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Original Article

Amixicile targets anaerobic bacteria within the oral microbiome

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ABSTRACT

Objectives: Anaerobic bacteria are the major causative agents of periodontal disease. However, so far, targeted therapy aimed at reducing those pathogens has not been widely implemented. We have previously reported on a novel antimicrobial, amixicile, that targets anaerobic bacteria through inhibition of the function of the major anaerobic metabolic enzyme pyruvate ferredoxin oxidoreductase (PFOR), while not affecting aerotolerant organisms. It effectively inhibited the growth of oral anaerobes both in monocultures as well as in mixed *in vitro* mixed cultured however, amixicile's activity in *in vivo*-like conditions remained to be established.
Methods: Here, we expand our study using an *ex vivo* oral microbiome combined with metagenomic sequencing to determine the effect of amixicile treatment on the composition of the microbiome and compare it to that of metronidazole.
Results: Our results show that in the complex microbiomes, anaerobic bacteria are selectively inhibited, while the growth of aerotolerant ones, such as *Streptococcus, Klebsiella, Neisseria,* and *Rothia* is unaffected. *Veillonella* was the most abundant anaerobic genus in our *ex vivo* microbiome, and we observed complete inhibition of its growth. In addition, growth of other anaerobes, *Fusobacterium* and *Prevotella,* was significantly inhibited. It is noteworthy that a change in abundance of bacteriophages, such as *Siphoviridae* and *Myoviridae*, associated with the oral microbiome was observed.
Conclusions: Collectively, our data expand on the so far reported inhibitory spectrum of amixicile and demonstrates that it inhibits anaerobic bacteria, including both clinical isolates and laboratory strains.

1. Introduction

Although known to be the major etiological agent of periodontal disease, anaerobic bacteria are yet to be specifically targeted as a means to prevent and treat periodontitis. The antimicrobial therapies used, both mechanical and adjunctive antibiotic treatments (tetracycline, clindamycin, or amoxicillin) are aimed at reducing the total load of the oral microbiome [1,2]. Similarly, preventive measures, such as chlorhexidine are also effective through their indiscriminative antimicrobial action [3]. As the oral cavity harbors over 50–70 billion bacteria with 700 different predominant taxa [4] with some being beneficial and even required for the development of a healthy immune system, and others being at the root of infectious conditions, such as caries and periodontal diseases [5], such indiscriminate treatment is not optimal. Periodontal diseases are triggered by bacteria that mainly include anaerobic types, such as *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia,* and *Treponema denticola* [6–16]. As anaerobic bacteria are the major periodontal pathogens, it would seem intuitive that the inhibition of the growth of these bacteria would reduce the pathogenic potential of the oral microbiome.

Metronidazole specifically inhibits anaerobic bacteria. However, due to its adverse side effects, such as neurotoxicity, opticneuropathy, peripheral neuropathy, encephalopathy, and changes in the gut microbiome (especially due to the high dose or long term use), it is not widely used for the treatment of periodontal diseases [17,18]. Importantly, it is usually used in combination with amoxicillin, thus, making this combination of antibiotics broad spectrum

Abbreviations: PFOR, pyruvate ferredoxin oxidoreductase; Sm, *Streptococcus mutans*; Va, *Veillonella atypica*; La, *Lactobacillus acidophilus*; BHI, Brain Heart Infusion; qPCR, quantitative PCR.

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[19]. Treatment with broad-spectrum antimicrobials is usually used as adjunctive therapy to a mechanical one and eliminates both pathogenic and beneficial bacteria, thus, leaving the treated sites ripe for re-infection with pathogens.

Recently, we reported on amixicile as a potential treatment strategy for oral anaerobes [20]. Amixicile inhibits pyruvate ferredoxin oxidoreductase (PFOR), an enzyme present only in anaerobic bacteria and parasites, such as *Cryptosporidium, Trichomonas, Entamoeba,* and *Giardia*, and does not affect bacteria relying on pyruvate dehydrogenase, and enzymes present in aerotolerant bacteria, as well as most eukaryotic organisms [20,50]. As stated above, most of the bacteria implicated in the development and progression of periodontal diseases are anaerobic and, thus, rely on PFOR for energy generation. Therefore, the inhibition of the enzyme is predicted to have a selective inhibition effect on the oral microbiome resulting in a reduction of growth of the periodontitis-associated bacteria while leaving many health-associated organisms unaffected. Our earlier work was conducted using an *in vitro* mixture of oral bacteria composed of six species: *P. gingivalis, Streptococcus gordonii, Fusobacterium nucleatum, T. forsythia, P. intermedia*, and *A. actinomycetemcomitans* [20]. Amixicile is also effective against the oral periodontal pathogen *T. denticola* [21]. However, amixicile is yet to be tested in the universal system, the salivary microbiome that is the major carrier of oral bacteria. Using the *ex vivo* salivary microbiome we were able to test a larger variety of bacteria derived from a clinical setting and expanded the diversity of the bacteria beyond the suspected periodontal pathogens [12–22].

2. Materials and methods

2.1. The sample used in this study

The stock microbiome was obtained from Dr. W. Shi (20 μl of the microbiome was diluted in 2 ml of SHI media) [22,23].

2.2. Microbiome cultures

A schematic representation of the design for this study is shown in Fig. 1. The oral salivary microbiome derived from human subjects was obtained from Dr. W. Shi [22,23]. This is a well-established microbiome derived from the saliva of healthy volunteers. SHI media, shown to reduce the overgrowth of streptococci and preserve the diversity of the oral microbiota, was used to grow the bacteria [24]. Bacteria were grown in an anaerobic chamber (Coy anaerobic chamber [Ann Arbor, MI, USA]) in an artificial atmosphere (composed of 80% N_2, 10% H_2, and 10% CO_2) overnight. A 1:100 dilution of the stock microbiome was obtained from Dr. W. Shi's laboratory (20 μl in 2 ml of SHI media) and was prepared and grown for 16 h in the presence of either 30 μg/mL (for low concentration) or 60 μg/mL (for high concentration) of amixicile (provided by Dr. Hoffman) or 25 μg/mL of metronidazole (MilliporeSigma, St. Louis, MO, USA). Cultures without antibiotics served as controls. Following overnight growth, the broth cultures were centrifuged at 8000 g for 20 min to pellet the bacterial cells. The supernatant was discarded, and the cell pellet was used to isolate DNA.

2.3. Bacterial strains and culture conditions for monoculture studies

Microbial strains *Streptococcus mutans* ATCC25175 (Sm), *Lactobacillus acidophilus* ATCC4356 (La), and *Veillonella atypica* ATCC17744 (Va) were cultured on blood agar plates (BAP) (BD BBL™, Thermo Fisher Scientific, Waltham, MA, USA) and in brain heart infusion (BHI) (BD Bacto™, Thermo Fisher Scientific, Waltham, MA, USA) broth containing 5 μg/mL hemin (MilliporeSigma, St. Louis, MO, USA) and 0.2 μg/mL menadione (vitamin K) (MilliporeSigma, St. Louis, MO, USA). The bacteria were maintained in an anaerobic chamber, as described above. For growth experiments, overnight cultures were inoculated into BHI broth containing a range of concentrations (0, 5, 25, and 100 μg/mL) of either amixicile or metronidazole to an OD_{600} of 0.05 under anaerobic conditions. Cultures grown in BHI without antibiotics were used as the control. The bacteria were grown for 24 h, and the growth of the bacteria was monitored by measuring OD_{600}.

2.4. Preparation of DNA

Cell pellets were suspended in 50 mM EDTA containing 10 mg/mL lysozyme and 100 U/mL mutanolysin (MilliporeSigma, St. Louis, MO, USA) and incubated at 37 °C for 1 h. DNA from microbial cell pellets was prepared using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The integrity of the DNA was verified by nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel. DNA was stored at −80 °C.

2.5. Quantitative PCR (qPCR)

The total bacteria (universal 16S rRNA primer) and species-specific 16S rRNA primer sequences used in this study are shown in Table 1 (Supplementary Table 1). Purified DNA (1 μL) and primers were added to the Fast SYBR Green Mastermix (Thermo-Fisher, Waltham MA, USA) and ran using standard cycle conditions: 95 °C for 20 s (1 cycle); 95 °C for 3 s, 60 °C for 30 s (40 cycles). The samples were run on the 7500 Fast Real-time PCR machine (Thermo-Fisher, Waltham MA, USA). The cycle threshold (Ct) data were collected and then converted to absolute fold change.

2.6. Metagenome sequencing

Shotgun metagenome sequencing was performed in this study. Samples for sequencing were prepared using the ThruPLEX DNA-seq Kit (Rubicon Genomics, Ann Arbor, MI USA). Libraries were barcoded for multiplexing in one lane and submitted to the VCU Nucleic Acid Core. Sequencing was done on the Illumina NextSeq 500 using the high-output kit, paired-end reads, 2 × 150 bp to generate 54 Gb of data. The samples were then deconvoluted, barcodes were trimmed, and short sequences (<100bp) were removed.

2.7. Data analysis

The data derived from the shotgun metagenomic sequencing was analyzed using MetaPhlAn2 (http://huttenhower.sph.harvard.edu/metaphlan) [25,44]. MetaPhlAn, a Metagenomic Phylogenetic Analysis method, is a robust tool that estimates the relative abundance of microbial cells using a set of clade-specific marker sequences. Those markers are derived from 2887 genomes available from the Integrated Microbial Genomes (IMG) system [45,46]. Abundance levels for each sample were obtained at the level of kingdom, phylum, class, order, family, genus, species, and strain. Heat maps were generated using the ggplot2 package in R. Genus relative abundance profiles (row-wise) were clustered using agglomerative hierarchical clustering. Euclidean distance was used as the distance metric.

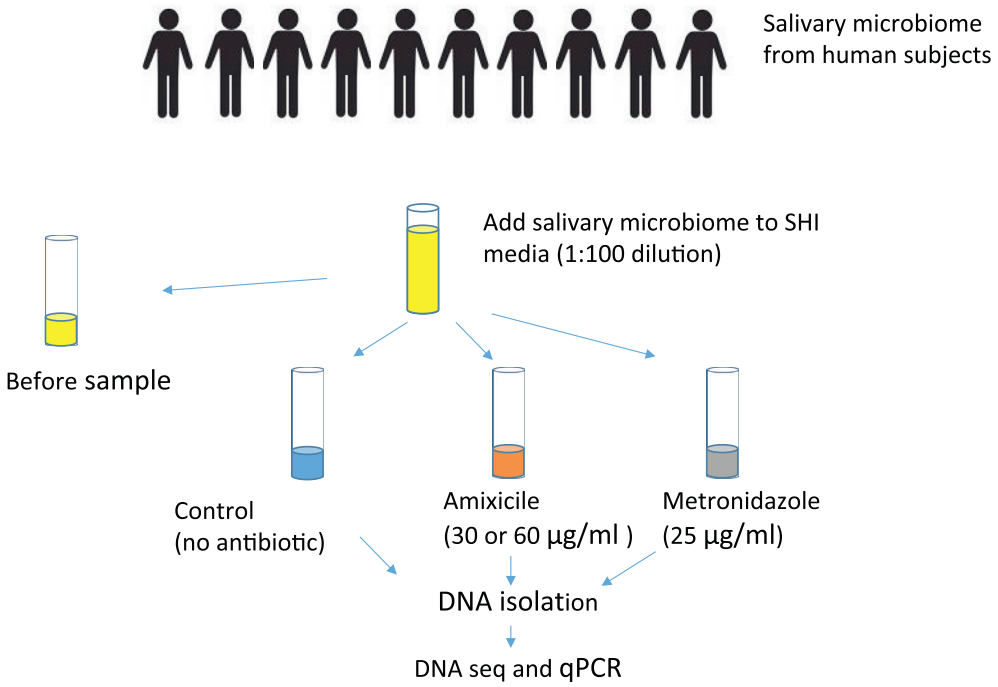


Fig. 1. Schematic representation of the workflow used for this study. The salivary microbiome (generously provided by the Shi laboratory) [22] was used as the baseline microbiome for this study. This microbiome was used as the starting material and was grown in SHI media overnight with or without antibiotics (amixicile 30 or 60 µg/ml and metronidazole 25 µg/ml). Following 16 h of growth, microbial cells were harvested, DNA was isolated, and the metagenome was determined using Illumina NextSeq.

Table 1
Effect of amixicile on bacterial abundance in salivary microbiomes.

Genus	Amix Mean[a]	Amix SD[a]	Control Mean[a]	Control SD[a]	P value[a]	Fold Change[a]
Gammaretrovirus	0.392	0.141	0.074	0.051	2.00E-04	5.294
Enterococcus	0.015	0.011	0.008	0.009	0.1416	1.892
Granulicatella	1.27	0.348	0.768	0.277	0.0164	1.653
Rothia	0.028	0.011	0.006	0.002	2.00E-04	4.588
Solobacterium	0	0.001	0.022	0.011	8.00E-04	0.01
Gemella	2.547	0.457	4.02	2.309	0.4079	0.634
Lactobacillus	0.531	0.368	27.45	16.046	2.00E-04	0.019
Prevotella	0.266	0.134	0.435	0.102	0.0549	0.612
Veillonella	0.383	0.157	6.005	2.711	2.00E-04	0.064
Parvimonas	0.038	0.014	0.046	0.028	0.536	0.822
Siphoviridae	0.324	0.312	1.248	0.525	0.0042	0.26
Alloprevotella	0.023	0.013	0.015	0.012	0.2105	1.555
Mitsuokella	0.029	0.02	0.005	0.004	0.0164	5.572
Peptostreptococcus	0.058	0.013	0.126	0.095	0.0549	0.462
Porphyromonas	0.001	0.003	0.205	0.245	8.00E-04	0.006
Abiotrophia	0.023	0.009	0.021	0.012	0.4584	1.055
Myoviridae	2.106	1.588	0.641	0.306	0.0311	3.287
Betaretrovirus	4.351	2.087	0.625	0.417	2.00E-04	6.963
Fusobacterium	0.264	0.088	0.439	0.307	0.4079	0.601
Klebsiella	24.11	3.529	4.771	2.575	2.00E-04	5.054
Campylobacter	0.027	0.013	0.013	0.005	0.0311	2.018
Streptococcus	60.481	7.41	52.657	12.605	0.4079	1.149
Haemophilus	0.084	0.032	0.028	0.01	7.00E-04	2.965
Neisseria	2.436	1.996	0.262	0.109	2.00E-04	9.299

[a] Results are derived from nine independent experiments for control and seven independent experiments for amixicile-treated samples. Amix; amixicile-treated samples, Control; samples grown without antibiotics, Fold change; ratio of Amix/Control.

2.8. Statistical analysis

Microbial abundance data obtained using high throughput sequencing were analyzed using the Wilcoxon-signed ranks test. *P* values were calculated for pairs where the abundance for each microbial genus grown in the presence of an antibiotic was compared to the abundance of the microbial genus in an untreated control sample. Growth study results were analyzed with a two-tail *t*-test. All experiments were performed in triplicate unless otherwise noted. *P* values for the statistical significance of the growth studies were calculated and compared to the untreated control.

2.9. Accession number

High throughput sequencing data were deposited to NCBI's Sequence Read Archive (SRA) with the accession number SUB5200315.

3. Results

3.1. Growth of oral microbiome bacteria under in vitro broth culture

We determined microbial abundance for the baseline microbiome (aliquots of the microbiome as obtained from Dr. Shi's laboratory prior to cultivation) and for the microbiome grown for 16 h anaerobically in SHI media (control microbiome). Eight independent libraries for the baseline microbiome (prepared on different days using the same stock microbiome obtained from Dr. Shi's laboratory, thus, serving as technical replicates) and nine independent libraries prepared using samples grown on different days for the control microbiome were prepared. The baseline microbiome contained significant levels of *Neisseria, Streptococcus*, and *Lactobacillus* (Fig. 2). It is noteworthy that it also contained significant levels of anaerobic bacteria such as *Veillonella, Prevotella, Fusobacterium*, and *Porphyromonas* (Fig. 2). Following overnight growth (16 h) the most abundant species were *Streptococcus, Lactobacillus,* and *Klebsiella* (Fig. 2). Levels of anaerobic bacteria were reduced when compared to the baseline microbiome. However, these were still present in significant amounts (Fig. 2B). Changes at a species level are shown in a figure included as supplementary information (Fig. A1).

3.2. Amixicile reduces the abundance of anaerobic bacteria

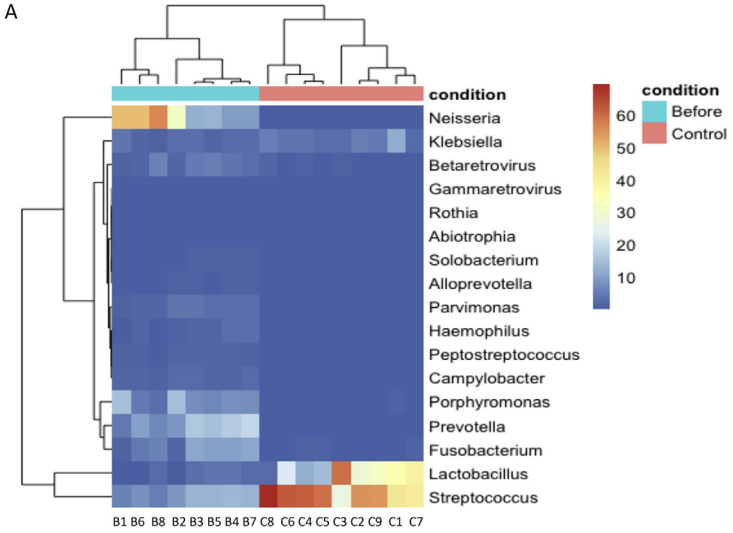
The abundance of different bacterial genera was reduced in the presence of amixicile. This reduction was mainly due to the reduction of the abundance of the anaerobic bacteria in favor of other aerobic ones. Therefore, in untreated (control) samples, the most abundant bacteria at the level of genus were *Streptococcus, Lactobacillus*, and *Klebsiella.* The most abundant anaerobic bacterium was *Veillonella* accounting for approximately 10% of the bacteria (Figs. 2 and 3). We also detected *Prevotella, Fusobacterium*, and at a lower level *Porphyromonas* (Fig. 3 and Table 1). When compared to samples treated with amixicile, the levels of *Veillonella, Prevotella*, and *Fusobacterium* were drastically reduced (fold change [amixicile/control] for the genera was 0.064, 0.612, and 0.601, respectively) while *Porphyromonas* was not detected in the amixicile-treated samples (Fig. 3 and Table 1). The most abundant in the treated samples were *Streptococcus* and *Klebsiella* (Fig. 3 and Table 1). We also noted an increase in the abundance of *Rothia* and *Neisseria.* The abundance of *Lactobacillus* was also high in samples grown in the presence of 30 μg/ml of amixicile but was significantly reduced when cultures were supplemented with 60 μg/ml of amixicile (Fig. 3 and Table 1).

We prepared nine independent biological replicates (cultures grown on different days) and observed a significant consistency between the samples (Fig. 3 and Table 1). There was some variation between samples, but the main trend where aerobic bacteria increased in proportional abundance while anaerobic ones were reduced was observed. The main difference between our samples was due to the use of two concentrations of amixicile; in seven experiments 60 μg/ml of amixicile was used (samples A2 – 8) while in two experiments lower levels of amixicile were used (30 μg/ml) (samples A1 and A9) (Fig. 3 and Table 1). In total, 60 μg/ml of amixicile resulted in the significant growth inhibition of *Lactobacillus,* while only slight growth inhibition was noted in the presence of the lower concentrations of the antibiotic (Fig. 3). Changes observed at a species level are shown in the Supplementary Figs. A2.

3.3. The effectiveness of amixicile is comparable to that of metronidazole

We also compared the effectiveness of metronidazole in reducing the growth of anaerobic bacteria in the complex oral microbiome. The major reduction in abundance was seen for

A



B

Genus	Baseline mean	Baseline SD	Control mean	Control SD
Gammaretrovirus	0.346	0.153	0.074	0.051
Enterococcus	0	0	0.008	0.009
Actinomyces	0.324	0.2	0.001	0.001
Granulicatella	1.896	0.866	0.768	0.277
Rothia	0.243	0.128	0.006	0.002
Aggregatibacter	0.564	0.199	0.001	0.001
Slackia	0.087	0.076	0	0
Solobacterium	0.595	0.215	0.022	0.011
Gallionellaceae_unclassified	0.236	0.165	0.007	0.009
Gemella	2.801	0.772	4.02	2.309
Lactobacillus	2.141	1.491	27.45	16.046
Clostridiales_Family_XIII_Incertae_Sedis_unclassified	0.417	0.098	0.019	0.014
Prevotella	12.176	5.209	0.435	0.102
Atopobium	0.484	0.196	0.019	0.016
Weissella	0	0	0.01	0.006
Veillonella	6.79	2.249	6.005	2.711
Parvimonas	2.71	0.909	0.046	0.028
Siphoviridae_noname	0.158	0.273	1.248	0.525
Alloprevotella	0.747	0.291	0.015	0.012
Mitsuokella	0.096	0.064	0.005	0.004
Peptostreptococcus	1.463	0.441	0.126	0.095
Oribacterium	0.216	0.101	0	0
Porphyromonas	8.272	4.114	0.205	0.245
Abiotrophia	0.265	0.083	0.021	0.012
Selenomonas	0.13	0.059	0.001	0.001
Myoviridae_noname	0.236	0.189	0.641	0.306
Betaretrovirus	3.281	1.703	0.625	0.417
Stomatobaculum	0.901	0.465	0.019	0.025
Fusobacterium	6.776	3.72	0.439	0.307
Klebsiella	2.427	0.881	4.771	2.575
Campylobacter	1.833	0.556	0.013	0.005
Streptococcus	9.982	3.234	52.657	12.605
Haemophilus	1.666	0.85	0.028	0.01
Megasphaera	0.134	0.049	0.004	0.007
Neisseria	28.9	19.124	0.262	0.109

Fig. 2. Bacterial abundance at the baseline and after 16 h of growth. (A) Hierarchical cluster analysis of data derived from the baseline (starting microbiome designated as Before: samples B1-8) and control (microbiome grown for 16 h designated as Control: samples C1-9). (B) Numerical representation of the microbial abundance for the baseline microbiome and control microbiome (Mean and SD quantification). Eight independently grown microbiomes (grown on different days) were used for the analysis. Control; samples grown without antibiotics. Fold change; ratio of Amix/Control.

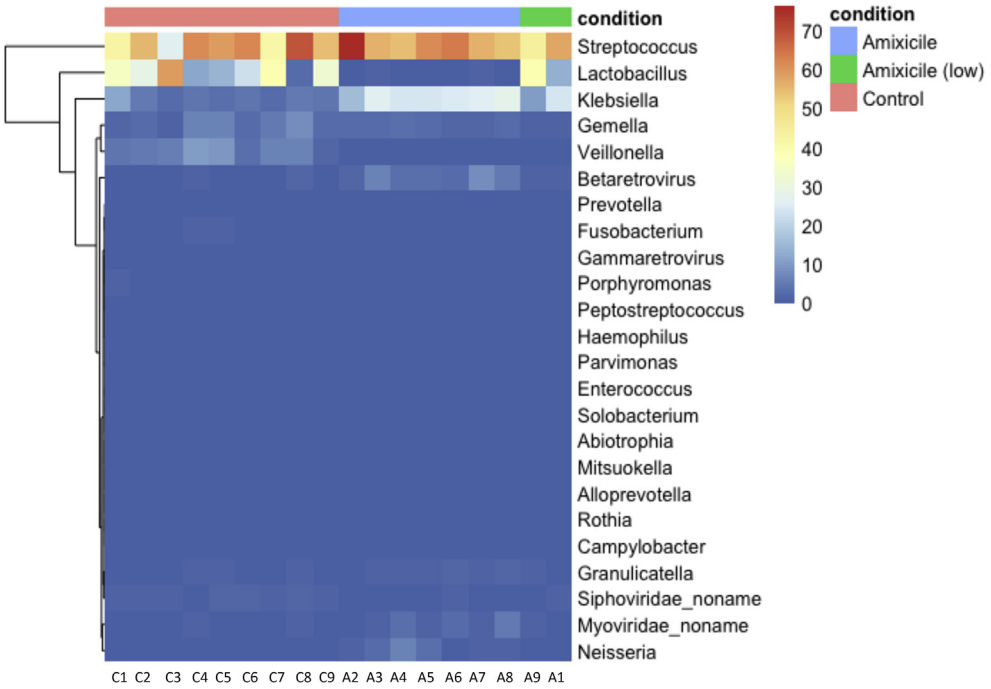


Fig. 3. Amixicile inhibits the growth of anaerobic bacteria. The salivary *ex vivo* baseline microbiome was used to prepare a culture at 1:80 dilution in SHI media. Nine independent experiments were performed. One group was grown in the presence of 30 μg/ml of amixicile (two experiments designated as Amixicile Low: samples 1 and A9) or 60 μg/ml of amixicile (seven experiments designated as Amixicile: samples C2-8) while the other was grown without antibiotics (designated as Control: samples C1-9).

Veillonella, Fusobacterium, Prevotella, and *Porphyromonas* (Fig. 4 and Supplementary Table A2) (fold change is a ratio metronidazole/control and for the genera, it was 0.011, 0.071, 0.043, and 0.001, respectively). This was associated with an increase in proportions of *Streptococcus, Klebsiella*, and *Lactobacillus* (Fig. 4 and Supplementary Table A2). In total, 1–5% of *Gemella* was observed in the treated samples. This corresponded to the abundance seen in both the control samples as well as in the amixicile-treated samples. The major difference was the lack of effect of metronidazole on the growth of *Lactobacillus* when compared with that observed for amixicile when used at 60 μg/ml (Figs. 4 and 5 and Supplementary Tables A2 and A3). However, this inhibitory effect was reduced when lower concentrations of amixicile were used (30 μg/ml). In addition, a similar abundance of *Klebsiella* and *Gemella* was

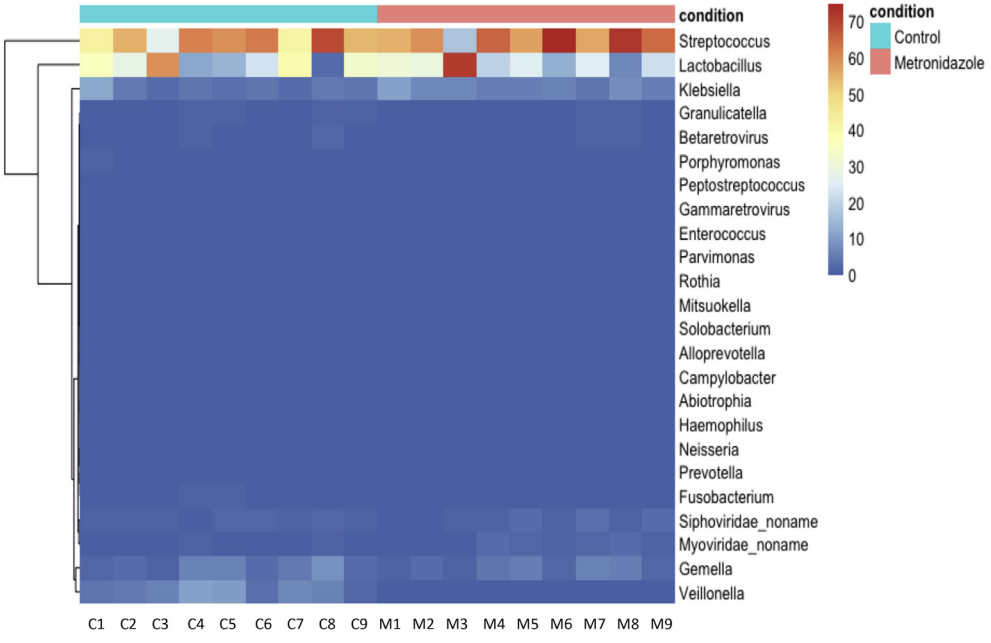


Fig. 4. Metronidazole inhibits the growth of anaerobic bacteria. The salivary *ex vivo* baseline microbiome was used to prepare a culture at 1:80 dilution in the SHI media. One aliquot was grown in the presence of 25 μg/ml of metronidazole while the other was grown without antibiotics. Nine independent experiments were prepared. The salivary microbiome was grown with metronidazole designated as Metronidazole: samples M1-9, or without antibiotics designated as Control: samples C1-9.

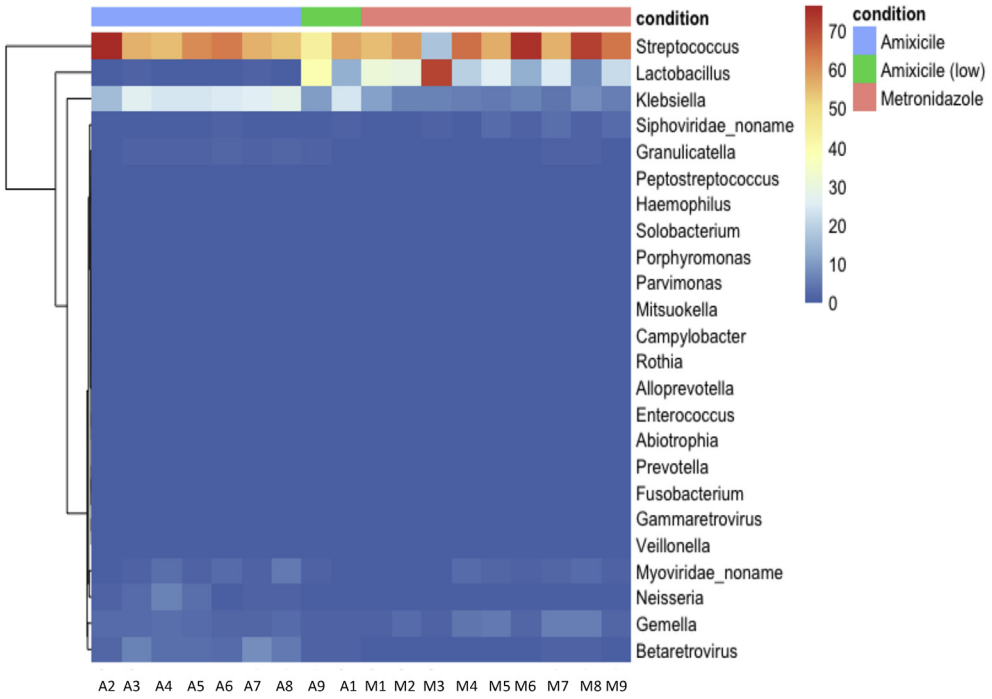


Fig. 5. Amixicile has a comparable inhibitory spectrum to that of metronidazole. The salivary *ex vivo* baseline microbiome was used to prepare a 1:80 dilution in SHI media. One aliquot was grown in the presence of 25 μg/ml of metronidazole while the other was grown in the presence of either 30 (two experiments; designated as Amixicile Low) or 60 (seven experiments; designated as Amixicile) μg/ml of amixicile. Nine independent experiments were prepared. The salivary microbiome grown with amixicile was designated as Amixicile: samples A1-9, or metronidazole designated as Metronidazole: samples M1-9.

observed in the amixicile treated samples compared to those grown in the presence of metronidazole (Figs. 4 and 5, Supplementary Tables A2 and A3). Therefore, further demonstrating a similar inhibitory spectrum for both antimicrobials. Species-level comparisons are shown in Supplementary Figs. A3 and A4 (Appendix).

3.4. Amixicile modulates bacteriophage abundance in metagenomic samples

It is noteworthy that the presence of several virus genera was also observed: *Betaretrovirus*, *Gammaretrovirus*, *Siphoviridae*, and *Myoviridae* (Figs. 2–5, Table 1, Supplementary Tables A2 and A3). Their abundance varied depending on the presence of the antibiotic. However, as viruses are not affected by antibiotics and, in this case, were most probably hosted by bacteria, the difference in their number also corresponded to the difference in specific groups of bacteria. *Siphoviridae* were significantly reduced in the amixicile-treated samples compared to the control samples (0.26 fold change), while *Myoviridae* were significantly elevated in abundance (3.3 fold change). *Myoviridae* were also elevated in metronidazole-treated samples, while sample dependent variation was observed for the *Siphoviridae* with metronidazole. The presence of both bacteriophage genera is in agreement with previously published data [26]. Overall, these data indicate that oral microflora carries a significant load of viral genomes. Based on the changes in bacterial abundance, some correlations can be drawn between *Betaretrovirus* and *Klebsiella* as well as between *Siphoviridae* and *Lactobacillus.*

3.5. Verification of metagenomic sequencing using qPCR

The reduction in the abundance of anaerobic bacteria following antibiotic treatment as detected using high throughput sequencing was also examined using qPCR. As shown in Fig. 6, the abundance of *F. nucleatum* was reduced by 4.47-fold in amixicile-treated samples compared to control samples. Additionally, *Prevotella intermedia* abundance was reduced by 10.57-fold in the amixicile-treated samples compared to the samples grown in the absence of amixicile. For bacteria grown with metronidazole, reduction was observed 11.72-fold and 21.39-fold for *F. nucleatum* and *P. intermedia*, respectively, compared to cultures grown without antibiotics. These results verify our data obtained using high throughput sequencing.

3.6. Verification of the microbiome analysis using bacterial monocultures

Three bacterial strains were used to verify the results obtained from our microbiome study: *S. mutans, V. atypica,* and *L. acidophilus* (Fig. 7A–C). For *S. mutans*, some growth inhibition (50% for amixicile and 30% for metronidazole) was observed at 6 h of growth when using the highest concentrations of the antibiotic (100 μg/ml) (Fig. 7A). This growth reduction was statistically significant, $P < 0.05$ (two-tail t-test). However, this inhibition was alleviated at 24 h, and no statistically significant differences in growth were observed at 24 h of growth [$P = 0.26$ (amixicile) and $P = 0.35$ (metronidazole) (two-tail t-test)]. *L. acidophilus* was inhibited by 45% at 6 h with the highest concentration of amixicile (100 μg/ml), and this inhibition deepened at 24 h (55%) (Fig. 7B). Slight inhibition was observed when using 25 μg/ml of amixicile. No statistically significant inhibition was observed in the presence of metronidazole [$p = 0.14$, 0.19, and 0.47 by two-tail t-test for metronidazole (5, 25, 100 μg/ml)]. The most drastic inhibition was seen for *V. atypica* in the presence of both antibiotics (Fig. 7C). Significant inhibition ($p < 0.0001$ by two-tail t-test) was observed at 4 and 6 h with all concentrations of antibiotics (5, 25, and 100 μg/ml). However, at 24 h *V. atypica* with 5 μg/ml of either amixicile or metronidazole grew to the level similar to the bacterium grown in the absence of antibiotics. Additionally, *V. atypica* was able to grow to higher levels

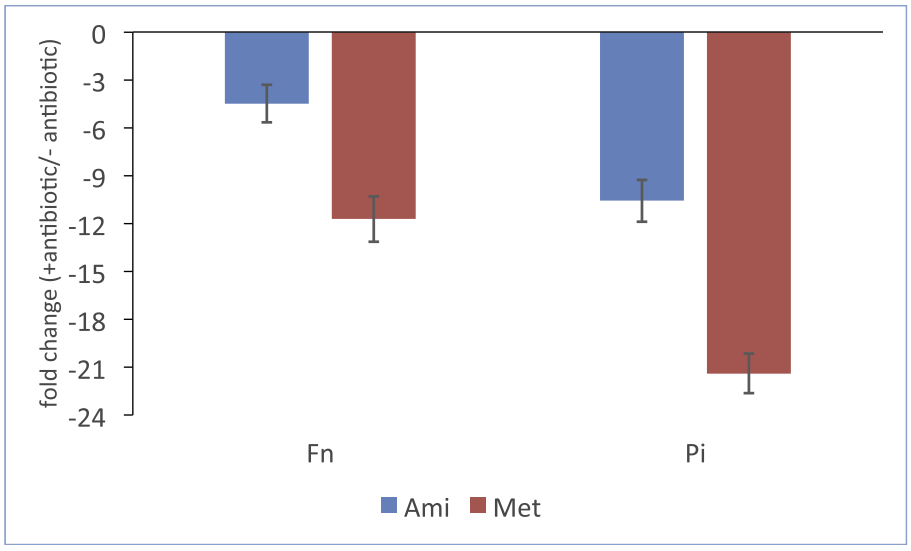


Fig. 6. Verification of the metagenomic sequencing data using qPCR. The DNA derived from the microbiome samples was subjected to qPCR analysis using primers specific for *P. intermedia* and *F. nucleatum*. The amount of DNA was standardized using a 16S rRNA universal primer. Ratios for the amounts of DNA in the samples were derived from the antibiotic supplemented cultures versus the cultures grown without antibiotics (control).

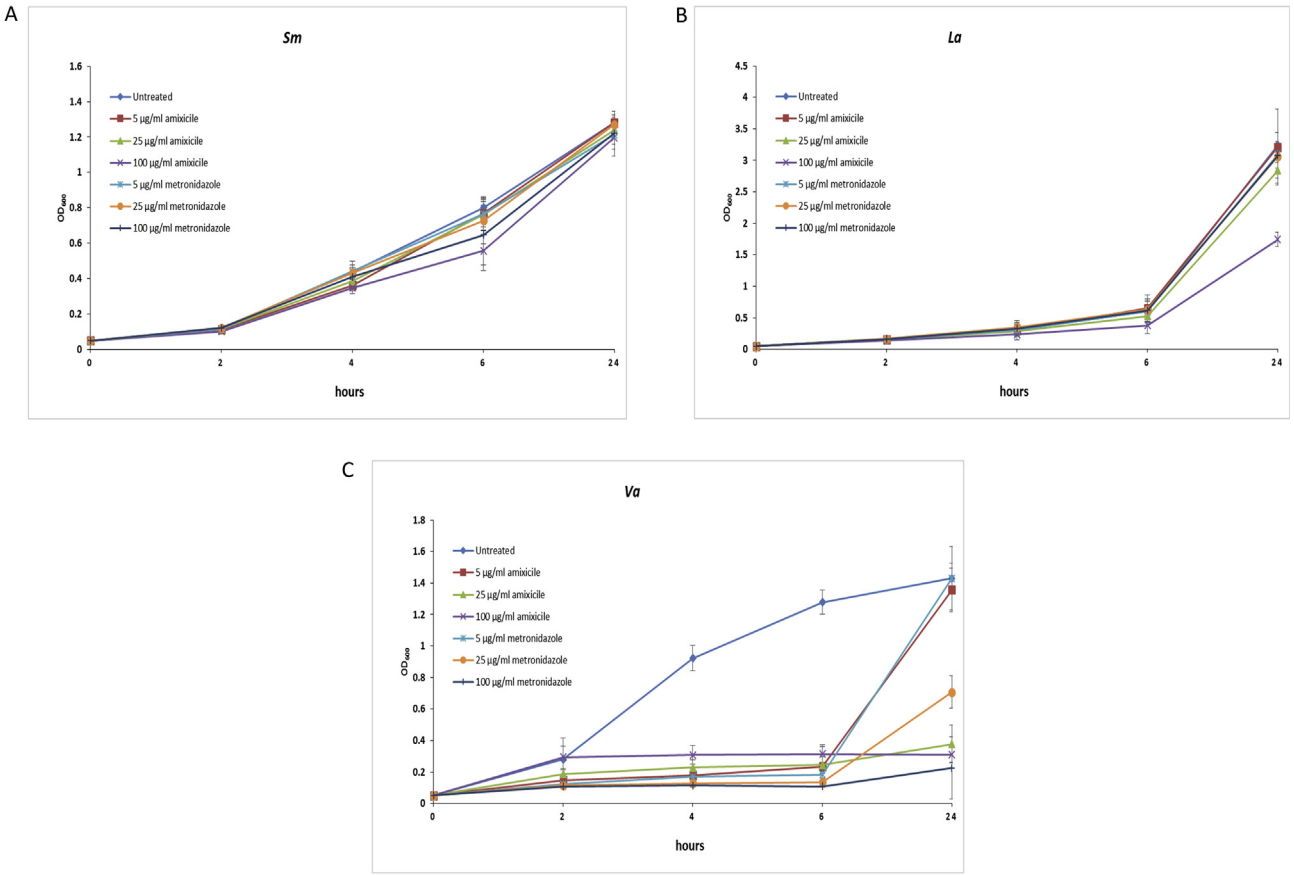


Fig. 7. The effect of amixicile and metronidazole concentrations on the growth of oral bacteria. Overnight cultures of oral bacterial species *Streptococcus mutans* ATCC25175 (A – Sm), *Lactobacillus acidophilus* ATCC4356 (B - La), and *Veillonella atypica* ATCC17744 (C - Va) were inoculated into the BHI broth containing either amixicile (0, 5, 25, or 100 μg/mL) or metronidazole (0, 5, 25, or 100 μg/mL) to an OD_{600} of 0.05 under anaerobic conditions. Cultures grown in BHI without antibiotics (untreated) served as the control. The bacteria were grown for 24 h, and the growth of the bacteria was monitored by measuring OD_{600} at 0, 2, 4, 6, and 24 h. The means and SD from two biological replicates performed in technical triplicates are shown.

with 25 μg/ml of metronidazole than with 100 μg/ml of metronidazole. In summary, the growth of this bacterium is completely inhibited with higher (25 μg/ml for amixicile or 100 μg/ml for metronidazole) antibiotic concentrations, thus, demonstrating the effectiveness of both antimicrobials against this anaerobic bacterium.

4. Discussion

Our data shows that amixicile specifically inhibits anaerobic bacteria in the salivary microbiome. Although the proportion of anaerobic bacteria in the salivary microbiome is lower compared with the periodontal pocket, given optimal conditions, these bacteria may overgrow and trigger periodontal disease [27]. Among the bacteria that had reduced abundance in the presence of the antimicrobial, were *Prevotella, Fusobacterium, Porphyromonas,* and *Veillonella.* While the role of *Porphyromonas* as a periodontopathogen is well established, the role of the other anaerobes is less established with respect to their ability to induce periodontitis. However, it is known that *P. gingivalis* has higher virulence when co-inoculated with *F. nucleatum* than when used as a mono-infection [28,29].

Additionally, *Prevotella,* although a member of the less virulent orange cluster, can alter its behavior and become more pathogenic in diseased sites [30–32]. Anaerobic bacteria, such as *Veillonella,* are highly active and contribute significant putative virulence factors in diseased sites when explored using a metatranscriptomic approach [33]. Although many studies do associate *Veillonella* with healthy sites [34], a higher prevalence of both *Prevotella* and *Veillonella* in the salivary microbiome has been associated with poor oral health [35] suggesting that those bacteria also play a role in maintenance of a dysbiotic microbiome. This has further been corroborated by Mashima et al., who have shown that an increase in *Veillonella* is associated with poor oral health in children [48]. Recently, *Veillonella* has also been shown to be present in higher levels in subjects with periodontal diseases and aggressive periodontitis when compared to healthy subjects, thus, indicating its potential role in the disease [47]. Finally, conversion of a healthy microbiome into a dysbiotic one under *in vitro* conditions is shown to be associated with an increase in levels of *Veillonella* [49].

The reduction of anaerobic periodontopathogens may also be of significance when designing preventive strategies targeting individuals at risk before the disease fully develops and becomes clinically noticeable. In addition, in periodontally diseased individuals, usually a mixture of diseased and healthy sites are present and, thus, use of an antibiotic will affect all sites. Our data show that amixicile reduces the incidence of periodontal pathogens without affecting health-associated bacteria such as *Streptococcus, Lactobacillus, Klebsiella, Neisseria*, and *Rothia*. This is consistent with the recently proposed model for a dysbiosis ratio where higher levels of both *Neisseria* and *Rothia* are associated with health [34]. Since amixicile does not affect health-promoting bacteria, it may also be a candidate for use as a preventive therapy, especially for individuals at risk.

As a shotgun metagenomic analysis was conducted in this study rather than the sequencing of 16S rRNA for microbial profiling, it was also able to detect other organisms in addition to bacteria. Of particular interest, is the variation in abundance of several groups of viruses; *Betaretrovirus*, *Gammaretrovirus*, *Siphoviridae*, and *Myoviridae*. Due to the high prevalence and diversity of bacteriophages within the oral cavity, our study may contribute additional clues with regards to the modulation of the oral microbiome [26]. Indeed, bacteriophages belonging to both *Siphoviruses* and *Myoviruses* groups were shown to be associated with differences in periodontal health status [36]. Finally, the finding regarding betaretroviruses is novel.

Our results indicate that amixicile at high concentrations (60 μg/ml) may have off-target effects and will guide our subsequent work that will include amixicile at concentrations lower than 30 μg/ml. Indeed, we have previously shown that amixicile is effective in inhibiting anaerobic bacteria at concentrations as low as 1 μg/ml for *P. gingivalis* and *F. nucleatum*, and 5 μg/ml for *P. intermedia* [20]. Supporting the systemic use of amixicile, pharmacokinetic studies indicate that oral amixicile, like metronidazole, is efficiently absorbed. However, unlike metronidazole, amixicile is eliminated via the renal system and does not concentrate in the feces [37]. Further study will be needed to verify that amixicile indeed concentrates sufficiently in periodontal pockets to be an effective therapeutic. So far amixicile has been validated in animal models for the treatment of colitis caused by *Clostridium difficile* and gastritis caused by *Helicobacter pylori* [37,38]. As opposed to metronidazole, no side effects were noted with concentrations as high as 300 mg/kg [37]. Amixicile also reverses weight loss in a protein-deficient mouse model of malnutrition and indirectly resolves infection by *Cryptosporidium parvum* [39].

Our studies also verify that amixicile specifically inhibits anaerobic bacteria at concentrations lower than 30 μg/ml. The 30 μg/ml concentration (and lower) would be used when administering amixicile systemically (in the form of a pill). The higher concentration reflects the amixicile concentration that would be achievable when administering amixicile topically (using gels supplemented with high concentrations of the antimicrobial). Based on our study, the selective inhibition of anaerobic bacteria in a complex oral microbiome is achieved with lower amixicile concentration.

So far, the only antimicrobial used to deplete the complex microbiome of specific bacteria was reported by the Shi laboratory [23]. This antimicrobial, peptide C16G2, was demonstrated to specifically target *S. mutans* [23]. Having amixicile allows the exploration of the contribution of anaerobes to driving periodontal disease as well as to delineate the mechanisms involved in the triggering of the inflammatory state of the host by this group of bacteria. As shown in our supplementary data (Figs. A2–A5), a reduction in the numbers of anaerobic bacteria affects the composition of the aerotolerant bacteria.

From a clinical perspective, the need for antimicrobials that specifically target anaerobes is further underscored by promising results obtained using metronidazole, where it reduced the use of periodontal surgery in patients for up to six years [40]. However, the risk of dysbiosis, as well as adverse effects, is associated with the use of metronidazole [17,18] and, thus, is not commonly used to treat periodontal disease. Such side effects are anticipated to be minimal when using amixicile as no side effects have been noted so far in preclinical studies when using up to 300 mg/kg of amixicile [37]. The added benefit of amixicile is that it concentrates at inflamed sites and has no effect on gut microbiota as it is absorbed in the upper intestine. Amixicile is also effective against internalized bacteria that usually serve as a reservoir for recurrent periodontitis [20].

Finally, it is realized that there are limitations to this study, especially regarding the fully-developed periodontal disease. As this is a salivary microbiome derived from healthy subjects, its composition may differ from that of the microbiome derived from periodontal pockets where anaerobic bacteria are more abundant [5,6,22,41,42]. Therefore, future work that will involve the characterization of the effect of amixicile and metronidazole on microbiomes derived from periodontal pockets of subjects with periodontal disease is needed. This will also be in line with the observation that amixicile is predicted to concentrate in periodontal pockets as a result of inflammation. Such a prediction will ultimately underscore the selectivity of amixicile over metronidazole that is found mainly in saliva in *in vivo* conditions [37].

Additionally, microbiomes grown in the form of a biofilm-like setting are yet to be tested using both anaerobic as well as microaerophilic conditions [43]. Finally, in our studies, a culture method was used to evaluate the efficacy of amixicile. Such a method does not reflect the complexity encountered in *in vivo* conditions, where

the environmental conditions (nutrition, oxygen and pH levels, and effect of host response) lead to the formation of a more complex microbiome. SHI media shown to preserve microbial diversity was used. However, more thorough studies using a suitable animal model with a microbiome similar in complexity to that encountered in a human host is needed.

5. Conclusions

Anaerobic bacteria are the culprit of periodontal disease, and amixicile, specifically targeting this group of bacteria, presents itself as a promising targeted therapy for periodontitis. Using an *ex vivo* salivary microbiome, this study shows that amixicile specifically inhibits the growth of PFOR containing bacteria. The reduction in abundance of anaerobic bacteria is associated with an increase in the proportion of aerotolerant bacteria that are mainly health-promoting organisms. Furthermore, the presence of bacteriophages was detected in the salivary microbiome, the abundance of which was also altered proportionally to the bacterial levels. Finally, the profile of amixicile is similar to that of metronidazole, an antimicrobial targeting anaerobic bacteria that has already been successfully shown to treat periodontal disease. Altogether, our studies further verify that amixicile has the potential to convert a complex microbiome into one with an overabundance of bacteria associated with periodontal health and, thus, shows promise for clinical application.

CRediT authorship contribution statement

Qin Gui: Investigation, Methodology, Validation, Visualization, Formal analysis, Conceptualization, Writing - review & editing. **Paul S. Hoffman:** Writing - review & editing, Resources. **Janina P. Lewis:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft.

Author contributions

Q. G. performed most of the experiments, analyzed the data, prepared figures for the manuscript, and contributed to the writing of the manuscript. J. P. L. conceived the idea for this project, performed growth studies, and supervised all of the work reported in this manuscript. J.P.L. also wrote the first draft and revised the manuscript. P. S. H. provided the amixicile as well as helpful comments for this work and revised the manuscript. All the authors reviewed the manuscript.

Ethical statement

Ethical approval is not required for this article.

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Appendix A. Supplementary data

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References

[1] Pretzl B, Sälzer S, Ehmke B, Schlagenhauf U, Dannewitz B, Dommisch H, et al. Administration of systemic antibiotics during non-surgical periodontal therapy-a consensus report. Clin Oral Investig 2018;23(7):3073–85. https://doi:10.1007/s00784-018-2727-0.
[2] Fritoli A, Gonçalves C, Faveri M, Figueiredo LC, Pérez-Chaparro PJ, Fermiano D, et al. The effect of systemic antibiotics administered during the active phase of non-surgical periodontal therapy or after the healing phase: a systematic review. J Appl Oral Sci Rev FOB 2015;23:249–54. https://doi.org/10.1590/1678-775720140453.
[3] Al-Kamel A, Baraniya D, Al-Hajj WA, Halboub E, Abdulrab S, Chen T, et al. Subgingival microbiome of experimental gingivitis: shifts associated with the use of chlorhexidine and N-acetyl cysteine mouthwashes. J Oral Microbiol 2019;11:1608141. https://doi.org/10.1080/20002297.2019.1608141.
[4] Krishnan K, Chen T, Paster BJ. A practical guide to the oral microbiome and its relation to health and disease. Oral Dis 2017;23:276–86. https://doi.org/10.1111/odi.12509.
[5] Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. Nat Rev Microbiol 2018;16:745–59. https://doi.org/10.1038/s41579-018-0089-x.
[6] Wade WG. The oral microbiome in health and disease. Pharmacol Res 2013;69:137–43. https://doi.org/10.1016/j.phrs.2012.11.006.
[7] Nakayama M, Ohara N. Molecular mechanisms of *Porphyromonas gingivalis*-host cell interaction on periodontal diseases. Jpn Dent Sci Rev 2017;53:134–40. https://doi.org/10.1016/j.jdsr.2017.06.001.
[8] Hajishengallis G, Lamont RJ. Breaking bad: manipulation of the host response by *Porphyromonas gingivalis*. Eur J Immunol 2014;44:328–38. https://doi.org/10.1002/eji.201344202.
[9] Deng Z-L, Szafrański SP, Jarek M, Bhuju S, Wagner-Döbler I. Dysbiosis in chronic periodontitis: key microbial players and interactions with the human host. Sci Rep 2017;7:3703. https://doi.org/10.1038/s41598-017-03804-8.
[10] Ibrahim M, Subramanian A, Anishetty S. Comparative pan genome analysis of oral *Prevotella* species implicated in periodontitis. Funct Integr Genom 2017;17:513–36. https://doi.org/10.1007/s10142-017-0550-3.
[11] Mysak J, Podzimek S, Sommerova P, Lyuya-Mi Y, Bartova J, Janatova T, et al. *Porphyromonas gingivalis*: major periodontopathic pathogen overview. J Immunol Res 2014;2014:476068. https://doi.org/10.1155/2014/476068.
[12] 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. https://doi.org/10.1111/j.1600-0757.2005.00113.x.
[13] Dashper SG, Seers CA, Tan KH, Reynolds EC. Virulence factors of the oral spirochete *Treponema denticola*. J Dent Res 2011;90:691–703. https://doi.org/10.1177/0022034510385242.
[14] Sharma A. Virulence mechanisms of *Tannerella forsythia*. Periodontol 2000 2010;54:106–16. https://doi.org/10.1111/j.1600-0757.2009.00332.x.
[15] Fenno JC, Hannam PM, Leung WK, Tamura M, Uitto VJ, McBride BC. Cytopathic effects of the major surface protein and the chymotrypsinlike protease of *Treponema denticola*. Infect Immun 1998;66:1869–77.
[16] Ishihara K. Virulence factors of *Treponema denticola*. Periodontol 2000 2010;54:117–35. https://doi.org/10.1111/j.1600-0757.2009.00345.x.
[17] Sørensen CG, Karlsson WK, Amin FM, Lindelof M. Metronidazole-induced encephalopathy: a systematic review. J Neurol 2018. https://doi.org/10.1007/s00415-018-9147-6.
[18] Dingsdag SA, Hunter N. Metronidazole: an update on metabolism, structure-cytotoxicity and resistance mechanisms. J Antimicrob Chemother 2018;73:265–79. https://doi.org/10.1093/jac/dkx351.
[19] McGowan K, McGowan T, Ivanovski S. Optimal dose and duration of amoxicillin-plus-metronidazole as an adjunct to non-surgical periodontal therapy: a systematic review and meta-analysis of randomized, placebo-controlled trials. J Clin Periodontol 2018;45:56–67. https://doi.org/10.1111/jcpe.12830.
[20] Hutcherson JA, Sinclair KM, Belvin BR, Gui Q, Hoffman PS, Lewis JP. Amixicile, a novel strategy for targeting oral anaerobic pathogens. Sci Rep 2017;7:10474. https://doi.org/10.1038/s41598-017-09616-0.
[21] 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;89:1467–74. https://doi.org/10.1002/JPER.17-0185.
[22] Edlund A, Yang Y, Hall AP, Guo L, Lux R, He X, et al. An in vitro biofilm model system maintaining a highly reproducible species and metabolic diversity approaching that of the human oral microbiome. Microbiome 2013;1:25. https://doi.org/10.1186/2049-2618-1-25.

[23] Guo L, McLean JS, Yang Y, Eckert R, Kaplan CW, Kyme P, et al. Precision-guided antimicrobial peptide as a targeted modulator of human microbial ecology. Proc Natl Acad Sci U S A 2015;112:7569–74. https://doi.org/10.1073/pnas.1506207112.
[24] Tian Y, He X, Torralba M, Yooseph S, Nelson KE, Lux R, et al. Using DGGE profiling to develop a novel culture medium suitable for oral microbial communities. Mol Oral Microbiol 2010;25:357–67. https://doi.org/10.1111/j.2041-1014.2010.00585.x.
[25] Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C. Metagenomic microbial community profiling using unique clade-specific marker genes. Nat Methods 2012;9:811–4. https://doi.org/10.1038/nmeth.2066.
[26] Pride DT, Salzman J, Haynes M, Rohwer F, Davis-Long C, White RA, et al. Evidence of a robust resident bacteriophage population revealed through analysis of the human salivary virome. ISME J 2012;6:915–26. https://doi.org/10.1038/ismej.2011.169.
[27] Naginyte M, Do T, Meade J, Devine DA, Marsh PD. Enrichment of periodontal pathogens from the biofilms of healthy adults. Sci Rep 2019;9:5491. https://doi.org/10.1038/s41598-019-41882-y.
[28] Metzger Z, Lin Y-Y, Dimeo F, Ambrose WW, Trope M, Arnold RR. Synergistic pathogenicity of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in the mouse subcutaneous chamber model. J Endod 2009;35:86–94. https://doi.org/10.1016/j.joen.2008.10.015.
[29] Polak D, Shapira L, Weiss EI, Houri-Haddad Y. The role of coaggregation between *Porphyromonas gingivalis* and *Fusobacterium nucleatum* on the host response to mixed infection. J Clin Periodontol 2012;39:617–25. https://doi.org/10.1111/j.1600-051X.2012.01889.x.
[30] Byrne DP, Potempa J, Olczak T, Smalley JW. Evidence of mutualism between two periodontal pathogens: co-operative haem acquisition by the HmuY haemophore of *Porphyromonas gingivalis* and the cysteine protease interpain A (InpA) of *Prevotella intermedia*. Mol Oral Microbiol 2013;28:219–29. https://doi.org/10.1111/omi.12018.
[31] Yanagisawa M, Kuriyama T, Williams DW, Nakagawa K, Karasawa T. Proteinase activity of *Prevotella* species associated with oral purulent infection. Curr Microbiol 2006;52:375–8. https://doi.org/10.1007/s00284-005-0261-1.
[32] Araki H, Kuriyama T, Nakagawa K, Karasawa T. The microbial synergy of *Peptostreptococcus micros* and *Prevotella intermedia* in a murine abscess model. Oral Microbiol Immunol 2004;19:177–81. https://doi.org/10.1111/j.0902-0055.2004.00138.x.
[33] Yost S, Duran-Pinedo AE, Teles R, Krishnan K, Frias-Lopez J. Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. Genome Med 2015;7:27. https://doi.org/10.1186/s13073-015-0153-3.
[34] Meuric V, Le Gall-David S, Boyer E, Acuña-Amador L, Martin B, Fong SB, et al. Signature of microbial dysbiosis in periodontitis. Appl Environ Microbiol 2017;83(14). e00462-17, https://doi:10.1128/AEM.e00462-17.
[35] Yamashita Y, Takeshita T. The oral microbiome and human health. J Oral Sci 2017;59:201–6. https://doi.org/10.2334/josnusd.16-0856.
[36] Santiago-Rodriguez TM, Naidu M, Abeles SR, Boehm TK, Ly M, Pride DT. Transcriptome analysis of bacteriophage communities in periodontal health and disease. BMC Genomics 2015;16:549. https://doi.org/10.1186/s12864-015-1781-0.
[37] Hoffman PS, Bruce AM, Olekhnovich I, Warren CA, Burgess SL, Hontecillas R, 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;58:4703–12. https://doi.org/10.1128/AAC.03112-14.
[38] Warren CA, van Opstal E, Ballard TE, Kennedy A, Wang X, Riggins M, et al. Amixicile, a novel inhibitor of pyruvate: ferredoxin oxidoreductase, shows efficacy against *Clostridium difficile* in a mouse infection model. Antimicrob Agents Chemother 2012;56:4103–11. https://doi.org/10.1128/AAC.00360-12.
[39] Bartelt LA, Bolick DT, Kolling GL, Stebbins E, Huston CD, Guerrant RL, et al. Amixicile reduces severity of Cryptosporidiosis but does not have in vitro activity against Cryptosporidium. Antimicrob Agents Chemother 2018;62(12). e00718-18, https://doi:10.1128/AAC.00718-18.
[40] Loesche WJ, Giordano JR, Soehren S, Kaciroti N. The nonsurgical treatment of patients with periodontal disease: results after 6.4 years. Gen Dent 2005;53:298–306. quiz 307.
[41] Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu W-H, et al. The human oral microbiome. J Bacteriol 2010;192:5002–17. https://doi.org/10.1128/JB.00542-10.
[42] Aruni AW, Dou Y, Mishra A, Fletcher HM. The biofilm community-rebels with a cause. Curr Oral Health Rep 2015;2:48–56. https://doi.org/10.1007/s40496-014-0044-5.
[43] Lewis JP, Iyer D, Anaya-Bergman C. Adaptation of *Porphyromonas gingivalis* to microaerophilic conditions involves increased consumption of formate and reduced utilization of lactate. Microbiol Read Engl 2009;155:3758–74. https://doi.org/10.1099/mic.0.027953-0.
[44] Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, et al. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. Nat Methods 2015;12:902–3.
[45] Markowitz VM, Chen IM, Palaniappan K, Chu K, Szeto E, Grechkin Y, et al. The integrated microbial genomes system: an expanding comparative analysis resource. Nucleic Acids Res 2010;38:D382–90.
[46] Markowitz VM, Chen IM, Palaniappan K, Chu K, Szeto E, Pillay M, et al. IMG 4 version of the integrated microbial genomes comparative analysis system. Nucleic Acids Res 2014;42:D560–7.
[47] Wei Y, Shi M, Zhen M, Wang C, Hu W, Nie Y, et al. Comparison of subgingival and buccal mucosa microbiome in chronic and aggressive periodontitis: a pilot study. Front Cell Infect Microbiol 2019;9:53.
[48] Mashima I, Theodorea CF, Thaweboon B, Thaweboon S, Scannapieco FA, Nakazawa F. Exploring the salivary microbiome of children stratified by the oral hygiene index. PLoS One 2017;12:e0185274.
[49] Naginyte M, Do T, Meade J, Devine DA, Marsh PD. Enrichment of periodontal pathogens from the biofilms of healthy adults. Sci Rep 2019;9:5491.
[50] Hoffman PS, Sisson G, Croxen MA, Welch K, Harman WD, Cremades N. Antiparasitic drug nitazoxanide inhibits the pyruvate oxidoreductases of *Helicobacter pylori*, selected anaerobic bacteria and parasites, and *Campylobacter jejuni*. Antimicrob Agents Chemother 2007;51:868–76.