Received: 8 March 2022 | Accepted: 23 September 2022
DOI: 10.1111/evj.13884

ORIGINAL ARTICLE

Equine sinusitis aetiology is linked to sinus microbiome by amplicon sequencing

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Abstract

Background: Information regarding the microbiome in sinusitis using genetic sequencing is lacking and more-in-depth understanding of the microbiome could improve antimicrobial selection and treatment outcomes for cases of primary sinusitis.

Objectives: To describe sinus microbiota in samples from horses with sinusitis and compare microbiota and the presence of antimicrobial resistance genes between primary, dental-related and other secondary causes of sinusitis.

Study design: Retrospective case series.

Methods: Records of equine sinusitis from 2017 to 2021 were reviewed and historical microbial amplicon sequence data were obtained from clinical diagnostic testing of sinus secretions. Following bioinformatic processing of bacterial and fungal sequence data, the sinus microbiota and importance of sinusitis aetiology among other factors were investigated from the perspectives of alpha diversity (e.g., number of operational taxonomic units [OTUs], Hill1 Diversity), beta diversity, and differentially abundant taxa. Quantitative PCR allowed for comparisons of estimated bacterial abundance and detection rate of common antibiotic resistance-associated genes. In a smaller subset, longitudinal analysis was performed to evaluate similarity in samples over time.

Results: Of 81 samples analysed from 70 horses, the bacterial microbiome was characterised in 66, and fungal in five. Only sinusitis aetiology was shown to significantly influence microbiome diversity and composition ($p < 0.05$). Dental-related sinusitis ($n = 44$) was associated with a significantly higher proportion of obligate anaerobic bacteria, whereas primary sinusitis ($n = 12$) and other ($n = 10$) groups were associated with fewer bacteria and higher proportions of facultative anaerobic and aerobic genera. Antimicrobial resistance genes and fungal components were exclusively identified in dental-related sinusitis.

Main limitations: Retrospective nature, incomplete prior antimicrobial administration data.

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Conclusions: Molecular characterisation in sinusitis identifies microbial species which may be difficult to isolate via culture, and microbiome profiling can differentiate sinusitis aetiology, which may inform further treatment, including antimicrobial therapy.

KEYWORDS
antimicrobial resistance genes, horse, microbiome, molecular sequencing, sinusitis

1 | INTRODUCTION

Sinusitis is the most common disease that affects the paranasal sinus cavities in the horse.[1] Sinusitis is commonly subclassified into either primary (PS) or secondary depending upon whether an underlying aetiology is identified. Dental-related sinusitis (DRS) is the most common secondary cause, but others such as tumours, trauma and sinonasal cysts are also reported.[1] The clinical signs of sinusitis may include nasal discharge, facial swelling, draining tracts exiting the maxilla and malodor.[2]

Previous studies estimate that between 62% to 84% of horses with sinusitis are treated with antibiotics prior to referral.[1,3] Antimicrobial therapy is selected empirically or based on culture and sensitivity results of specimens collected from the affected sinus compartments. The presence of anaerobic bacteria has historically been associated with DRS, and *Streptococcus equi zooepidemicus* with PS, although studies reporting culture are conflicted on this association.[4,5] Molecular sequencing is better able to characterise the microbiome of the sinuses compared to routine culture, because of the uncultivable nature of many species present.[6] This has been demonstrated in humans, and more recently in sinus samples from healthy horses.[4,7] Therefore, traditional culture methods do not accurately identify sinus microbiota in health or disease. While molecular sequencing does not provide specific antimicrobial sensitivity information unlike culture, appropriate therapeutics can be further guided by databases incorporating knowledge relevant to antibiotic selection (e.g., bacterial morphology). In addition, the presence of common antimicrobial resistance-associated genes (ARGs) can be examined during the amplification stage of molecular sequencing.

Studies in humans using molecular sequencing have shown that significant changes occur in the sinus microbiome during times of disease.[7–9] To the best of the authors' knowledge, there are no studies using next generation sequencing (NGS) to evaluate the microbiome of equine paranasal sinusitis. Thus, the purpose of this study is to describe the microbiota of clinical cases of sinusitis using NGS, to compare the microbiota between PS, DRS and other causes of sinusitis (OS), and to report the presence of ARGs within the sampled population.

2 | MATERIALS AND METHODS

2.1 | Case selection

A medical records search was performed to identify a convenience sample of horses presented to the Marion duPont Scott Equine Medical Center for investigation of sinusitis between 1 August 2017 and 15 August 2021. Horses were included if they had a sample aseptically collected from one or more paranasal sinus compartments during surgery that was submitted for NGS. All horses had standing computed tomography of the head performed prior to surgery, particularly to rule in or out dental-related sinusitis. Diagnosis of other secondary causes of sinusitis was based on surgical findings and results of samples submitted for histopathology. Information recorded included signalment, previous antibiotic administration, sinusitis aetiology (PS, DRS or OS), and whether previous sinus surgery had been performed. Horses were excluded if samples were collected from outside of the paranasal sinuses, or were not collected at the time of surgery.

2.2 | Collection of sinus secretions

All horses received flunixin meglumine (1.1 mg/kg iv, Covetrus), and pre-operative antimicrobial therapy was provided with either ceftiofur (2.2 mg/kg iv, Naxcel, Zoetis) and gentamicin (6.6 mg/kg iv, VetOne), or ceftiofur crystalline-free (6.6 mg/kg im, Excede, Zoetis). Horses were sedated with either detomidine (0.01 mg/kg iv, Zoetis) or xylazine (0.4 mg/kg iv, Covetrus) and restrained in standing stocks. Sinus trephination was performed as previously described using a 19 or 25 mm galt trephined (Sontec Instruments).[10] Samples were collected on dry polyester flock swabs (Puritan Medical Products Company LLC) and held at room temperature (20°C–23°C) for up to 6 h, prior to overnight shipping without cold packing to the processing laboratory.

2.3 | Microbiome community profiling

DNA extraction, PCR amplification, library preparation and sequencing of 16S rRNA regions V1-V2 on Illumina Miseq platform was conducted as previously performed by MicroGenDX.[11,12] Briefly, DNA extraction was performed with a Qiagen TissueLyser and Zymo MagBead 96 DNA/RNA kit (Zymo Research). Samples were mechanically lysed using Zirconium oxide beads (0.5 mm) and the Qiagen TissueLyser. The lysate was extracted for total DNA following the Zymo MagBead 96 DNA/RNA kit's protocol on the KingFisher FLEX (Thermo Fisher Scientific). PCR amplification was selective for the 16S rRNA hypervariable regions V1-V2 using primers 28F (GAGTTTGATCNTGGCTCAG)

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and 388R (GCTGCCTCCCGTAGGAGT). PCR reactions were conducted on ABI Veriti thermocyclers (Applied Biosystems) with a thermal profile consisting of 5-min denaturation step at 95°C, 35 cycles of 94°C for 30 s, 52°C for 40 s and 72°C for 60 s, and a final extension step of 72°C for 10 min. PCR products were combined based on qualitative band strength to form the pooled amplicon libraries and size selection was performed using Agencourt AMPure XP beads (Beckman Coulter) and Qiagen Minelute Kits. Pooled libraries were quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific). Sequencing was conducted on an Illumina MiSeq. Not previously reported, ITS3-4 amplification was attempted to identify fungal lineages using otherwise same protocol as in 16S profiling and, upon positive confirmation from gel image, ITS3-4 target amplicons were sequenced to characterise fungal communities following standard MicroGenDX diagnostic procedures, using ITS3F (GCATCGATGAAGAACGCAGC) and ITS4R (GCATCGATGAAGAACGCAGC) primers. Positive controls, negative extraction controls and no template controls were also included for sequencing. A proprietary quantitative PCR (qPCR) panel using Roche Lightcycler 480 platform (Roche Diagnostics) was used to estimate total bacterial load and test for presence of common ARGs (potentiated sulphonamides [Sul I/Sul II], beta lactams [SHV/TEM/CTX-M], carbapenem [NDM/KPC/OXA], macrolides [ermB], methicillin [mecA], quinolones [qnr/gyrA], tetracycline [tetB/tetM] and vancomycin [vanA]). Bioinformatic curation, quality control filtering and analysis of data were subsequently performed by RTL Genomics, a division of MicroGenDX. Denoising of sequence reads, chimera detection and paired read assembly were conducted using Usearch7,[13] UCHIME[14] and PEAR,[15] respectively. Quality filtered and assembled reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity threshold using the UPARSE algorithm.[16] OTU assignment then used an in-house curated taxonomic reference database. Multiple sequence alignment and phylogenetic tree construction for downstream analysis was performed using MUSCLE,[17] and FastTree.[18] Sequence data were uploaded into NCBI sequence reads archive (SRA) Submission number PRJNA807287.

2.4 | Data analysis

Statistical analyses were conducted in R version 4.1.0 (R Core Team, R Foundation for Statistical Computing). OTUs corresponding to 'no hit' and suspected contaminants (e.g., *Ralstonia* sp., *Pelomonas*

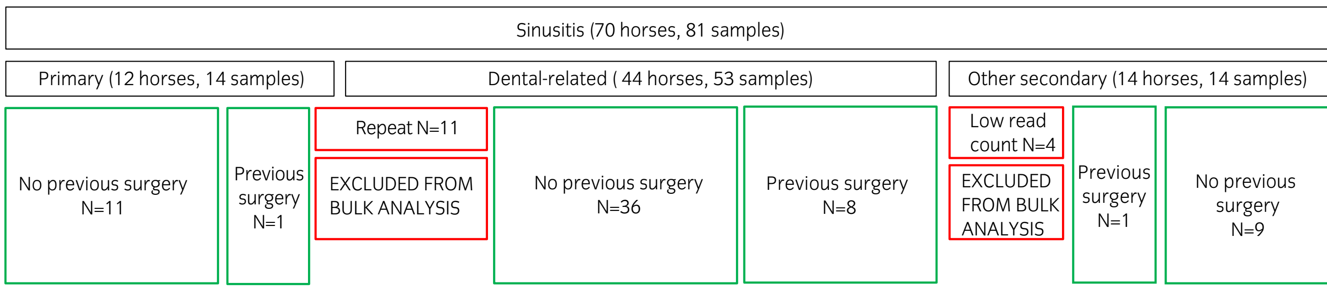

FIGURE 1 Distribution of cases across the three aetiologic groupings of sinusitis, and those excluded from the bulk analysis. N, number of samples

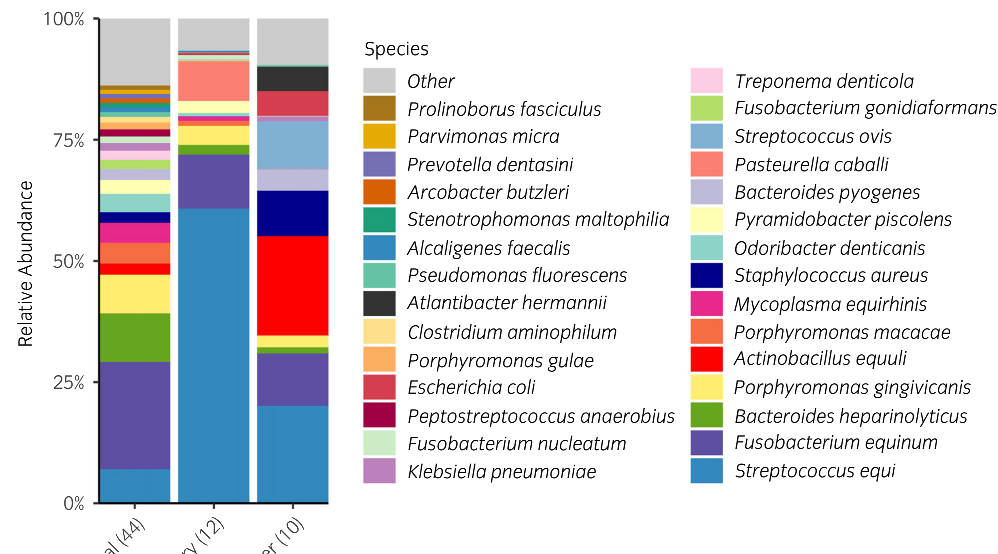

FIGURE 2 Mean relative abundance of 29 most abundantly quantified species, with all others collapsed into 'other' and shown per sinusitis group. Additionally, the number of samples included in analysis per group is shown in parentheses

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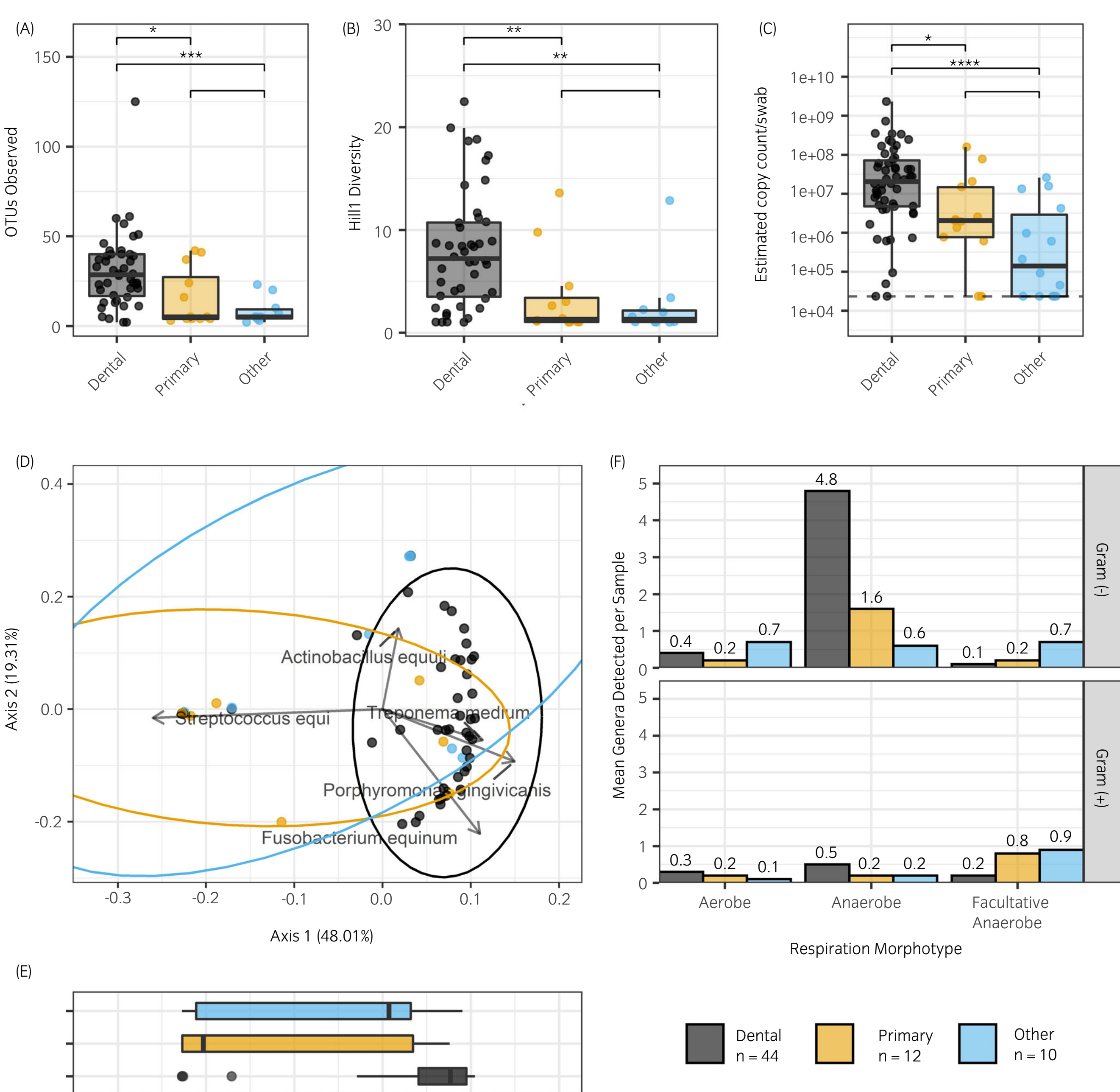

FIGURE 3 Microbial profiles are strongly associated with the cause of sinusitis. Box and whisker plots show distribution of (A) normalised OTU count, (B) Hill1 diversity, and (C) estimated bacterial burden per sample, where boxes indicate median, 1st and 3rd quartiles. In (C), a horizontal dashed line indicates the lower limit of detection (D) bacterial profile dissimilarity was summarised via weighted uniFrac distance and assessed by principal coordinate analysis (PCoA). The 5 species which were most strongly associated with the top 2 axes are depicted in biplot, where length of arrow is proportional to correlation strength. In (E), a box and whisker plot paired to (D) shows the degree of separation along Axis 1 by each group. Lastly, (F) OTUs were reduced to genus level, and all detected above 2% relative abundance were kept to show the mean number of bacteria detected per sample, per known gram stain and aerotolerance characteristics. Significance annotation for (A–C) as follows: * = ($p < 0.05$), ** = ($p < 0.01$), *** = ($p < 0.001$), and **** = ($p < 0.0001$).

saccharophila) based on inspection of controls were removed prior to analysis. Rarefaction survey suggested that sequencing depth was uneven across samples (Figure S1). Thus, read counts were normalised to 1125 counts per sample (the lowest post-QC read count above 1000 reads) and samples below that count were discarded. Count normalisation was achieved using Scaling with Ranked Subsampling approach.[19]

Alpha diversity was investigated by number of OTUs and Hill1 Diversity. Study case factors (age, breed, sex, previous antibiotic usage, chronicity and sinusitis aetiology) were evaluated for their influence on alpha diversity using analysis of variance (ANOVA). Backward feature selection was used to eliminate study factors that were not indicated to relate to alpha diversity. Beta diversity among

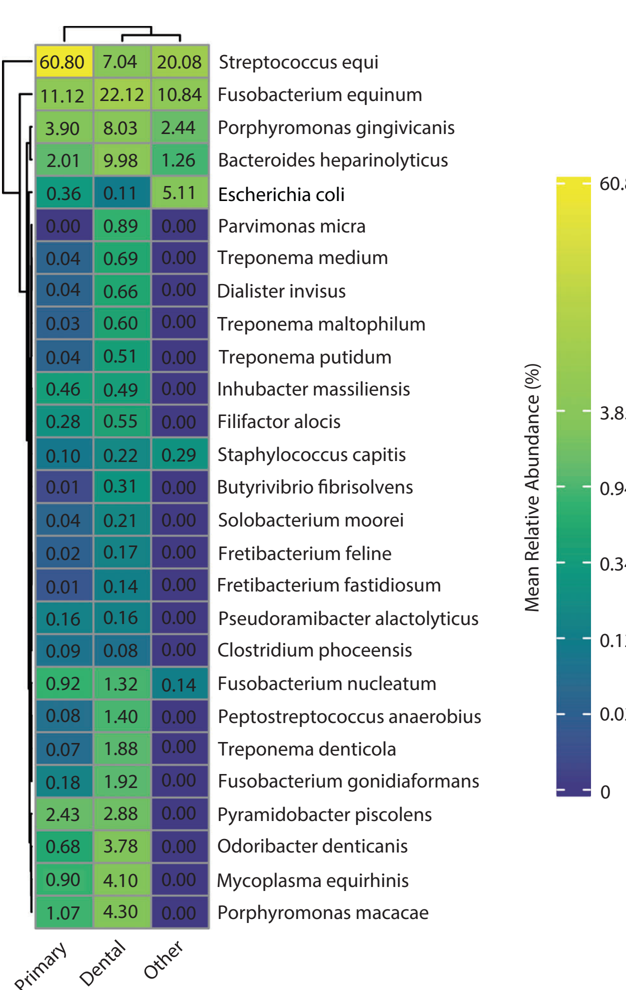

FIGURE 4 27 species identified by ANCOM procedure to vary significantly between groups are shown in heatmap. Values provided in cells and colours correspond to mean relative abundance per sinusitis origin. Cells filled in black may be considered zero, or very close. Row-wise dendrogram indicates species clustering based on abundance pattern. Column-wise clustering and dendrogram indicate group similarity, based on this subset of species

samples was summarised using Weighted UniFrac, with group-wise significance testing conducted using permutational multivariate analysis of variance (PERMANOVA), as implemented in the R function ADONIS.[20] Principal coordinates analysis (PCoA) was used to visualise compositional similarity among microbiome communities. To evaluate differences in the relative abundances of taxa between cohorts, analysis of composition of microbiota (ANCOM) procedure was carried out on taxa present in at least 20% of samples and using moderate correction (multcorr = 2).[21] Weighted UniFrac dissimilarity scores were calculated to summarise the compositional dissimilarity between longitudinal samples. Welch's *t*-test was used to compare similarity between longitudinal samples to random pairs of horses, and linear regression to analyse the effect of time on sample composition.

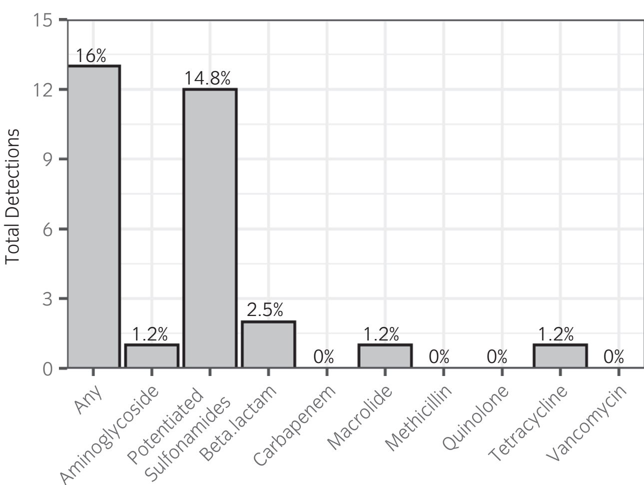

FIGURE 5 All samples ($n = 81$) were screened for ARG by commercial quantitative PCR panel. All positive ARG detections were found within the DRS group and number of detections shown per bar plot. A percentage shown above each bar indicates the proportional frequency in the overall sample set

3 | RESULTS

3.1 | Patient demographics

A total of 70 horses were included in the study, with 81 samples collected for NGS. Mean age was 15.7 years (range 8 months to 29 years). There were 42 geldings and 28 mares. Breeds included Thoroughbreds (28 horses), Warmbloods (20 horses), cross breeds (10 horses), Quarter Horses (5 horses) and seven others. There were 53 samples (66%, 44 horses) of DRS, 14 samples (17%, 12 horses) of PS and 14 samples (17%, 14 horses) of OS (Figure 1). There were 10 horses that had previously undergone sinus surgery to diagnose (1 horse) or treat (9 horses) their sinusitis. Average time elapsed between historic surgery and initial sample collection was 84.8 days (Range 8–180). Ten horses had serial samples collected at subsequent sinus surgeries (11 samples, Table S1).

3.2 | Previous antimicrobial administration

Data on previous antimicrobial administration was unavailable for 12 horses, and incomplete (missing duration or temporal relationship to presentation) in 25 horses (Table S1). Of the 58 horses with antimicrobial information, 48 horses (83%) were treated prior to presentation and 14 horses were receiving antimicrobials at the time of initial sample collection. The average number of antimicrobial classes used prior to presentation was 1.2 (Range 0–4), and potentiated sulphonamides (30 horses) were most commonly administered.

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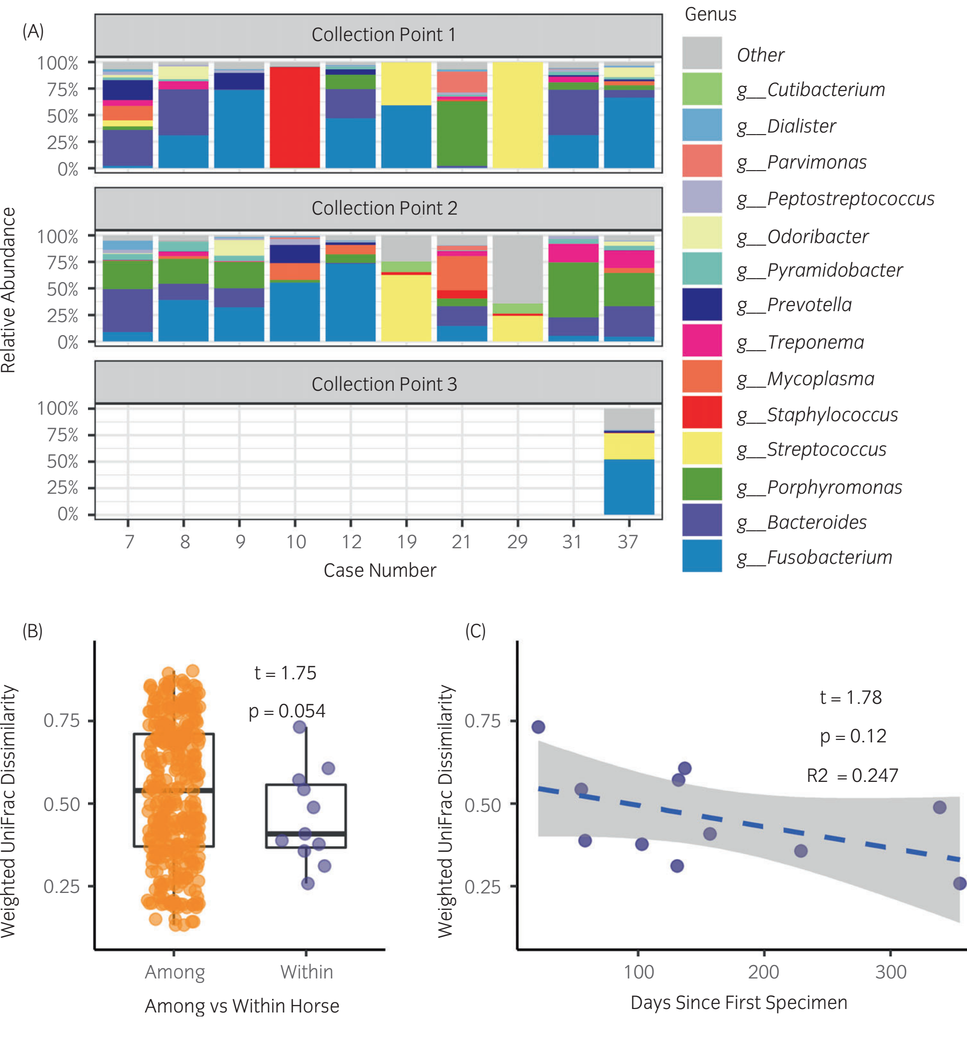

FIGURE 6 Longitudinal analysis investigated continuity of microbial profiles in 10 horses with data available at more than one time point. (A) Bar plots follow genus-level bacterial composition at baseline and subsequent time points. Weighted UniFrac was used to investigate sample dissimilarity (B) and (C), a score of zero indicates complete microbial overlap between samples and increasing scores indicate fewer lineages in common. (B) Microbiota compared from samples collected from the same horse are modestly more similar than comparisons among random horses. (C) Scatterplot shows relationship between dissimilarity score and days since baseline collection. Statistics provided per Welch's non-parametric *t*-test (B) and linear regression (C)

3.3 | Microbial community analysis

Of the 81 samples analysed, 77 passed quality control for bacteria, and five for fungal, with 489 OTUs and 12 OTUs identified, respectively. All samples with positive fungal reads also had positive bacterial reads. Four samples were excluded due to low read counts, which were exclusively from OS horses (Figure 1). Bacterial profiles and only baseline sampling points were considered for bulk of analyses, resulting in 66 samples. Nine horses had one follow-up sample and one horse had two follow-up samples (Table S1).

Good's coverage estimate was found to be at mean 99.5% and 99.4% before and after normalisation,[22] which suggests sufficient sequencing depth to characterise these microbial communities. Following count normalisation, five species accounted for 50% of read counts including *Streptococcus equi*, *Fusobacterium equinum*, *Bacteroides heparinolyticus*, *Poryphromonas gingivalis* and *Actinobacillus equuli* (Figure 2). Of these, *S. equi* was the most common to dominate more than 90% of a sample's relative abundance and did so in 11 samples (11 horses).

Of the variables investigated for effect on alpha diversity, DRS samples were found to have significantly more OTUs per sample (Anova; $F = 7.8$; df $= 2, 57$; $p = 0.001$; $R^2 = 0.186$; Figure 3A) and significantly higher Hill1 diversity ($F = 6.61$; $p = 0.002$; $R^2 = 0.173$, Figure 3B) compared to other aetiologies. Horse breed was found to explain a suggestive amount of variation in the number of OTUs observed ($F = 2.2$; df $= 5, 57$; $p = 0.07$; $R^2 = 0.13$) though less so in Hill1 diversity ($F = 1.25$; $p = 0.3$; $R^2 = 0.82$).

In addition to alpha metrics, 16S qPCR was used to estimate the number of bacterial cells per sample, and it was found that DRS samples had significantly higher bacterial burden ($F = 13.7$; df $= 2, 78$; $p < 0.0001$; $R^2 = 0.261$; Figure 3C), with a 5.3- and 26.4-fold mean increase from PS and OS, respectively. Furthermore, a global significance test was used to investigate the importance of various study factors to overall bacterial composition, which indicated that aetiology was a significant driver of overall composition (Adonis; $F = 6.55$; df $= 2, 57$; $p = 0.001$; $R^2 = 0.18$, Figures 3D,E).

General morphologies were mapped according to microbial genera (Figure 3F). A total of 195 genera were detected in this study and this set was trimmed down to 58 genera to exclude taxa detected in low abundance (less than 2% sample-wise relative abundance). Twelve genera could not be readily mapped, though all were less common and all with mean relative abundance less than 1%.

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Anaerobic genera were common to all groups though more abundant in DRS, with a mean 5.3 and 1.2 anaerobic genera detected in DRS and PS samples, respectively (Figure 3F).

The ANCOM procedure was used to identify 27 species which differed significantly between sinusitis aetiology (Figure 4, Table S2).[21] PS was significantly more abundant in *S. equi*. DRS had multiple species detected which were unique to the group (e.g., *Parvimonas micra*, *Treponema* [3 spp.]), and more that were commonly detected among all groups, but present to a greater degree in DRS (e.g., *F. equinum*, *Bacteroides heparinolyticus*). Except for *Mycoplasma equirhinus*, all species which were mapped to phenotypes and were most abundant in DRS were considered obligate anaerobes.

3.4 | Fungal community analysis

Only 6% of samples had positive fungal detections (five samples, five horses). Four out of the five samples were from horses that had previous antimicrobial treatment, including potentiated sulphonamides (3/5) and cephalosporins (1/5). The antimicrobials utilised were unspecified in one horse. The most abundant fungal genera observed were *Scedosporium* (2 spp., 3/5), *Fusarium* (4 spp., 2/5), *Aspergillus* (3 spp., 2/5), *Humicola* (1 spp, 1/5) and *Alternaria* (1 spp, 1/5). Three species accounted for 85% of all fungal read counts, including *S. boydii*, *S. apiospermum* and *F. proliferatum*. All five fungal instances were associated with DRS.

3.5 | Antimicrobial resistance-associated gene detections

Across 81 samples eligible for antimicrobial resistance testing, 13 samples were positive for detection of at least one ARG (10 horses, Figure 5). All but one of these samples had a potentiated sulphonamide-ARG detected, which was the most common and found in 15% of all 81 samples. All samples with a positive detection corresponded to DRS and could be found in 25% of samples from this group (X2 = 7.04, $p = 0.03$, Table S1). Samples with positive ARG detection tended to have increased bacterial burden (Anova; $F = 4.28$; df = 1, 79; $p = 0.04$, $R^2 = 0.051$).

3.6 | Longitudinal analysis of bacterial profiles

Ten horses had two separate samples collected on different dates, and one horse had three samples collected ($n = 21$ samples, Table S1). Mean time between sample collection in these horses was 156 days (Range 21–355). Qualitative inspection of plots suggests that abundant species observed at baseline are often observed again at follow-up, though at different proportions (Figure 6A). Samples measured within the same horse at differing time points exhibited increased similarity compared to random pairs of horses, though this was non-significant (Welch's *t*-test; $t = 1.75$, adj.df = 11.3, $p = 0.054$, Figure 6B). Increased time elapsed between consecutive sampling events was found to have a non-significant negative association to weighted UniFrac dissimilarity, meaning samples collected in closer proximity tended to be more distinct from the previous measure (Linear Regression; $t = 1.78$, df = 9, $p = 0.1$, $R^2 = 0.247$, Figure 6C).

4 | DISCUSSION

This study provides important new information describing the sinusitis microbiota and identifies significant differences in the microbiome between sinusitis aetiologies. The presence of 21 bacterial species were associated with the diagnosis of DRS, including multiple species of *Fusobacterium*, *Odorobacter*, *Bacteroides* and *Mycoplasma*. These genera have been previously identified within the oral cavity of horses using genetic sequencing techniques, and *Fusobacterium* and *Bacteroides* were specifically associated with periodontal disease[23,24] When compared to other sinusitis aetiologies, the microbiome of DRS is significantly more diverse, possesses a higher bioburden, and is less likely to be dominated by one or two species. In contrast, PS is associated with decreased alpha diversity and dominance of a single bacterial species. The majority of PS samples had one species account for over 90% of the microbiome, most often *Streptococcus equi*. These differences in aetiologic microbial profiles could be utilised by primary care veterinarians to guide the need for additional diagnostics to identify dental disease, and possible candidates for earlier referral and surgical treatment.

The OS group had the most genetic variation between samples. This high intra-group variance is unsurprising, since these samples represent an unfocused group with a large range of aetiologic causes for sinusitis. Horses with OS usually had cystic structures or masses present within the sinus cavity, which were preferentially sampled, and may account for their lower bioburden.

A recent study examined the healthy paranasal sinuses in horses using a comparable 16 s rRNA gene sequencing method.[4] A significantly larger number of phyla and genera were identified in the sinuses of diseased versus healthy horses. The current study identified bacteria from seven different phyla and 58 genera, whereas only four phyla and 12 genera were detected in healthy sinuses.[4] These differences are mainly bacteria associated with DRS. The most common phylum in healthy horses was Proteobacteria and anaerobes were not a significant feature of the microbiome.[4] In comparison, the two most identified phyla in the current study were Firmicutes and Bacteroidetes which are mainly obligate anaerobes. While Streptococcal species were commonly identified, no bacteria within the healthy sinuses were present at the high relative abundance characteristic of PS.[4] This indicates that PS may be associated with overgrowth of a single commensal organism, in many cases *Streptococcus equi*.

Fungal species were only detected in five of the 81 samples analysed in this study, whereas sinus washes from healthy horses had fungi identified in all samples.[4] Direct comparisons between the two studies are difficult because of differences in laboratory processing methods, PCR primer target and sequence, taxonomic reference

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database, and bioinformatic processing. However, the current results do support a greater fungal component to DRS compared to other aetiologies. The top two reported genera in this study, *Scedosporium* and *Fusarium*, were not identified within the sinuses of healthy horses.[4] Both genera have been previously identified within horses diagnosed with mycotic sinusitis.[25–27] The majority of fungal positive samples were collected from horses with a history of previous antimicrobial usage. The numbers and types of antimicrobials utilised are similar to those utilised in the entire group of sinusitis cases. However, due to small group size statistical analysis on these factors was not performed. While the effect of length of disease on microbiome is beyond the scope of this study, no fungal species were identified in repeat samples, or horses that had previous surgery, which would indicate that chronicity and fungal sinusitis are not correlated.

There were individual PS samples in this study that had a large alpha diversity and multiple obligate anaerobic bacteria present, which appears more consistent with a profile of DRS. Certain cases of DRS with equivocal apical dental changes on computed tomography may have been misdiagnosed as PS and resolved with medical treatment alone, as has been previously reported.[28]

The findings of this study are in agreement with published culture results of equine sinusitis, which also found DRS to be associated with higher diversity and an increased component of obligate anaerobic bacteria, while PS was associated with *Streptoccus equi*.[5] On average, culture of sinus secretions identified half as many species per sample than NGS identified genera. Only the presence of the family Enterobacteriaceae was associated with DRS when evaluated by culture,[5] while the current study identified 21 species associated with this diagnosis.

The most commonly detected ARG was for potentiated sulphonamides, which was also the most utilised antimicrobial in this study. ARGs can be passed between microbes through horizontal gene transfer or can increase in prevalence when a resistant community is treated with an antibiotic, causing those tolerant microbes to become more dominant.[29] Furthermore, animals are likely frequently exposed to colonising microbes which may carry harmful traits as they interact with the environment.[29,30] Accurate diagnosis and prompt treatment is critical to decrease antimicrobial dependence in cases of DRS.

The longitudinal investigation suggests that there is microbial continuity between chronic sinusitis microbiome profiles measured at different time points. As expected, follow-up bacterial profiles tended to be more similar to baseline samples when compared to random horses of the same cohort, though unexpected was the observation that given more time, the community may revert to a state more like the baseline infection. This observation was based on a small subset of horses and there is little to compare to in the realm of chronic sinus disease-associated microbiota. Targeted management of a suspected microbial infection may require repeated diagnostics to capture the dynamic nature of infection.

Limitations of this study are typical for retrospective studies (incomplete data and selection bias). The relatively small sample size of OS and PS groups may have limited the ability to detect statistical significance of various causal factors on the composition of the sinus microbiome. All the samples analysed in this study were from horses residing in one general geographic region of the United States, and the microbiota may not be representative of those seen in horses from other parts of the country or world. However, a recent multicentre study on sinonasal samples from humans residing in 13 locations across five continents did not identify significant shifts in microbiome composition based on geography.[31]

The effect of storage temperature on the microbial composition of sinus samples is not completely understood at this time. Previous studies have evaluated room temperature storage on human faecal samples, skin swabs and soil samples for up to 14 days. Results were mixed, and ranged from no significant change in microbial profiles, to up to a 10% shift in relative abundance of the most abundant bacterial genera present.[32,33] Based on these studies, 24-h room temperature storage is likely to have a minor effect at most on the bacterial composition of the samples analysed in this study. However, specific studies analysing the effect of storage conditions of equine sinus samples would be required to determine the true effect of storage conditions on microbial profiles.

The horses in this study received pre-operative antimicrobials with gentamicin and a 3rd generation cephalosporin to prevent complications associated with the surgical procedure itself. Bacteremia has been shown to occur following dental extractions in horses,[34] and endoscopic surgery for chronic rhinosinusitis in humans.[35] Broad-spectrum antibiotics which covered the most likely bacteria present in the sinus were thus utilised, including a 3rd generation cephalosporin due to the superior anaerobic coverage when compared to penicillin. These antibiotics were not utilised to treat the sinusitis in these horses, and were not continued long term as surgical therapy to remove the inciting cause (inspissated material, sinus cyst, infected tooth, etc.) and high-volume lavage were considered more critical to resolution of disease.

This study reports novel data regarding the microbiome of equine sinusitis and demonstrates that aetiology is highly associated to unique microbial patterns dominated by anaerobic species which are not identified using traditional culturing techniques. NGS allowed for the identification of a greater number and diversity of bacterial species than has previously been reported for culture of equine sinus secretions. DRS was significantly associated with an increased obligate anerobic component, increased diversity, and bioburden, and had a higher prevalence of fungal species and ARG detection. In contrast, PS was more commonly associated with overwhelming dominance of a single bacterial species, most often *Streptococcus equi*. These findings suggest that the composition of the sinus microbiome could be indicative of sinusitis aetiology in individual cases and further document the dynamic nature of the sinus microbiome through time.

AUTHOR CONTRIBUTIONS

Megan E. Lowman, James A. Brown and Alexandra L. Labordère all contributed to study design and data collection. Craig D. Tipton analysed the data. Megan E. Lowman, Craig D. Tipton and James A. Brown were involved in manuscript preparation. All authors were involved in manuscript review and editing and gave their final approval of the manuscript.

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ACKNOWLEDGEMENTS

We thank members of RTL Genomics and MicroGenDX team for their logistical support and technical expertise throughout this research endeavour, especially Lars Koenig, Courtney Jarvis and Laith LeMaster.

CONFLICT OF INTEREST

C. Tipton is employed by RTL Genomics, a subsidiary of MicroGenDX.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/evj.13884.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available at the NCBI sequence reads archive (SRA) at https://dataview.ncbi.nlm.nih.gov/object/PRJNA807287?reviewer=asp9anivh96vhmr6t74oliubd0, Submission number PRJNA807287.

ETHICS STATEMENT

Research ethics committee oversight not required by this journal: retrospective analysis of clinical data.

INFORMED CONSENT

Explicit owner consent for animals' inclusion in the study was not stated.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Lowman ME, Tipton CD, Labordère AL, Brown JA. Equine sinusitis aetiology is linked to sinus microbiome by amplicon sequencing. Equine Vet J. 2022. https://doi.org/10.1111/evj.13884

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