THE RELATIONSHIP BETWEEN TOOTH EXTRACTION AND CHANGES IN BLOOD MICROBIOME IN HORSES

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by

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DEDICATION

This thesis is dedicated to Chancey, the kind, fuzzy, energetic Morgan horse that started it all. Not only did you fascinate me and spark my love of horses, but you taught me how to ride, how to care for horses, and how to act as a mini veterinarian when I was a child. This thesis is also dedicated to all of the old, ailing horses of the world with dental disease. You are the horses that I grew up with and inspired me to become a veterinarian and, specifically, an equine internal medicine specialist.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variants
BWT	Body Weight
CFU	Colony Forming Units
CRI	Constant Rate Infusion
CVID	Common Variable Immunodeficiency
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
dsDNA	Double-Stranded Deoxyribonucleic Acid
EB	Elution Buffer
EDTA	Ethylenediaminetetraacetic Acid
EOTRH	Equine Odontoclastic Tooth Reabsorption and Hypercementosis
GC	Guanine-Cytosine
IV	Intravenous
NGS	Next Generation Sequencing
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PPID	Pituitary Pars Intermedia Dysfunction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation

THE RELATIONSHIP OF TOOTH EXTRACTION AND CHANGES IN BLOOD MICROBIOME IN HORSES

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ABSTRACT

Bacteremia resulting from dental surgery is increasingly recognized as a health risk, especially in older and immunocompromised patients (in human medicine and also now in equine veterinary medicine). Dentistry-associated bacteremia can lead to remote infections, as exemplified by valvular endocarditis. Emerging evidence points to a novel role played by oral cavity commensals in the pathogenesis of diabetes, respiratory disease, cardiovascular disease, and adverse pregnancy outcomes. Whether dental extraction, a commonly undertaken procedure in old horses, causes bacteremia has not been reported extensively. In a prospective clinical study using next generation sequencing (based on bacterial 16S rRNA), the circulating blood microbiome was characterized before and at 1 hour following extraction of incisor, canine or cheek teeth from 29 adult horses with dental disease. 16S rRNA gene sequencing results from the blood microbiome were compared with those from gingival swab samples obtained prior to extraction at the location of the diseased tooth.

Univariate data were first tested for normality using the Shapiro-Wilk method. Non-normally distributed data were then tested using a Kruskal-Wallis analysis of variance (ANOVA) on ranks, followed by *post hoc* pairwise comparisons using Dunn's method, with significance defined by $p < 0.05$. Multivariate data were compared using permutational multivariate ANOVA (PERMANOVA) based on Jaccard similarities, using Past3 software. Bacteremia associated with translocated gingival commensals was

demonstrated in horses undergoing exodontia and was, in some cases, still evident one hour post-operatively.

CHAPTER 1: INTRODUCTION

Oral Cavity Microbiome, Dental Disease, and Systemic Implications

In human medicine, it has long been accepted that there exists a relationship between dental disease and systemic health. There have been numerous reports over the last 50 years of periodontal disease and its pathophysiological association with conditions such as diabetes mellitus, atherosclerotic cardiovascular disease, metabolic syndrome, chronic diseases (i.e. rheumatoid arthritis, cancer, respiratory diseases, Alzheimer's disease), and adverse pregnancy outcomes [1]. Recently, veterinarians have also begun to recognize and characterize pathophysiological associations between systemic health and periodontal disease in canine and feline species [2]. These relationships have led to broader recognition of the importance of routine oral examination and preventive hygiene in both human medicine and veterinary medicine.

Domestic animal species and humans commonly suffer from both gingivitis and periodontal disease. The prevalence of periodontal disease in dogs has been estimated at approximately 80% [3, 4]. In human medicine, mild periodontitis affects about 50% of adults and increases to over 60% in people over 65 years of age [1]. Periodontitis in its severe form is the sixth most common human disease (estimated to affect 11.2% of the global adult population) [5]. As dental disease has come into the spotlight in human and small animal medicine, substantial advances in veterinary dentistry and preventive health care have also been made in the specific field of equine dentistry. Although equine dental abnormalities and ailments have been recognized since antiquity, it was not until approximately the last 15 years that there have been major developments in routine examinations and in both simple and advanced dentistry procedures in this species [6].

Human Perspective

There exists a complex relationship between inhabitant microbiota of the oral cavity, dental/gingival disease, and systemic health. The healthy oral microbiome is an important part of the overall human microbiome and is comprised of several hundred to several thousand diverse species. As a normal component of the oral cavity, the oral microbiome functions to protect against colonization by extrinsic pathogenic bacteria that could cause local disease and adversely compromise systemic health [7]. It is now widely recognized that *abnormal bacterial influence is central to* the pathogenesis of the most commonly encountered oral diseases in people, including gingivitis, periodontitis, and dental caries [8].

The earliest recognition of an oral microbiome is attributed to Antony van Leeuwenhoek. Using a microscope of his own design, he reported that a diverse array of microorganisms is present in plaque that develops on dental surfaces and, remarkably, he deduced that individual differences in these microbial populations were related to the health of the oral cavity [8]. It is now recognized that oral microorganisms are organized into biofilms and that greater than 500 bacterial species inhabit the oral cavity, constituting the oral microbiome [9-11]. Biofilm is an aggregate of microorganisms in which cells adhere to each other and/or to a surface.

In general, dental disease (gingivitis, periodontitis, and caries) results initially from the development and advancement of dental plaque (also known as microbial plaque, oral biofilm, dental biofilm, dental plaque biofilm, or bacterial plaque biofilm) [12]. Disease initiation and propagation results from a dysbiosis of the commensal oral microbiota (dental plaque), which then interacts with host immune defenses and leads to

inflammation and disease. For example, caries (dental decay) is a disease of the hard tissues of the teeth resulting from an imbalance in the interactions between cariogenic bacteria in dental plaque and fermentable carbohydrates (mainly sugars) that are retained in the mouth from the diet [13]. Disease prevention is facilitated by daily self-performed oral hygiene (tooth brushing and flossing) and regular professional removal of accumulated microbial biofilm (scaling performed by a dentist) [12].

Dental plaque is a biofilm or aggregation of bacteria that normally accumulates on hard surfaces within the oral cavity. It develops on interdental, lingual/palatal, and buccal/labial surfaces of teeth, including chewing surfaces, along the gum line, and below gum line margins [14]. In the healthy state, plaque is present as a thin layer that is continuously being restricted by the natural cleaning activities that result from chewing, saliva production, and physical actions of the tongue. In the absence of sufficient and effective cleaning or saliva production, plaque accumulates and becomes mineralized (calculus).

Formation of a bacterial plaque biofilm on newly erupted or cleaned dental surfaces is preceded by formation of pellicle, a layer of saliva that is composed mainly of glycoproteins that provide an adhesive role. Bacteria then attach to the pellicle layer, forming microcolonies, and subsequently maturing into a dental plaque biofilm. In excess of five hundred distinct bacterial species have been identified in dental plaque. The composition of dental surface microbiota is variable; different bacterial species are found during early surface colonization (early colonizers) compared with those identified in later stages of disease (late colonizers) [15]. Early colonizing bacteria include predominantly Streptococcal species (especially *Streptococcus mutans*) and other

anaerobes (Fusobacteria and Actinobacteria); late colonizing species include *Actinobacillus* spp., *Prevotella intermedia, Eubacterium* spp., *Treponema* spp., *Porphyromonas gingivalis,* and *Fusobacterium nucleatum* [16]. Specific microbial biofilm composition also varies by location within the oral cavity.

Biofilm bacteria are normally found in the healthy oral cavity and are usually harmless. Supragingival biofilm, plaque that develops above the gums, is the first kind of plaque to form following dental brushing. Supragingival biofilm is comprised of mostly aerobic bacteria, needing oxygen to survive [14]. Anaerobic bacteria grow in (deeper) supragingival biofilms that remain on dental surfaces for longer periods of time. Biofilm bacteria in closest proximity to dental surfaces generally obtain energy through fermentation of dietary sucrose, leading to the production of acids [15].

Subgingival biofilm is plaque that is located under the gums. Subgingival biofilm develops after establishment of supragingival biofilm through local extension. Subgingival biofilm plaque is predominantly comprised of anaerobic bacteria; bacteria that only survive in the absence of oxygen. The subgingival location serves to protect these anaerobes from oxygen. The major local ecological factors within the oral cavity that influence and contribute to plaque formation include pH, saliva, temperature, and redox reaction status [17, 18]. In health, the natural oral cavity environment supported by saliva is optimal for bacterial growth in dental plaque [19].

Unfettered progression and build-up of a thick layer of dental plaque and calculus represent the principal causes for the common human dental diseases including gingivitis, periodontitis, and dental caries. Plaque removal and control is routinely achieved by regular tooth brushing and the employment of interdental cleaning devices such as dental

floss and interdental brushes. Accumulated calculus cannot be removed by tooth brushing and can only be removed through professional cleaning (scaling) [20].

Gingivitis is the result of a host inflammatory reaction to local, excessive biofilm bacteria. In its early stages, gingivitis itself can be neutralized by simple plaque removal. However, if plaque accretion is unrecognized or untreated, gingivitis progresses to degradation of local supporting tissues, causing periodontitis. Plaque bacteria release enzymes that cause local osteolysis and promote establishment and progression of periodontitis [20].

Disruption of the gingival-blood barrier as a result of plaque biofilm bacteria-induced gingivitis/periodontitis potentially facilitates the translocation of bacteria and bacterial products into the circulation, which could lead to various systemic diseases [21]. Various diverse human diseases that have been attributed to this phenomenon have included adverse pregnancy outcomes, diabetes mellitus, respiratory disease, cardiovascular disease, and atheroma [22-24]. Antibodies to the oral pathogens *Campylobacter rectus*, *Veillonella parvula*, *Prevotella melaninogenica* have been specifically associated with hypertension [25].

Fusobacterium nucleatum is a fastidious Gram-negative anaerobe and an emerging pathogen for which laboratory cultivation is challenging. However, using culture-independent methods, such as Next Generation Sequencing (NGS) technology, an emerging role for this common plaque biofilm inhabitant in numerous human systemic diseases (adverse pregnancy outcomes [chorioamnionitis, preterm birth, stillbirth, neonatal sepsis, and preeclampsia], gastrointestinal disorders [colorectal cancer, inflammatory bowel disease, and appendicitis], cardiovascular disease, rheumatoid

arthritis, respiratory tract infections, Lemierre's syndrome, and Alzheimer's disease) is becoming increasingly apparent [24]. Whereas *F. nucleatum* is regarded as ubiquitous in the oral cavity, it is absent or infrequently detected elsewhere in the body under normal conditions [26, 27]. However, under disease conditions, *F. nucleatum* is one of the most prevalent species found at extra-oral sites [28]. The role of *F. nucleatum* in some of the conditions noted above is presently at the stage of association and proven cause-and-effect is yet lacking.

Dental caries is an infectious disease caused principally by *Streptococcus mutans*. Organic acids released from dental plaque bacteria cause demineralization of the adjacent dental surface, and consequently promote dental caries. The development of dental caries is predominantly dependent on the availability of fermentable sugars, other local environmental conditions, bacteria, and host factors. Although current understanding regarding microorganisms involved in the initiation and progression of caries is incomplete, the most relevant acidogenic-aciduric bacterial species recognized presently are *Streptococcus mutans*, bifidobacteria, and lactobacilli [29]. Mutans streptococci are initiators of disease progress and bifidobacteria and lactobacilli facilitate disease progression. Enhancing factors for pathogenic microbial activity include specific environmental conditions, such as the presence of fermentable dietary sugars and the absence of oxygen [29]. The present-day western diet is particularly cariogenic, not just because of the high amount of fermentable carbohydrates but also because food intake occurs with high frequency throughout the day [16].

Oral sepsis and oral or maxillofacial surgery (including exodontia) have been recognized as risk factors for bacteremia and endocarditis in human medicine since the

1930s [30, 31]. Because of the close association of teeth to the gingival capillaries, it is logical to deduce that some bacteria located in the periodontal space could move directly into the bloodstream when the area is disrupted (by disease or surgery).

In human medicine, bacteremia has been confirmed as a potential sequel to dental treatment and is even associated with routine daily activities such as chewing of hard foodstuffs or tooth brushing [32]. The risk of developing bacteremia is reported to be as high as 100% following exodontia procedures in human patients [33]. Usually, bacteremia, particularly if it occurs during ordinary activities, does not result in complicating infections because those bacteria typically are present only in small numbers and are rapidly removed from the bloodstream by an effective immune system. It has been reported that bacteremia experienced under those circumstances results from only a low number of bacteria and is only present for up to 30 minutes [33]. However, if bacteria are present for long enough and in large enough numbers, particularly in people who have a weakened immune system, bacteremia can lead to other infections (various locations) and sometimes trigger a serious body-wide response (systemic inflammation and sepsis) [32]. Risk of transient bacteremia in people is related to both oral cavity bacterial load and to the severity of inflammation (gingivitis/periodontitis) in the oral cavity.

Transient bacteremia is especially important for patients with generalized poor health, underlying immunodeficiency, or other comorbidities because dental procedures may cause severe complications in this population [32]. Antimicrobial treatment is specifically recommended for people undergoing dental procedures that may cause bleeding, and therefore allowing overt ingress of bacteria into the bloodstream and

circulation [32]. Other medical conditions warranting antimicrobial prophylaxis in humans could potentially be relatable to equine or veterinary patients as a whole. A few of those diseases and populations of patients include those affected with: endocarditis, obstructive cardiomyopathy, glomerulonephritis, labile diabetes mellitus, cancer, previous adverse pregnancy outcomes, and patients undergoing immunosuppressive treatment [1, 26, 32].

Veterinary Perspective

Due to developing of interest in equine dentistry and preventive medicine for horses, it has been recognized that many of the same dental conditions that afflict small animal species and humans are present in horses. Gingivitis, periodontitis, and dental caries can all lead to weakness and decay of teeth that can result in tooth fractures, necessitating exodontia [34].

Although reports of severe, systemic complications following exodontia in horses are uncommon, a few have been reported in the literature in the last several decades and revolve around the diagnosis of post-operative endocarditis [35]. In most reports of equine bacterial endocarditis, no primary source of infection is identified (however, several horses with tricuspid valve lesions had septic jugular vein thrombophlebitis as the primary cause) [35]. Although descriptions of horses with bacterial endocarditis resulting definitively from periodontal or gingival disease have not been reported, it is possible that bacteremia from these conditions could predispose horses to infection. In a 2016 study performed by Kern *et al*., bacteremia before, during, and after exodontia was described in a group of 20 adult horses. In that study, it was reported that 90% of the sampled patient population developed a positive blood culture result during at least one time point

throughout the course of surgery [36]. To the author's knowledge, Kern *et al*. were the first and only group to publish data on bacteremia in horses, post-exodontia. This finding confirmed the concept that horses also sometimes develop transient bacteremia during tooth extraction, as had been demonstrated in humans and veterinary species [33, 36].

Periodontal disease and resulting bacteremia are known to lead to renal, hepatic, or cardiac disease in human and canine species and, therefore, they may also be a risk factor for horses developing these undesirable sequelae [37]. Specifically, Semedo-Lemsaddek *et al*. (2016) described a conclusive cause-and-effect relationship between enterococcal infective endocarditis following periodontal disease in dogs [38]. Although endocarditis is a rare complication, effective treatment options are limited and it is often difficult or impossible to treat or resolve it in veterinary species. Since this has been reported rarely in horses, it is logical to use caution in equine patients that have compromised health status when planning elective and necessary equine dental procedures. Examples of equine-specific compromised health status that should be considered in this regard include: pituitary pars intermedia dysfunction (PPID), advanced age, emaciation, severe endoparasitism, protein-energy malnutrition, and immunodeficiency disorders such as common variable immunodeficiency (CVID) [39-41].

Equine dental procedures, including invasive extractions, are being employed more commonly in the last decade due to advancements in equipment and increased recognition of the importance of oral health (and its relationship to systemic health). In parallel with increased interest in equine dentistry, the discovery and characterization of a novel syndrome, equine odontoclastic tooth reabsorption and hypercementosis (EOTRH),

was recently reported [42]. EOTRH is a chronic, painful, degenerative, debilitating disease of the canine and incisor teeth affecting middle-aged to older horses that is currently best treated by the removal of affected teeth, thus maintaining comfort and function [42]. Although not originally recognized as part of the condition, recent work by Sykora *et al*. demonstrated that there may be an association with oral microbiome changes and EOTRH. Specifically, some novel *Treponema* and *Tannerella* spp. were isolated in association with EOTRH-related periodontal disease [34].

Due to the increased extent to which dental extractions are being performed by equine veterinarians in recent years (facilitated by improved equipment, more expertise, and better recognition of disease), it seems likely that more complications will be encountered/reported in horses associated with the post-surgical period following exodontia. Improved follow-up examinations on cases and communications between veterinarians and owners may also lead to an increase in reported cases, as associations and diagnoses are made based on history of recent dental procedure. The complications related to bacteremia seen in dogs (endocarditis, etc.) may become more prevalent in horses due to an increase in the number of dental extraction procedures being attempted and performed.

Using Next Generation Sequencing to Characterize Microbiomes and Uncultivable Bacteria

Recently, NGS approaches (polymerase chain reaction [PCR] technology) can be used to identify bacteria based on their 16S rRNA signature. In order to perform this type of analysis, samples of biological material must be collected and total DNA extracted using a combination of mechanical and enzymatic disruption [43, 44]. Once the DNA has

been extracted, comparison of genomic regions between collected samples and reference data is undertaken. When choosing genetic markers for phylogenetic analysis, the 16S rRNA gene is chosen due to its stability and the fact that it is present in all Bacteria and Archaea [43]. This gene is approximately 1,550 base pairs long and is composed of nine hypervariable regions and several more highly conserved regions. Within the hypervariable regions, sequence differences characterizing specific organisms allow for the taxonomic identification of the bacteria present in a sample. This method, 16S rRNA-based sequencing, is the current gold standard for sequence-based bacterial analysis [43].

Detecting bacteria based on their 16S rRNA signature does not require bacterial culturing and allows for both identification and characterization of bacteria in various samples, such as surface swabs, gastrointestinal contents, and blood [43, 45]. The 16S rRNA signature detection does not distinguish between living bacteria, dead bacteria, and bacterial products or fragments containing RNA [43]. The microbiome present in the bloodstream of the horse has not been well studied in relation to blood culture, but based on human medical literature, it is assumed that a significant amount of the bacteria comprising the microbiome present in horse blood are not detectable with conventional techniques.

Although bacteremia (as characterized with blood culture) [35] has been demonstrated in horses undergoing exodontia, there are currently no reports (to the authors' knowledge) that characterize microbiome analysis of blood samples obtained before and after exodontia [36]. Reliance on blood culturing approaches to identify contaminating bacteremia is an insensitive method because a majority of bacteria are uncultivable and antimicrobial components of blood are inhibitory to those bacterial

species that are cultivable *in vitro* [36]. Moreover, the number of bacteria present in the bloodstream following minor dental procedures is very small. For example, only approximately 10 CFU/mL were reported in the blood for approximately 30 minutes following minor procedures in people [33]. PCR-based NGS approaches have recently been used to analyze the population of bacteria associated with dental caries in horses [46]. The 16S rRNA gene sequence analysis has been used to characterize equine dental caries, as it has been proposed that at least 50% of oral bacteria are presently regarded as non-cultivable using current standard techniques [46].

Use of Antimicrobials in the Context of Equine Dentistry

Although bacteremia has been reported in horses during and after exodontia, the use of systemic antimicrobial drugs is inconsistent and there are no current guidelines on when and for how long they should be utilized [36]. In dogs, there has been an association between periodontal disease and histopathological changes in the kidneys, liver, and myocardium [2]. Endocarditis has specifically been associated with periodontal disease in dogs and is proposed to be a potential risk in horses with periodontal disease and exodontia [38]. In the study by Kern *et al*. it was noted that two horses developed bacteremia (characterized by positive blood culture) after the completion of extraction procedures [36]. Although antimicrobials are not always used in human dentistry, they are sometimes employed under certain circumstances (more invasive procedures or if the patient is immunocompromised). One study demonstrated that dentists were more likely to prescribe prophylactic antibiotics to patients if they are believed to be at risk of developing an infection, including those undergoing invasive oral health procedures such as surgical endodontic therapy or apicectomies, nonsurgical endodontic therapy, and

extractions, especially surgical extractions and conventional extractions taking more than 5 minutes [47]. It has been observed that patients with a systemic disease, periodontitis, poor oral hygiene, or a smoking habit more commonly received prescriptions for prophylactic antibiotics when undergoing dental surgical procedures [31, 47].

Commonly employed antimicrobials used in equine medicine in this context include amoxicillin, ampicillin, clindamycin, first-generation cephalosporins, and macrolide antibiotics [32, 48]. Bacteremia and right-sided endocarditis have been reported as sequelae of dental extraction in the horse [35]. Serious systemic complications following exodontia have not been commonly reported in the literature so it is logical to question the validity of antimicrobial treatments for exodontia in horses, especially at a time when objective antimicrobial stewardship is so important. Using NGS and identification of bacteria based on their 16S rRNA signature, the population of bacteria present normally in the equine bloodstream and the population of bacteria after exodontia should be further characterized, thus helping guide future antimicrobial use protocols for different types of dental procedures in this species. In the current climate, it is important to remember that, whenever practical *"we must develop narrow-spectrum antibiotics, ideally aimed at a single taxon, to avoid the collateral effects of broad-spectrum antibiotics on "innocent bystanders" in the microbiota*"[49].

CHAPTER 2: STUDY

Hypotheses and Specific Aims

In this study, we aimed to quantify and identify the bacterial 16S rRNA signature present on the tooth to be extracted and its surrounding gingiva prior to surgery, in the bloodstream prior to surgery, and in the bloodstream one hour after completion of various exodontia procedures undertaken in tranquilized (standing) adult horses. We hypothesized that the bloodstream will contain a significantly higher number and more diverse population of bacteria (as differentiated by 16S rRNA signature) one-hour post-surgery, and that identified bacteria will be representative of the population that is detected on the gingiva prior to starting the procedure.

Materials and Methods

Animals

The study group consisted of 29 adult horses (with several horses requiring multiple procedures), including 22 geldings and 12 mares. The study population had a mean ± SD age of 19.4 ± 5.6 years (range: 3 to 32 years) and mean ± SD weight of 479.3 ± 107.1 kg (range: 99.0 to 621.0 kg), presented to the University of Missouri Veterinary Health Center for dental examination and dental extraction. There were a variety of breeds, including 7 Thoroughbreds, 7 American Quarter Horses, 3 American Paint Horses, 2 Hanoverians, and one each of the following breeds: Tennessee Walking Horse, Standardbred, Saddlebred, Oldenburg, American Miniature Horse, Haflinger, National Show Horse, Welsh pony, Missouri Fox Trotting Horse, and Arabian. None of the horses had received antimicrobial drugs for at least 1 week prior to presentation. All horses

received both a physical examination and an oral cavity examination. Oral endoscopic and radiographic examinations were used, if indicated.

Preparation and Medication

Horses were placed in stocks. The left jugular vein was subjected to aseptic preparation by clipping and scrubbing with 4% chlorhexidine gluconate that was rinsed using 70% isopropanol. Immediately following skin disinfection, a blood sample (20 mL) was collected from the left jugular vein using a vacutainer needle and immediately transferred into two 10 mL tubes containing ethylenediaminetetraacetic acid (EDTA).

Subsequently, an indwelling intravenous (IV) catheter was placed into the left jugular vein for drug administration and secured with monofilament suture material. For sedation, horses were given a bolus of detomidine hydrochloride[a] at 0.01 mg/kg bodyweight (BWT) IV and butorphanol tartrate[b] at 0.01 mg/kg BWT IV followed by a constant rate infusion (CRI) of detomidine hydrochloride at 0.005 mg/kg BWT/hour IV/butorphanol tartrate at 0.005 mg/kg BWT/hour CRI IV in saline. Prior to administration of local anesthesia, the gingiva adjacent to both the lingual and buccal aspects of extracted teeth was sampled using a sterile cotton swab that was then placed into a semi-solid transport medium.[c]

Additionally, anesthesia of the mental, infraorbital, mandibular, or maxillary nerves (as appropriate for location of tooth to be extracted) and local infiltration of the gingiva surrounding the diseased tooth were performed using 2% lidocaine hydrochloride.[d] No antimicrobials were given prior to or during extractions.

The oral extraction of cheek, canine, or incisor teeth was performed in a standardized manner as described elsewhere [50-52]. One hour following delivery of the last tooth and cessation of all surgical manipulations, blood was aseptically drawn from the left jugular catheter. The first 10 mL of blood were discarded, and the next 20 mL were collected and transferred into two 10 mL tubes containing EDTA. All blood samples and gingival swabs collected were immediately frozen until further processing. All dental procedures were performed by the same veterinarian. Horse-owners gave informed consent for their animals' inclusion in this study, which was approved by the institutional Animal Care and Use Committee (MU ACUC# 9233).

DNA Extraction

DNA was extracted from 750 µL whole blood and dental/gingival swabs using PowerFecal kits[e] according to the manufacturer's instructions, with the exception that, rather than performing the initial homogenization of samples using the vortex adapter described in the protocol, samples were homogenized in the provided bead tubes using a TissueLyser II[e] for three minutes at 30/second, before proceeding according to the protocol and eluting with 100 µL of elution buffer[e]. DNA yields were quantified via fluorometry[f] using quant-iT BR dsDNA reagent kits.[f] As negative and positive controls respectively, blank reagents (n = 10) and one mock bacterial community standard[g] were processed alongside experimental samples.

16S rRNA Library Preparation and Sequencing

Extracted blood and gingival swab DNA was processed at the University of Missouri DNA Core Facility. Bacterial 16S rRNA amplicons were constructed via

amplification of the V4 region of the 16S rRNA gene with universal primers (U515F/806R) previously developed against the V4 region, flanked by Illumina standard adapter sequences [53, 54]. Oligonucleotide sequences are available at proBase [55]. Dual-indexed forward and reverse primers were used in all reactions. PCR was performed in 50 µL reactions containing 100 ng metagenomic DNA, primers (0.2 µM each), dNTPs (200 µM each), and Phusion high-fidelity DNA polymerase (1 U). Amplification parameters were $98°C^{(3\ min)}$ + [$98°C^{(15\ sec)}$ + $50°C^{(30\ sec)}$ + $72°C^{(30\ sec)}$] × 40 cycles +$72°C^{(7\ min)}$. Amplicon pools (5 µL/reaction) were combined, thoroughly mixed, and then purified by addition of Axygen Axyprep MagPCR clean-up beads to an equal volume of 50 µL of amplicons and incubated for 15 minutes at room temperature. Products were then washed multiple times with 80% ethanol, and the dried pellet was re-suspended in 32.5 µL EB buffer, incubated for two minutes at room temperature, and then placed on the magnetic stand for five minutes. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified using quant-iT HS dsDNA reagent kits, and diluted according to Illumina's standard protocol for sequencing on the MiSeq instrument.

Bioinformatics Analysis

The DNA sequences were assembled and annotated at the MU Informatics Research Core Facility. Primers were designed to match the 5' ends of the forward and reverse reads. Cutadapt (version 2.6; https://github.com/marcelm/cutadapt) was used to remove the primer from the 5' end of the forward read [56]. If found, the reverse complement of the primer to the reverse read was then removed from the forward read as were all bases downstream. Thus, a forward read could be trimmed at both ends if the

insert was shorter than the amplicon length. The same approach was used on the reverse read, but with the primers in the opposite roles. Read pairs were rejected if one read or the other did not match a 5' primer, and an error-rate of 0.1 was allowed. Two passes were made over each read to ensure removal of the second primer. A minimal overlap of three with the 3' end of the primer sequence was required for removal.

The Qiime2 [57] DADA2 [58] plugin (version 1.10.0) was used to de-noise, de-replicate, and count ASVs, incorporating the following parameters: 1) forward and reverse reads were truncated to 150 bases, 2) forward and reverse reads with number of expected errors higher than 2.0 were discarded, and 3) chimeras were detected using the "consensus" method and removed. R version 3.5.1 and Biom version 2.1.7 were used in Qiime2. Taxonomies were assigned to final sequences using the Silva.v132 database [59], using the classify-sklearn procedure.

Hierarchical clustering was performed using an unweighted pair group method with arithmetic mean (UPGMA) approach based on unweighted Jaccard similarities. Similarly, principal coordinate analysis was performed using Jaccard similarities. Clustering approaches were executed using Past3 software [60], downloaded on August 20, 2019. All methods were carried out in accordance with relevant guidelines and regulations.

Statistical analysis

Univariate data were first tested for normality using the Shapiro-Wilk method. Non-normally distributed data were then tested using a Kruskal-Wallis ANOVA on ranks, followed by *post hoc* pairwise comparisons using Dunn’s method, with

significance defined by $p < 0.05$. Multivariate data were compared using PERMANOVA based on Jaccard similarities, using Past3 software [60].

Results

Of the 29 horses, most were determined to be healthy (n = 24) or affected with paranasal sinusitis (n = 6), PPID (n = 3), or asthma (n = 1). A total of 34 procedures were performed on 29 horses (some horses returned for a second exodontia procedure at least one month after completion of the first). Procedures included those requiring cheek tooth extraction (n = 25) and those requiring incisor or canine tooth extraction (n = 9). Justification for exodontia included: apical tooth root abscessation (n = 9), slab fracture (n = 9), EOTRH syndrome (n = 8), infundibular caries (n = 5), crown fractures (n = 3), and fractured incisive bone (n = 1). Retropulsion of teeth was needed for extraction in three cases and standard intra-oral tooth extraction was performed in the remaining 31 cases. Sinus lavage was performed post-procedurally in all cases with comorbid sinusitis. Detailed signalment and procedural information are presented in **Table 1**.

To qualitatively assess the validity of microbial signatures detected via 16S rRNA sequencing, DNA amplification (quantified by total number of reads for a given sample among a shared sequencing flow cell) was compared between pre- and post-exodontia blood samples, gingival swabs, negative reagent controls, and a commercially available bacterial community standard. As anticipated, the swabs yielded higher sequence numbers than either group of blood samples, and the mock community standard yielded higher coverage, by an order of magnitude, than negative reagent controls and most blood samples (**Figure 1**). Notably however, five blood samples collected post-exodontia yielded unexpectedly deep coverage, ranging from 187,130 to 669,731 sequences per

sample. While sequencing coverage is not absolutely quantitative of starting microbial biomass, these results suggested the presence of increased bacterial biomass in a subset of blood samples collected post-exodontia. Moreover, the validity of the remaining blood samples and a few swabs samples that amplified poorly was brought into question.

Recognizing that the differences in sample coverage would likely skew comparisons of bacterial composition, all samples yielding fewer than 1,055 sequences were removed from the following analyses, and the remaining data were rarefied randomly to a uniform read depth of 1,054 reads/sample. The original sequencing coverage of those samples (**Figure 2A**) is reflective in the hierarchical clustering of samples based on the rarefied dataset, with those same five highly amplified post-exodontia blood samples clustering with the gingival swabs, along with two other post-exodontia and one pre-exodontia blood samples with lower coverage (**Figure 2B**). These relationships were also visualized using principal coordinate analysis (PCoA), which demonstrated a similar pattern with the same post-exodontia blood samples clustering close to the gingival swabs (**Figure 3**). One-way permutational multivariate ANOVA confirmed significant differences between swabs and pre-exodontia blood ($p \leq 0.0001$, F = 6.6), swabs and post-exodontia blood ($p \leq 0.0001$, F = 4.7), and between pre- and post-exodontia blood ($p = 0.039$, F = 1.3). All three groups were significantly different from negative reagent controls ($p \leq 0.0001$; F = 2.6, 2.6, and 6.2 for pre- and post-exodontia blood and swabs, respectively). Collectively, we interpreted these results to indicate compositional similarities between the microbial communities present on the gingiva and those detected in post-exodontia blood in a subset of horses, including those whose samples yielded high sequence counts.

To identify the taxonomies contributing to the differences between swabs and pre- and post-exodontia blood, data from control samples were removed, and serial ANOVA testing was performed on all detected Amplicon Sequence Variants (ASV). Based on those ASVs returning the 50 lowest *p* values, hierarchical clustering was repeated and visualized using a heatmap (**Figure 4**). The same post-exodontia blood samples clustered with the gingival swabs, due to the shared presence of multiple taxa associated with the oral cavity including members of the genera *Actinobacillus*, *Fusobacterium*, *Leptotrichia*, *Porphyromonas*, *Prevotella*, *Streptococcus*, and *Veillonella*. Notably, these same taxa linking a subset of post-exodontia blood samples to the gingival microbiota represent the dominant taxa in the gingival microbiota (**Figure 5**). Thus, we interpreted the extremely high coverage selectively observed in a subset of post-exodontia samples, and compositional similarities between those samples and the oral cavity microbiota, as compelling evidence of bacteremia resulting from translocated gingival microbiota in horses undergoing exodontia procedures.

CHAPTER 3: DISCUSSION

To the authors' knowledge, there have been no previous characterizations of an equine blood microbiome, either in health or disease. The present study is the first to provide information about the equine blood microbiome in adult horses before and after exodontia. 16S rRNA gene profiling has consistently yielded greater microbial diversity in samples with an anticipated low microbial biomass (such as amniotic fluid and blood) than appreciated based on culture-dependent methods [28]. We adopted an approach that had been successfully employed to improve 16S rRNA sequencing in several types of samples, including murine blood [61]. The method entailed increasing the PCR cycle number during library preparation from 25 to 40 cycles and was highly effective in the present study, yielding detection of many ASVs in blood of horses both before and after exodontia. While the requisite reagent controls yielded greater coverage than many of the blood samples, the marked increases observed in a subset of post-exodontia samples, along with the compositional similarity to oral microbiota in those same samples, demonstrate the utility of increased cycle number for similar samples with low microbial biomass.

Post-exodontia bacteremia has been well documented in non-equine species and is associated with various potential health complications [1, 2, 28, 31-33, 36, 48, 62-64]. Results of earlier studies have varied based on the specific surgical treatment undertaken, method used for bacterial identification, immune system responsiveness, and whether antimicrobial drugs were present in sampled blood at the time of collection [33]. In most instances, distant site bacterial infections (such as bacterial endocarditis) were attributed to bacteria originating from the oral microbiome (including *Streptococcus mitis* and

Streptococcus oralis in the human medical context) [9]. Although there have been few reports of distant site infections associated with post-exodontia bacterial showering in horses, implicated pathogenic bacteria were also likely derived from oral cavity microbiota [65, 66]. Results of the present study show that the 16S rRNA signatures of bacteria present at the gingiva in proximity to an extracted (diseased) tooth are similar to those detected in the blood following exodontia. Moreover, the results of the present study indicate that bacteremia by oral commensal bacteria may still be evident one hour following exodontia, in some cases.

Prolonged bacteremia post-exodontia would likely increase the chances of adverse health complications, especially in immunocompromised individuals. If the immune system is ineffective at clearing bacteria from the bloodstream for up to one hour post-operatively from an exodontia procedure, bacteria have greater opportunity to become disseminated throughout the body and potentially colonize and infect a remote location. Since the present study is the first to report bacteremia up to one hour post-operatively, there is a need to examine this phenomenon further to evaluate how long it could potentially last and to investigation the applicability of systemic prophylactic or continued post-operative antimicrobial therapy.

Whereas the human oral microbiome (reportedly the most extensively studied human microflora) has been extensively characterized [9, 11], only a few descriptions of the equine oral microbiome have been published [6, 34, 46, 67]. Approximately 500 prevalent bacterial species have been identified in the human oral cavity based on culture-independent molecular methods [9]. Specifically, using culture-independent 16S

rRNA gene clonal analyses, a majority of bacterial species present in the oral cavity are currently deemed uncultivable [10, 11, 68].

Uncultivable bacteria have been increasingly identified due to the widespread application of 16S rRNA gene cloning and sequencing methods to identify microorganisms in natural samples [69]. The advent of such techniques has revealed an extensive and somewhat unsuspected diversity within bacterial phyla thought to be previously well known [69]. It is estimated that less than 2% of bacteria can be cultured in the laboratory using conventional techniques and *in vitro* culturing techniques and conditions may not allow growth of all the bacteria in a sample [70]. It is also possible that *in vitro* conditions do not replicate environmental bacterial cytokine networks that mediate signaling (may be important for coordinating growth) and biofilm formation [70]. As science progresses and more is learned about individual bacterial composition and requirements for growth, some of the uncultivable bacteria today may be readily cultivable in the future using novel techniques and by targeting specific bacterial requirements.

Earlier investigations of the equine oral cavity microbiota using bacteriological culturing methods showed that Gram positive cocci (mainly Streptococci, Micrococci, and starch hydrolyzers) represent prevalent colonizers in healthy horses [46, 71, 72]. Both *Gemella* spp. and *Actinobacillus* spp. are also frequently associated with periodontal health in horses [34, 36, 46]. *Corynebacterium* spp. and *Moraxella* spp. have also been identified in the oral cavity of healthy horses [46]. In another study, *Actinobacillus* spp. and an unclassified *Pasteurellaceae* sp. were the most abundant taxa present in healthy subgingival plaque samples from horses [67]. In that study, *Gammaproteobacteria,*

Firmicutes, and *Bacteroidetes* (with *Treponema*, *Tannerella*, and *Porphyromonas* species detected at low levels) represented the predominant bacterial phyla identified in the healthy equine subgingival microbiome [6, 67].

16S rRNA gene sequencing was used to show that periodontitis is associated with disruption of the oral cavity microbiota (dysbiosis) in horses [6]. Whereas bacteria in the healthy oral cavity included *Prevotella* spp., *Veillonella* spp., *Gemella* spp., and *Actinobacillus* spp., both *Tannerella* and *Treponema* genera were significantly increased when periodontitis was identified [6]. 16S rRNA PCR was also used to show that acidogenic and aciduric bacteria, including *Streptococcus* species, are associated with peripheral caries in horses, as has been reported in other species [43]. Novel red complex bacteria, *Treponema* and *Tannerella* species, were also identified through their DNA signatures from the gingiva of EOTRH-affected horses [34]. It was determined that *Treponema* and/or *Tannerella* DNA was present in 100% of horses with periodontitis and only 52.2% of healthy horses, suggesting that the aforementioned bacterial species have a pathogenic role in the equine mouth [34]. In another study, 18 of 20 horses developed positive blood cultures following exodontia and, in some of those horses, gingival elevation alone resulted in bacteremia [36]. The most commonly identified bacteria on blood culture in that study were *Streptococcus* spp., *Actinomyces* spp., *Fusobacterium* spp., and *Prevotella* spp.; bacterial genera isolated from swab samples of extracted teeth were similar to those detected in the blood, emphasizing that bacteremia resulted from translocation of oral cavity bacteria [36]. However, it should also be noted that results of bacteriological culturing likely underestimate the extent of bacteremia because most oral cavity bacteria are presently uncultivable [73].

Collectively, these studies demonstrate commonalities in oral microbiota composition between diverse species (human, canine, and feline) and that the equine oral microbiome appears to be broadly similar at the taxonomic level of genus and higher [6, 67]. Consistent with previous publications, predominant genera that were identified in the oral cavity of horses in the present study included *Actinobacillus*, *Fusobacterium*, *Leptotrichia*, *Porphyromonas*, *Prevotella*, *Streptococcus*, and *Veillonella*. Moreover, these same taxa were identified in the five post-exodontia blood samples that yielded unexpectedly deep coverage (prolonged bacterial DNA presence). Four of those horses were also affected with sinusitis, suggesting that post-exodontia bacteremia may be more significant when exodontia is undertaken in horses with comorbid sinusitis. This may be due to local inflammation already being present in the area, allowing for a leakier blood vessel barrier for bacteria to penetrate, or it could possibly be due to the larger number of bacteria present locally at and around the surgical site. Although sinusitis in this study appeared to have some correlation with increased bacterial 16S rRNA signature retrieval from the bloodstream, a larger number of cases would need to be studied to validate this association. Sinusitis is a commonly encountered clinical sign in horses with periapical tooth root infection due to the close association of several of the maxillary cheek teeth with the sinuses [52, 74]. Specifically, when dealing with secondary sinusitis, the most commonly afflicted teeth include the first molar (109, 209), fourth premolar (108, 208), and third premolar (107, 207) [74].

The use of 16S rRNA gene cloning and sequencing methods has led to the emerging realization that many diverse bacterial phyla that were previously unrecognized or considered unimportant do play a significant role in some diseases [69]. It is becoming

increasingly evident that commensal bacteria from the oral cavity microbiome are important in the pathogenesis of post-exodontia complications in people following dental surgery [11, 31, 62, 68, 73]. Although 16S rRNA gene cloning and sequencing methods do not differentiate living bacteria from residual bacterial nucleic acid, even residual microbial DNA (in the absence of viable bacterial cells) can serve as an inflammatory signal via innate immune mechanisms including various Toll-like receptors [75]. In light of the fact that a majority of identified bacteria not readily cultivable, it is not possible to conclude which, if any, of the identified bacteria are playing a clinically important role in the pathogenesis of exodontia-associated disease based solely on their respective 16S rRNA signatures [73].

It has long been recognized that bacteremia resulting from either dental infection or dental surgery can lead to distant infection (such as bacterial endocarditis), especially in immunocompromised individuals [35, 38, 48, 76-78]. Disruption of the gingival-blood barrier as a result of disease or surgical intervention potentially facilitates translocation of bacteria and bacterial products into the circulation, potentially leading to systemic diseases [21]. Moreover, there is emerging realization that anaerobic commensal bacteria from the oral cavity might, given access to the circulation, play a role in the pathogenesis of a remarkable and diverse inventory of extra-oral diseases. Various (human) diseases that have been attributed to this phenomenon include diabetes mellitus, respiratory disease, cardiovascular disease, and atheroma [22-24]. Of special interest in this regard is *Fusobacterium nucleatum*, which has been associated with dental disease, various adverse pregnancy outcomes (chorioamnionitis, preterm birth, stillbirth, neonatal sepsis, and preeclampsia), neoplastic and inflammatory gastrointestinal diseases, and various

other infections in human patients [24]. In 1998, Socransky *et al*. defined a set of three species showing strong associations with periodontal disease and with each other: *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* which was further verified using PCR technology in 2012 [79, 80]. Although it remains to be seen whether these oral cavity anaerobic commensals might contribute to systemic disease in a hitherto unrecognized manner in horses, the fact that periodontal disease is very common in aging horses and that *Fusobacteria* were prominently identified in post-exodontia blood in the present study suggests that parallel equine studies should be undertaken [81]. Considering potential bacterial associations between systemic diseases known to be associated with dental disease (i.e. endocarditis, adverse pregnancy outcomes) using NGS may reveal patterns similar to what has been observed in human medicine.

The extent to which post-procedural bacteremia persists has not been extensively reported. In one (human) investigation it was reported that *viridans* group streptococci were rapidly (within 10 minutes) eliminated from 42 of 46 patients undergoing various oral surgical procedures [33]. In one equine study, two blood samples yielded positive cultures following exodontia (samples obtained 10 minutes after the termination of surgery), providing evidence for short term persistence of bacteremia [36]. Those authors speculated that persistence of bacteremia could have resulted from a greater number of bacteria (quantitative bacterial counts were not performed) or a result of immune function variations between individual horses (two horses in that study were bacteremic prior to the surgical procedure) [36]. Results of earlier work in other species suggest that intravascular bacteria are rapidly cleared from the circulation by the reticuloendothelial system (within 10–20 minutes) [82]. Our results show that significant post-exodontia

bacteremia is still evident at 60 minutes following conclusion of surgery in some horses. The immune status of the horses in this study was not examined, but future investigations could incorporate an evaluation of the immune system (such as routine hematology and PPID testing) for horses receiving exodontia surgery. Specialized tests of innate immune system function are available to veterinarians at specialized laboratories that involve assessing neutrophil function. Further studies might also evaluate additional time points beyond one hour for evidence of longer-persisting bacteremia.

The use of prophylactic antimicrobials in the perioperative period is restricted to more invasive dental procedures in human dentistry, especially for individuals affected with immunocompromising comorbidities or those with cardiac disease or implants [83, 84]. Antimicrobials are used under the assumption that they do not prevent bacteremia but inhibit bacterial propagation and bacterial adherence to tissues/implants [83, 84]. Specific guidelines for antimicrobial use in horses receiving exodontia have not been published. Results of the present study showing marked post-exodontia bacteremia persisting for at least one hour suggest that antimicrobial use might be important in this setting, especially for immunocompromised horses. In the present study, age and immune status (horses previously diagnosed with PPID) were not identified as positive risk factors for post-operative bacteremia. In future studies, it may be relevant to examine a larger cohort of animals and perform PPID screening tests to look for associations of age and immune status.

Using only a solitary time point for blood sampling post-exodontia (one hour post-operatively) was a limitation of this study and the results imply significant post-procedural bacteremia may persist beyond this timeframe and is deserving of further

investigation. Although time expended with each exodontia was not measured, it is reasonable to assume that difficult extractions requiring more time could be associated with increased post-procedural bacteremia when compared with more expeditiously concluded procedures. Other limitations include the limited number of cases and the lack of age-matched controls. Blood microbiome results do not necessarily reflect a normal population as all recruited horses were affected with dental disease necessitating exodontia and pre-exodontia blood microbiomes may have been influenced by the presence of dental infection. It should be emphasized that 16S rRNA gene sequencing results are relative, meaning that the actual quantity of bacteria in a given sample is uncertain [85]. It is also possible that each 16S rRNA gene may not amplify with equal efficiency during PCR reactions due to differential primer affinity and GC content and taxonomy assignment is conditional upon the completeness of reference databases [85]. Moreover, multiple studies have demonstrated that increased PCR cycle numbers during library preparation are likely to increase the error rate and potentially introduce bias [61, 86, 87]. The use of such methods should therefore be based on the sample type and goals of the study, and results interpreted appropriately.

Conclusions

The results of this study affirm that bacteremia resulting from translocated oral cavity commensals occurs in horses following dental extraction. Additionally, post-exodontia bacteremia is still evident in some individuals for up to one hour, which is much longer than had been previously documented. These results include the first extensive documentation of a blood microbiome based on 16S rRNA gene sequencing in adult horses. The extent of post-exodontia bacteremia, especially as pertains to

uncultivable commensal bacteria and their propensity to contribute to extra-oral disease, is deserving of further investigation in horses.

ILLUSTRATIONS

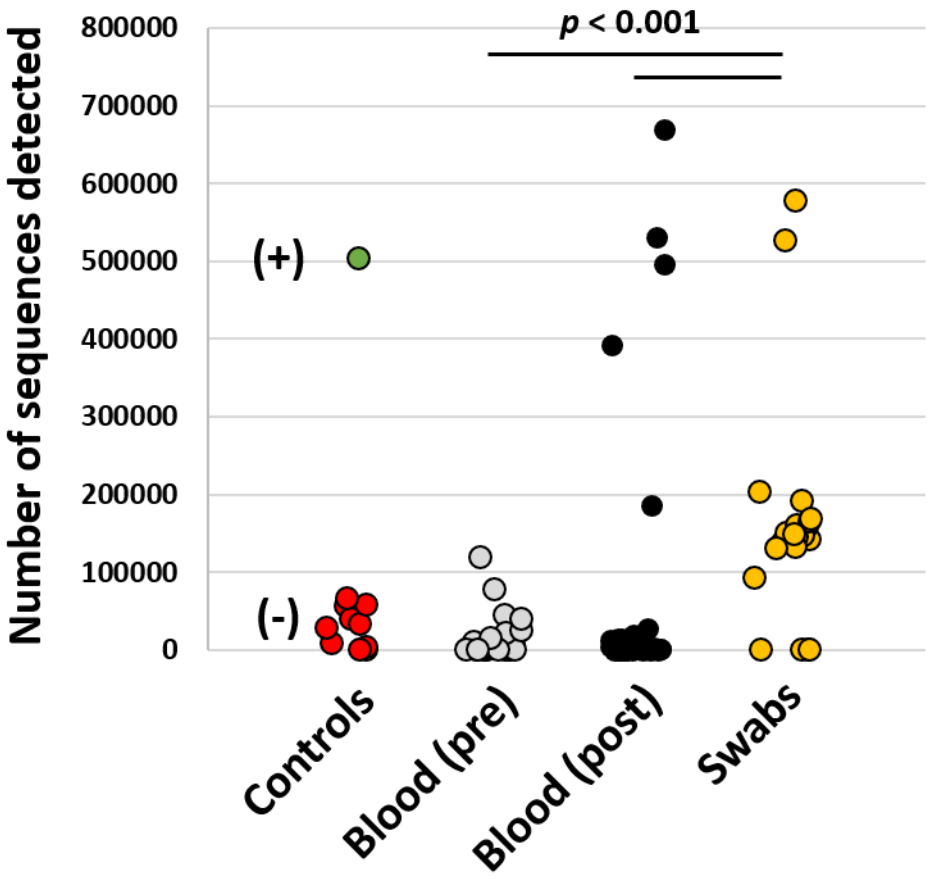

Figure 1: Dot plots showing the total number of 16S rRNA amplicon sequences resulting from amplification and sequencing on a shared flow cell, of negative (-) and positive (+) controls, peripheral blood collected aseptically pre- and post-exodontia procedure, and dental/gingival swabs.

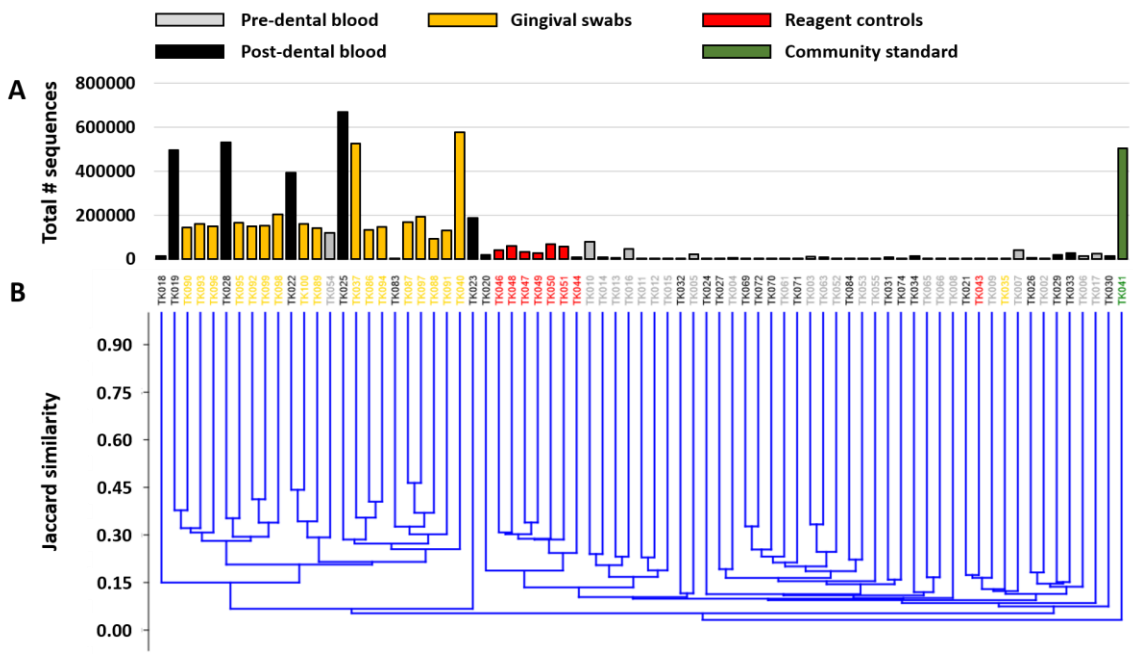

Figure 2: Bar chart showing sample coverage in those samples yielding > 1054 sequences, legend at top (A), and a dendrogram generated from those data, rarefied to a uniform coverage of 1054 sequences/sample (B).

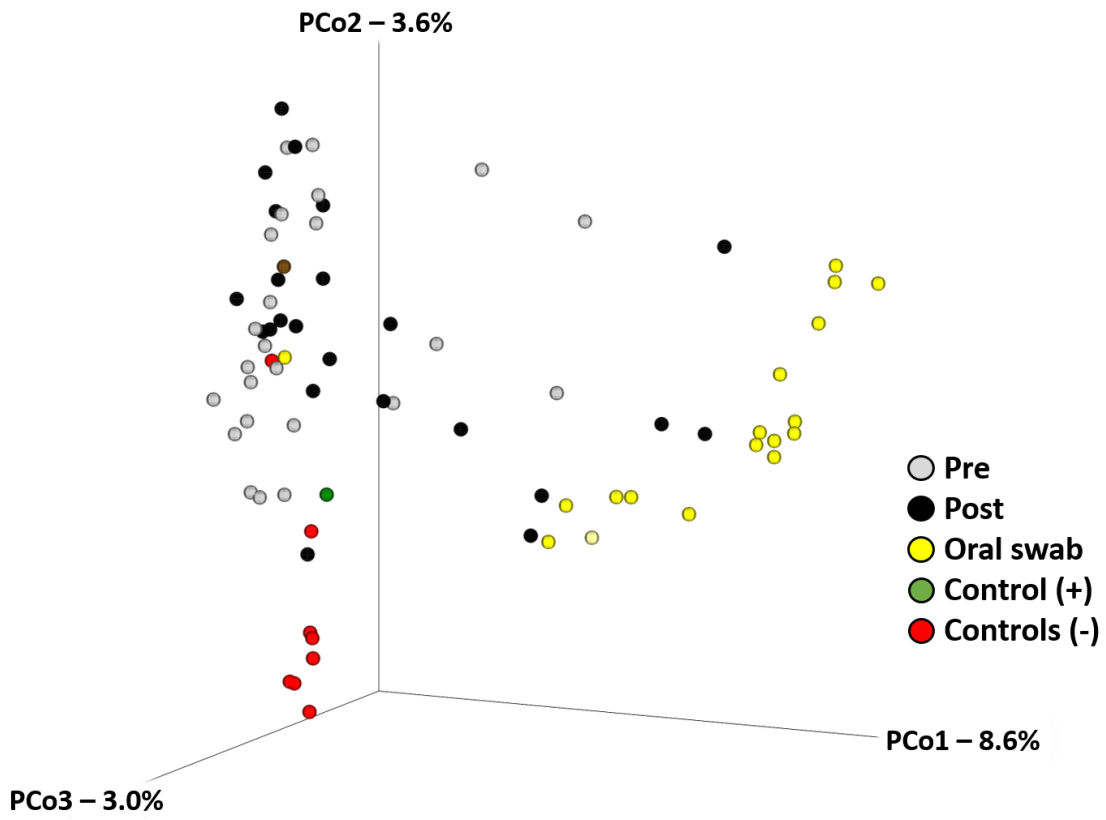

Figure 3: Principal coordinate analysis based on Jaccard similarities and generated using a rarefied dataset (1054 sequences/sample).

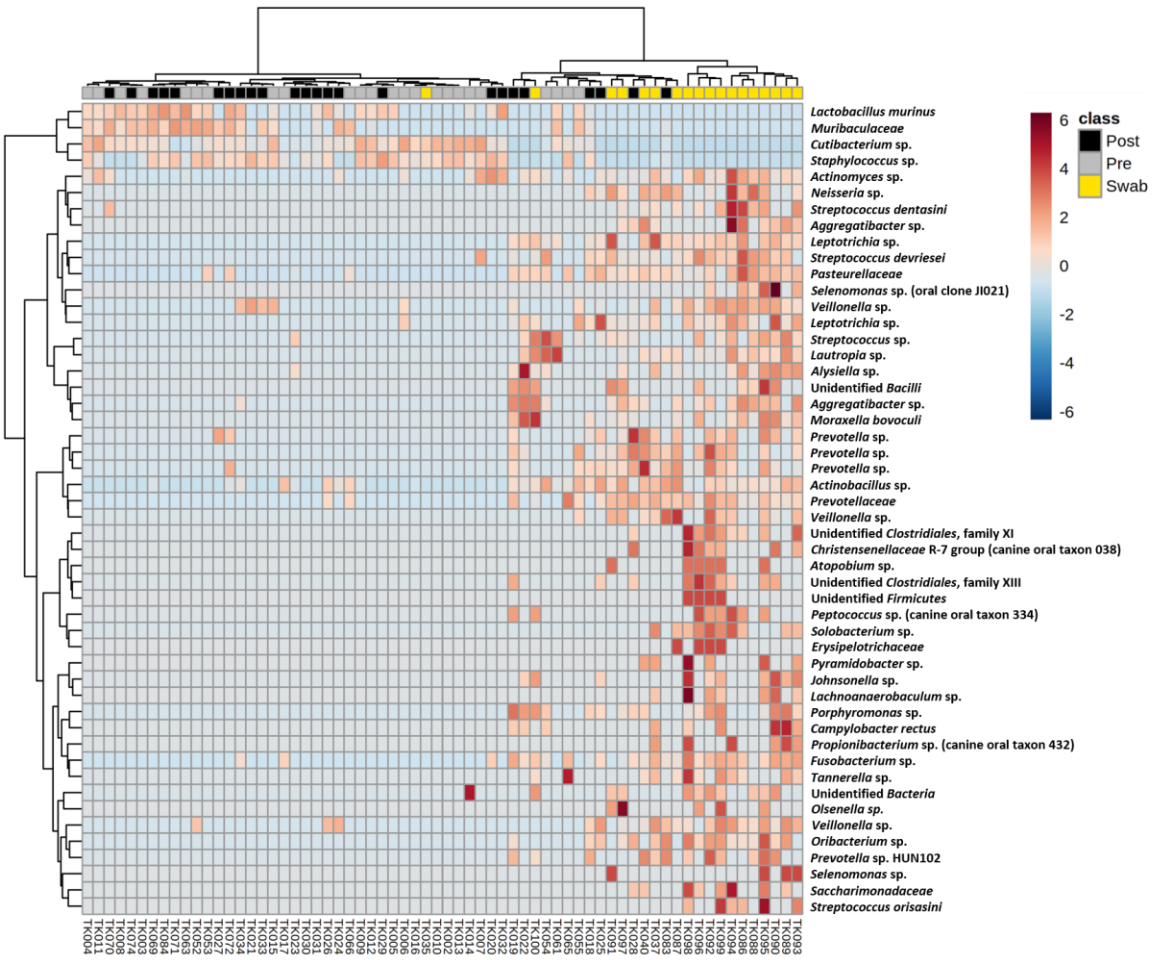

Figure 4: Heatmap generated via hierarchical clustering of samples based on the relative abundance of the 50 ASVs yielding the lowest p values following ANOVA of all ASVs comparing pre- and post- exodontia blood and swabs.

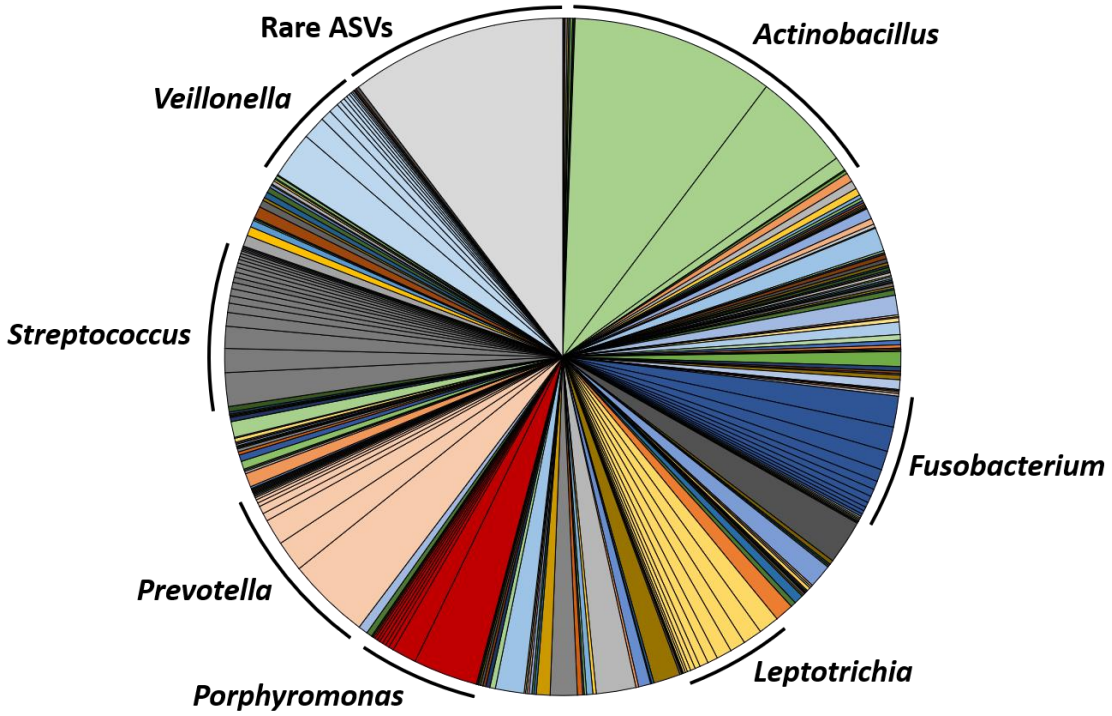

Figure 5. Pie chart showing the mean relative abundance of ASVs detected in the gingival swabs, with dominant genera labeled. The grey portion represents a total of 8544 rare ASVs, comprising roughly 10% of any given sample.

TABLES

Patient	Tooth/Teeth	Diagnosis	Procedure	Healthy	PPID	Asthma	Sinusitis
Horse 1[a]	111	Slab fracture 111	Removal of 111	x			
Horse 1[b]	110, 211	Recessed and missing parts of crown 110, slab fracture 211	Removal of 110 and removal of palatal slab of 211	x			
Horse 2[a]	Mandibular incisors	EOTRH	Removal of mandibular incisors	x			
Horse 2[b]	Maxillary incisors	EOTRH	Removal of maxillary incisors	x			
Horse 3	109, 209	Nasosinus fistula, sinusitis, apical abscess 109,209, nasal cyst	Removal of 209, 109, trephination and sinus lavage			x	x
Horse 4	202,203,302,303	EOTRH	Removal of 202, 203, 302, 303		x		
Horse 5	209	Slab fracture 209	Removal of 209	x			
Horse 6	209, 210	Fractured 209, sinusitis, apical abscess 210	Removal of 209, 210, rostral maxillary lavage				x
Horse 7	108, 109	Apical abscessation 108, blunted roots 109	Removal of 108, 109	x			
Horse 8	208, 209	Apical abscessation, sinusitis	Removal of 208, 209, trephanation and sinus lavage				x
Horse 9	204, 404	EOTRH	Removal of 204, 404		x		
Horse 10	Maxillary incisors	EOTRH	Removal of maxillary incisors	x			
Horse 11	209, 210	Slab fracture 209, 210	Retropulsion of 209, 210 and sinus lavage				x
Horse 12	108	Infundibular caries	Removal of 108	x			
Horse 13	108	Sinusitis of the frontal and maxillary sinus (right sided),associated with 108	Removal of 107, 108 caps and sinus lavage				x
Horse 14	310, 410	Slab fractures 310, 410	Attempted retropulsion of 410 - failed	x			
Horse 15	111, 211	Slab fractures 111, 211	Removal of 111	x			
Horse 16	107, 108, 209	Apical root abscessation 107, 108, 209	Removal of 107, 108, 209	x			
Horse 17	101, 102, 103	Traumatic fracture of incisive bone and tooth roots	Removal of 101, 102, 103	x			
Horse 18	108	Crown fracture with periodontal disease	Removal of 108	x			
Horse 19	209	Infundibular caries	Removal of 209	x			
Horse 20[a]	Maxillary incisors	EOTRH	Removal of maxillary incisors	x			
Horse 20[b]	308, 409	Apical abscessation 308, 409	Removal of 308, 409		x		
Horse 21[a]	Maxillary incisors	EOTRH	Removal of maxillary incisors	x			
Horse 21[b]	Mandibular incisors	EOTRH	Removal of mandibular incisors	x			
Horse 22	209	Infundibular caries, fracture through pulp horn	Removal of 209	x			
Horse 23	110	Slab fracture, periapical abscess 110	Retropulsion of 110 and sinus lavage				x
Horse 24	109, 209	Apical abscessation, periodontal disease 109, 209	Removal of 109, 209	x			
Horse 25	208	Infundibular caries	Removal of 208	x			
Horse 26	206, 306	Complicated crown fracture with pulp exposure 206, fractured fragment of 306	Removal of 206, 306	x			
Horse 27	301	Fractured 301 (suspect traumatic)	Removal of 301	x			
Horse 28[a]	109, 208	Infundibular caries 109, slab fracture 208	Removal of 109	x			
Horse 28[b]	208	Slab fracture	Removal of 208	x			
Horse 29	309, 310	Apical root abscessation 309, 310	Removal of 309, 310	x			

Table 1: Table depicting individual horse information including teeth extracted, diagnosis, surgical procedure, and health designation (healthy, affected with PPID, equine asthma, sinusitis). Teeth were numbered using the Modified Triadan System.

[a] refers to the first visit, [b] refers to second visit

BIBLIOGRAPHY

1. Genco, R.J. and M. Sanz, *Clinical and public health implications of periodontal and systemic diseases: An overview.* Periodontol 2000, 2020. **83**(1): p. 7-13.
2. DeBowes, L.J., *The effects of dental disease on systemic disease.* Vet Clin North Am Small Anim Pract, 1998. **28**(5): p. 1057-62.
3. Marshall, M.D., et al., *A longitudinal assessment of periodontal disease in 52 Miniature Schnauzers.* BMC Vet Res, 2014. **10**: p. 166.
4. Stella, J.L., A.E. Bauer, and C.C. Croney, *A cross-sectional study to estimate prevalence of periodontal disease in a population of dogs (Canis familiaris) in commercial breeding facilities in Indiana and Illinois.* PLoS One, 2018. **13**(1): p. e0191395.
5. Vos, T., et al., *Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013.* The Lancet, 2015. **386**(9995): p. 743-800.
6. Kennedy, R., et al., *The microbiome associated with equine periodontitis and oral health.* Vet Res, 2016. **47**: p. 49.
7. Arweiler, N.B. and L. Netuschil, *The Oral Microbiota.* Adv Exp Med Biol, 2016. **902**: p. 45-60.
8. Yamashita, Y. and T. Takeshita, *The oral microbiome and human health.* J Oral Sci, 2017. **59**(2): p. 201-206.
9. Aas, J.A., et al., *Defining the normal bacterial flora of the oral cavity.* J Clin Microbiol, 2005. **43**(11): p. 5721-32.
10. Dewhirst, F.E., et al., *The human oral microbiome.* J Bacteriol, 2010. **192**(19): p. 5002-17.
11. Chen, T., et al., *The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information.* Database (Oxford), 2010. **2010**: p. baq013.
12. Kinane, D.F., P.G. Stathopoulou, and P.N. Papapanou, *Periodontal diseases.* Nat Rev Dis Primers, 2017. **3**: p. 17038.
13. Walsh, T., et al., *Fluoride toothpastes of different concentrations for preventing dental caries.* Cochrane Database Syst Rev, 2019. **3**(3): p. Cd007868.
14. Chetrus, V. and I. Ion, *Dental Plaque - Classification, Formation, and Identification.* International Journal of Medical Dentistry., 2013. **17**(2): p. 139-143.
15. Kolenbrander, P.E., *Oral microbial communities: biofilms, interactions, and genetic systems.* Annu Rev Microbiol, 2000. **54**: p. 413-37.
16. ten Cate, J.M., *Biofilms, a new approach to the microbiology of dental plaque.* Odontology, 2006. **94**(1): p. 1-9.
17. Marsh, P.D., *Are dental diseases examples of ecological catastrophes?* Microbiology (Reading), 2003. **149**(Pt 2): p. 279-294.

18. Marsh, P.D. and D.A. Devine, *How is the development of dental biofilms influenced by the host?* J Clin Periodontol, 2011. **38 Suppl 11**: p. 28-35.
19. Humphrey, S.P. and R.T. Williamson, *A review of saliva: normal composition, flow, and function.* J Prosthet Dent, 2001. **85**(2): p. 162-9.
20. Tonetti, M.S., et al., *Principles in prevention of periodontal diseases: Consensus report of group 1 of the 11th European Workshop on Periodontology on effective prevention of periodontal and peri-implant diseases.* J Clin Periodontol, 2015. **42 Suppl 16**: p. S5-11.
21. Gulati, M., et al., *Essentials of periodontal medicine in preventive medicine.* Int J Prev Med, 2013. **4**(9): p. 988-94.
22. Allen-Vercoe, E., J. Strauss, and K. Chadee, *Fusobacterium nucleatum: an emerging gut pathogen?* Gut Microbes, 2011. **2**(5): p. 294-8.
23. Field, C.A., et al., *Investigation and quantification of key periodontal pathogens in patients with type 2 diabetes.* J Periodontal Res, 2012. **47**(4): p. 470-8.
24. Han, Y.W., *Fusobacterium nucleatum: a commensal-turned pathogen.* Curr Opin Microbiol, 2015. **23**: p. 141-7.
25. Pietropaoli, D., et al., *Definition of hypertension-associated oral pathogens in NHANES.* J Periodontol, 2019. **90**(8): p. 866-876.
26. Aagaard, K., et al., *A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy.* PLoS One, 2012. **7**(6): p. e36466.
27. Segata, N., et al., *Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples.* Genome Biol, 2012. **13**(6): p. R42.
28. Han, Y.W. and X. Wang, *Mobile microbiome: oral bacteria in extra-oral infections and inflammation.* J Dent Res, 2013. **92**(6): p. 485-91.
29. Conrads, G. and I. About, *Pathophysiology of Dental Caries.* Monogr Oral Sci, 2018. **27**: p. 1-10.
30. Okell, C. and T.S. Elliott, *Bacteriaemia and oral sepsis with special reference to aetiology of subacute endocarditis.* Lancet, 1935. **226**: p. 869-872.
31. Takai, S., et al., *Incidence and bacteriology of bacteremia associated with various oral and maxillofacial surgical procedures.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2005. **99**(3): p. 292-8.
32. Meurman, J.H. and P. Hämäläinen, *Oral health and morbidity--implications of oral infections on the elderly.* Gerodontology, 2006. **23**(1): p. 3-16.
33. Heimdahl, A., et al., *Detection and quantitation by lysis-filtration of bacteremia after different oral surgical procedures.* J Clin Microbiol, 1990. **28**(10): p. 2205-9.
34. Sykora, S., et al., *Isolation of Treponema and Tannerella spp. from equine odontoclastic tooth resorption and hypercementosis related periodontal disease.* Equine Vet J, 2014. **46**(3): p. 358-63.
35. Maxson, A.D. and V.B. Reef, *Bacterial endocarditis in horses: ten cases (1984-1995).* Equine Vet J, 1997. **29**(5): p. 394-9.
36. Kern, I., et al., *Bacteraemia before, during and after tooth extraction in horses in the absence of antimicrobial administration.* Equine Vet J, 2017. **49**(2): p. 178-182.

37. Pereira Dos Santos, J.D., et al., *Relation between periodontal disease and systemic diseases in dogs.* Res Vet Sci, 2019. **125**: p. 136-140.
38. Semedo-Lemsaddek, T., et al., *Enterococcal Infective Endocarditis following Periodontal Disease in Dogs.* PLoS One, 2016. **11**(1): p. e0146860.
39. McGowan, T.W., G.P. Pinchbeck, and C.M. McGowan, *Prevalence, risk factors and clinical signs predictive for equine pituitary pars intermedia dysfunction in aged horses.* Equine Vet J, 2013. **45**(1): p. 74-9.
40. Ireland, J.L. and C.M. McGowan, *Epidemiology of pituitary pars intermedia dysfunction: A systematic literature review of clinical presentation, disease prevalence and risk factors.* Vet J, 2018. **235**: p. 22-33.
41. Muirhead, T.L., et al., *The effect of age on serum antibody titers after rabies and influenza vaccination in healthy horses.* J Vet Intern Med, 2008. **22**(3): p. 654-61.
42. Staszyk, C., et al., *Equine odontoclastic tooth resorption and hypercementosis.* Vet J, 2008. **178**(3): p. 372-9.
43. Tyler, A.D., M.I. Smith, and M.S. Silverberg, *Analyzing the human microbiome: a "how to" guide for physicians.* Am J Gastroenterol, 2014. **109**(7): p. 983-93.
44. Salonen, A., et al., *Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis.* J Microbiol Methods, 2010. **81**(2): p. 127-34.
45. Païssé, S., et al., *Comprehensive description of blood microbiome from healthy donors assessed by 16S targeted metagenomic sequencing.* Transfusion, 2016. **56**(5): p. 1138-47.
46. Borkent, D., et al., *A microbiome analysis of equine peripheral dental caries using next generation sequencing.* Equine Vet J, 2020. **52**(1): p. 67-75.
47. Stein, K., et al., *The use and misuse of antibiotics in dentistry: A scoping review.* J Am Dent Assoc, 2018. **149**(10): p. 869-884.e5.
48. Ito, H.O., *Infective endocarditis and dental procedures: evidence, pathogenesis, and prevention.* J Med Invest, 2006. **53**(3-4): p. 189-98.
49. Blaser, M.J., *The Past and Future Biology of the Human Microbiome in an Age of Extinctions.* Cell, 2018. **172**(6): p. 1173-1177.
50. Tremaine, W.H. and J. Schumacher, *Exodontia. In Equine Dentistry*. Third Edition ed. Exodontia, ed. J. Easly, P. Dixon, and J. Schumacher. 2011, Philadelphia: Saunders Ltd. 424.
51. Tremaine, W.H., *Oral extraction of equine cheek teeth.* 2004. **16**(3): p. 151-158.
52. Caramello, V., et al., *Equine cheek tooth extraction: Comparison of outcomes for five extraction methods.* Equine Vet J, 2020. **52**(2): p. 181-186.
53. Walters, W.A., et al., *PrimerProspector: de novo design and taxonomic analysis of barcoded polymerase chain reaction primers.* Bioinformatics, 2011. **27**(8): p. 1159-61.
54. Caporaso, J.G., et al., *Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample.* Proc Natl Acad Sci U S A, 2011. **108 Suppl 1**: p. 4516-22.
55. Loy, A., et al., *probeBase--an online resource for rRNA-targeted oligonucleotide probes: new features 2007.* Nucleic Acids Res, 2007. **35**(Database issue): p. D800-4.

56. Martin, M., *Cutadapt removes adapter sequences from high-throughput sequencing reads.* 2011, 2011. **17**(1): p. 3 %J EMBnet.journal.
57. Bolyen, E., et al., *Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.* Nat Biotechnol, 2019. **37**(8): p. 852-857.
58. Callahan, B.J., et al., *DADA2: High-resolution sample inference from Illumina amplicon data.* Nature Methods, 2016. **13**(7): p. 581-583.
59. Quast, C., et al., *The SILVA ribosomal RNA gene database project: improved data processing and web-based tools.* Nucleic Acids Res, 2013. **41**(Database issue): p. D590-6.
60. Hammer, O., D.A.T. Harper, and P.D. Ryan, *Past: Paleontological Statistics Software Package for Education and Data Analysis.* Palaeontol Electron, 2001. **4**(1): p. 9.
61. Witzke, M.C., et al., *Influence of PCR cycle number on 16S rRNA gene amplicon sequencing of low biomass samples.* J Microbiol Methods, 2020. **176**: p. 106033.
62. Olsen, I., *Update on bacteraemia related to dental procedures.* Transfus Apher Sci, 2008. **39**(2): p. 173-8.
63. Potgieter, M., et al., *The dormant blood microbiome in chronic, inflammatory diseases.* FEMS Microbiol Rev, 2015. **39**(4): p. 567-91.
64. Rawlinson, J.E., et al., *Association of periodontal disease with systemic health indices in dogs and the systemic response to treatment of periodontal disease.* J Am Vet Med Assoc, 2011. **238**(5): p. 601-9.
65. Verdegaal, E.J.M.M., et al., *A right-sided bacterial endocarditis of dental origin in a horse.* 2006. **18**(4): p. 191-195.
66. Bartmann, C., et al., *Dentogene Sinusitis durch gramnegative Anaerobier beim Pferd.* Tierärztliche Praxis. Ausgabe G, Grosstiere/Nutztiere, 2002. **30**: p. 178-183.
67. Gao, W., et al., *In-depth snapshot of the equine subgingival microbiome.* Microb Pathog, 2016. **94**: p. 76-89.
68. Curtis, M.A., P.I. Diaz, and T.E. Van Dyke, *The role of the microbiota in periodontal disease.* Periodontol 2000, 2020. **83**(1): p. 14-25.
69. Rappé, M.S. and S.J. Giovannoni, *The Uncultured Microbial Majority.* 2003. **57**(1): p. 369-394.
70. Wade, W., *Unculturable bacteria--the uncharacterized organisms that cause oral infections.* J R Soc Med, 2002. **95**(2): p. 81-3.
71. Baker, G.J., *Some aspects of equine dental decay.* Equine Vet J, 1974. **6**(3): p. 127-30.
72. Lundström, T., et al., *Equine saliva components during mastication, and in vivo pH changes in the oral biofilm of sound and carious tooth surfaces after sucrose exposure.* Acta Vet Scand, 2020. **62**(1): p. 21.
73. Epstein, S.S., *The phenomenon of microbial uncultivability.* Curr Opin Microbiol, 2013. **16**(5): p. 636-42.
74. Freeman, D.E., *Sinus disease.* Veterinary Clinics of North America: Equine Practice, 2003. **19**(1): p. 209-243.

75. Crump, K.E. and S.E. Sahingur, *Microbial Nucleic Acid Sensing in Oral and Systemic Diseases.* J Dent Res, 2016. **95**(1): p. 17-25.
76. Fülöp, T., et al., *The Role of Immunosenescence in the Development of Age-Related Diseases.* Rev Invest Clin, 2016. **68**(2): p. 84-91.
77. Glickman, L.T., et al., *Evaluation of the risk of endocarditis and other cardiovascular events on the basis of the severity of periodontal disease in dogs.* J Am Vet Med Assoc, 2009. **234**(4): p. 486-94.
78. Parahitiyawa, N.B., et al., *Microbiology of odontogenic bacteremia: beyond endocarditis.* Clin Microbiol Rev, 2009. **22**(1): p. 46-64, Table of Contents.
79. Socransky, S.S., et al., *Microbial complexes in subgingival plaque.* J Clin Periodontol, 1998. **25**(2): p. 134-44.
80. Griffen, A.L., et al., *Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing.* Isme j, 2012. **6**(6): p. 1176-85.
81. Nuttall, H.E. and P.J. Ravenhill, *Prevalence and analysis of equine periodontal disease, diastemata and peripheral caries in a first-opinion horse population in the UK.* Vet J, 2019. **246**: p. 98-102.
82. Silver, J.G., L. Martin, and B.C. McBride, *Recovery and clearance rates of oral microorganisms following experimental bacteraemias in dogs.* Arch Oral Biol, 1975. **20**(10): p. 675-9.
83. Wilson, W., et al., *Prevention of infective endocarditis: guidelines from the American Heart Association: a guideline from the American Heart Association Rheumatic Fever, Endocarditis and Kawasaki Disease Committee, Council on Cardiovascular Disease in the Young, and the Council on Clinical Cardiology, Council on Cardiovascular Surgery and Anesthesia, and the Quality of Care and Outcomes Research Interdisciplinary Working Group.* J Am Dent Assoc, 2008. **139 Suppl**: p. 3s-24s.
84. Kuong, E.E., et al., *Antibiotic prophylaxis after total joint replacements.* Hong Kong Med J, 2009. **15**(6): p. 458-62.
85. Jo, J.H., E.A. Kennedy, and H.H. Kong, *Research Techniques Made Simple: Bacterial 16S Ribosomal RNA Gene Sequencing in Cutaneous Research.* J Invest Dermatol, 2016. **136**(3): p. e23-e27.
86. Wu, J.Y., et al., *Effects of polymerase, template dilution and cycle number on PCR based 16 S rRNA diversity analysis using the deep sequencing method.* BMC Microbiol, 2010. **10**: p. 255.
87. Sze, M.A. and P.D. Schloss, *The Impact of DNA Polymerase and Number of Rounds of Amplification in PCR on 16S rRNA Gene Sequence Data.* 2019. **4**(3): p. e00163-19.

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