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The Effects of Amixicile on Sub-gingival Biofilm Cultured from Humans

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

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Abstract

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By Kian Azarnoush, DMD

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Abstract: Periodontitis is an inflammatory disease of the oral cavity induced by anaerobic bacteria, that remains to be the primary cause of tooth loss in adults worldwide. Finding an anti-microbial therapeutic to selectively target periodontal pathogens has proven to be difficult, and current treatment modalities only provide a transient benefit. Amixicile is a non-toxic, readily bioavailable novel antimicrobial that targets strict anaerobes through inhibition of the activity of Pyruvate Ferredoxin Oxidoreductase (PFOR), a major enzyme mediating oxidative decarboxylation of pyruvate, a critical step in metabolism. Our study aimed to evaluate the efficacy of amixicile in inhibiting the growth of bacteria harvested from the complex sub-gingival biofilm of patients with chronic periodontitis. We hypothesize that amixicile will selectively inhibit pathogenic anaerobic bacteria collected from patients, with the same efficacy as metronidazole, the current accepted treatment modality.

Plaque samples were harvested from patients with severe chronic periodontitis and cultured under anaerobic conditions. The microbiomes were grown in the presence of amixicile and metronidazole and the growth was compared to that of bacteria grown in the absence of the antimicrobials. Following 24 hour incubation, bacterial DNA was isolated and bacterial quantity was evaluated by quantitative PCR (qPCR) using primers specific for 12 bacterial species: *P. gingivalis* (Pg), *P. intermedia* (Pi), *F.nucleatum* (Fn), *S.gordonii* (Sg), *S. anginosus* (Sa), *V. atypical* (Va), *L. acidophilus* (La), *A.actinomycetemcomitans* (Aa), *T.denticola* (Td), *S.mutans* (Sm), *S.sanguis* (Ss), and 16s. Individual qPCR runs were combined to represent an overall average of CT value differences.

Amixicile treatment groups exhibited statistical significant reductions (P<.001) for several anaerobic bacteria: *P. intermedia, F. nucleatum* and *Veillonella atypical*. When comparing amixicile to metronidazole, amixicile performed with similar efficacy with the largest effect seen for PFOR bacteria. Our conclusion supports amixicile as a potent inhibitor of anaerobic bacteria, and could be a potential new therapeutic antimicrobial in the treatment of periodontal disease.

Keywords: amixicile, metronidazole, mico-biofilms, periodontitis, q-PCR analysis

INTRODUCTION

Chronic periodontitis is an inflammatory disease of the oral cavity, induced by bacterial biofilm in a susceptible host. Research conducted in the last decade has revealed the complexity of this oral bacterial biofilm, it can no longer be viewed as a conglomeration of bacteria attached to the diseased root surface. Rather it is an organized and structured three-dimensional assembly of over 600 bacterial species, which develop multicellular units forming specific scaffolds and passageways allowing for fluid flow for nutrition and capacity to share genes for antibiotic resistance[1]. Furthermore, there appears to be a sequential acquisition of certain species within the biofilm that lay the framework for greater pathogenic potential[2]. Keystone pathogens such as *Porphyromonas gingivalis* can therefore play a significant role in orchestrating pathogenesis while only making up a fraction of the biofilm population[3,4]. These bacteria produce pro-inflammatory antigens and virulence factors such as lipopolysaccharide (LPS), altering the local environment to one more suitable for disease progression[4]. Once the host response modulation is initiated, the inflammation can spread beyond the marginal gingiva, lead to irreversible destruction of tooth supporting tissues and ultimately bone loss[5].

In fact, classic studies have already demonstrated that dental plaque and calculus are major etiologic agents in the progression of periodontal disease[6], even before the mechanism was completely understood. With an increase in the quantity of bacteria in the oral cavity, there is a shift in the microflora. In health, the predominant bacterial species is aerobic gram-positive cocci which includes the *Streptococcus* species. However, in periodontitis the predominant species are anaerobic gram-negative rods which include organisms such as *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum*, and *Tannerella forsythia*[7,8].

Disease progression in periodontitis can best be described by the polymicrobial synergy and dysbiosis model. In this model, colonizing bacteria form communities that with the aid of the host inflammatory system, can enhance the colonization and/or virulence of other bacteria (polymicrobial synergy). Eventually this results in a dysbiotic community, a state of imbalance in the relative abundance and influence of certain species on the inflammatory response. In a susceptible host, a profound and "ill advised" immune response allows the biofilm to cause enough inflammation to cause irreversible damage to the local environment[4]. During an inflammatory response, there is an activation of T and B cells along with an increase in the production cytokines, chemokines, and other mediators. Ultimately, expression levels of a protein called receptor activator of nuclear factor-kappa B ligand (RANKL) increase. When RANKL expression is enhanced relative to its competitor osteoprotegrin (OPG), RANKL is available to bind RANK receptor on osteoclast precursor cells activating osteoclast formation and bone resorption[9].

Based on this model, it is abundantly clear that although the bacterial biofilm does not directly cause bone and tissue destruction, its presence is the primary etiology of plaque induced periodontitis. Therefore, the first phase of treatment in periodontal disease is mechanical therapy, which aims to reduce bacterial biofilm present at the site of infection. This is accomplished by scaling and root planning with hand and ultrasonic instruments in an attempt to debride the teeth and soft tissue. Studies have confirmed the efficacy scaling and root planing accompanied with improved oral hygiene, resulting in a shift away from disease and back to a healthy state[10]. Scaling and root planing however does have its limitations, namely the initial pocket depth, the anatomy of the tooth root surface and the number of roots present[11,12].

Generally speaking, the deeper the probing the depth, the less likelihood of complete removal of plaque and calculus[13]. Furthermore, despite meticulous mechanical therapy, persistent bacteria can still remain due their ability to invade host cells, survive and replicate, and then serve as a reservoir for future re-infections[14].

Based on the infectious nature of periodontal disease, some clinicians have advocated the use of antibiotics as an adjunct to mechanical therapy in order to further decrease the bacterial load. Ideally targeting specific periodontal pathogens and not commensal species[15]. A 2003 systematic review analyzing the clinical benefits of antibiotics as both an adjunct to mechanical debridement and a sole therapy concluded that systemic antibiotics were uniformly beneficial in providing improvement in attachment loss when used as adjuncts to scaling and root planing; with borderline significance when used as stand-alone therapy. The clinical benefits of antibiotics however only surmounted to about 0.3mm to 0.4mm mean "gain" in attachment, indicating only a slight advantage over mechanical debridement with no antibiotics[7]. The results of that paper provide support for judicious application of antibiotics rather than routine use with periodontal therapy. A 2004 position paper on systemic antibiotic use in periodontics published by the American Academy of Periodontics further supported this notion by concluding that systemic antibiotics is only appropriated for patients that do not respond to adequate mechanical therapy, manifest acute periodontal infections, as a prophylaxis for medically compromised patients and as an adjunct to both surgical and non-surgical therapy[16]. A recent study conducted on 400 patients with chronic periodontitis being treated in the United States revealed that 74.2% patients had at least one periodontal pathogen exhibit resistance to the therapeutic concentrations of antibiotics commonly used in clinical periodontal practice. One or more periodontal

pathogens exhibited resistance to doxycycline in 220 (55.0%) patients, to amoxicillin in 173 (43.3%) patients, to metronidazole in 121 (30.3%) patients, and to clindamycin in 106 (26.5%) patients. In addition, 60 (15.0%) of the study patients harbored subgingival test periodontal pathogens resistant in vitro to both amoxicillin and metronidazole[17]. With the publication of these influential studies, it can be concluded that the risks of routinely prescribing broad spectrum antibiotics only used to treat periodontal pathogens heavily outweighs the benefits.

Yet the subgingival bacterial biofilm remains an alluring target for the treatment of periodontal disease because of its influence in dysbiosis and the subsequent progression of disease. This has led the periodontal community to seek the ideal antibiotic, one that could target only the periodontal pathogens and marginalize the chances of bacterial resistance. In the field of medicine, amixicile is a promising novel antimicrobial that affects strict anaerobes by targeting the cofactors of essential enzymatic reactions necessary for metabolism. It selectively targets the disease promoting bacteria by affecting pyruvate:ferredoxin oxidoreductase (PFOR) enzyme. PFOR catalyzes the conversion of pyruvate and Coenzyme A (CoA) to CO_2 and Acetyl-CoA and is an important component of many metabolic pathways found in anaerobic bacteria and parasites. This pathway is highly conserved, and therefore resistance to Amixicile by mutation is conceptually impossible[18–20]. In a mouse model, Amixicile was shown to have an inhibitory effect on *Clostridium difficile* infection, less systemic side effects, and reduced number of resistant bacteria when compared to vancomycin and fidaxomicin[21].

Amixicile was shown to be effective specifically against anaerobic bacteria, therefore it should also be effective against specific anaerobic bacteria present in periodontal disease. To test this

hypothesis, our lab examined the effects of amixicile on the growth of oral anaerobic pathogens associated with periodontal disease. Amixicile was able to inhibit the growth of laboratory strains of *P. gingivalis, P. intermedia* and *F. nulceatum*. This warranted further studies on multispecies broth cultures that contained equal amounts of *P.gingivalis, P. intermedia, A. actinomycetemcomitans, F. nuleatum, T. forsythia* and *S. gordonii*. DNA was isolated and qPCR analysis has shown that amixicile inhibited the growth of PFOR-containing bacteria *P. gingivalis, P. intermedia, F. nucleatum* and *T. forsythia.* The amount of inhibition was comparable to cultures treated with metronidazole, the current treatment of choice for anaerobic periodontal pathogens[22].

Our current study aimed to evaluate the efficacy of amixicile on a complex microbiome harvested from sulcus of healthy patients and the periodontal pocket of patients with severe chronic periodontitis. Our hypothesis is that within the microbiome model, amixicile will selectively inhibit specific pathogens associated with periodontal disease and spare commensal bacteria. We hypothesize that Amixicile will selectively inhibit PFOR bacteria and have similar effects on select bacterial species when compared to Metronidazole.

MATERIALS AND METHODS

Study Population

All of the samples harvested in this study came from patients of record at VCU Graduate Periodontics Clinic. All participants of the study completed a comprehensive periodontal exam at the VCU Department of Periodontology, and received informed consent prior to plaque harvest. Our inclusion criteria for all participants was as follows:

1. Adult patients (age 21+)
2. Non-diabetics
3. The patient cannot have taken antibiotics within the 6 months
4. Patient has not received periodontal therapy in the 6 months
5. Non-pregnant patients
6. Non-smokers
7. No patients who required premedication prophylaxis due to joint replacement
8. No aggressive periodontitis

The diagnosis of disease severity was based on full mouth periodontal charting and clinical attachment levels. Severe chronic periodontitis was defined as inflammation of the periodontium with attachment loss of 5mm or more in conjunction with radiographic bone loss. Health was considered probing depth of 3mm or less with no clinical signs of inflammation.

Biofilm Sample Collection

Bacterial samples were harvested from the pocket originating from the mesial of first molars. Local anesthesia was provided to all patients for comfort. All sites were air dried, and cotton roll isolation was used. Supra-gingival plaque was gently removed from the tooth, so that the free gingival margin was not disturbed. The sample was harvested sub-gingivally via a sterile curette and stored in 500 µl of SHI medium. Sample was immediately transported into anaerobic chamber and another 500 µl of SHI medium, was added to lower the oxygen level of the sample. Samples were incubated overnight in an artificial atmosphere (composed of 80% N, 10% H, and 10% CO2) at 37 ^{o}C using a Coy anaerobic chamber (Ann Arbor, MI), and then aliquoted to 100 µl and stored in -80 ^{o}C with 10% of glycerol. Sample aliquots from ten patients were pooled together and aliquoted to 50 µl of each for the following study.

Antimicrobial Treatment

50 µl of pooled sample was added to 4 mL of BHI with 10% of filtered human serum (Valley Biomedical), then separated into four containers. One was centrifuged and the pellet was kept at -20 ^{o}C for DNA isolation as baseline. The others were incubated at 37 ^{o}C in the anaerobic chamber with or without antimicrobial treatment. The concentrations of amixicile and metronidazole (Sigma) used in this study are 25 µg/mL. Pellets from the overnight cultures were obtained for DNA preparation. These steps were then repeated exactly for the healthy samples. As a result, there are four groups of samples for the diseased and four groups of samples for the healthy groups. The "before group" (B) which is the sample bacteria harvested but never incubated *in vitro*. The "control group" (C) which is the sample of bacteria harvested and then incubated for 24 hours in the anaerobic chamber. The "metronidazole group" (MET) which is

the sample of bacteria harvested and then incubated in the presence of 5 µl/mL of metronidazole. The "amixicile group" (AMX) which is the sample of bacteria harvested and then incubated in the presence of 5 µl/mL of amixicile.

DNA isolation and qPCR

Cell pellets were re-suspended in 50 mM EDTA containing 10 mg/mL lysozyme and 100 U/mL mutanolysin (Sigma) and incubated at 37°C for 1 hr. DNA was isolated using the Wizard Genomic DNA purification kit (Promega) according to manufacturer's instructions. The DNA was then used to quantify the presence of bacterial species in the various samples using a 7500 Fast Real-time PCR machine (Thermo-Fisher). Purified DNA (1 µL) and species-specific primers were added to Fast SYBR Green Mastermix (Thermo-Fisher) and 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 species-specific 16S rDNA primer sequences used in this study are shown in below. The cycle threshold (Ct) data were collected and then converted to absolute fold change. This process was completed three individual times to provide triplicates results from the data.

DNAseq library generation

1µg of purified gDNA was fragmented by covaris S2 ultrasonicator following the settings for Whole-genome Resequencing. ThruPLEX DNA-seq Kit (Rubicon Genomics) was used for library preparation according to manufacturer's instructions. Library samples were run on the Bioanalyzer to check the quantity and quality, then processed for next generation sequencing through Nucleic Acids Research Facilities in VCU.

16S rDNA primers

Porphyromonas gingivalis (Pg) HmuY F: GTGGCGAAAGTGGTAAGGGA HmuY R: TCAGCACCACGAACGAAGAA	*Lactobacillus acidophilus* (La) La F: GGATAGAGGTAGTAACTGGCCTTTATT La R: CAGTTTCCGATGCAGTTCCTCG
Prevotella intermedia (Pi) Pi F: CCATCAGGTTATGCTGGGCA Pi R: GTTGCAGACCTCAGTCCGAA	*Aggregatibacter actinomycetemcomitans* (Aa) Aa F: AGTCGGACGGTAGCAGGTAA Aa R: GCTTGGTAGGCCTTTACCCC
Fusobacterium nucleatum (Fn) Fn F: CTGGCTCAGGATGAACGC Fn R: ATGGGACGCAAAGCTCTCTC	*Treponema denticola* (Td) Td F: AGCATGCAAGTCGAACGGTA Td R: AACTAGCTAATGGGACGCGG
Tannerella forsythia Tf F: AGGATGACTGCCCTATGGGT Tf R: AAGCGACAAACTTTCACCGC	*Veillonella atypical* (Va) Va F: CGGCTACTGATCATCGCCTT Va R: ATCTTAGTGGCGAACGGGTG
Streptococcus gordonii (Sg) Sg F: GCAATTGCACCACTACCAGA Sg R: TGCTCGGTCAGACTTTCGTC	*Streptococcus mutans* (Sm) Sm F: GCACACCGTGTTTTCTTGAGTCG Sm R: CGGCTATGTATCGTCGCCTT
Streptococcus anginosus (Sa) Sa F: GAGTGCTAGGTGTTGGGTCC Sa R: TGTTCCGAAGAAACTTCCTATCTCT	16S universal F: AGAGTTTGATCCTGGCTCAG 16S universal R: GCTGCCTCCCGTAGGAGT

Statistical Analysis

Each run used two antimicrobials (amixicile, and metronidazole—each in duplicate) with 16s targeted and 12 bacterial species targeted (Pg, Pi, Fn, La, Aa, Td, Tf, Va, Sa, Sm, and Sg). CT values were also measured before incubation on the 12+1 targets. The after incubation CT values were normalized by subtracting each 16s value difference with the non-controls. The corrected CT values were analyzed using a mixed-model ANOVA with the following factors: Antimicrobial treatment, bacterial species-a repeated, within-sample factor, and the Antimicrobial*Species interaction. The before incubation CT values (un-normalized) were also compared to the control values.

RESULTS

Plaque Harvest and Growth

Before incubation samples (B) were compared to the Control samples (C). Figure 1 displays a comparison between (B) before and (C) Control. The lower the CT value, the more bacteria are present in the sample. The diseased group (D sample) harvested from the chronic periodontitis patients and the healthy group (H sample) harvested from the healthy non chronic periodontitis patients. PCR analysis was performed three times for the D samples and the H samples, creating triplicates. **Error! Reference source not found.** shows the difference in CT values from the before groups (B) and the control groups (C) in both the diseased samples (D1-3) and healthy samples (H1-3). This analysis shows an increase in nearly all of the bacteria tested, which is indicated by a decrease in the CT value. Based on this data, the incubation methods employed were successful in culturing and growing the bacteria harvested from patients.

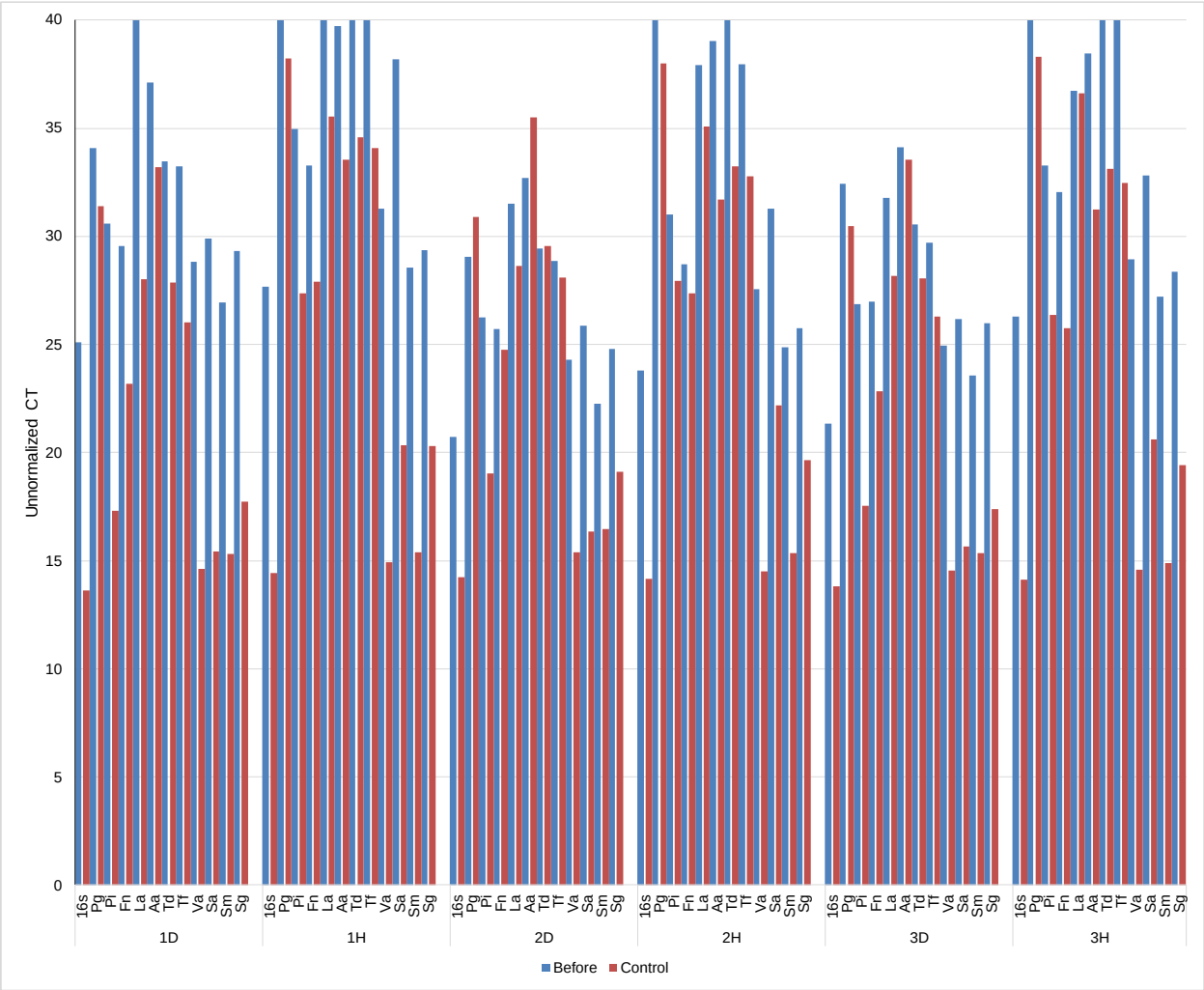

Figure 1. Control group: Comparison of Bacteria Before and After Incubation

Figure 1 displays the comparison of CT values before incubation plaque samples harvested (B Group) in blue and the control samples incubated for 24 hours in the anaerobic chamber (C group) in red. The decrease in CT value corresponds to a greater quantity of bacteria in the sample.

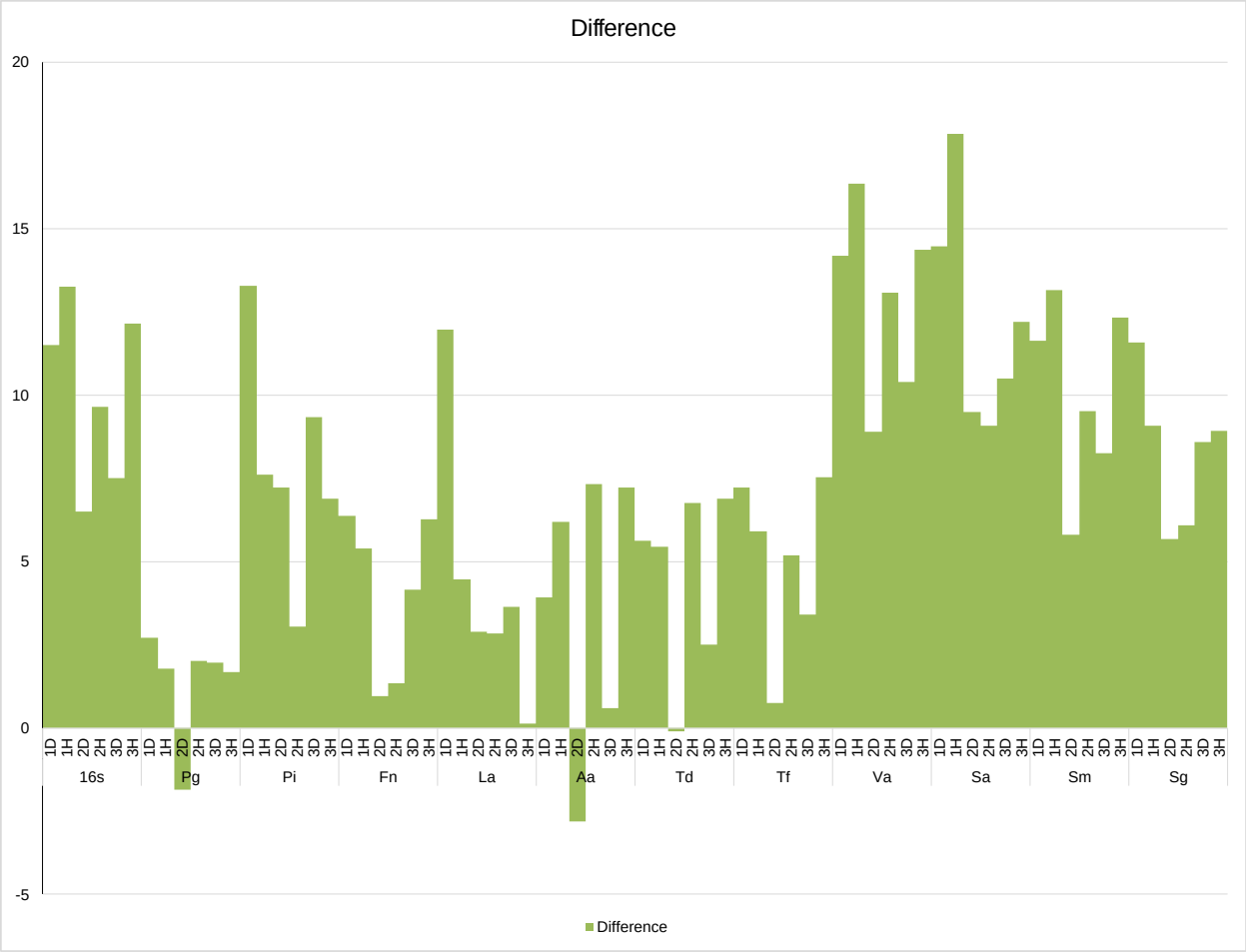

Figure 2. Control: Difference in Bacteria Before and After Incubation

Figure 2 is the difference in CT values of the B group and C group for each D and H sample. This data shows that with the exception of *Pg* in the 2D group and *Aa* in the 2D group, the incubation method utilized in this study resulted in successful growth of the bacteria harvested from patients.

Total Bacteria in Healthy Runs

The three individual runs were analyzed as one combined experiment. This was accomplished by adding an additional factor to the ANOVA model: "H Combined" (1H, 2H, 3H). This permits each run to have a different mean level. **Table 1** displays the corrected CT means compiled from the three individual qPCR runs. From Table 1 and

Figure 2, within each bacterial species, there were differences in the relative abundance under the three antimicrobial conditions. High abundant species which is reflected by a low CT value were seen for: *Pi, Fn, Va, Sa, Va, Sm* and *Ss.* Whereas bacterial species *Pg, La, Aa, Td,* and *Tf* displayed a decreased abundance which is reflected by a higher CT value.

From Table 1 and

Figure 2 there were statistical significant differences for *Pg* (P=.001), *Pi* (P<.001), *Va* (P<.001), and *Sm* (P<.001). For the 3 treatment groups, there are 3 paired comparisons—2 with the control and 1 for amixicile vs metronidazole. For those with an overall difference, an individually identifiable difference is declared if the p-value for the comparison is less than 0.05/3—a correction for multiple comparisons. In the table, if the active antimicrobial is significantly different from the control, then the active antimicrobial is labeled with a "-c" and if amixicile is different than metronidazole then each antimicrobial is labeled with "-x". From **Table 1** and **Figure 2** it demonstrates a difference from the control and amixicile in the following bacterial species: *Pi, Va,* and *Sm.* A difference was seen from the control and metronidazole in the following bacterial species: *Pi, Va,* and *Sm.* Lastly between amixicile and metronidazole, differences were observed for bacterial primers *Pg* and *Sm*

Table 1. Corrected CT mean estimates for the three healthy runs combined (H samples)

		Corrected CT		
Bacterial species	Antimicrobials	Estimate	95% CI	
Pg (P=.001)	Control	38.18	37.39	38.98
	Amixicile-x	39.08	38.28	39.87
	Metronidazole-x	36.80	36.00	37.59
Pi (P<.001)	Control	27.23	26.43	28.02
	Amixicile-c	29.46	28.66	30.25
	Metronidazole-c	29.10	28.31	29.90
Fn (P=.553)	Control	27.01	26.21	27.80
	Amixicile	26.79	26.00	27.58
	Metronidazole	26.41	25.62	27.20
La (P=.008)	Control	35.74	34.94	36.53
	Amixicile-x	35.70	34.91	36.49
	Metronidazole-cx	37.33	36.54	38.13
Aa (P=.048)	Control	32.15	31.36	32.94
	Amixicile	31.50	30.71	32.30
	Metronidazole-c	30.73	29.93	31.52
Td (P=.041)	Control	33.64	32.84	34.43
	Amixicile	34.96	34.16	35.75
	Metronidazole	33.74	32.95	34.54
Tf (P=.030)	Control	33.11	32.32	33.91
	Amixicile	31.87	31.07	32.66
	Metronidazole-c	31.69	30.90	32.49
Va (P<.001)	Control	14.67	13.88	15.46
	Amixicile-c	21.79	21.00	22.59
	Metronidazole-c	20.84	20.05	21.63
Sa (P=.744)	Control	21.04	20.25	21.84
	Amixicile	21.44	20.65	22.24
	Metronidazole	21.36	20.57	22.15
Sm (P<.001)	Control	15.21	14.41	16.00
	Amixicile-cx	18.34	17.55	19.14
	Metronidazole-cx	16.63	15.84	17.43
Sg (P=.022)	Control	19.79	18.99	20.58
	Amixicile-c	21.37	20.58	22.17
	Metronidazole	20.84	20.05	21.64

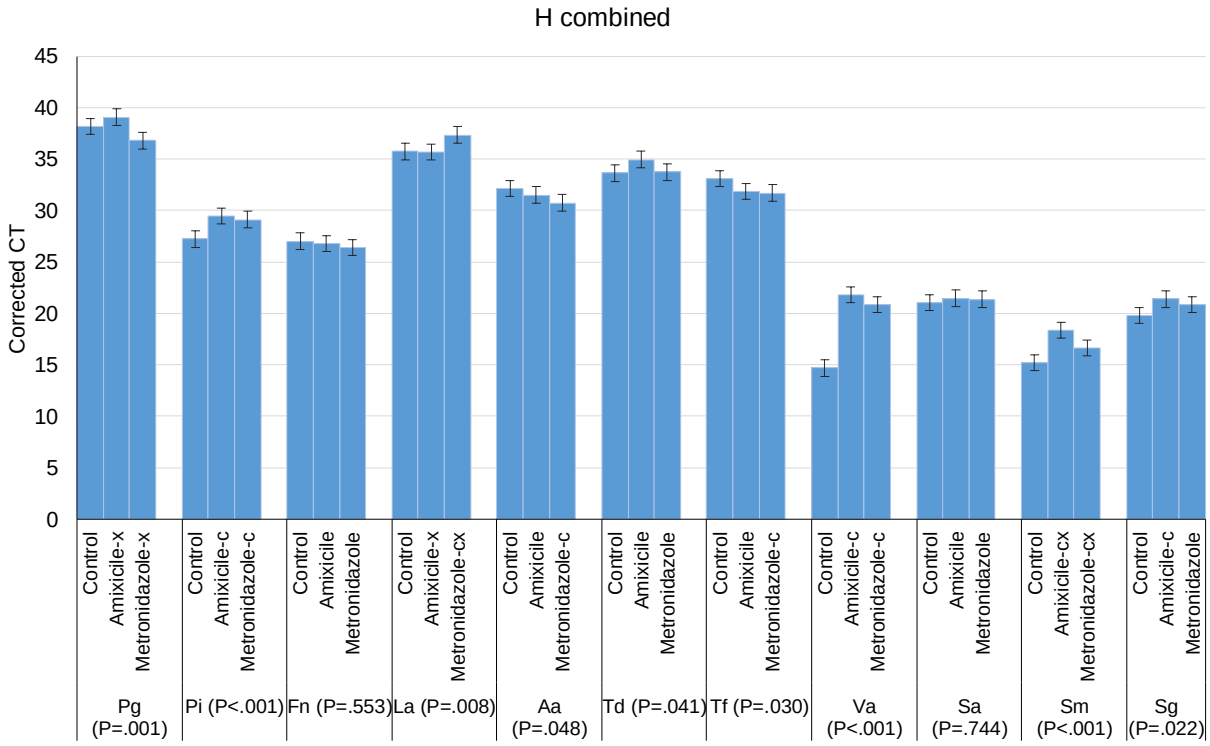

Figure 3. Corrected CT mean estimates for the three healthy runs combined (H samples)

Figure 3 includes the average CT values taken from the C, AMX and MET (microbiomes prepared on different days) each run in triplicate (n=9). ANOVA analysis was performed and applied to compare the control group to amixicile, control group to metronidazole and lastly compare amixicile and metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from amixicile and metronidazole.

The results for comparing each of the antimicrobials, separately within each bacterial species is shown in Appendix A **Table 14** and these differences may be transformed into a fold-change by taking the differences with controls and exponentiating the difference. Exponentiating the 95% confidence intervals on the differences yields the 95% CI estimate for the fold estimate (and so, the CI's are not symmetric around the fold estimate).Table 2 and Figure 4 display the fold changes observed for all of the H sample runs combined. Statistically significant reductions were seen for *Pi* (<.001), *Va* (<.001), and *Sm* (<.001). No statistically significant increases were seen for *Pg, Fn, La, Aa, Td, Tf, Sa* and *Sg* (P>.001). A fold change decrease was observed for amixicile on *Pi, Va* and *Sm* but a fold change decrease for metronidazole was only observed for Va.

Table 2. Fold change for the three healthy runs combined (H samples)

Bacterial species	Antimicrobials	Fold Estimate	95% CI	
Pg	Amixicile (P=.115)	0.539	0.247	1.173
	Metronidazole (P=.017)	2.610	1.199	5.685
Pi	Amixicile (P<.001)	0.213	0.098	0.465
	Metronidazole (P=.002)	0.273	0.125	0.594
Fn	Amixicile (P=.697)	1.162	0.533	2.530
	Metronidazole (P=.287)	1.512	0.694	3.293
La	Amixicile (P=.948)	1.025	0.471	2.233
	Metronidazole (P=.007)	0.330	0.152	0.719
Aa	Amixicile (P=.248)	1.567	0.720	3.413
	Metronidazole (P=.015)	2.680	1.231	5.836
Td	Amixicile (P=.023)	0.401	0.184	0.874
	Metronidazole (P=.852)	0.931	0.427	2.027
Tf	Amixicile (P=.031)	2.372	1.089	5.166
	Metronidazole (P=.015)	2.673	1.227	5.821
Va	Amixicile (P<.001)	0.007	0.003	0.016
	Metronidazole (P<.001)	0.014	0.006	0.030
Sa	Amixicile (P=.470)	0.757	0.347	1.647
	Metronidazole (P=.567)	0.802	0.368	1.746
Sm	Amixicile (P<.001)	0.114	0.052	0.248
	Metronidazole (P=.015)	0.373	0.171	0.812
Sg	Amixicile (P=.007)	0.333	0.153	0.725
	Metronidazole (P=.064)	0.480	0.221	1.046

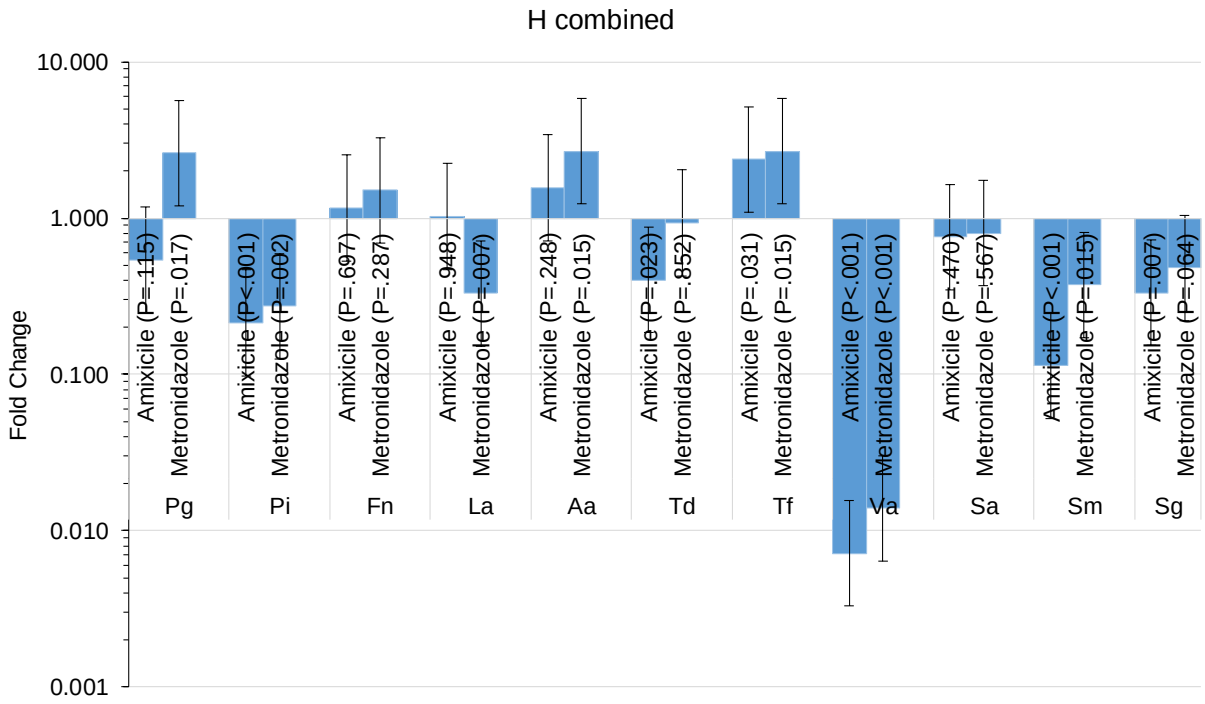

Figure 4. Fold changes observed for the three healthy runs combined (H samples)

Figure 4 represents the fold change in CT values taken from the AMX and MET (microbiomes prepared on different days) each run in triplicate. A P Value <.001 represented a statistical significant change in the numbers of bacteria from the control and antimicrobial treatment.

Total Bacteria in Diseased Runs

The three individual diseased samples (D samples) were also analyzed as one combined experiment. The same data processing and analysis were performed on the H samples data was also performed on the D samples data. The average corrected CT estimates are shown in

Table 3 and Figure 5. Corrected CT mean estimates for the three diseased runs combined (D samples)

. Similar trends were observed in regards to the abundance levels seen in the H samples, and certain bacteria were present in high abundance relative to others. Higher abundant species represented by a low control CT value included *Pi, Fn, Va, Sa, Sg* and *Sm.* Whereas a higher CT control value reflected lower abundant species and included *Pg, La, Aa, Td* and *Tf.*

Statistical significant differences were observed for *Pi* (P<.001), *Fn* (P<.001), *Va* (<.001) and *La* (P<.001). Within the three treatment groups, there are 3 paired comparisons—2 with the control and 1 for amixicile vs metronidazole. From Table 2 it demonstrates a difference from the control and amxicile in the following bacterial species: *Pi, Fn, Sg, Va, La*, and *Td.* A difference was seen from the control and metronidazole in the following bacterial species: *Pi, Fn, Va, La* and *Sm.* Lastly between amixicile and metronidazole, a difference was observed for *Td* species.

The results for comparing each of the antimicrobials, separately within each bacterial species is shown in Appendix A Table 24. And these differences may be transformed into a fold-change by

taking the differences with controls and exponentiating the difference. Exponentiating the 95% confidence intervals on the differences yields the 95% CI estimate for the fold estimate (and so, the CI's are not symmetric around the fold estimate). **Error! Reference source not found.** and **Error! Reference source not found.**

Table 3. Corrected CT mean estimates for the three diseased runs combined (D samples)

Bacterial species	Antimicrobials	Corrected CT Estimate	95% CI	
Pg (P<.001)	Control	30.92	30.55	31.29
	Amixicile-c	29.71	29.34	30.08
	Metronidazole-c	29.59	29.23	29.96
Pi (P<.001)	Control	17.95	17.58	18.32
	Amixicile-cx	25.63	25.26	26.00
	Metronidazole-cx	26.73	26.36	27.10
Fn (P<.001)	Control	23.59	23.22	23.96
	Amixicile-cx	24.66	24.30	25.03
	Metronidazole-cx	25.37	25.00	25.73
La (P<.001)	Control	28.27	27.90	28.63
	Amixicile-c	27.04	26.67	27.40
	Metronidazole-c	26.85	26.48	27.22
Aa (P=.029)	Control	34.08	33.71	34.44
	Amixicile-c	33.36	32.99	33.73
	Metronidazole	33.77	33.40	34.14
Td (P=.088)	Control	28.48	28.11	28.84
	Amixicile	27.90	27.53	28.27
	Metronidazole	28.27	27.90	28.64
Tf (P<.001)	Control	26.80	26.43	27.17
	Amixicile-x	27.17	26.80	27.54
	Metronidazole-cx	28.09	27.72	28.45
Va (P<.001)	Control	14.85	14.49	15.22
	Amixicile-cx	23.42	23.05	23.79
	Metronidazole-cx	25.02	24.66	25.39
Sa (P=.267)	Control	15.81	15.44	16.18
	Amixicile	15.42	15.05	15.79
	Metronidazole	15.47	15.10	15.84
Sm (P=.314)	Control	15.70	15.33	16.06
	Amixicile	15.56	15.19	15.92
	Metronidazole	15.95	15.58	16.31
Sg (P<.001)	Control	18.07	17.70	18.43
	Amixicile-cx	17.29	16.92	17.66
	Metronidazole-x	18.46	18.10	18.83

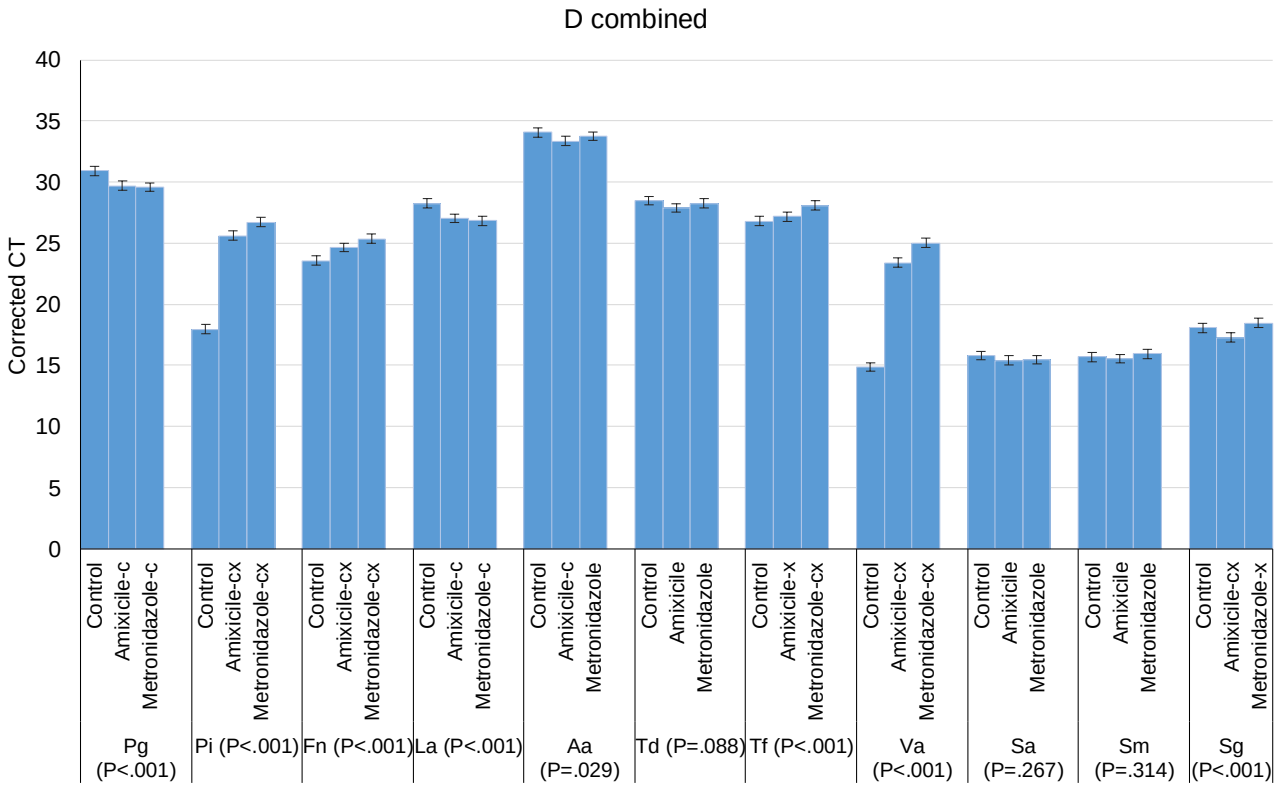

Figure 5. Corrected CT mean estimates for the three diseased runs combined (D samples)

Figure 5 includes the average CT values taken from the C, AMX and MET groups (microbiomes prepared on different days) each run in triplicate (n=9). ANOVA analysis was performed and applied to compare the control group to amixicile, control group to metronidazole and lastly compare amixicile and metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from amixicile and metronidazole.

Table 4. Fold change for the three diseased runs combined (D samples)

Bacterial species	Antimicrobials	Fold Estimate	95% CI	
Pg	Amixicile (P<.001)	2.311	1.611	3.313
	Metronidazole (P<.001)	2.508	1.749	3.597
Pi	Amixicile (P<.001)	0.005	0.003	0.007
	Metronidazole (P<.001)	0.002	0.002	0.003
Fn	Amixicile (P<.001)	0.475	0.331	0.681
	Metronidazole (P<.001)	0.292	0.204	0.419
La	Amixicile (P<.001)	2.345	1.636	3.362
	Metronidazole (P<.001)	2.671	1.863	3.829
Aa	Amixicile (P=.008)	1.645	1.147	2.358
	Metronidazole (P=.237)	1.237	0.863	1.774
Td	Amixicile (P=.031)	1.492	1.040	2.139
	Metronidazole (P=.420)	1.155	0.806	1.656
Tf	Amixicile (P=.160)	0.775	0.541	1.112
	Metronidazole (P<.001)	0.411	0.287	0.589
Va	Amixicile (P<.001)	0.003	0.002	0.004
	Metronidazole (P<.001)	0.001	0.001	0.001
Sa	Amixicile (P=.138)	1.309	0.913	1.877
	Metronidazole (P=.193)	1.265	0.882	1.814
Sm	Amixicile (P=.583)	1.103	0.769	1.582
	Metronidazole (P=.336)	0.842	0.587	1.207
Sg	Amixicile (P=.005)	1.713	1.195	2.456
	Metronidazole (P=.128)	0.758	0.529	1.088

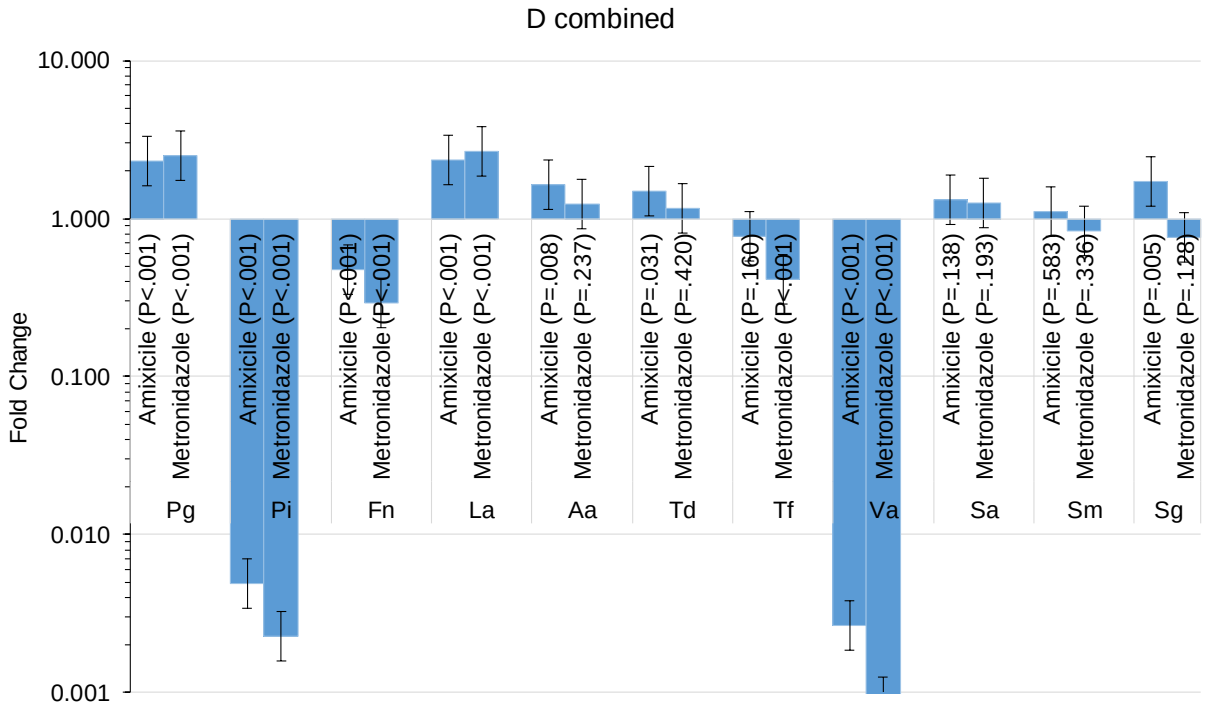

Figure 6. Fold changes observed for the three diseased samples combined (D samples)

Figure 6 represents the fold change in CT values taken from the AMX and MET (microbiomes prepared on different days) each run in triplicate. A P Value <.001 represented a statistical significant change in the numbers of bacteria from the control and antimicrobial treatment.

DISCUSSION

Chronic periodontitis is a an inflammatory disease induced by a sub-gingival biofilm often associated with gram negative anaerobic bacteria such as *Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola*[7]. The sub-gingival biofilm enable bacteria to flourish in a layered ecosystem that involves adherence to a solid surface (the tooth), surrounded by microbial polysaccharides and protein matrix. This complex eco-system provides numerous protective advantages to the bacteria including: nutrient availability and uptake, removal of potentially harmful metabolic products, evasion of the host immune system and ability to share genes particularly ones that provide resistance to antibiotics[4]. Socransky identified six groups of oral bacterial species and grouped them according their spatial relationships which include; yellow, green, purple, orange and red complexes. These complexes represent a group of distinct bacterial species that tend to aggregate together and contribute to the collective survival of the complex within the micro-biofilm. Complexes green and purple act as early colonizers, and have the ability to attach directly to the tooth. Orange and red complexes tend to be associated with pathogenic bacteria that cause periodontal destruction[7].

Periodontitis is first managed with mechanical therapy aimed at reducing the overall quantity of bacteria and implementing better oral hygiene practices to the patient. Numerous studies have showcased the benefits of mechanical therapy in the treatment of periodontal disease such as reduction in inflammation and bleeding in probing, along with decreases in probing depths, detoxification of root surfaces and clinical attachment gain [11,12,14,23]. Despite its effectiveness,

mechanical therapy is unable to remove all pathogens associated with disease. The trend observed in clinical practice is that as disease severity increases the odds of effective removal decrease[11,12]. Additionally bacterial re-contamination following debridement can take place in as little as 42 days, therefore strict maintenance schedules are required for all patients presenting with periodontal disease[24,25].

The undeniable microbial etiology of periodontal diseases provides the rationale for the use of antimicrobial agents in the treatment and resolution of both microbes and the inflammation they induce. A systematic review published in 2003, showed that systemic antibiotics when used as an adjunct to scaling and root planing was shown to be "uniformly beneficial" in providing improvement in attachment loss[26]. Certain antibiotics are considered "ideal" for periodontal infections based on their ability to target anaerobic bacteria, or ability to concentrate in the gingival fluid[27]. The primary drawback to systemic antibiotics is the well-documented problem of bacterial resistance. Meaning that once exposed, certain strains of bacteria are able to survive, and then pass their resistant genes onto the next generation. In a 2014 study, which sought to measure the antibiotic resistance in human chronic periodontitis microbiota, researchers found that "patients with chronic periodontitis frequently yielded sub gingival periodontal pathogen resistance to *in vitro* concentrations of antibiotics commonly (amoxicillin, clindamycin and metronidazole) used in clinical periodontal practice[17]."

Amoxicillin is a medium spectrum bacteriolytic, β-lactam antibiotic that targets susceptible gram positive and gram negative bacteria. Amoxicillin inhibits the cross-linkage between the linear peptidoglycan polymer chains that make up a major component of the cell wall of gram-positive

and a minor component of the gram-negative bacteria. In a double blinded, placebo controlled randomized clinical trial, *Winkel et al* investigated the effects of conventional initial periodontal therapy followed by systemic amoxicillin and clavulanic acid in adult periodontitis patients in a double blinded, placebo-controlled randomized clinical trial. Patients received 10 days of systemic antibiotic or placebo after completion of thorough initial periodontal therapy. *Winkel et al* concluded that in comparison to placebo, adjunctive amoxicillin plus clavulanic acid does not provide additional clinical and microbiological effects in the treatment of adult periodontitis patients. 12 months after therapy, there were no differences in plaque index, bleeding on probing, gingival index, probing depths or clinical attachment levels[28]. Of the 400 patients studied in the *Rams et al* investigation, 173 or 43.3% of the patients exhibited periodontal pathogens with resistance to amoxicillin[17].

Clindamycin is a broad spectrum bacteriostatic antibiotic that targets both aerobic and anaerobic bacteria via inhibition of protein synthesis. *Gordon et al* evaluated the efficacy of clindamycin as an adjunct to conventional periodontal therapy in the treatment of patients who had previously been unsuccessfully treated with scaling, periodontal surgery and use of tetracycline. At 12 and 24 months in the group of 13 patients, the annual rate of active disease progression reduced 10.7% to 0.5%. Bleeding on probing reduced from 33% to 8% and gingival inflammation decreased from 36% to 1% in patients receiving clindamycin plus scaling compared to scaling alone. This was accompanied by a reduction in probing depths along with microbial flora[29,30]. Although effective against anaerobes, the broad spectrum nature of clindamycin puts patients at risk for pseudomembranous colitis, which is accompanied by an overgrowth of *Clostridium difficile* which is inherently resistant to clindamycin. This results in the production of toxins that

cause adverse effects such as diarrhea, colitis and toxic megacolon[31,32]. Of the 400 patients studied in the *Rams et al* investigation, 106 or 26.5% of the patients exhibited periodontal pathogens with resistance to clindamycin[17]. Due to the potential harm of adverse side effects and high rate of resistance, it is recommended that clindamycin be used with great caution.

Metronidazole is a limited spectrum antibiotic compound of the nitroimidazole class, it inhibits nucleic acid synthesis by disrupting the DNA of microbial cells. Considered a pro-drug, metronidazole is activated only in anaerobic cells, where partially reduced and begin to function as a bactericidal antibiotic[33]. It is considered the gold standard, and has been shown to be effective in reducing the periodontal pathogens in moderate to severe chronic periodontal pockets, in particular as an adjunct to scaling and root planning[34]. *Loesche et al*, provided patients with metronidazole during initial therapy, and found that even after 6.4 years follow up time, patients receiving this adjunct therapy had less need for surgery. Despite its ability to target strict anaerobes associated with disease, Metronidazole has several harmful side effects including: nausea, gastrointestinal disturbances, disulfram reaction, and neuropathies[34]. It has been linked to outbreaks in Stevens-Johnson syndrome as well as sudden death due to ethanol interactions[35–37]. Of the 400 patients studied in the *Rams et al* investigation, 121 or 30.3% of the patients exhibited periodontal pathogens with resistance to metronidazole[17]. The potential for harmful side effects is very high with the use of metronidazole, and as a result its popularity among prescribers has been declining.

The American Academy of Periodontology's position paper on the use of systemic antibiotics in periodontics states that the prime candidates for systemic antibiotic therapy are patients who

exhibit continuing loss of periodontal attachment despite diligent conventional mechanical periodontal therapy. They advocate the conservative use of systemic antibiotics with particular attention to be paid to the patient, the pathogenic microbiota and the drug administered[16]. Based on these recommendations, it may be necessary to seek an antimicrobial agent, which can specifically target "keystone" pathogens, avoid bacterial resistance and all the while not harm the host.

Amixicile is newly discovered potent inhibitor of *Clostridium difficile*, a gram-positive obligate anaerobe that is associated with pseudomembranous colitis in patients receiving long-term broad-spectrum antibiotics. Its mechanism is through the inhibition of pyruvate:ferredoxin oxidoreductase, a critical enzyme involved in the vitamin synthesis pathway shared by many anaerobes. Because this pathway is highly conserved and essential, resistance to this novel therapeutic agent is not compatible with life. As a result, amixicile is showing great promise to patients suffering from pseudomembranous colitis and are unable to take any other antibiotics[21].

Like metronidazole, amixicile targets specific anaerobic bacteria, however it differs in it mechanism of action. Amixicile targets and inhibits the pyruvate:ferredoxin oxidoreductase (PFOR), an essential enzyme for central metabolism. PFOR catalyzes the conversion of pyruvate and Coenzyme A (CoA) to CO_2 and Acetyl-CoA. Once Acetly-CoA has been produced, it is then reduced to Acetate producing ATP in the process. Amixicile targets the thiamine pyrophosphate (TPP) vitamin cofactor of PFOR by outcompeting the substrate pyruvate by nearly 2 orders of magnitude[19,38]. Animal research models have evaluated the effects when administering systemic Amixicile in the treatment of a *Clostriudum difficile* infection and

compared it to traditional Vancomycin. Researchers found Amixicile was efficacious in eradicating the disease, but also displayed low toxicity, excellent drug metabolism, and an absence of mutation-based drug resistance[21]. They concluded that Amixicile could be a potential new drug to be used in infections caused by PFOR-expressing bacteria. *P. gingivalis, P. intermedia, F. nucleatum* and *T. forsythia* area all periodontal pathogens that express the PFOR enzyme, and are therefore novel targets to amixicile.

Lewis et al, in a 2017 publication found that amixicile was effective on the growth of oral anaerobic pathogens associated with periodontal disease. Amixicile showed a minimum inhibitory concentration of 1μg/mL to laboratory strains of *P. gingivalis, F. nulceatum* and *T. forsythia*. A higher dose of 5 μg/mL, was required to inhibit growth of *P. intermedia*. Amixicile was then tested on multispecies broth cultures that contained equal amounts of *P.gingivalis, P. intermedia, A. actinomycetemcomitans, F. nuleatum, T. forsythia* and *S. gordonii*. DNA was isolated and qPCR analysis and amixicile inhibited the growth of PFOR-containing bacteria *P. gingivalis*, *P. intermedia*, *F. nucleatum* and *T. forsythia.* Moreover, the inhibition measured was comparable to cultures treated with metronidazole, the current treatment of choice for anaerobic periodontal pathogens[22].

Our study aimed to evaluate how an oral microbiome cultured from patients with periodontal disease and would respond to amixicile compared to healthy samples. To our knowledge this is the first study to investigate the effects of amixicile on a microbiome collected from human subjects. Amixicile testing on a microbiome sample cultured from patients with severe chronic

periodontal disease will provide more clinically relevant results compared to single species cultures previously tested.

Our hypothesis is that amixicile will selectively inhibit PFOR utilizing anaerobic bacteria, and reduce their prevalence in the biofilm. Secondly, we hypothesized that when compared to metronidazole, amixicile would act with similar efficacy in reducing the quantities of anaerobic bacteria. We found that in the biofilm cultured from patients with severe chronic periodontitis, amixicile treatment, exhibited a statistical significant ($P<.001$) reduction in: *P. intermedia, F. nucleatum* and *Veillonella atypical*. All of these bacterial species utilize the PFOR pathway. When the data was evaluated to determine fold changes that occurred in the given bacterial species, both Amixicile and Metronidazole displayed a statistically significant ($P<.001$) decrease in the relative quantities of *P. intermedia, F. nucleatum* and *Veillonella atypical*.

The data supports the notion that amixicile targets specific anaerobic bacteria within an oral microbiome and performs with a similar degree of efficacy to metronidazole. All of the species that were affected have been implicated in the development and progression of periodontal disease[7]. *Prevotella intermedia* is a gram negative obligate anaerobe, associated with gingivitis, pregnancy gingivitis, periodontitis, acute necrotizing ulcerative gingivitis as well as dental abscesses[39]. *Fusobacterium nucleatum* is a microbe associated with initiation of the microbial shift from a primarily gram + to gram – biofilm[8]. This microbial shift is crucial in the development of periodontal disease, and the clinical attachment loss that follows. In vitro analysis has confirmed that *F. nucleatum* coaggregates with all of the following bacteria: *P. gingivalis, Treponema denticola, A. actinomycetemcomitans, P. intermedia, Eubacterium*

species, *Selenomonas* species and *Actinomyces* species[40]. In theory, if *F.nucleatum* could be targeted at an earlier stage, it could prevent the transition for a gram + to gram – micro-biofilm. This could potentially reduce the harmful effects the micro-biofilm causes in periodontal disease. *Veillonella* species have shown the ability to co-aggregate with other bacterial strains, and could provide an importance role in the initiation of bacterial colonization and biofilm formation[41].

When plaque samples from healthy patients were incubated in the presence of amixicile there was a statistically significant reduction (P<.001) for *P. intermedia, Veillonella atypical* and *Streptococcus mutans*. When the data was evaluated to determine fold changes that occurred in the given bacterial species, both amixicile and metronidazole displayed a statistically significant (P<.001) decrease in the relative quantities of *P. intermedia, Veillonella atypical* and *Streptococcus mutans* for amixicile, while metronidazole displayed reductions only in *P. intermedia* and *Veillonella atypical*

The results from this study provide support for additional research to be performed regarding the use of amixicile as a potential new antimicrobial in the treatment of periodontal disease. While this study is only *in vitro*, it demonstrates that amixicile targets strict anaerobes and reduces their quantity in samples derived from biofilms. While antibiotics have forever changed the practice of medicine, the issues with increasing drug resistance cannot be ignored. Within oral biofilms, resistance to amoxicillin, clindamycin, tetracycline, and metronidazole has been reported at surprisingly high rates[17]. Amixicile targets a highly conserved pathway within anaerobes, therefore drug resistance as the result of mutation is conceptually impossible.

As the Academy of Periodontology outlines, antibiotic therapy needs to be considered for patients presenting with severe disease. Ideally, thorough mechanical debridement should be performed with subsequent re-evaluation. If inflammation persists even after mechanical therapy, then microbiological testing can be performed to determine the types of bacteria present. Sites with bleeding and deep probing depths, have been associated with specific periodontal pathogens including *P.gingivalis*, *A. actinomycetemcomitans*, and *Fusobacterium* species. As most bacteria associated with severe periodontal disease belong to anaerobic phyla, treatment with amixicile could provide additional benefits to patients and possibly reduce the need for surgical therapy in the future.

Limitations to this research include a lack of effect seen with *P.gingivalis*. *P.gingivalis* did not respond to either Amixicile or Metronidazole treatment. *P.gingivalis* has been regarded as a "keystone pathogen" and its presence has been linked with active disease in periodontal pockets[3]. Ideally Amixicile and Metronidazole should both have an effect on *P.gingivalis* because *P.gingivalis* is a gram negative anaerobe. However little change was observed from the control and the antimicrobial treatment groups. Multiple factors could explain this finding, *P.gingivalis* is a difficult anaerobe to grow in laboratory conditions. It grows more slowly than other species within a biofilm, therefore after DNA isolation was performed the higher CT values would indicate a lower overall quantity of DNA. It is likely that the *P. gingivalis* collected from patients has enough genetic variety that the traditional primers used for DNA detection would not accurately measure its presence.

Future research involving amixicile should focus on the effects it would have on periodontal disease, and other anaerobic infections in animal models. The systemic side effects, optimal dosing, and overall effect on periodontal disease remain to be determined with future research. Ultimately randomized clinical trials in human subjects would be needed in order to allow amixicile to be FDA approved in the treatment of periodontal disease, and possibly other diseases that are the result of anaerobic dominated infections.

CONCLUSIONS

Amixicile is a promising new antimicrobial in the treatment of anaerobic bacterial infections. The effect of amixicile and metronidazole was dependent on the bacteria being analyzed. amixicile and metronidazole had an effect on PFOR-containing bacteria, specifically changes were seen for *P. intermedia, F. nucleatum* and *Veillonella atypical*. When comparing amixicile to metronidazole, amixicile performed with similar efficacy with the largest effect seen for PFOR bacteria. The data supports the notion that amixicile targets specific anaerobic bacteria within an oral micro-biofilm and performs with a similar degree of efficacy to metronidazole. Such a specific, non-toxic and bioavailable antimicrobial would be highly desirable for the treatment of periodontal disease.

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Appendices

Table 5. Corrected CT mean estimates for Set 1H

Bacterial species	Antimicrobials	Corrected CT Estimate	95% CI	
Pg (P=.153)	Control	38.23	37.26	39.20
	Amixicile	39.57	38.60	40.55
	Metronidazole	38.80	37.83	39.78
Pi (P<.001)	Control	27.36	26.39	28.33
	Amixicile-c	30.37	29.40	31.34
	Metronidazole-c	30.14	29.16	31.11
Fn (P=.384)	Control	27.89	26.92	28.86
	Amixicile	27.77	26.80	28.74
	Metronidazole	27.02	26.04	27.99
La (P<.001)	Control	35.54	34.57	36.51
	Amixicile-x	35.87	34.90	36.84
	Metronidazole-cx	39.97	38.99	40.94
Aa (P=.033)	Control	33.52	32.55	34.50
	Amixicile	31.93	30.96	32.91
	Metronidazole	31.88	30.91	32.85
Td (P=.798)	Control	34.56	33.59	35.54
	Amixicile	34.93	33.96	35.90
	Metronidazole	34.98	34.01	35.95
Tf (P=.052)	Control	34.09	33.12	35.06
	Amixicile	32.93	31.95	33.90
	Metronidazole	32.41	31.44	33.38
Va (P<.001)	Control	14.92	13.95	15.89
	Amixicile-c	21.55	20.57	22.52
	Metronidazole-c	20.06	19.08	21.03
Sa (P<.001)	Control	20.33	19.36	21.31
	Amixicile-cx	23.34	22.37	24.31
	Metronidazole-x	20.84	19.87	21.81
Sm (P<.001)	Control	15.39	14.42	16.36
	Amixicile-cx	19.51	18.54	20.48
	Metronidazole-x	16.06	15.08	17.03
Sg (P=.005)	Control	20.30	19.32	21.27
	Amixicile-cx	22.52	21.55	23.49
	Metronidazole-x	20.67	19.70	21.64

Least-squares means estimates from ANOVA analysis are shown. The analysis was applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference between Amxicile and Metronidazole.

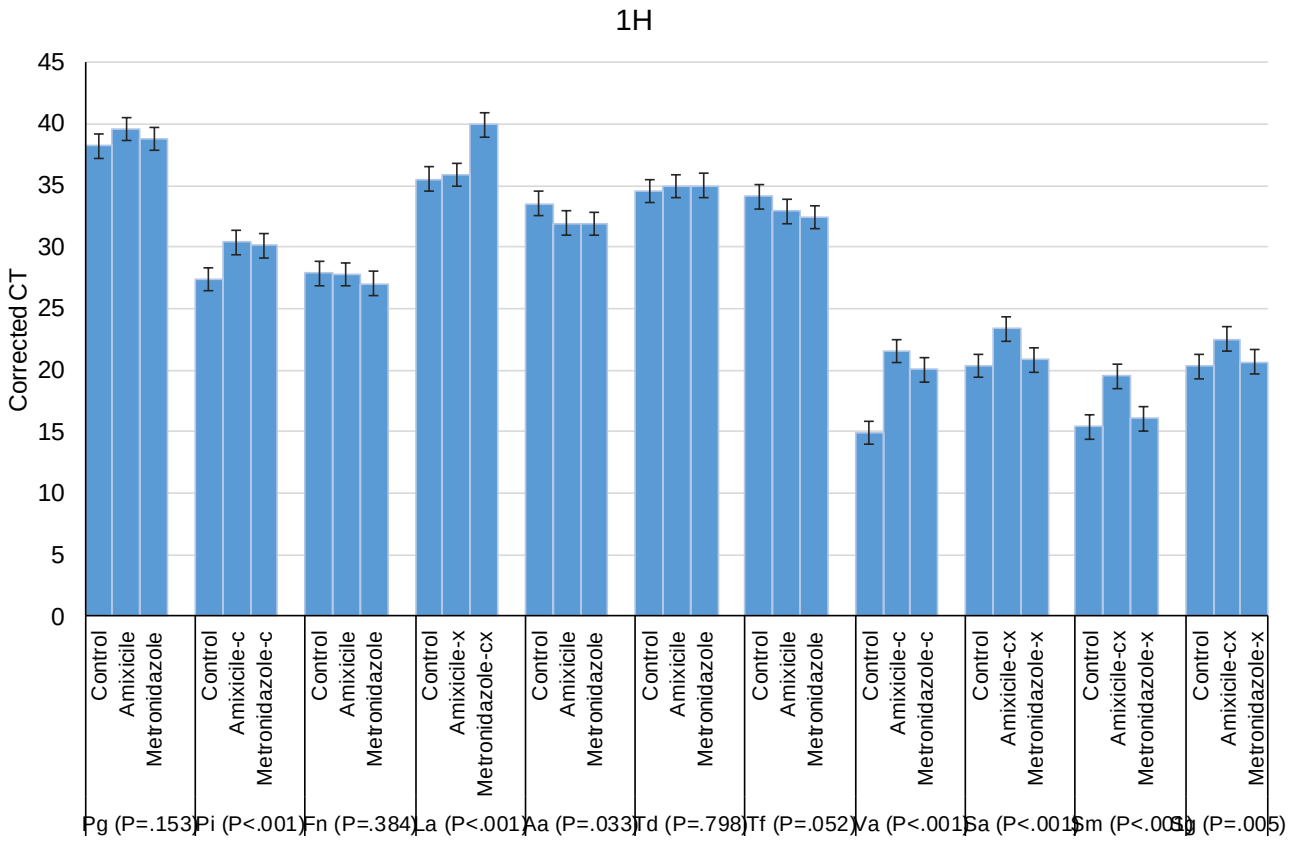

Figure 7. Corrected CT mean estimates for Set 1H (95% CIs)

Figure 7 represents the average CT values taken of Set 1H. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference between Amxicile and Metronidazole.

Table 6. Differences in the Corrected CT mean estimates for Set 1H

Bacterial species	Compare	Corrected CT Estimate	95% CI	
Pg	CvA (P=.055)	-1.342	-2.717	0.032
	CvM (P=.401)	-0.573	-1.947	0.801
	AvM (P=.262)	0.769	-0.605	2.144
Pi	CvA (P<.001)	-3.011	-4.386	-1.637
	CvM (P<.001)	-2.776	-4.150	-1.401
	AvM (P=.729)	0.236	-1.139	1.610
Fn	CvA (P=.865)	0.115	-1.259	1.490
	CvM (P=.205)	0.871	-0.503	2.246
	AvM (P=.270)	0.756	-0.618	2.130
La	CvA (P=.629)	-0.328	-1.703	1.046
	CvM (P<.001)	-4.428	-5.802	-3.053
	AvM (P<.001)	-4.100	-5.474	-2.725
Aa	CvA (P=.025)	1.589	0.215	2.963
	CvM (P=.021)	1.641	0.267	3.016
	AvM (P=.939)	0.052	-1.322	1.427
Td	CvA (P=.590)	-0.366	-1.741	1.008
	CvM (P=.543)	-0.414	-1.789	0.960
	AvM (P=.944)	-0.048	-1.422	1.326
Tf	CvA (P=.095)	1.161	-0.213	2.535
	CvM (P=.018)	1.678	0.304	3.053
	AvM (P=.448)	0.517	-0.857	1.891
Va	CvA (P<.001)	-6.626	-8.000	-5.252
	CvM (P<.001)	-5.136	-6.510	-3.761
	AvM (P=.035)	1.490	0.116	2.865
Sa	CvA (P<.001)	-3.004	-4.379	-1.630
	CvM (P=.457)	-0.507	-1.881	0.867
	AvM (P<.001)	2.497	1.123	3.872
Sm	CvA (P<.001)	-4.118	-5.492	-2.744
	CvM (P=.331)	-0.666	-2.040	0.709
	AvM (P<.001)	3.452	2.078	4.827
Sg	CvA (P=.002)	-2.227	-3.601	-0.852
	CvM (P=.583)	-0.373	-1.748	1.001
	AvM (P=.010)	1.854	0.479	3.228

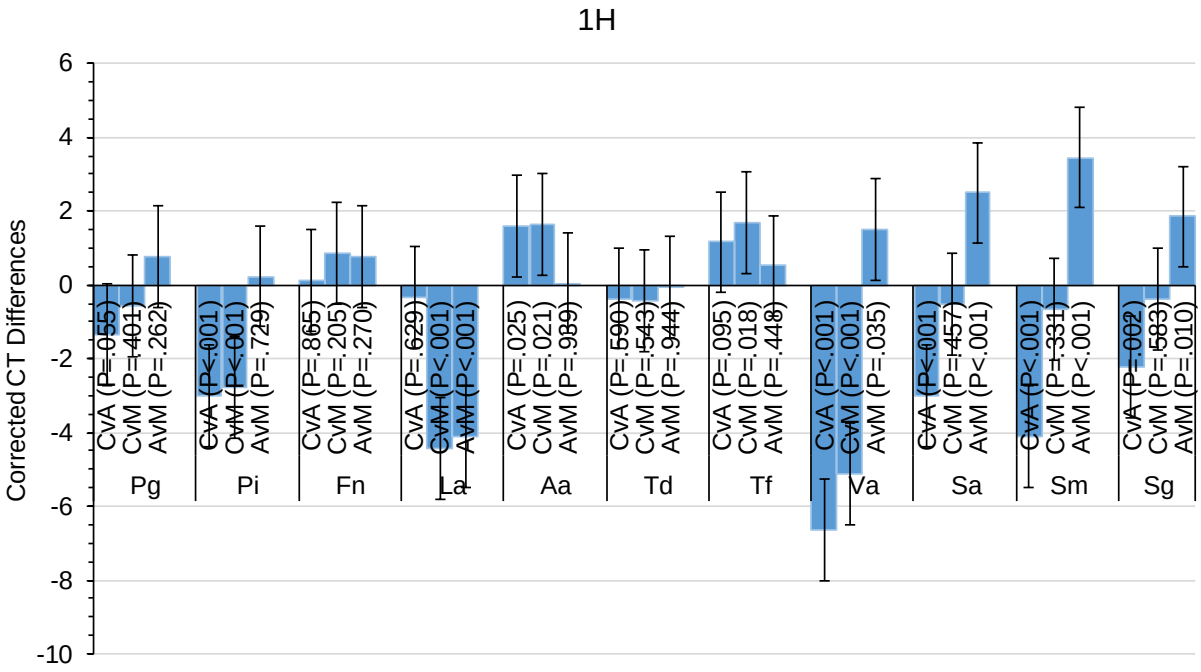

Figure 8. Differences in the Corrected CT mean estimates for Set 1H (95% CIs)

Figure 10 represents the differences in corrected CT mean estimates from the original CT values after standardization with 16s primer for Set 1H. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference between Amixicile and Metronidazole.

Table 7. Fold Estimates for Set 1H

Bacterial species	Antimicrobials	Fold Estimate	95% CI	
Pg	Amixicile (P=.055)	0.394	0.152	1.023
	Metronidazole (P=.401)	0.672	0.259	1.743
Pi	Amixicile (P<.001)	0.124	0.048	0.322
	Metronidazole (P<.001)	0.146	0.056	0.379
Fn	Amixicile (P=.865)	1.083	0.418	2.808
	Metronidazole (P=.205)	1.829	0.706	4.742
La	Amixicile (P=.629)	0.797	0.307	2.065
	Metronidazole (P<.001)	0.046	0.018	0.120
Aa	Amixicile (P=.025)	3.009	1.160	7.800
	Metronidazole (P=.021)	3.120	1.203	8.088
Td	Amixicile (P=.590)	0.776	0.299	2.011
	Metronidazole (P=.543)	0.750	0.289	1.945
Tf	Amixicile (P=.095)	2.236	0.863	5.798
	Metronidazole (P=.018)	3.200	1.234	8.297
Va	Amixicile (P<.001)	0.010	0.004	0.026
	Metronidazole (P<.001)	0.028	0.011	0.074
Sa	Amixicile (P<.001)	0.125	0.048	0.323
	Metronidazole (P=.457)	0.704	0.271	1.824
Sm	Amixicile (P<.001)	0.058	0.022	0.149
	Metronidazole (P=.331)	0.630	0.243	1.634
Sg	Amixicile (P=.002)	0.214	0.082	0.554
	Metronidazole (P=.583)	0.772	0.298	2.002

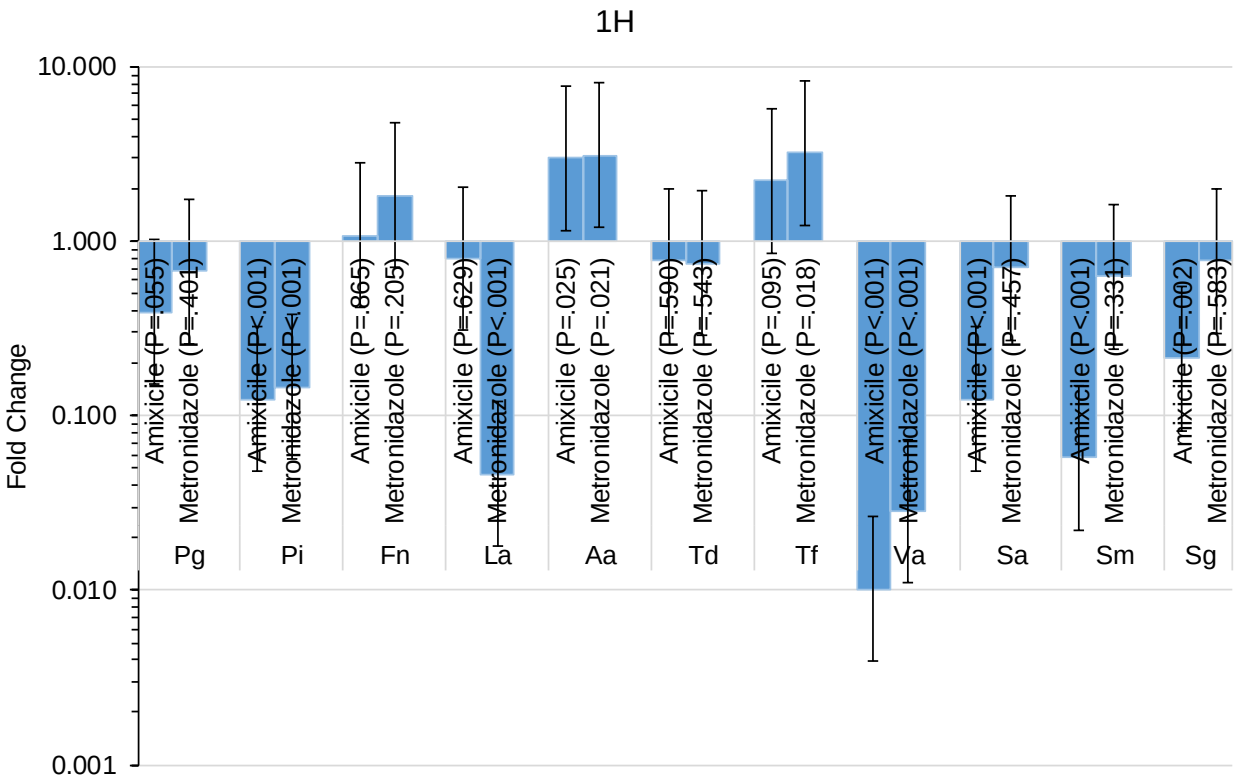

Figure 9. Fold Estimates for Set 1H (95% CIs)

Figure 11 represents the fold change observed for Set 1H for bacterial species after treatment of either Amixicile or Metronidazole. A P Value <.001 represented a statistically significant change in the numbers of bacteria from the control and antimicrobial treatment.

Table 8. Corrected CT mean estimates for Set 2

Bacterial species	Antimicrobials	Corrected CT Estimate	95% CI	
Pg (P=.006)	Control	38.00	36.80	39.20
	Amixicile-x	38.38	37.18	39.58
	Metronidazole-cx	35.70	34.50	36.91
Pi (P=.316)	Control	27.95	26.75	29.16
	Amixicile	28.67	27.47	29.87
	Metronidazole	29.24	28.04	30.44
Fn (P=.346)	Control	27.37	26.17	28.57
	Amixicile	26.15	24.94	27.35
	Metronidazole	26.88	25.68	28.08
La (P=.081)	Control	35.07	33.87	36.27
	Amixicile	35.54	34.34	36.74
	Metronidazole	36.94	35.74	38.14
Aa (P=.313)	Control	31.69	30.49	32.89
	Amixicile	31.09	29.89	32.30
	Metronidazole	30.40	29.20	31.60
Td (P=.220)	Control	33.24	32.04	34.45
	Amixicile	34.65	33.45	35.85
	Metronidazole	33.53	32.32	34.73
Tf (P=.016)	Control	32.78	31.58	33.99
	Amixicile-c	30.33	29.13	31.53
	Metronidazole	32.19	30.99	33.39
Va (P<.001)	Control	14.50	13.29	15.70
	Amixicile-c	22.64	21.43	23.84
	Metronidazole-c	22.07	20.87	23.27
Sa (P=.033)	Control	22.19	20.99	23.39
	Amixicile-x	20.67	19.47	21.87
	Metronidazole-x	22.93	21.72	24.13
Sm (P<.001)	Control	15.36	14.15	16.56
	Amixicile-c	19.25	18.05	20.45
	Metronidazole-c	17.49	16.29	18.70
Sg (P=.067)	Control	19.65	18.45	20.85
	Amixicile	21.42	20.21	22.62
	Metronidazole	21.39	20.19	22.59

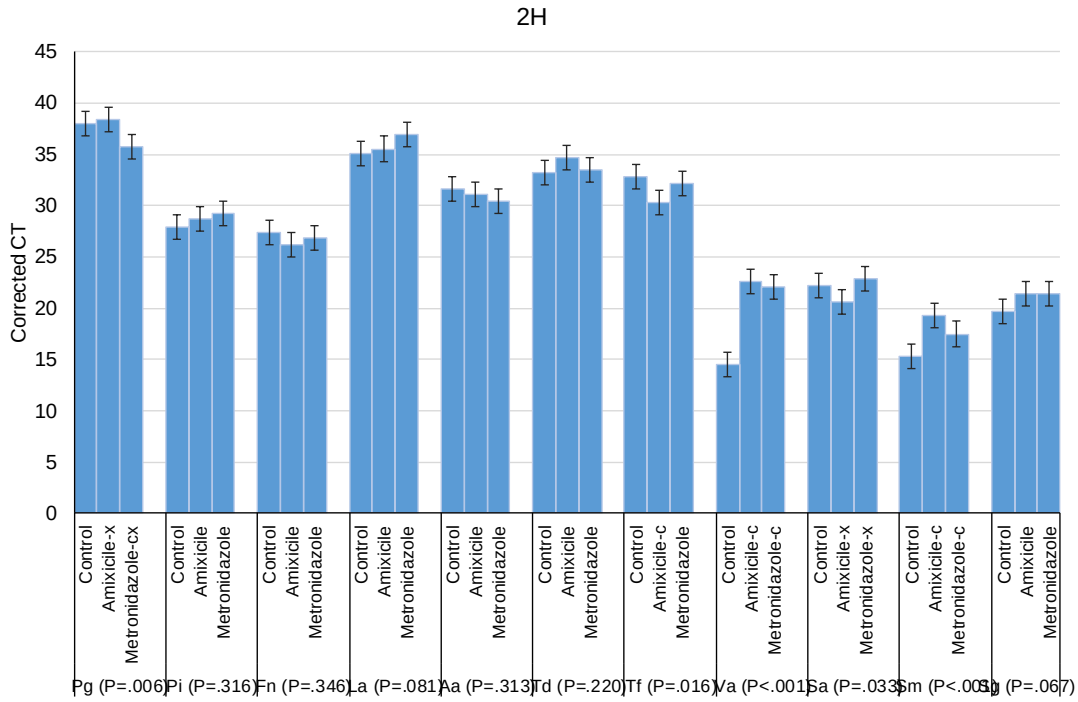

Figure 10. Corrected CT mean estimates for Set 2H (95% CIs)

Figure 12 represents the average CT values taken of Set 2H. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference between Amxicile and Metronidazole.

Table 9. Differences in the Corrected CT mean estimates for Set 2H

Bacterial species	Compare	Corrected CT Estimate	95% CI	
Pg	CvA (P=.650)	-0.381	-2.080	1.318
	CvM (P=.010)	2.296	0.597	3.995
	AvM (P=.003)	2.677	0.977	4.376
Pi	CvA (P=.397)	-0.715	-2.414	0.984
	CvM (P=.133)	-1.285	-2.985	0.414
	AvM (P=.498)	-0.570	-2.270	1.129
Fn	CvA (P=.151)	1.226	-0.474	2.925
	CvM (P=.558)	0.493	-1.206	2.192
	AvM (P=.385)	-0.733	-2.432	0.966
La	CvA (P=.576)	-0.471	-2.170	1.229
	CvM (P=.032)	-1.872	-3.572	-0.173
	AvM (P=.102)	-1.402	-3.101	0.298
Aa	CvA (P=.481)	0.594	-1.105	2.293
	CvM (P=.131)	1.291	-0.408	2.991
	AvM (P=.409)	0.697	-1.002	2.397
Td	CvA (P=.102)	-1.404	-3.104	0.295
	CvM (P=.738)	-0.281	-1.980	1.418
	AvM (P=.187)	1.123	-0.576	2.823
Tf	CvA (P=.006)	2.451	0.752	4.150
	CvM (P=.481)	0.593	-1.106	2.293
	AvM (P=.033)	-1.858	-3.557	-0.158
Va	CvA (P<.001)	-8.140	-9.839	-6.441
	CvM (P<.001)	-7.572	-9.271	-5.873
	AvM (P=.500)	0.568	-1.131	2.267
Sa	CvA (P=.078)	1.520	-0.179	3.219
	CvM (P=.385)	-0.733	-2.432	0.966
	AvM (P=.011)	-2.253	-3.952	-0.553
Sm	CvA (P<.001)	-3.894	-5.594	-2.195
	CvM (P=.015)	-2.138	-3.838	-0.439
	AvM (P=.043)	1.756	0.057	3.455
Sg	CvA (P=.042)	-1.766	-3.465	-0.067
	CvM (P=.045)	-1.740	-3.439	-0.040
	AvM (P=.975)	0.026	-1.673	1.726

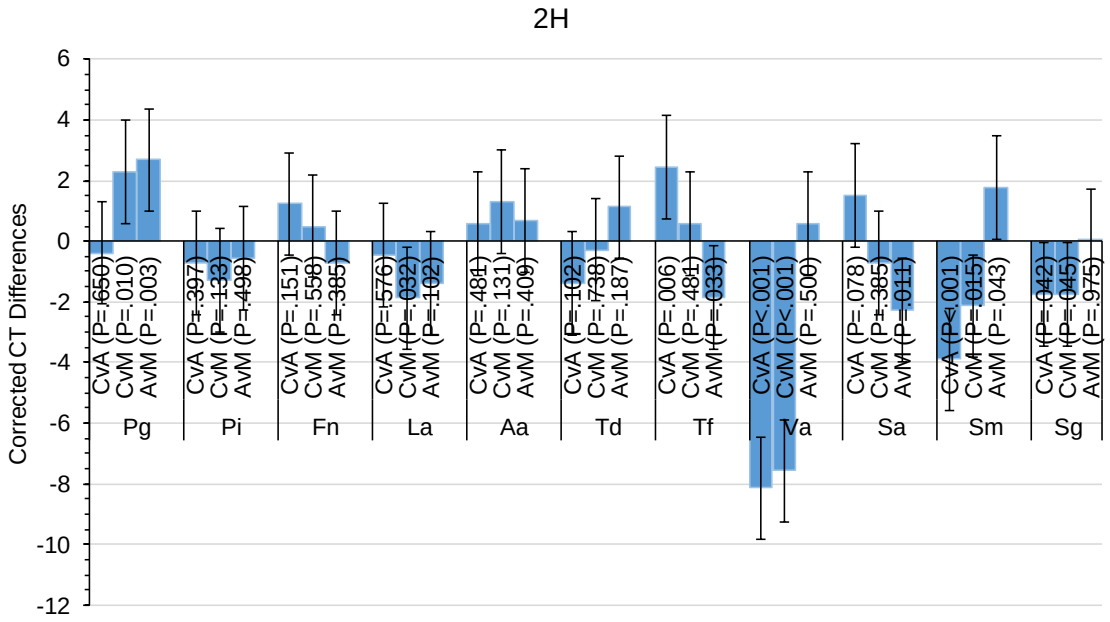

Figure 11. Differences in the Corrected CT mean estimates for Set 2H (95% CIs)

Figure 13 represents the differences in corrected CT mean estimates from the original CT values after standardization with 16s primer for Set 2H. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amixicile and Metronidazole.

Table 10. Fold Estimates for Set 2H

Bacterial	Antimicrobials	Fold Estimate	95% CI	
Pg	Amixicile (P=.650)	0.768	0.237	2.494
	Metronidazole (P=.01	4.910	1.512	15.945
Pi	Amixicile (P=.397)	0.609	0.188	1.978
	Metronidazole (P=.13	0.410	0.126	1.332
Fn	Amixicile (P=.151)	2.339	0.720	7.594
	Metronidazole (P=.55	1.407	0.433	4.570
La	Amixicile (P=.576)	0.722	0.222	2.343
	Metronidazole (P=.03	0.273	0.084	0.887
Aa	Amixicile (P=.481)	1.509	0.465	4.902
	Metronidazole (P=.13	2.448	0.754	7.949
Td	Amixicile (P=.102)	0.378	0.116	1.227
	Metronidazole (P=.73	0.823	0.253	2.673
Tf	Amixicile (P=.006)	5.468	1.684	17.756
	Metronidazole (P=.48	1.509	0.465	4.899
Va	Amixicile (P<.001)	0.004	0.001	0.012
	Metronidazole (P<.00	0.005	0.002	0.017
Sa	Amixicile (P=.078)	2.868	0.883	9.313
	Metronidazole (P=.38	0.602	0.185	1.954
Sm	Amixicile (P<.001)	0.067	0.021	0.218
	Metronidazole (P=.01	0.227	0.070	0.738
Sg	Amixicile (P=.042)	0.294	0.091	0.955
	Metronidazole (P=.04	0.299	0.092	0.972

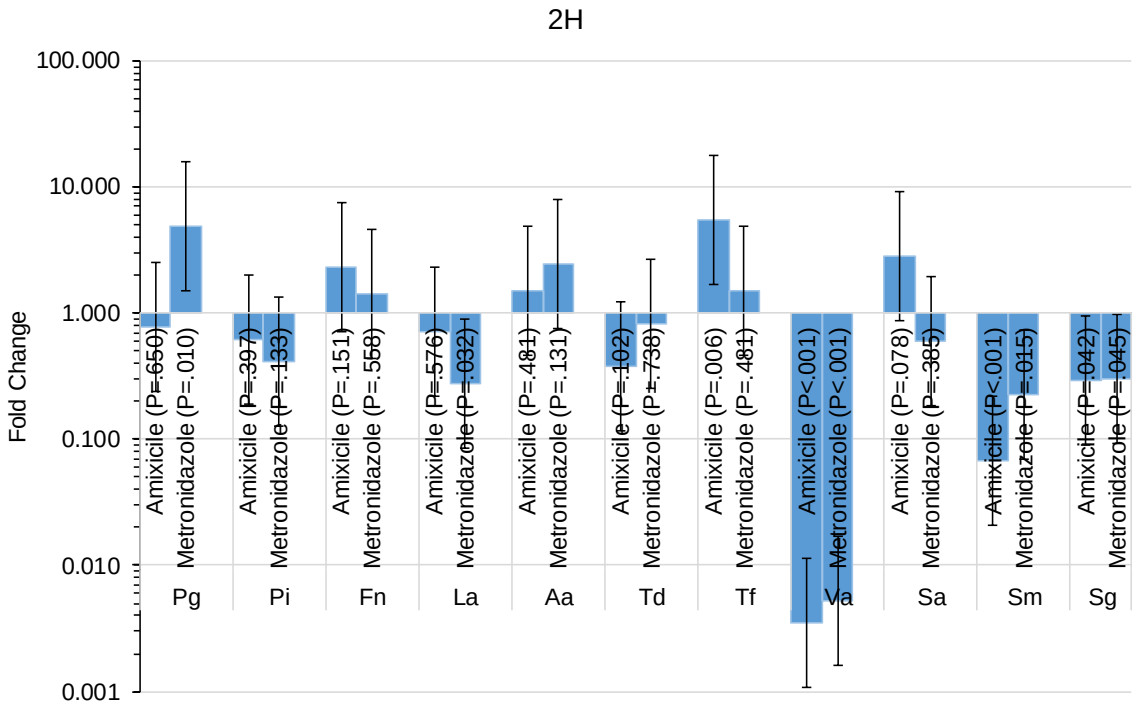

Figure 12. Fold Estimates for Set 2H (95% CIs)

Figure 14 represents the fold change observed for Set 2H for bacterial species after treatment of either Amixicile or Metronidazole. A P Value <.001 represented a statistical significant change in the numbers of bacteria from the control and antimicrobial treatment.

Table 11. Corrected CT mean estimates for Set 3H

Bacterial species	Antimicrobials	Corrected CT Estimate	95% CI	
Pg (P<.001)	Control	38.32	37.30	39.33
	Amixicile-x	39.27	38.26	40.29
	Metronidazole-cx	35.89	34.87	36.90
Pi (P<.001)	Control	26.37	25.36	27.39
	Amixicile-c	29.33	28.32	30.35
	Metronidazole	27.94	26.92	28.95
Fn (P=.290)	Control	25.76	24.75	26.78
	Amixicile	26.45	25.44	27.47
	Metronidazole	25.34	24.32	26.35
La (P=.114)	Control	36.60	35.59	37.61
	Amixicile	35.69	34.68	36.71
	Metronidazole	35.09	34.08	36.11
Aa (P=.070)	Control	31.24	30.22	32.25
	Amixicile	31.48	30.46	32.49
	Metronidazole	29.90	28.89	30.92
Td (P=.002)	Control	33.11	32.09	34.12
	Amixicile-cx	35.29	34.27	36.30
	Metronidazole-x	32.72	31.71	33.74
Tf (P=.013)	Control	32.47	31.45	33.48
	Amixicile-x	32.34	31.33	33.35
	Metronidazole-cx	30.48	29.47	31.50
Va (P<.001)	Control	14.59	13.58	15.61
	Amixicile-c	21.20	20.18	22.21
	Metronidazole-c	20.39	19.38	21.41
Sa (P=.899)	Control	20.60	19.59	21.61
	Amixicile	20.32	19.31	21.34
	Metronidazole	20.32	19.30	21.33
Sm (P=.079)	Control	14.88	13.87	15.90
	Amixicile	16.28	15.26	17.29
	Metronidazole	16.34	15.33	17.36
Sg (P=.310)	Control	19.41	18.39	20.42
	Amixicile	20.18	19.16	21.19
	Metronidazole	20.47	19.46	21.49

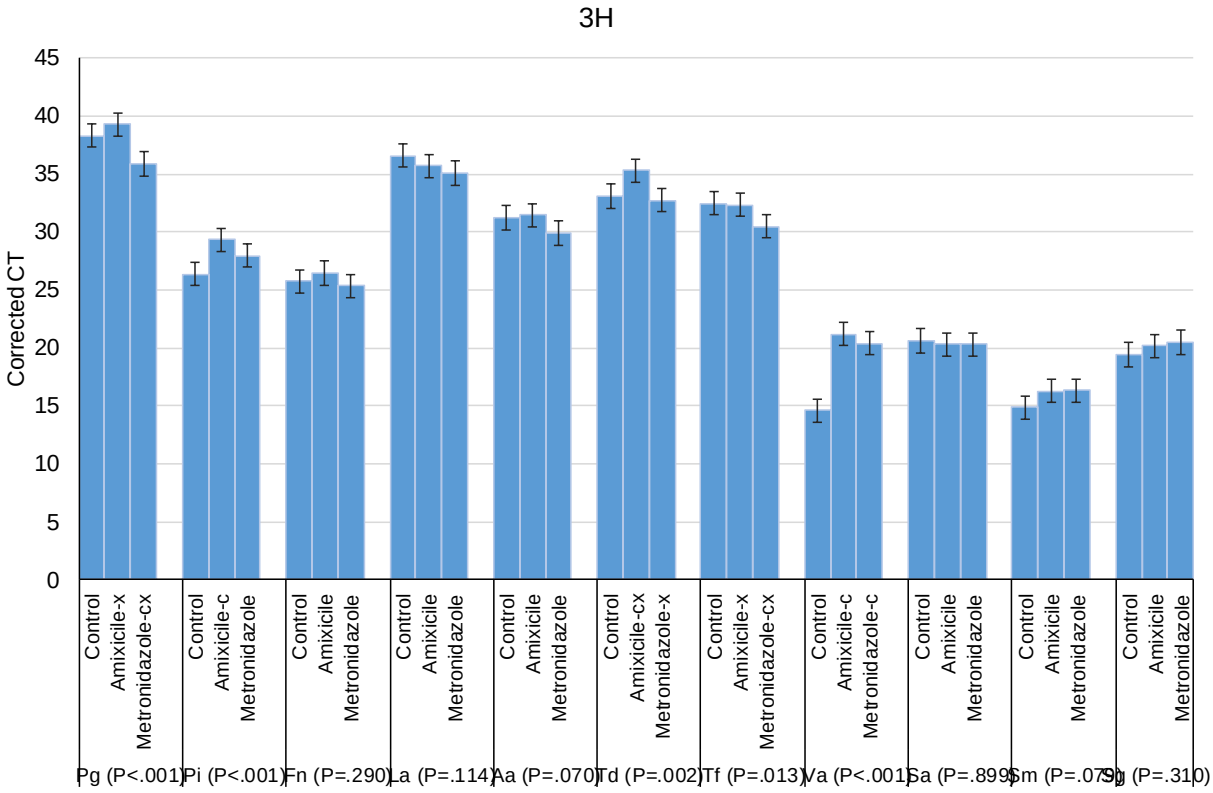

Figure 13. Corrected CT mean estimates for Set 3H (95% CIs)

Figure 15 represents the average CT values taken of Set 3H. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amxicile and Metronidazole.

Table 12. Differences in the Corrected CT mean estimates for Set 3H

Bacterial species	Compare	Corrected CT Estimate	95% CI	
Pg	CvA (P=.184)	-0.955	-2.389	0.479
	CvM (P=.002)	2.430	0.995	3.864
	AvM (P<.001)	3.385	1.951	4.819
Pi	CvA (P<.001)	-2.961	-4.395	-1.526
	CvM (P=.034)	-1.564	-2.999	-0.130
	AvM (P=.056)	1.396	-0.038	2.831
Fn	CvA (P=.332)	-0.693	-2.127	0.742
	CvM (P=.549)	0.425	-1.009	1.860
	AvM (P=.122)	1.118	-0.316	2.552
La	CvA (P=.207)	0.907	-0.528	2.341
	CvM (P=.040)	1.507	0.073	2.941
	AvM (P=.400)	0.600	-0.834	2.034
Aa	CvA (P=.737)	-0.238	-1.672	1.196
	CvM (P=.067)	1.334	-0.100	2.768
	AvM (P=.033)	1.572	0.138	3.006
Td	CvA (P=.004)	-2.182	-3.617	-0.748
	CvM (P=.588)	0.385	-1.049	1.819
	AvM (P<.001)	2.567	1.133	4.002
Tf	CvA (P=.858)	0.127	-1.308	1.561
	CvM (P=.008)	1.984	0.549	3.418
	AvM (P=.013)	1.857	0.423	3.292
Va	CvA (P<.001)	-6.606	-8.040	-5.172
	CvM (P<.001)	-5.802	-7.237	-4.368
	AvM (P=.261)	0.804	-0.631	2.238
Sa	CvA (P=.696)	0.277	-1.158	1.711
	CvM (P=.689)	0.284	-1.150	1.718
	AvM (P=.992)	0.007	-1.427	1.442
Sm	CvA (P=.056)	-1.396	-2.830	0.039
	CvM (P=.046)	-1.463	-2.898	-0.029
	AvM (P=.924)	-0.068	-1.502	1.366
Sg	CvA (P=.284)	-0.767	-2.201	0.668
	CvM (P=.141)	-1.062	-2.496	0.372
	AvM (P=.677)	-0.295	-1.730	1.139

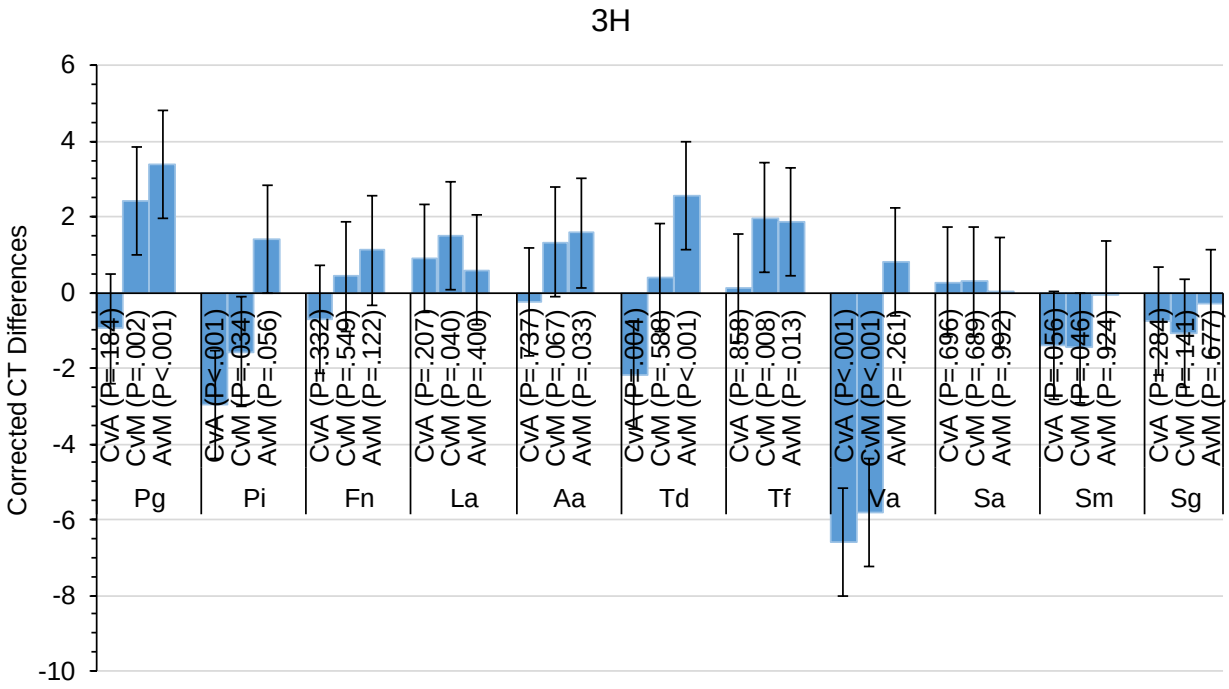

Figure 14. Differences in the Corrected CT mean estimates for Set 3H (95% CIs)

Figure 16 represents the differences in corrected CT mean estimates from the original CT values after standardization with 16s primer for Set 3H. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amixicile and Metronidazole.

Table 13. Fold Estimates for Set 3H

Bacterial species	Antimicrobials	Fold Estimate	95% CI	
Pg	Amixicile (P=.184)	0.516	0.191	1.394
	Metronidazole (P=.002)	5.388	1.994	14.563
Pi	Amixicile (P<.001)	0.128	0.048	0.347
	Metronidazole (P=.034)	0.338	0.125	0.914
Fn	Amixicile (P=.332)	0.619	0.229	1.672
	Metronidazole (P=.549)	1.343	0.497	3.629
La	Amixicile (P=.207)	1.875	0.694	5.067
	Metronidazole (P=.040)	2.842	1.052	7.681
Aa	Amixicile (P=.737)	0.848	0.314	2.292
	Metronidazole (P=.067)	2.521	0.933	6.813
Td	Amixicile (P=.004)	0.220	0.082	0.595
	Metronidazole (P=.588)	1.306	0.483	3.529
Tf	Amixicile (P=.858)	1.092	0.404	2.950
	Metronidazole (P=.008)	3.955	1.464	10.690
Va	Amixicile (P<.001)	0.010	0.004	0.028
	Metronidazole (P<.001)	0.018	0.007	0.048
Sa	Amixicile (P=.696)	1.212	0.448	3.274
	Metronidazole (P=.689)	1.218	0.451	3.291
Sm	Amixicile (P=.056)	0.380	0.141	1.027
	Metronidazole (P=.046)	0.363	0.134	0.980
Sg	Amixicile (P=.284)	0.588	0.217	1.589
	Metronidazole (P=.141)	0.479	0.177	1.295

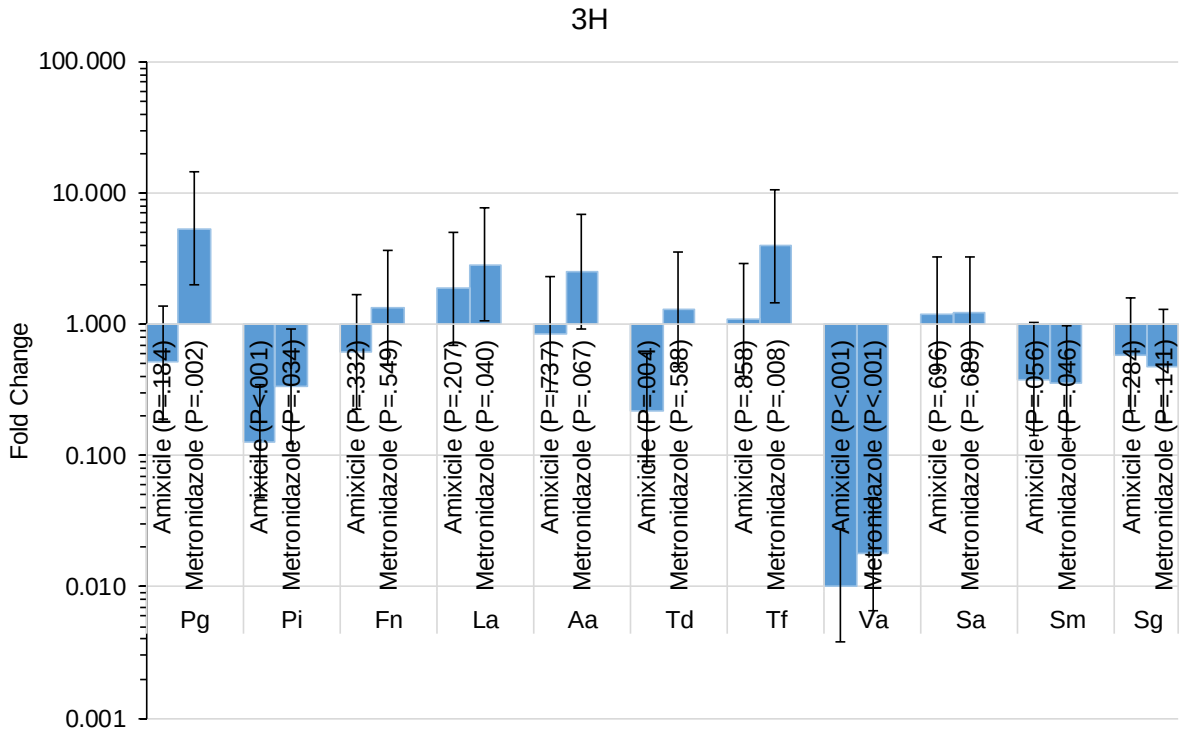

Figure 15. Fold Estimates for Set 3H (95% CIs)

Figure 17 represents the fold change observed for Set 3H for bacterial species after treatment of either Amixicile or Metronidazole. A P Value <.001 represented a statistical significant change in the numbers of bacteria from the control and antimicrobial treatment.

Table 14. Differences in the Corrected CT mean estimates for three H Sets

Bacterial species	Compare	Corrected CT Estimate	95% CI	
Pg	CvA (P=.115)	-0.893	-2.015	0.230
	CvM (P=.017)	1.384	0.261	2.507
	AvM (P<.001)	2.277	1.154	3.400
Pi	CvA (P<.001)	-2.229	-3.352	-1.106
	CvM (P=.002)	-1.875	-2.998	-0.752
	AvM (P=.525)	0.354	-0.769	1.477
Fn	CvA (P=.697)	0.216	-0.907	1.339
	CvM (P=.287)	0.596	-0.526	1.719
	AvM (P=.494)	0.380	-0.742	1.503
La	CvA (P=.948)	0.036	-1.087	1.159
	CvM (P=.007)	-1.598	-2.721	-0.475
	AvM (P=.006)	-1.634	-2.757	-0.511
Aa	CvA (P=.248)	0.648	-0.474	1.771
	CvM (P=.015)	1.422	0.299	2.545
	AvM (P=.170)	0.774	-0.349	1.897
Td	CvA (P=.023)	-1.318	-2.441	-0.195
	CvM (P=.852)	-0.104	-1.226	1.019
	AvM (P=.035)	1.214	0.091	2.337
Tf	CvA (P=.031)	1.246	0.123	2.369
	CvM (P=.015)	1.418	0.296	2.541
	AvM (P=.756)	0.172	-0.951	1.295
Va	CvA (P<.001)	-7.124	-8.247	-6.001
	CvM (P<.001)	-6.170	-7.293	-5.047
	AvM (P=.093)	0.954	-0.169	2.077
Sa	CvA (P=.470)	-0.403	-1.525	0.720
	CvM (P=.567)	-0.319	-1.441	0.804
	AvM (P=.880)	0.084	-1.039	1.207
Sm	CvA (P<.001)	-3.136	-4.259	-2.013
	CvM (P=.015)	-1.422	-2.545	-0.300
	AvM (P=.004)	1.714	0.591	2.836
Sg	CvA (P=.007)	-1.586	-2.709	-0.464
	CvM (P=.064)	-1.058	-2.181	0.065
	AvM (P=.344)	0.528	-0.595	1.651

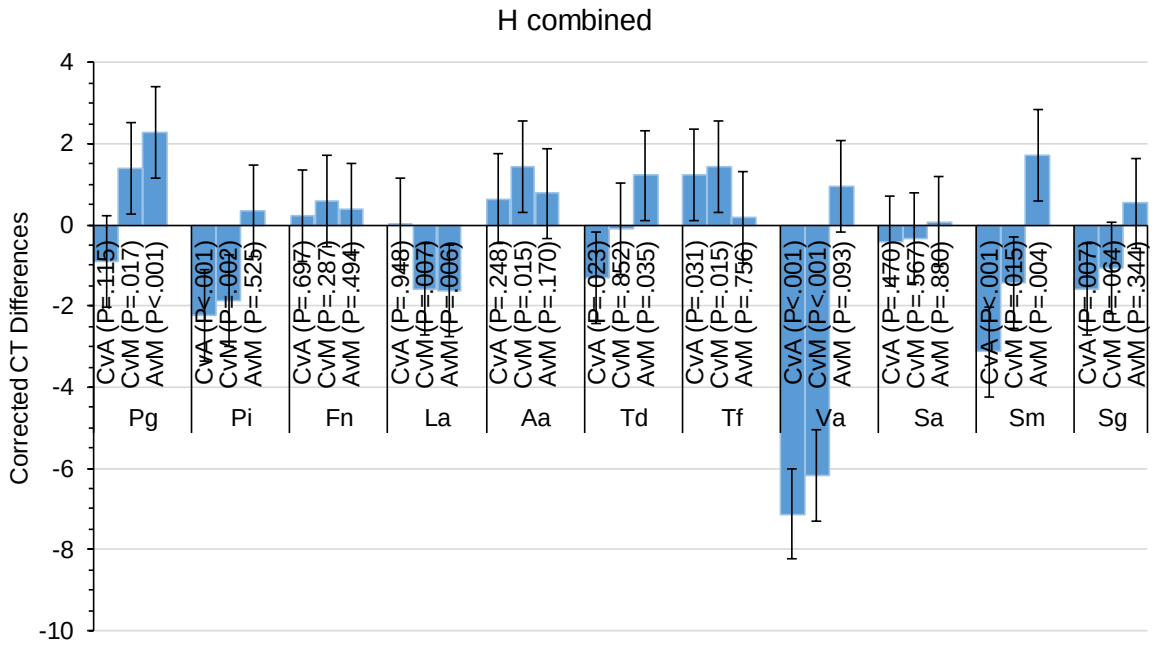

Figure 16. Differences in the Corrected CT mean estimates for three H Sets (95% CIs)

Figure 18 represents the differences in corrected CT mean estimates from the original CT values after standardization with 16s primer for Sets 1H, 2H, and 3H combined. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amixicile and Metronidazole.

Table 15. Corrected CT mean estimates for Set 1D

	Corrected CT		
Antimicrobials	Estimate	95% CI	
Control	31.39	31.16	31.62
Amixicile-c	29.09	28.86	29.32
Metronidazole-c	28.82	28.59	29.05
Control	17.29	17.06	17.52
Amixicile-cx	25.13	24.90	25.36
Metronidazole-cx	25.98	25.75	26.21
Control	23.19	22.96	23.42
Amixicile-cx	24.34	24.11	24.57
Metronidazole-cx	24.87	24.64	25.09
Control	28.03	27.80	28.25
Amixicile-cx	27.47	27.24	27.70
Metronidazole-cx	26.61	26.38	26.84
Control	33.19	32.96	33.42
Amixicile-cx	32.47	32.24	32.70
Metronidazole-x	33.16	32.93	33.39
Control	27.84	27.61	28.07
Amixicile	27.81	27.58	28.04
Metronidazole	27.72	27.49	27.95
Control	26.02	25.79	26.25
Amixicile-cx	27.21	26.98	27.44
Metronidazole-cx	27.71	27.48	27.94
Control	14.62	14.39	14.85
Amixicile-cx	22.72	22.49	22.95
Metronidazole-cx	24.10	23.87	24.33
Control	15.42	15.19	15.65
Amixicile-c	14.95	14.72	15.18
Metronidazole-c	14.88	14.65	15.11
Control	15.32	15.09	15.55
Amixicile-x	15.12	14.89	15.35
Metronidazole-cx	15.74	15.51	15.97
Control	17.71	17.48	17.94
Amixicile-cx	16.74	16.51	16.97
Metronidazole-x	18.03	17.80	18.26

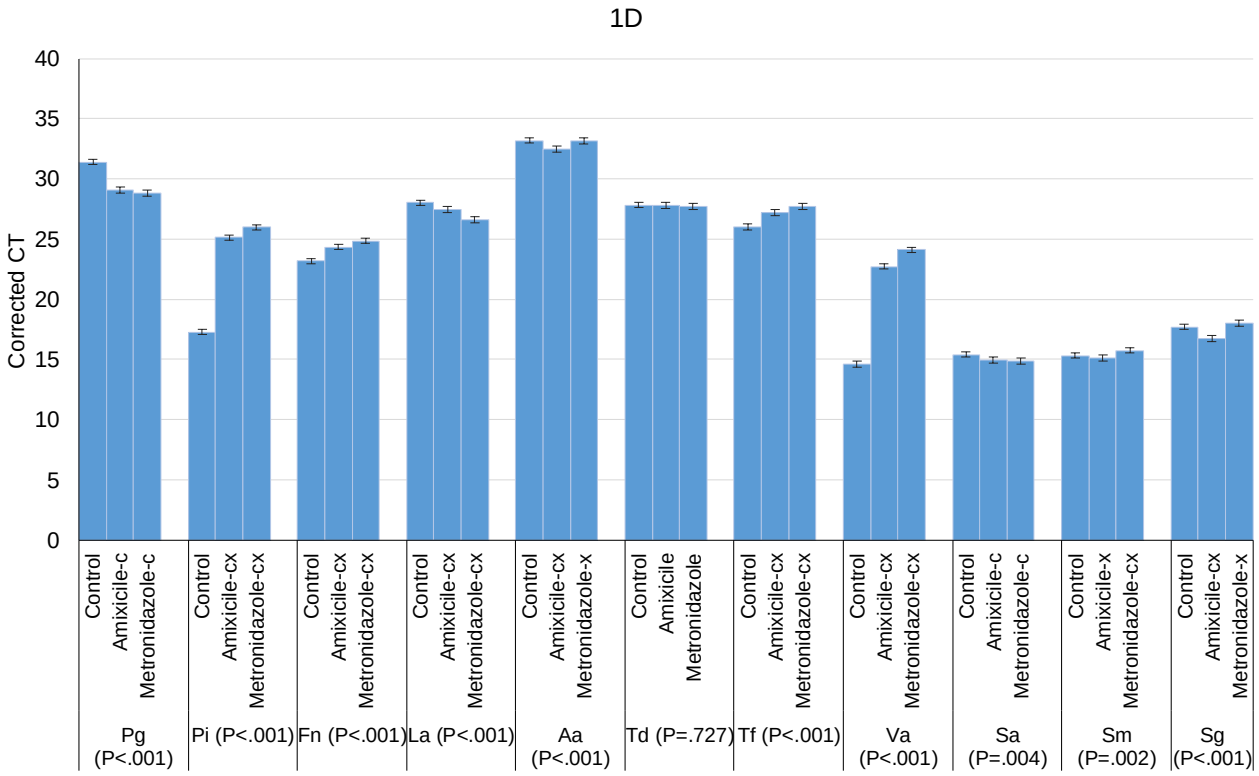

Figure 17. Corrected CT mean estimates for Set 1D (95% CIs)

Figure 19 represents the average CT values taken of Set 1D. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amxicile and Metronidazole.

Table 16. Differences in the Corrected CT mean estimates for Set 1D

Bacterial species	Compare	Corrected CT Estimate	95% CI	
Pg	CvA (P<.001)	2.300	1.976	2.624
	CvM (P<.001)	2.572	2.248	2.896
	AvM (P=.097)	0.272	-0.052	0.596
Pi	CvA (P<.001)	-7.840	-8.164	-7.515
	CvM (P<.001)	-8.689	-9.013	-8.364
	AvM (P<.001)	-0.849	-1.173	-0.525
Fn	CvA (P<.001)	-1.156	-1.481	-0.832
	CvM (P<.001)	-1.678	-2.002	-1.354
	AvM (P=.003)	-0.522	-0.846	-0.197
La	CvA (P=.002)	0.555	0.231	0.879
	CvM (P<.001)	1.418	1.094	1.742
	AvM (P<.001)	0.863	0.539	1.187
Aa	CvA (P<.001)	0.721	0.396	1.045
	CvM (P=.839)	0.032	-0.292	0.357
	AvM (P<.001)	-0.688	-1.012	-0.364
Td	CvA (P=.815)	0.037	-0.287	0.362
	CvM (P=.440)	0.124	-0.200	0.449
	AvM (P=.588)	0.087	-0.237	0.411
Tf	CvA (P<.001)	-1.196	-1.520	-0.872
	CvM (P<.001)	-1.694	-2.018	-1.369
	AvM (P=.004)	-0.498	-0.822	-0.173
Va	CvA (P<.001)	-8.100	-8.425	-7.776
	CvM (P<.001)	-9.478	-9.802	-9.153
	AvM (P<.001)	-1.377	-1.702	-1.053
Sa	CvA (P=.006)	0.472	0.148	0.796
	CvM (P=.002)	0.536	0.212	0.861
	AvM (P=.687)	0.065	-0.260	0.389
Sm	CvA (P=.222)	0.198	-0.126	0.522
	CvM (P=.013)	-0.420	-0.745	-0.096
	AvM (P<.001)	-0.618	-0.943	-0.294
Sg	CvA (P<.001)	0.973	0.649	1.298
	CvM (P=.057)	-0.314	-0.639	0.010
	AvM (P<.001)	-1.288	-1.612	-0.964

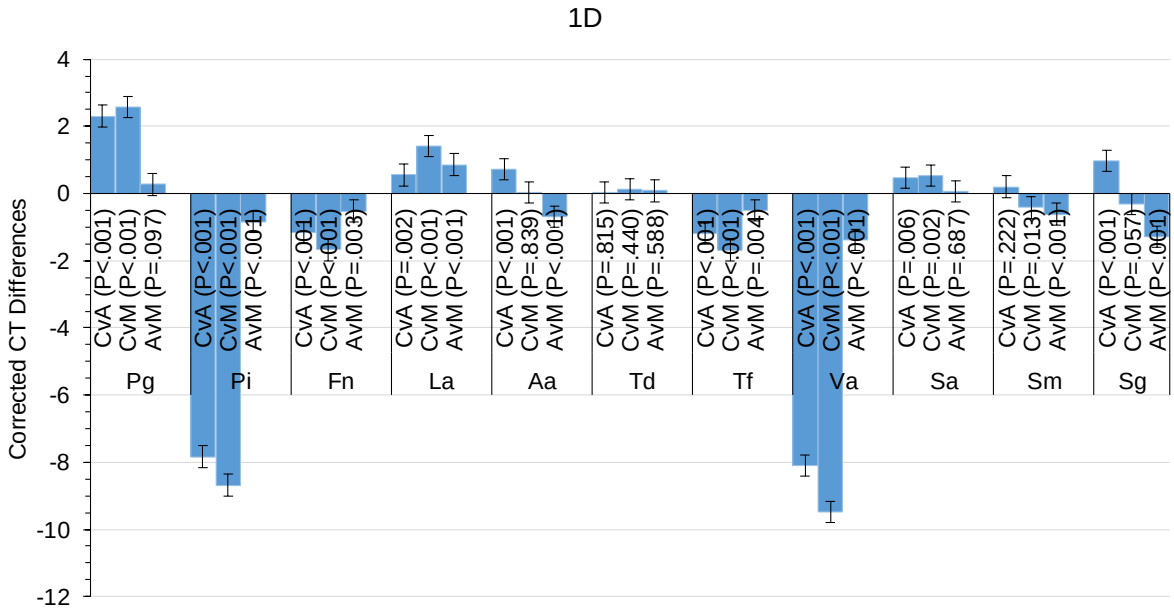

Figure 18. Differences in the Corrected CT mean estimates for Set 1D (95% CIs)

Figure 20 represents the differences in corrected CT mean estimates from the original CT values after standardization with 16s primer for Set 1D. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amixicile and Metronidazole.

Table 17. Fold Estimates for Set 1D

Bacterial species	Antimicrobials	Fold Estimate	95% CI	
Pg	Amixicile (P<.001)	4.925	3.934	6.167
	Metronidazole (P<.001)	5.947	4.750	7.446
Pi	Amixicile (P<.001)	0.004	0.003	0.005
	Metronidazole (P<.001)	0.002	0.002	0.003
Fn	Amixicile (P<.001)	0.449	0.358	0.562
	Metronidazole (P<.001)	0.312	0.250	0.391
La	Amixicile (P=.002)	1.469	1.173	1.839
	Metronidazole (P<.001)	2.672	2.134	3.345
Aa	Amixicile (P<.001)	1.648	1.316	2.063
	Metronidazole (P=.839)	1.023	0.817	1.281
Td	Amixicile (P=.815)	1.026	0.820	1.285
	Metronidazole (P=.440)	1.090	0.871	1.365
Tf	Amixicile (P<.001)	0.436	0.349	0.546
	Metronidazole (P<.001)	0.309	0.247	0.387
Va	Amixicile (P<.001)	0.004	0.003	0.005
	Metronidazole (P<.001)	0.001	0.001	0.002
Sa	Amixicile (P=.006)	1.387	1.108	1.736
	Metronidazole (P=.002)	1.450	1.158	1.816
Sm	Amixicile (P=.222)	1.147	0.916	1.436
	Metronidazole (P=.013)	0.747	0.597	0.936
Sg	Amixicile (P<.001)	1.964	1.568	2.458
	Metronidazole (P=.057)	0.804	0.642	1.007

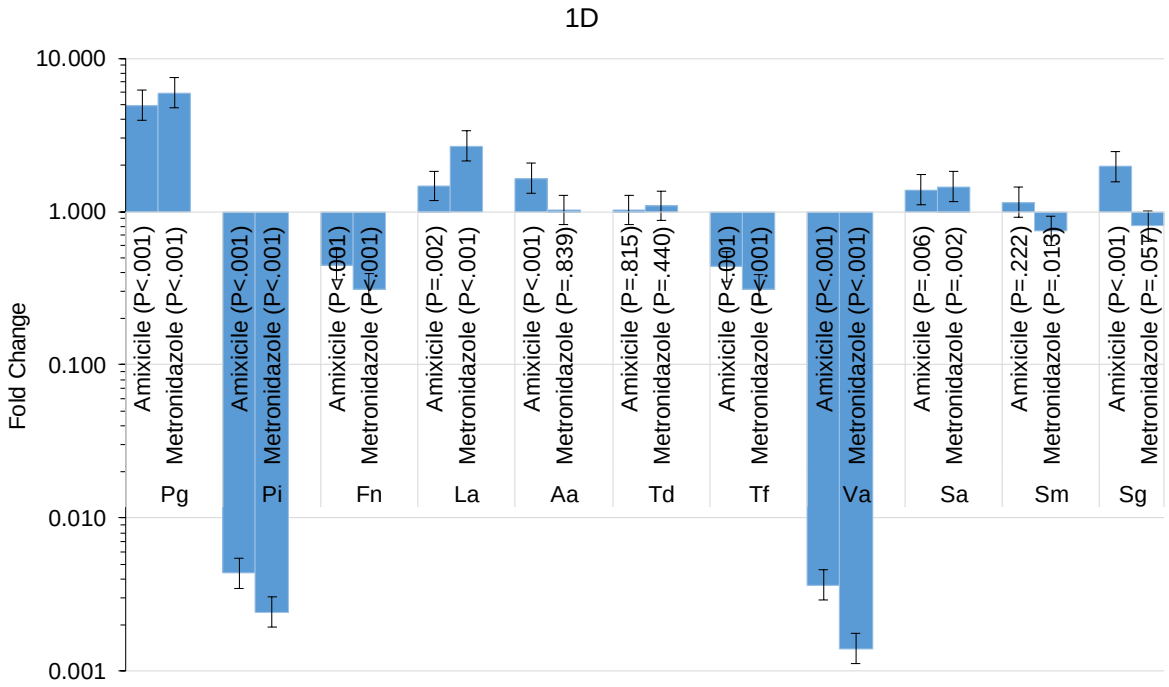

Figure 19. Fold Estimates for Set 1D (95% CIs)

Figure 21 represents the fold change observed for Set 1D for bacterial species after treatment of either Amixicile or Metronidazole. A P Value <.001 represented a statistical significant change in the numbers of bacteria from the control and antimicrobial treatment.

Table 18. Corrected CT mean estimates for Set 2D

Bacterial species	Antimicrobials	Corrected CT Estimate	95% CI	
Pg (P<.001)	Control	31.39	31.16	31.62
	Amixicile-c	29.09	28.86	29.32
	Metronidazole-c	28.82	28.59	29.05
Pi (P<.001)	Control	17.29	17.06	17.52
	Amixicile-cx	25.13	24.90	25.36
	Metronidazole-cx	25.98	25.75	26.21
Fn (P<.001)	Control	23.19	22.96	23.42
	Amixicile-cx	24.34	24.11	24.57
	Metronidazole-cx	24.87	24.64	25.09
La (P<.001)	Control	28.03	27.80	28.25
	Amixicile-cx	27.47	27.24	27.70
	Metronidazole-cx	26.61	26.38	26.84
Aa (P<.001)	Control	33.19	32.96	33.42
	Amixicile-cx	32.47	32.24	32.70
	Metronidazole-x	33.16	32.93	33.39
Td (P=.727)	Control	27.84	27.61	28.07
	Amixicile	27.81	27.58	28.04
	Metronidazole	27.72	27.49	27.95
Tf (P<.001)	Control	26.02	25.79	26.25
	Amixicile-cx	27.21	26.98	27.44
	Metronidazole-cx	27.71	27.48	27.94
Va (P<.001)	Control	14.62	14.39	14.85
	Amixicile-cx	22.72	22.49	22.95
	Metronidazole-cx	24.10	23.87	24.33
Sa (P=.004)	Control	15.42	15.19	15.65
	Amixicile-c	14.95	14.72	15.18
	Metronidazole-c	14.88	14.65	15.11
Sm (P=.002)	Control	15.32	15.09	15.55
	Amixicile-x	15.12	14.89	15.35
	Metronidazole-cx	15.74	15.51	15.97
Sg (P<.001)	Control	17.71	17.48	17.94
	Amixicile-cx	16.74	16.51	16.97
	Metronidazole-x	18.03	17.80	18.26

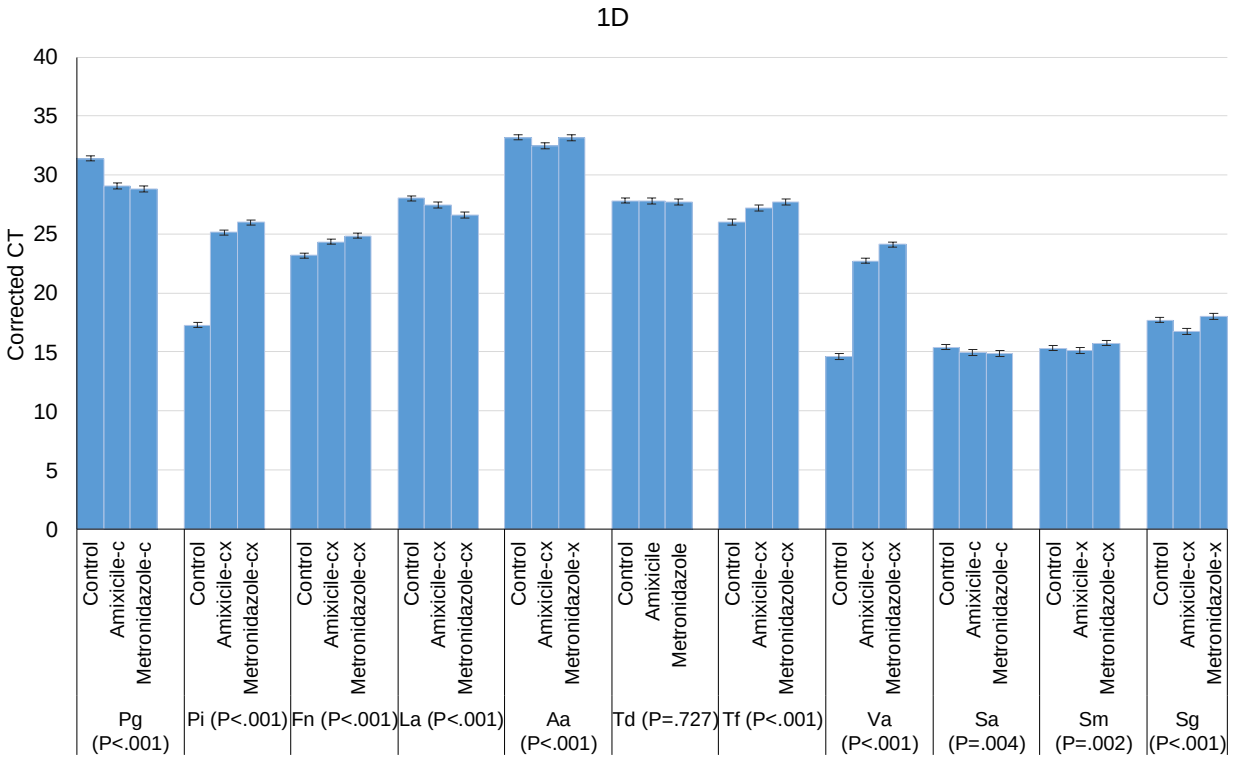

Figure 20. Corrected CT mean estimates for Set 2D (95% CIs)

Figure 22 represents the average CT values taken of Set 2D. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amxicile and Metronidazole.

Table 19: Differences in the Corrected CT mean estimates for Set 2D

Bacterial species	Compare	Corrected CT Estimate	95% CI	
Pg	CvA (P<.001)	2.300	1.976	2.624
	CvM (P<.001)	2.572	2.248	2.896
	AvM (P=.097)	0.272	-0.052	0.596
Pi	CvA (P<.001)	-7.840	-8.164	-7.515
	CvM (P<.001)	-8.689	-9.013	-8.364
	AvM (P<.001)	-0.849	-1.173	-0.525
Fn	CvA (P<.001)	-1.156	-1.481	-0.832
	CvM (P<.001)	-1.678	-2.002	-1.354
	AvM (P=.003)	-0.522	-0.846	-0.197
La	CvA (P=.002)	0.555	0.231	0.879
	CvM (P<.001)	1.418	1.094	1.742
	AvM (P<.001)	0.863	0.539	1.187
Aa	CvA (P<.001)	0.721	0.396	1.045
	CvM (P=.839)	0.032	-0.292	0.357
	AvM (P<.001)	-0.688	-1.012	-0.364
Td	CvA (P=.815)	0.037	-0.287	0.362
	CvM (P=.440)	0.124	-0.200	0.449
	AvM (P=.588)	0.087	-0.237	0.411
Tf	CvA (P<.001)	-1.196	-1.520	-0.872
	CvM (P<.001)	-1.694	-2.018	-1.369
	AvM (P=.004)	-0.498	-0.822	-0.173
Va	CvA (P<.001)	-8.100	-8.425	-7.776
	CvM (P<.001)	-9.478	-9.802	-9.153
	AvM (P<.001)	-1.377	-1.702	-1.053
Sa	CvA (P=.006)	0.472	0.148	0.796
	CvM (P=.002)	0.536	0.212	0.861
	AvM (P=.687)	0.065	-0.260	0.389
Sm	CvA (P=.222)	0.198	-0.126	0.522
	CvM (P=.013)	-0.420	-0.745	-0.096
	AvM (P<.001)	-0.618	-0.943	-0.294
Sg	CvA (P<.001)	0.973	0.649	1.298
	CvM (P=.057)	-0.314	-0.639	0.010
	AvM (P<.001)	-1.288	-1.612	-0.964

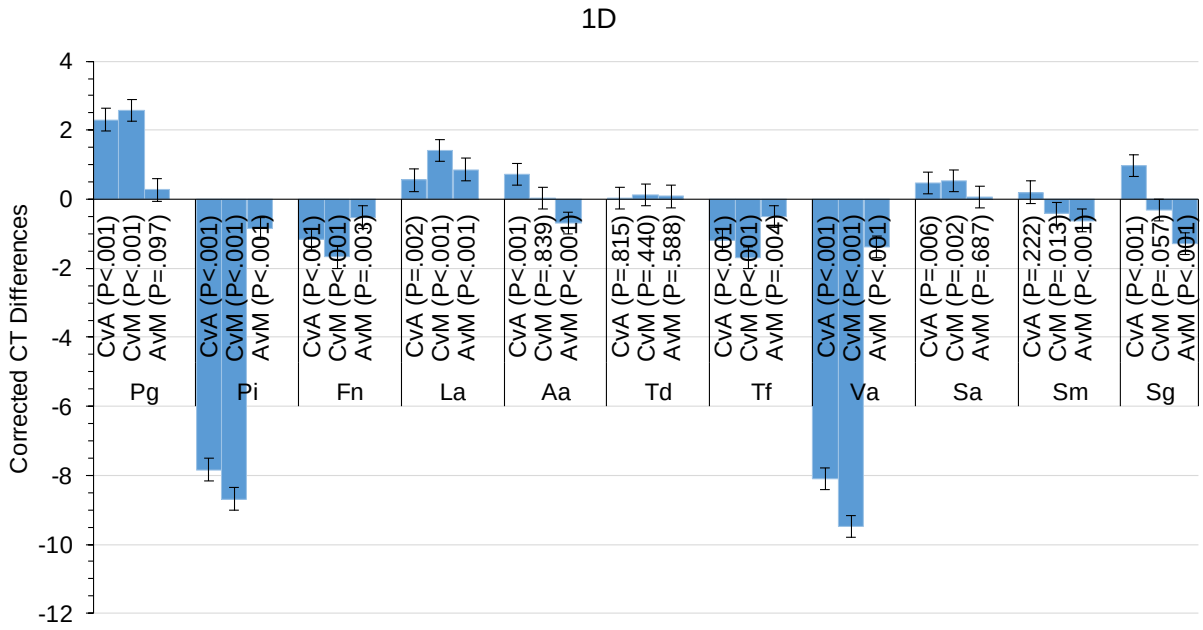

Figure 21. Differences in the Corrected CT mean estimates for Set 2D (95% CIs)

Figure 23 represents the differences in corrected CT mean estimates from the original CT values after standardization with 16s primer for Set 2D. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amixicile and Metronidazole.

Table 20. Fold Estimates for Set 2D

Bacterial species	Antimicrobials	Fold Estimate	95% CI	
Pg	Amixicile (P<.001)	4.925	3.934	6.167
	Metronidazole (P<.001)	5.947	4.750	7.446
Pi	Amixicile (P<.001)	0.004	0.003	0.005
	Metronidazole (P<.001)	0.002	0.002	0.003
Fn	Amixicile (P<.001)	0.449	0.358	0.562
	Metronidazole (P<.001)	0.312	0.250	0.391
La	Amixicile (P=.002)	1.469	1.173	1.839
	Metronidazole (P<.001)	2.672	2.134	3.345
Aa	Amixicile (P<.001)	1.648	1.316	2.063
	Metronidazole (P=.839)	1.023	0.817	1.281
Td	Amixicile (P=.815)	1.026	0.820	1.285
	Metronidazole (P=.440)	1.090	0.871	1.365
Tf	Amixicile (P<.001)	0.436	0.349	0.546
	Metronidazole (P<.001)	0.309	0.247	0.387
Va	Amixicile (P<.001)	0.004	0.003	0.005
	Metronidazole (P<.001)	0.001	0.001	0.002
Sa	Amixicile (P=.006)	1.387	1.108	1.736
	Metronidazole (P=.002)	1.450	1.158	1.816
Sm	Amixicile (P=.222)	1.147	0.916	1.436
	Metronidazole (P=.013)	0.747	0.597	0.936
Sg	Amixicile (P<.001)	1.964	1.568	2.458
	Metronidazole (P=.057)	0.804	0.642	1.007

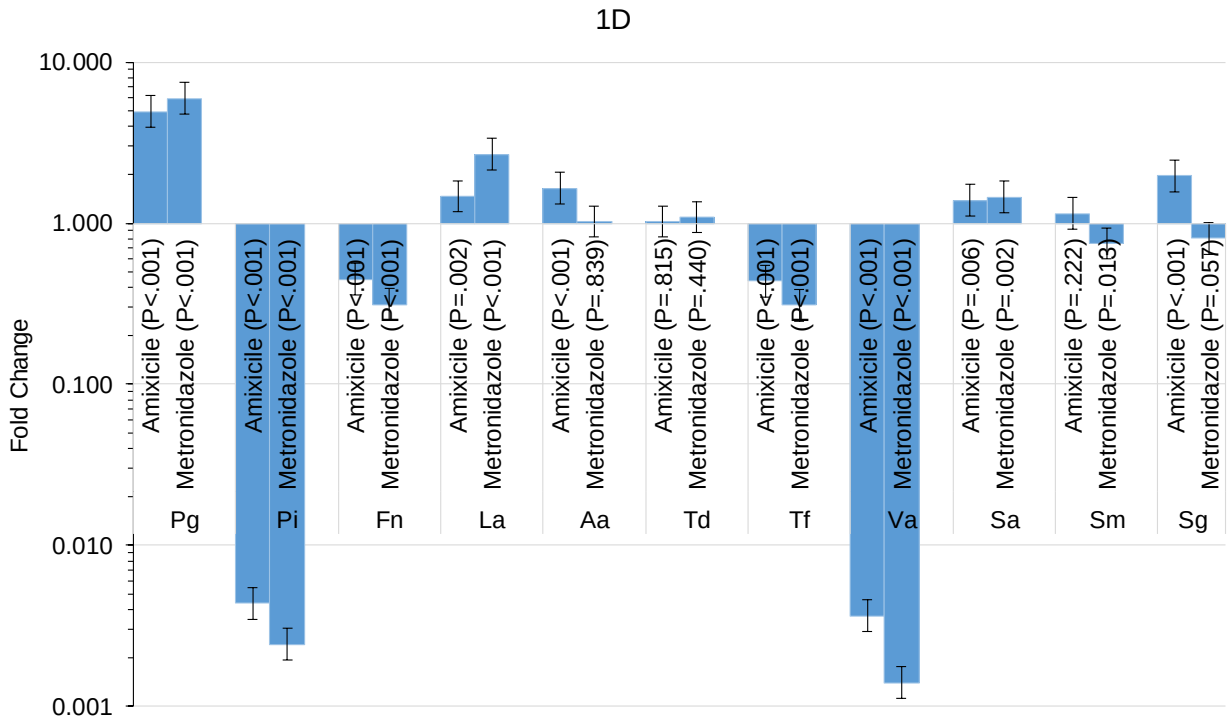

Figure 22. Fold Estimates for Set 2D (95% CIs)

Figure 24 represents the fold change observed for Set 2D for bacterial species after treatment of either Amixicile or Metronidazole. A P Value <.001 represented a statistical significant change in the numbers of bacteria from the control and antimicrobial treatment.

Table 21. Corrected CT mean estimates for Set 3D

Bacterial species	Antimicrobials	Corrected CT Estimate	95% CI	
Pg (P=.369)	Control	30.46	29.84	31.08
	Amixicile	29.95	29.34	30.57
	Metronidazole	29.91	29.29	30.52
Pi (P<.001)	Control	17.52	16.90	18.13
	Amixicile-cx	25.55	24.94	26.17
	Metronidazole-cx	26.87	26.25	27.49
Fn (P<.001)	Control	22.83	22.21	23.45
	Amixicile-c	24.40	23.78	25.02
	Metronidazole-c	25.19	24.57	25.80
La (P=.011)	Control	28.15	27.53	28.77
	Amixicile	27.07	26.45	27.69
	Metronidazole-c	26.87	26.25	27.49
Aa (P=.397)	Control	33.53	32.92	34.15
	Amixicile	33.60	32.98	34.22
	Metronidazole	34.08	33.46	34.69
Td (P=.595)	Control	28.03	27.42	28.65
	Amixicile	27.71	27.09	28.32
	Metronidazole	28.12	27.51	28.74
Tf (P=.005)	Control	26.29	25.67	26.91
	Amixicile-c	27.55	26.94	28.17
	Metronidazole-c	27.65	27.03	28.27
Va (P<.001)	Control	14.54	13.92	15.16
	Amixicile-cx	23.76	23.14	24.37
	Metronidazole-cx	25.35	24.74	25.97
Sa (P=.821)	Control	15.65	15.03	16.26
	Amixicile	15.88	15.26	16.50
	Metronidazole	15.88	15.26	16.49
Sm (P=.300)	Control	15.33	14.71	15.95
	Amixicile	15.25	14.63	15.86
	Metronidazole	15.87	15.25	16.49
Sg (P=.001)	Control	17.37	16.76	17.99
	Amixicile-x	17.01	16.39	17.62
	Metronidazole-cx	18.66	18.04	19.28

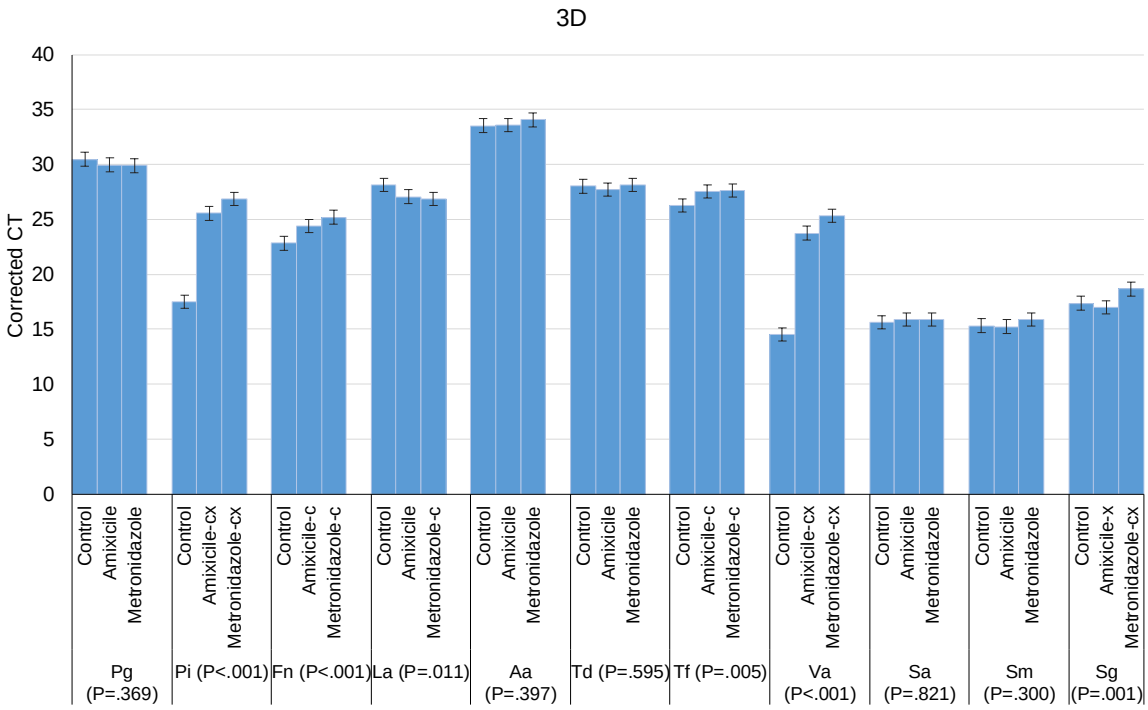

Figure 23. Corrected CT mean estimates for Set 3D (95% CIs)

Figure 25 represents the average CT values taken of Set 3D. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amxicile and Metronidazole.

Table 22. Differences in the Corrected CT mean estimates for Set 3D

Bacterial species	Compare	Corrected CT Estimate	95% CI	
Pg	CvA (P=.245)	0.507	-0.367	1.381
	CvM (P=.205)	0.554	-0.320	1.427
	AvM (P=.914)	0.047	-0.827	0.920
Pi	CvA (P<.001)	-8.038	-8.911	-7.164
	CvM (P<.001)	-9.352	-10.225	-8.478
	AvM (P=.004)	-1.314	-2.188	-0.440
Fn	CvA (P<.001)	-1.574	-2.447	-0.700
	CvM (P<.001)	-2.359	-3.233	-1.485
	AvM (P=.076)	-0.786	-1.659	0.088
La	CvA (P=.017)	1.084	0.210	1.958
	CvM (P=.005)	1.285	0.411	2.158
	AvM (P=.643)	0.201	-0.673	1.074
Aa	CvA (P=.872)	-0.070	-0.943	0.804
	CvM (P=.214)	-0.543	-1.416	0.331
	AvM (P=.277)	-0.473	-1.347	0.400
Td	CvA (P=.450)	0.328	-0.546	1.201
	CvM (P=.834)	-0.091	-0.964	0.783
	AvM (P=.336)	-0.418	-1.292	0.455
Tf	CvA (P=.006)	-1.264	-2.138	-0.391
	CvM (P=.003)	-1.360	-2.233	-0.486
	AvM (P=.825)	-0.095	-0.969	0.778
Va	CvA (P<.001)	-9.216	-10.090	-8.342
	CvM (P<.001)	-10.812	-11.686	-9.939
	AvM (P<.001)	-1.597	-2.470	-0.723
Sa	CvA (P=.585)	-0.236	-1.110	0.637
	CvM (P=.593)	-0.231	-1.105	0.642
	AvM (P=.991)	0.005	-0.869	0.879
Sm	CvA (P=.845)	0.084	-0.789	0.958
	CvM (P=.216)	-0.540	-1.414	0.333
	AvM (P=.155)	-0.625	-1.498	0.249
Sg	CvA (P=.397)	0.368	-0.506	1.241
	CvM (P=.005)	-1.289	-2.162	-0.415
	AvM (P<.001)	-1.656	-2.530	-0.782

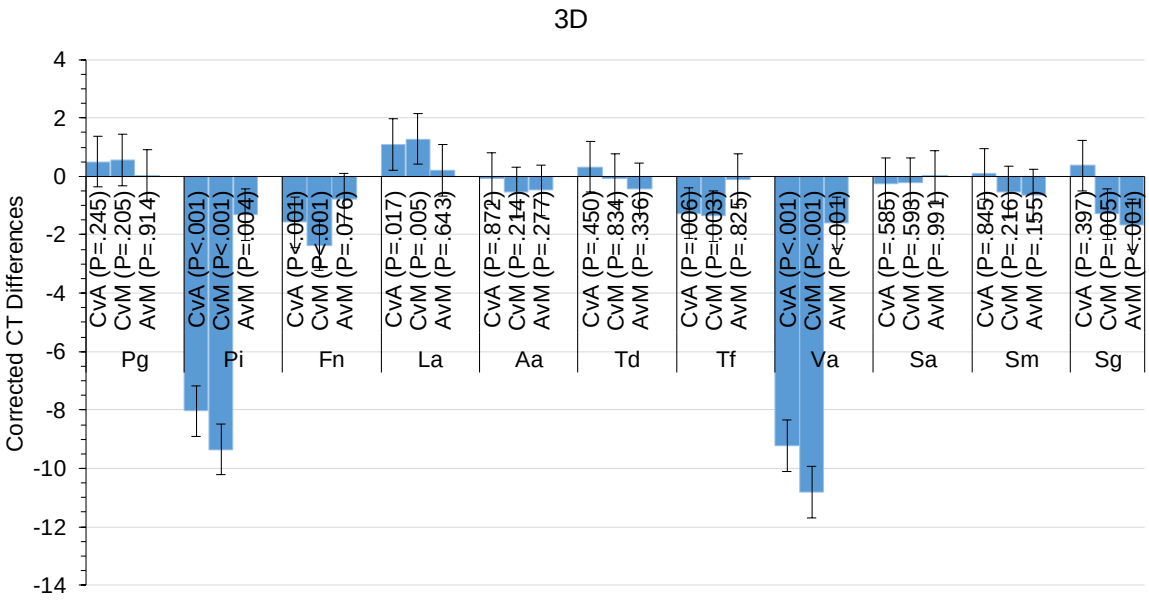

Figure 24. Differences in the Corrected CT mean estimates for Set 3D (95% CIs)

Figure 26 represents the differences in corrected CT mean estimates from the original CT values after standardization with 16s primer for Set 3D. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amixicile and Metronidazole.

Table 23. Fold Estimates for Set 3D

Bacterial species	Antimicrobials	Fold Estimate	95% CI	
Pg	Amixicile (P=.245)	1.421	0.776	2.604
	Metronidazole (P=.205)	1.468	0.801	2.689
Pi	Amixicile (P<.001)	0.004	0.002	0.007
	Metronidazole (P<.001)	0.002	0.001	0.003
Fn	Amixicile (P<.001)	0.336	0.183	0.616
	Metronidazole (P<.001)	0.195	0.106	0.357
La	Amixicile (P=.017)	2.120	1.157	3.885
	Metronidazole (P=.005)	2.436	1.330	4.464
Aa	Amixicile (P=.872)	0.953	0.520	1.746
	Metronidazole (P=.214)	0.686	0.375	1.258
Td	Amixicile (P=.450)	1.255	0.685	2.300
	Metronidazole (P=.834)	0.939	0.513	1.721
Tf	Amixicile (P=.006)	0.416	0.227	0.763
	Metronidazole (P=.003)	0.390	0.213	0.714
Va	Amixicile (P<.001)	0.002	0.001	0.003
	Metronidazole (P<.001)	0.001	0.000	0.001
Sa	Amixicile (P=.585)	0.849	0.463	1.556
	Metronidazole (P=.593)	0.852	0.465	1.561
Sm	Amixicile (P=.845)	1.060	0.579	1.942
	Metronidazole (P=.216)	0.688	0.375	1.260
Sg	Amixicile (P=.397)	1.290	0.704	2.364
	Metronidazole (P=.005)	0.409	0.223	0.750

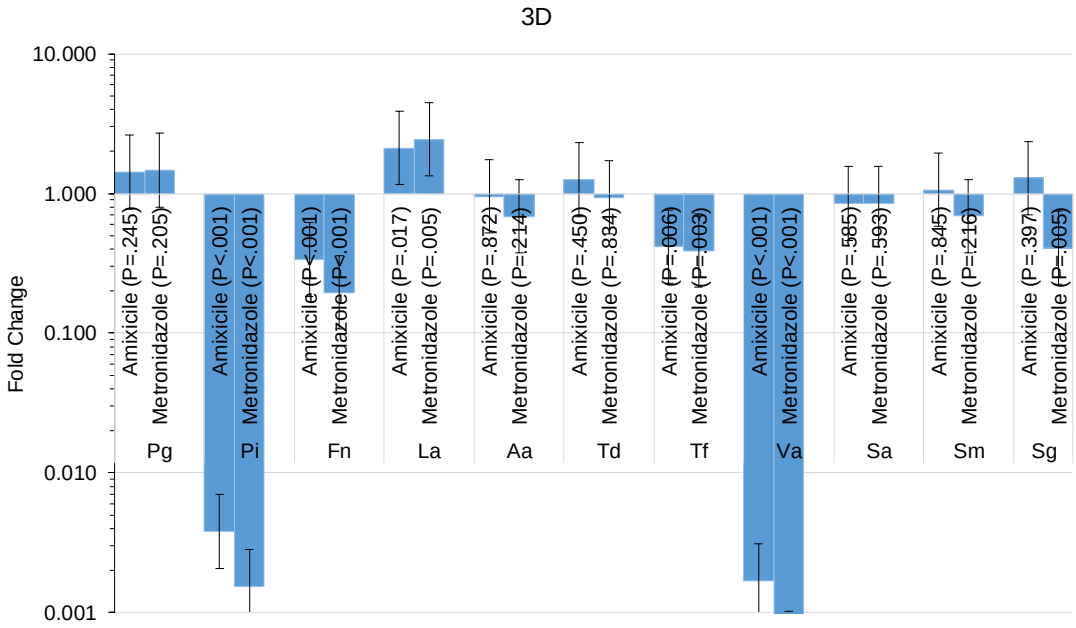

Figure 25. Fold Estimates for Set 3D (95% CIs)

Figure 27 represents the fold change observed for Set 3D for bacterial species after treatment of either Amixicile or Metronidazole. A P Value <.001 represented a statistical significant change in the numbers of bacteria from the control and antimicrobial treatment.

Table 24. Differences in the Corrected CT mean estimates for Sets 1D, 2D, 3D combined

Bacterial species	Compare	Corrected CT Estimate	95% CI	
Pg	CvA (P<.001)	1.208	0.688	1.728
	CvM (P<.001)	1.327	0.807	1.847
	AvM (P=.645)	0.119	-0.401	0.638
Pi	CvA (P<.001)	-7.677	-8.197	-7.157
	CvM (P<.001)	-8.779	-9.299	-8.259
	AvM (P<.001)	-1.102	-1.622	-0.582
Fn	CvA (P<.001)	-1.074	-1.594	-0.554
	CvM (P<.001)	-1.776	-2.295	-1.256
	AvM (P=.010)	-0.701	-1.221	-0.181
La	CvA (P<.001)	1.230	0.710	1.750
	CvM (P<.001)	1.417	0.897	1.937
	AvM (P=.467)	0.188	-0.332	0.707
Aa	CvA (P=.008)	0.718	0.198	1.238
	CvM (P=.237)	0.307	-0.213	0.827
	AvM (P=.117)	-0.411	-0.930	0.109
Td	CvA (P=.031)	0.577	0.057	1.097
	CvM (P=.420)	0.208	-0.312	0.728
	AvM (P=.158)	-0.369	-0.889	0.151
Tf	CvA (P=.160)	-0.367	-0.887	0.153
	CvM (P<.001)	-1.283	-1.803	-0.764
	AvM (P=.001)	-0.916	-1.436	-0.396
Va	CvA (P<.001)	-8.564	-9.083	-8.044
	CvM (P<.001)	-10.170	-10.690	-9.650
	AvM (P<.001)	-1.607	-2.127	-1.087
Sa	CvA (P=.138)	0.388	-0.131	0.908
	CvM (P=.193)	0.339	-0.181	0.859
	AvM (P=.848)	-0.049	-0.569	0.471
Sm	CvA (P=.583)	0.141	-0.378	0.661
	CvM (P=.336)	-0.249	-0.769	0.271
	AvM (P=.136)	-0.390	-0.910	0.130
Sg	CvA (P=.005)	0.776	0.257	1.296
	CvM (P=.128)	-0.399	-0.919	0.121
	AvM (P<.001)	-1.175	-1.695	-0.655

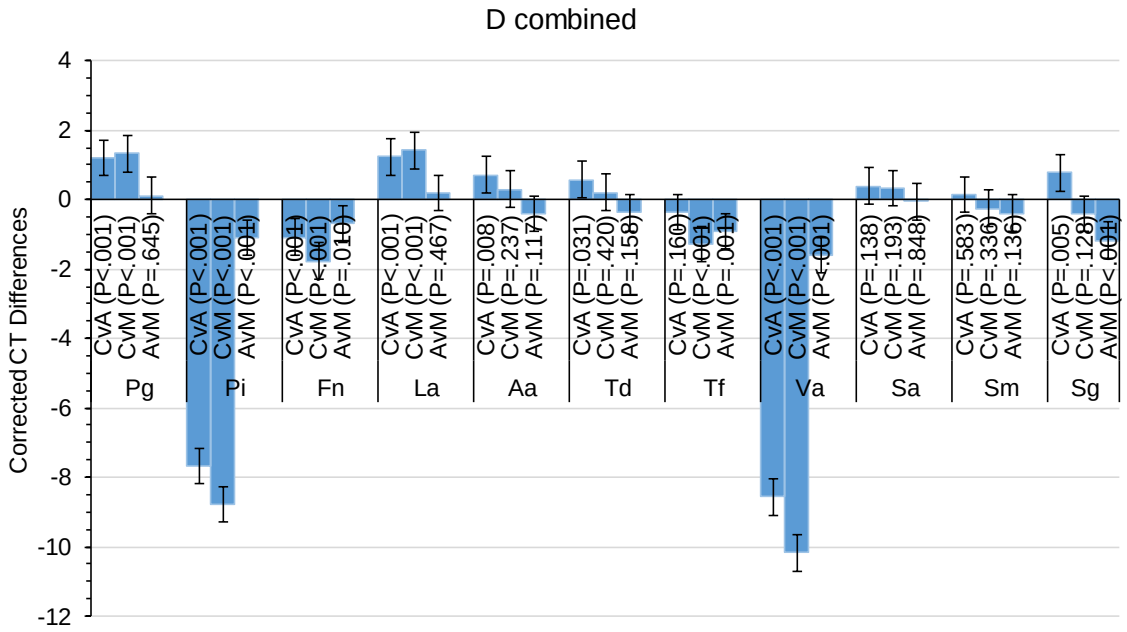

Figure 26. Differences in the Corrected CT mean estimates for Sets 1D, 2D, 3D combined

Figure 28 represents the differences in corrected CT mean estimates from the original CT values after standardization with 16s primer for Sets 1D, 2D, and 3D combined. ANOVA analysis was performed and applied to compare the control group to Amixicile, control group to Metronidazole and lastly compare Amixicile and Metronidazole. A “c” represents a statistically significant difference from control and antimicrobial. An “x” represents a statistically significant difference from Amixicile and Metronidazole.