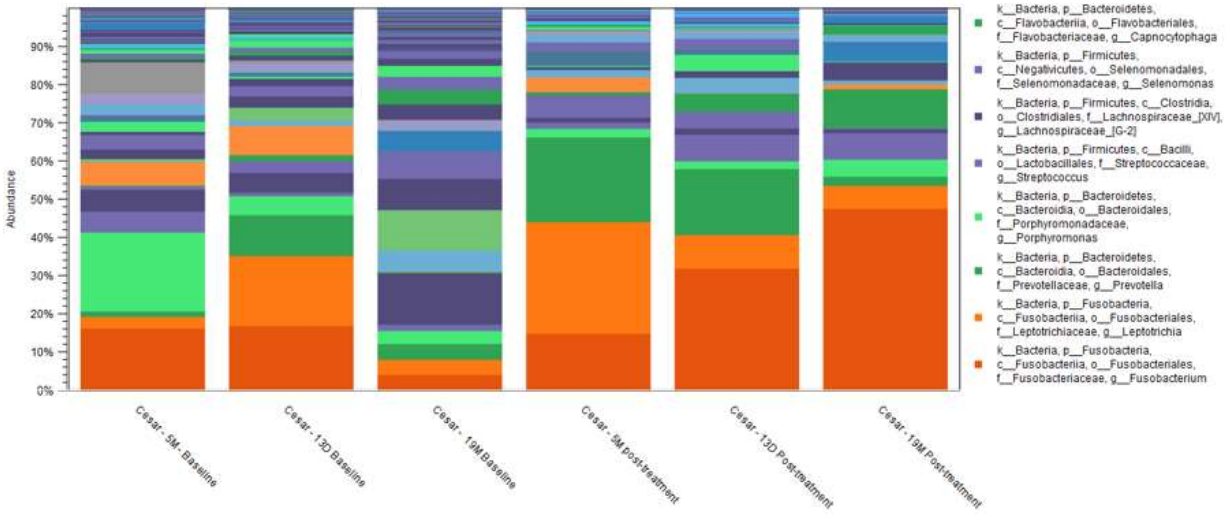

Figure 13. Subgingival plaque composition at a genus level of control animal C. Microbial composition at baseline and immediately post-treatment.

C – subgingival plaque (family level)

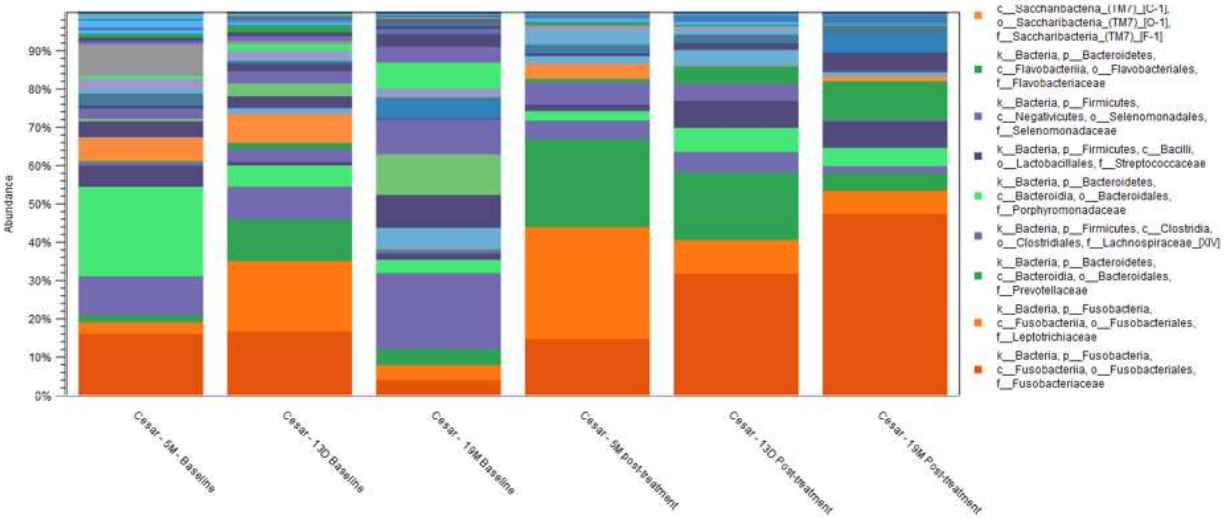

Figure 14. Subgingival plaque composition at a family level of control animal C. Microbial composition at baseline and immediately post-treatment.

S – subgingival plaque (genus level)

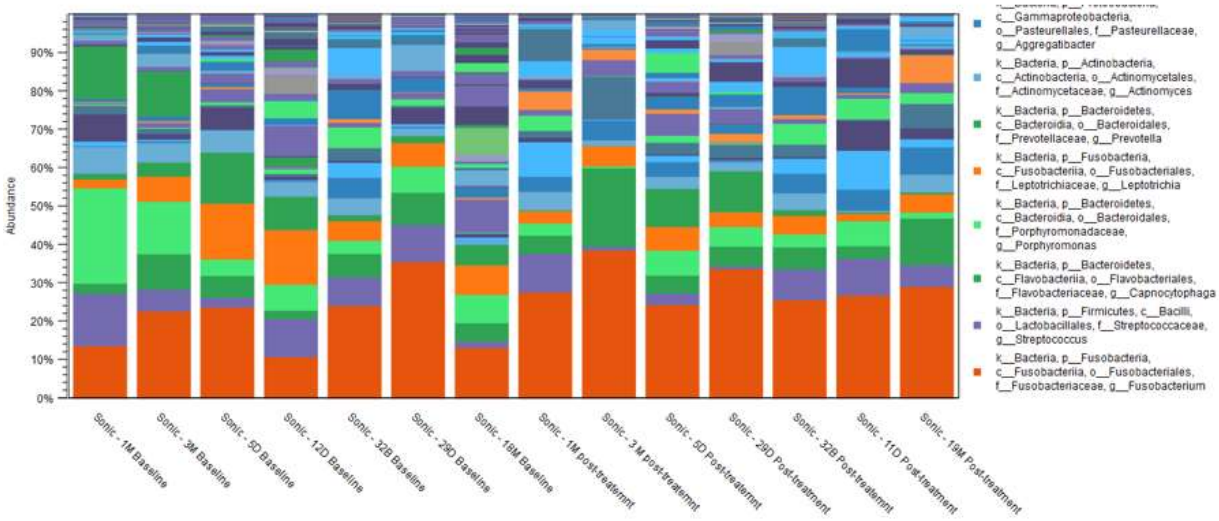

Figure 15. Subgingival plaque composition at a genus level of experimental animal S. Microbial composition at baseline and immediately post-treatment.

S – subgingival plaque (family level)

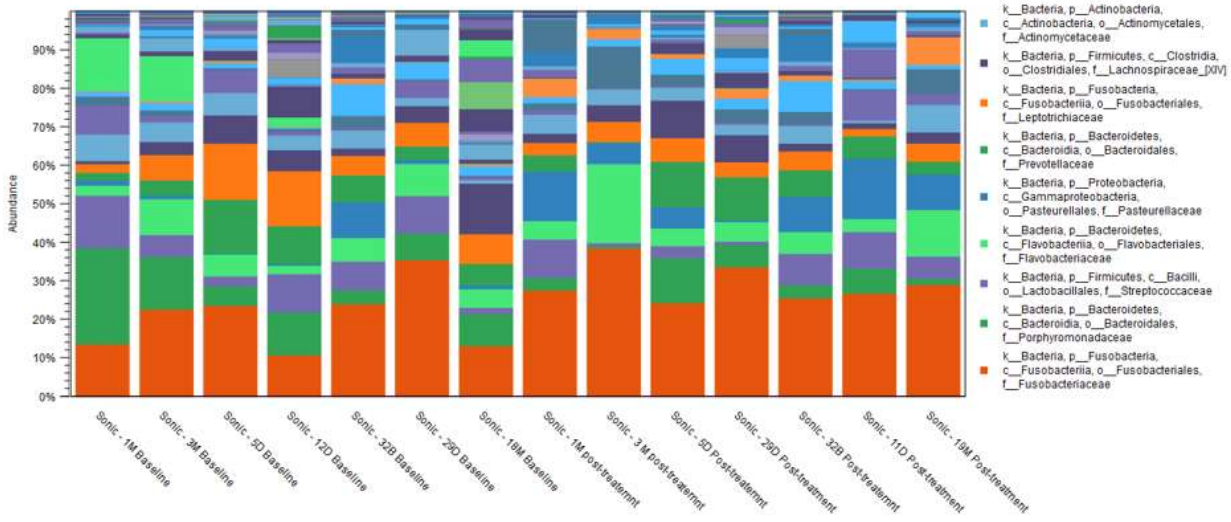

Figure 16. Subgingival plaque composition at a family level of experimental animal S. Microbial composition at baseline and immediately post-treatment.

To summarize the metagenomics sequencing data, experimental animal S demonstrated a reduction in anaerobic bacteria belonging to the genera *Porphyromonas* with a concomitant increase in the aerobic genera *Haemophilus*. In comparison, control animal C demonstrated an increase in anaerobic genera *Porphyromonas* and *Veillonella*, no change in genera *Streptococcus*, and a decrease in aerobic genera *Haemophilus*. A large amount of variability in microbial composition between individual subgingival sites existed within the same animal and between different animals, which is expected but makes comparison difficult.

Quantitative PCR — *Control Animal L, Experimental Animal E*

Salivary Microbiome. Analysis of the salivary microbiome of control animal L and experimental animal E is represented by “site 13” as seen in Figure 14. For control animal L, a fold change of -2.0, -3.9, and -2.3 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to post-treatment. For experimental animal E, a fold change of -10.4, -29.4, and -8.1 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to the immediate post-treatment. Overall, both control animal L and experimental animal E showed a reduction in the abundance of *Prevotella*, *Fusobacterium*, and *Streptococcus*; however, the experimental animal showed a greater reduction in abundance for all three bacteria studied.

Subgingival Plaque Microbiome. Analysis of the subgingival plaque microbiome of control animal L and experimental animal E is represented by “site 2” and

“site 4” as seen in Figure 14. “Site 2” is representative of samples collected from tooth #2 mesial, and “site 4” is representative of samples collected from tooth #31 mesial.

For tooth #2 mesial in control animal L, a fold change of 1.8, -2.0, and -15.5 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to post-treatment. For tooth #31 mesial in control animal L, a fold change of 1.2, 1.5, and -2.6 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to post-treatment.

For tooth #2 mesial in experimental animal E, a fold change of -18.4, -10.4, and -7.1 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to the immediate post-treatment. For tooth #31 mesial in experimental animal E, a fold change of 1.0, -2.1, and -3.8 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to post-treatment.

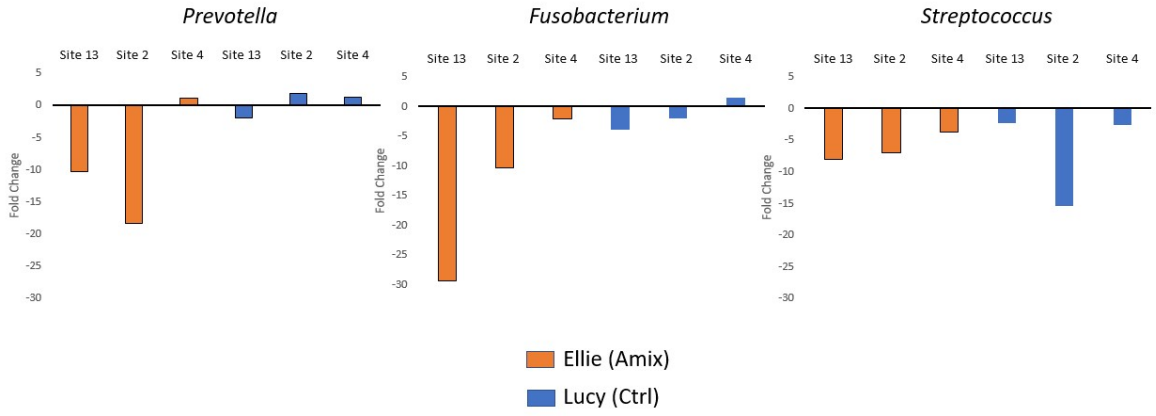

Figure 17. Fold change baseline versus post-treatment for control animal L and experimental animal E

Quantitative PCR — *Control Animal K, Experimental Animal J*

Salivary Microbiome. Analysis of the salivary microbiome of control animal K and experimental animal J is represented by “site 13” as seen in Figure 15. For control animal K, a fold change of -5.7, -11.6, and 13.6 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to post-treatment. For experimental animal J, a fold change of -5.4, -2.1, and -4.7 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to the immediate post-treatment. Overall, both control animal K and experimental animal J showed a reduction in the abundance of *Prevotella* and *Fusobacterium*. For *Streptococcus*, the control showed an increase whereas the experimental showed a decrease in abundance.

Subgingival Plaque Microbiome. Analysis of the subgingival plaque microbiome of control animal K and experimental animal J is represented by “site 2” and “site 4” as seen in Figure 15. “Site 2” is representative of samples collected from tooth #2 mesial, and “site 4” is representative of samples collected from tooth #31 mesial.

For tooth #2 mesial in control animal K, a fold change of 1.2, 94.9, and 47.9 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to post-treatment. For tooth #31 mesial in control animal K, a fold change of 1.8, 1.8, and -1.0 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to post-treatment.

For tooth #2 mesial in experimental animal J, a fold change of 4.2, -30.2, and -1.6 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when

comparing pre-treatment to the immediate post-treatment. For tooth #31 mesial in experimental animal J, a fold change of -17.7, -4.5, and -5.1 was seen for *Prevotella*, *Fusobacterium*, and *Streptococcus* respectively when comparing pre-treatment to post-treatment.

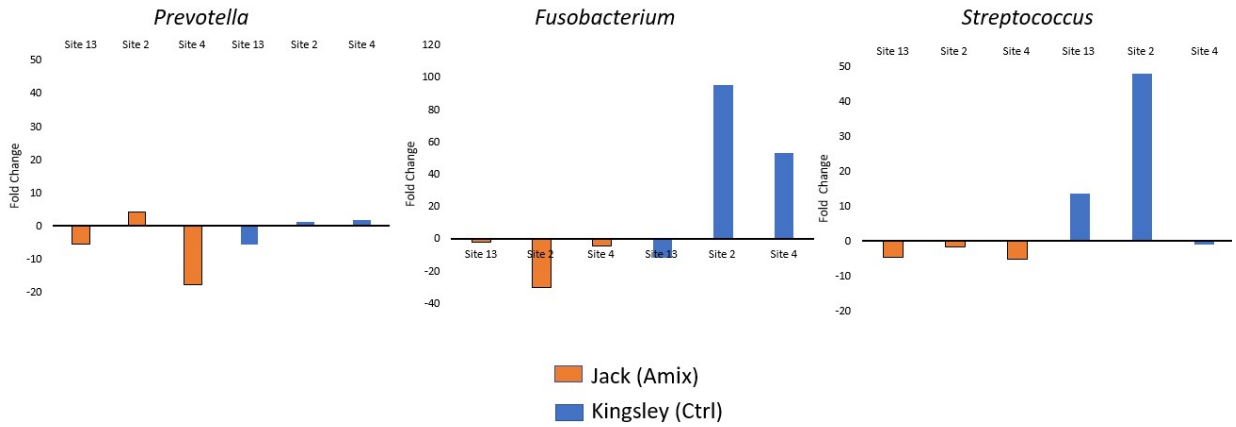

Figure 18. Fold change baseline versus post-treatment for control animal K and experimental animal J

To summarize the qPCR data, it was noted that Gram-negative, anaerobic *Prevotella* and *Fusobacterium* tended to decrease drastically in the experimental animals whereas in the control animals levels either increased or at times decreased less drastically. For aerobic *Streptococcus*, findings were more diverse and varied. Generally, both experimental and control animals tended to show a decrease in *Streptococcus*; however, for laboratory “site 2” and “site 13” in control animal K an increase was seen immediately post-treatment.

Correlation between Microbiological Data and Clinical Findings

Table 11. Animal and site-specific correlation between microbiological data and clinical findings.

	Name	Study Group	Clinical Data	Microbiological Data
Pair 1	**Animal C**	**Control**	• Site #5M— Baseline 2mm PD (+) BOP (+) plaque (+) calculus Immediate Post-Tx 2mm PD (+) BOP (+) plaque (+) calculus • Site #13D— Baseline 3mm PD (+) BOP (+) plaque (+) calculus Immediate Post-Tx 2mm PD* (+) BOP (+) plaque (+) calculus • Site #19M— Baseline 2mm PD (+) BOP (-) plaque (+) calculus Immediate Post-Tx 2mm PD (+) BOP (+) plaque* (-) calculus*	• Site #5M— **Increase in:** *Leptotrichia, Prevotella* **Decrease in:** *Porphyromonas, Streptococcus* **Minimal-to-no change in:** *Fusobacterium* • Site #13D— **Increase in:** *Fusobacterium, Prevotella, Streptococcus* **Decrease in:** *Leptotrichia, Porphyromonas* • Site #19M— **Increase in:** *Fusobacterium, Streptococcus, Leptotrichia* **Minimal-to-no change in:** *Prevotella, Porphyromonas*

	Animal S	**Experimental**	• Site #1M— Baseline 3mm PD (+) BOP (+) plaque (+) calculus Immediate Post-Tx 2mm PD* (+) BOP (+) plaque (-) calculus* • Site #3M— Baseline 3mm PD (+) BOP (+) plaque (+) calculus Immediate Post-Tx 2mm PD* (-) BOP* (+) plaque (-) calculus* • Site #5D— Baseline 3mm PD (+) BOP (-) plaque (+) calculus Immediate Post-Tx 2mm PD* (+) BOP (+) plaque* (-) calculus* • Site #29D— Baseline 2mm PD (+) BOP (+) plaque	• Site #1M— **Increase in:** *Fusobacterium, Capnocytophaga* **Decrease in:** *Streptococcus, Porphyromonas* • Site #3M— **Increase in:** *Fusobacterium, Capnocytophaga* **Decrease in:** *Streptococcus, Porphyromonas* • Site #5D— **Decrease in:** *Leptotrichia, Capnocytophaga* **Minimal-to-no change in:** *Fusobacterium, Streptococcus, Porphyromonas* • Site #29D— **Increase in:** *Prevotella* **Decrease in:** *Streptococcus, Capnocytophaga, Porphyromonas, Leptotrichia* **Minimal-to-no change in:** *Fusobacterium* • Site #32B— **Minimal-to-no change in:** *Fusobacterium, Streptococcus, Capnocytophaga, Porphyromonas, Leptotrichia*

			(+) calculus Immediate Post-Tx 2mm PD (+) BOP (+) plaque (+) calculus • Site #32B— Baseline 3mm PD (-) BOP (+) plaque (+) calculus Immediate Post-Tx 2mm PD* (-) BOP (+) plaque (+) calculus	
Pair 2	**Animal L**	**Control**	• Site #2M— Baseline 3mm PD (+) BOP (+) plaque (+) calculus Immediate Post-Tx 3mm PD (-) BOP* (+) plaque (+) calculus • Site #31M— Baseline 3mm PD (+) BOP (+) plaque (+) calculus Immediate Post-Tx 3mm PD (+) BOP (+) plaque	• Site #2M— **Increase in:** *Prevotella* **Decrease in:** *Fusobacterium, Streptococcus* • Site #31M— **Increase in:** *Prevotella, Fusobacterium* **Decrease in:** *Streptococcus*

			(+) calculus	
	Animal E	**Experimental**	• Site #2M— 3mm PD (+) BOP (+) plaque (+) calculus <u>Immediate Post-Tx</u> 3mm PD (-) BOP* (+) plaque (+) calculus • Site #31M— <u>Baseline</u> 2mm PD (+) BOP (+) plaque (+) calculus <u>Immediate Post-Tx</u> 3mm PD* (+) BOP (+) plaque (+) calculus	• Site #2M— **Decrease in:** *Prevotella, Fusobacterium, Streptococcus* • Site #31M— **Increase in:** *Prevotella* **Decrease in:** *Fusobacterium, Streptococcus*
Pair 3	**Animal K**	**Control**	• Site #2M— <u>Baseline</u> 3mm PD (+) BOP (+) plaque (+) calculus <u>Immediate Post-Tx</u> 3mm PD (-) BOP* (+) plaque (+) calculus • Site #31M— <u>Baseline</u> 3mm PD (+) BOP	• Site #2M— **Increase in:** *Prevotella, Fusobacterium, Streptococcus* • Site #31M— **Increase in:** *Prevotella, Fusobacterium* **Decrease in:** *Streptococcus*

			(+) plaque (+) calculus <u>Immediate Post-Tx</u> 2mm PD* (+) BOP (+) plaque (+) calculus	
	Animal J	**Experimental**	• Site #2M— <u>Baseline</u> 3mm PD (+) BOP (+) plaque (-) calculus <u>Immediate Post-Tx</u> 3mm PD (+) BOP (+) plaque (+) calculus* • Site #31M— <u>Baseline</u> 3mm PD (+) BOP (+) plaque (+) calculus <u>Immediate Post-Tx</u> 3mm PD (-) BOP* (+) plaque (+) calculus	• Site #2M— **Increase in:** *Prevotella* **Decrease in:** *Fusobacterium, Streptococcus* • Site #31M— **Decrease in:** *Prevotella, Fusobacterium, Streptococcus*

*notes a change from baseline to immediate post-treatment examination

Discussion

As previously stated, the aim of this study was to expand upon the referenced *in-vitro* research studies[11, 13, 14, 15] and the pilot study at Virginia Commonwealth University, Department of Periodontics and Philips Institute[16], in which there were no control subjects, by testing the effects of amixicile in *in-vivo* conditions in six rhesus macaque monkeys. The outcomes evaluated in this study included both clinical indices and microbiological markers. Generally, amixicile was tolerated well by all three experimental animals S, E, and J. No adverse reactions, such as diarrhea or other gastrointestinal upset, was noted from any of the animals that were administered amixicile. This, as the previous pilot study completed[16], establishes the safety of amixicile when given at an appropriate dose, which in this study was 20 mg/kg.

One of the initial concerns and limitations in this study was the lack of periodontal disease in these animals at baseline. No statistically significant changes were seen in the periodontal health measures, including probing depth, bleeding points, plaque sites, calculus sites, and GI scores, in part due to the fact that all six animals were relatively periodontally healthy from the baseline exam and throughout the study. Although not statistically significant, a trend seemed to be apparent in the number of bleeding sites when comparing the controls versus the experimental animals. As seen in Figure 5, all animals seem to have a decrease in bleeding sites from the baseline exam to the 2 week exam even though all other periodontal health indices measuring etiology (i.e.

presence of plaque and/or calculus) stayed comparable among the groups throughout. BOP behaved in a different pattern and showed a unique trend during the therapeutic interval. The controls (C, L, K) number of bleeding sites increase immediately after the 2 week exam whereas the experimental animals (S, E, J) seem to have a more sustained decrease in bleeding sites at the 1 month exam and even beyond. As seen in classic periodontal literature, the absence of bleeding on probing is considered an indicator of periodontal stability, and these findings in the amixicile-treated animals could be demonstrating this[22]. It could be possible that this difference could be more pronounced if animals with more severe forms of periodontal disease were included and studied.

Additionally, periodontal literature has demonstrated that certain bacterial species believed to be etiologically related to periodontitis have been found to be associated with bleeding on probing even in sites with minimal attachment loss[23]. Examples of these include *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*. Additional bacterial species that have demonstrated a weak positive association to bleeding on probing were *Campylobacter rectus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Micromonas micros*, and *Prevotella intermedia*. In contrast, bacterial species typically associated with periodontal health have been found to have an inverse association with bleeding on probing. In our study specifically, control animals showed a trend of having a rebound in bleeding sites during the therapeutic interval, which was after amixicile therapy (for experimental animals) through the one month examination. During this time, control animals, also, demonstrated an increase in periodontal pathogenic bacteria in contrast

to experimental animals. Additionally, although we did not specifically analyze oral treponemes (spirochetes) in this study, we can speculate that amixicile likely had an influence on this group of bacteria in the experimental animals based on periodontal literature that demonstrated amixicile's ability to inhibit growth of all oral treponeme species *in vitro*[13]. Therefore, our clinical results specifically relating to bleeding sites seem to correlate well with our microbiological findings.

Furthermore, the random assignment for the first and third pairs placed the more alpha and dominant males (animals C, K) in the control group while leaving the less dominant males (animals S, J) to be experimental subjects. The possible increased psychological stress of the less dominant males could have had an effect on the efficacy of amixicile. It has been shown recently in periodontal literature that humans with increased stress, depression, and anxiety as well as those exhibiting negative coping strategies demonstrated worsened outcomes to non-surgical periodontal treatment (i.e. scaling and root planing)[24]. It may be possible that the clinical and/or microbiological outcomes could have been different or shown a more dramatic effect from amixicile if both experimental subjects had not been less dominant, more stressed animals.

Even though the periodontal clinical indices did not change significantly when comparing control versus experimental and pre-treatment versus post-treatment, there still was microbiological evidence that allowed us to gain insight into the effectiveness of amixicile as a novel antimicrobial agent and its ability to affect the oral microbiome. For the first pair (control animal C and experimental animal S) funding allowed metagenomics 16S rDNA sequencing to be performed. In experimental animal S, a

reduction in bacteria belonging to the anaerobic genera *Porphyromonas* was seen while an increase in the aerobic genera *Haemophilus* was seen concomitantly. Comparatively in control animal C, an increase in anaerobic genera *Porphyromonas* and *Veillonella* was observed while no change was seen in genera *Streptococcus* and a decrease in aerobic genera *Haemophilus* was observed. These results demonstrate amixicile's ability to effectively reduce the levels of anaerobic bacteria present in the salivary microbiome of an NHP model while leaving the aerobic bacteria unaffected for the most part. A large amount of variability in microbial composition between individual subgingival sites was noted within the same animal and between different animals, which is expected but makes comparison difficult. Microbial composition likely varies based on the pocket depth as well as various environmental factors such as self-cleansability and chewing function. For the second pair (control animal L and experimental animal E) as well as for the third pair (control animal K and experimental animal J) lack of funding mid-study prevented sequencing from being completed; however, qPCR was able to be done which did give an insight into relative bacterial abundance pre-treatment compared to post-treatment specifically for *Prevotella*, *Fusobacterium*, and *Streptococcus*. Comparing the microbiological data for the second pair and the third pair, it was noted that *Prevotella* and *Fusobacterium*, both of which are Gram negative anaerobic bacteria, tended to either increase or at times decrease slightly in the control animals versus decrease more dramatically in the experimental animals thus possibly contributing to the reduction of bleeding sites in the experimental animals. Reduction in these specific bacteria at different time intervals aligns with the reduced number of bleeding sites noted. For *Streptococcus*, findings were more diverse

and varied. Generally, all subjects tended to show a decrease in *Streptococcus*; however, for laboratory “site 2” and “site 13” in control animal K an increase was seen post-treatment. The reduction of aerobic *Streptococcus* specifically in the experimental animals was likely in part due to the overall reduction in bacterial load that is a result of amixicile treatment.

Another major limitation in this study was the lack of funding and extreme limitation of laboratory resources mid-study, which prevented us from evaluating and studying the entire picture of the microbiological data that was hoped for. Fortunately, we were able to obtain metagenomics 16S rDNA sequencing for the first pair (control animal C and experimental animal S), but only for the samples from the baseline exam and the immediate post-treatment exam. As mentioned previously, sequencing was not able to be obtained for the second and third pairs (control animal L and experimental animal E, control animal K and experimental animal J); however, we were able to obtain data using qPCR. This essentially gave us a small snippet of the entire microbiological picture since we were only evaluating the three specific bacteria- *Prevotella*, *Fusobacterium*, and *Streptococcus*. The hope for future studies involving this novel antimicrobial agent is that funding will allow for full sequencing of all samples from all subjects at all time points to observe the bigger picture that is occurring post-amixicile treatment. Additionally, the lack of funding did inhibit the GCF samples from being processed and evaluated. Therefore, there is no data to present regarding this. Likewise, the hope for future research is that funding allows for measurement and evaluation of the concentration of amixicile or any inflammatory markers in the GCF or serum to determine how well it was able to localize to the gingival sulcus.

Conclusion

In conclusion, amixicile has been shown to selectively inhibit anaerobic bacteria associated with periodontal disease, including *Prevotella spp.*, *Fusobacterium spp.*, and *Porphyromonas spp.*, while not drastically altering the abundance of aerobic bacteria associated with periodontal health such as *Streptococcus spp.* and *Haemophilus spp*. Amixicile seems to be a strong, viable candidate as a novel antimicrobial agent to be used to alter one's oral microbiome from that of disease to that of health and possibly for the management of periodontal disease. Further research at a larger scale and in animals with more severe forms of periodontal disease is needed to bring amixicile closer to clinical trials in humans.

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