Equine oral microbiology

Chapter 8

Oral Microbiology

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Introduction

Until recently, equine oral microbiology was a relatively unexplored field. The role of bacteria in both oral health and in oral diseases such as periodontitis and dental caries has long been acknowledged in brachydont species such as humans, dogs and cats.

The ecological community of bacteria, both commensal and pathogenic, inhabiting the oral cavity is known as the oral microbiome and has been well studied in humans. [1] By 2010, the Human Oral Microbiome Database (HOMD) included approximately 700 prokaryote species that can be present in the human oral cavity. [2] Recent work has also began to characterise the equine [3-5] and donkey [6] oral microbiome in order to gain a better understanding how these microbial communities are involved in maintaining oral health and also their potential role in the pathogenesis of a variety of equine oral diseases. Technical advances, especially the increased availability of molecular microbiology techniques have allowed insights into this previously neglected area.

Bacteria, particularly anaerobes have been noted to play a major role in human, canine and feline periodontal disease and similar species have been detected in equine periodontal pockets. [3] In addition, acid-producing bacteria have long been implicated in the pathogenesis of human dental caries [7] and recent work by Borkent et al. [5] has also linked acidogenic bacteria with peripheral caries (PC) in the horse. An understanding how oral cavity microbial communities function and interact with the host is important not only in managing oral health but also in understanding and treating dental disease.

The oral microbiome

The term *microbiome* was initially described by Lederberg and McCray [8] and is used to 'signify the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease'. The oral cavity has been shown to contain several distinct microbial habitats with the teeth surface, gingival sulcus, gingiva, lip, buccal mucosa and tongue each supporting distinct

microbial populations. [9] As noted, over 700 species of bacteria have been identified in humans although full characterisation of the human oral microbiome is incomplete [1]. This microbial population is incredibly complex and dynamic, with many species of bacteria interacting not only with each other but also the host's immune system.

Oral biofilms (pellicle, plaque and bacteria)

Oral bacteria are exposed to difficult survival conditions, being constantly washed with host saliva that also contains antimicrobial constituents. They are also challenged with mechanical abrasion by the masticatory movements of food. This is especially true in the horse, a species which may masticate coarse forage for up to 15 hours per day. [10] In response to this environment, oral cavity bacteria have evolved techniques to allow them to firmly adhere to the surface of oral tissues within a biofilm that partially protects them, from these oral cavity conditions.

A biofilm has been defined by Costerton et al. [11] as a 'biopolymer matrix-enclosed bacterial population adherent to each other and other surfaces'. Multispecies bacterial communities such as those which exist in the oral biofilm are supported and protected by the surrounding matrix. A biofilm adhering to the tooth surface is termed a *pellicle* or *plaque*, depending on the thickness and constitution of the layer. The initial phase of pellicle formation starts within seconds of a tooth being exposed to saliva. Acquired pellicle (AP) is a thin (0.5-1µm thick) proteinaceous layer, composed of proteins, carbohydrates and lipids that forms on the surface of teeth. The sources of these compounds are salivary secretions, gingival crevicular fluid, oral epithelial cell products, and microbial products. [12, 13] Bacteria can adhere to acquired pellicle within three minutes of exposure of teeth to saliva [14] and proteins in the pellicle have specific receptors for *bacterial adhesins* that facilitate this process. [14, 15] This acquired pellicle plays an important role in oral lubrication, regulation of dental mineral homeostasis and host antimicrobial defense. [13]

The exact composition of an oral biofilm is dependent upon its position within the oral cavity and bacterial populations may significantly differ between different locations. Human tooth-associated biofilms have been categorised according to location by Kolenbrander et al. [16] with supragingival biofilms adhering to the clinical crown surface and subgingival biofilms adhering below the gingival margin. The latter may be situated within a normal (shallow) gingival sulcus or within a (deep) diseased periodontal pocket.

In the initial stages of biofilm formation, early bacterial colonisers which are well adapted to community formation and multispecies growth adhere to the salivary pellicle. Following adhesion and proliferation of these early pioneer microbial colonisers, these bacteria create a micro-environment suitable for the attachment and growth of other micro-organisms. Bacteria, genetically distinct to the early colonising species later attach to their surface layer. [17]

Multiple bacterial species then become surrounded and supported by the biofilm matrix, and may interact with each other via cell surface components which is termed the co-aggregation phase. Over time, the biofilm (pellicle) becomes increasingly complex and eventually matures into a thicker dental plaque. Dental plaque is a thick biofilm that mainly consists of an organic matrix of salivary mucins (mucopolysaccharides, the major glycoprotein components of mucus) and extracellular polysaccharide polymers with attached micro-organisms. [18] As the biofilm matures further, the microbial community becomes more complex. The rate of growth of dental plaque depends on the availability of nutrients, competition with other micro-organisms and environmental conditions in the biofilm. [19] In humans, the microbial community of supragingival plaque differs from that of subgingival plaque. [20] Predilection sites for plaque to accumulate are often at mechanically protected areas. [21] In horses these sites are mainly abnormally wide interdental (interproximal) spaces. In an equine dental peripheral caries study Erridge et al. [22] termed a biofilm greater than 10 µm thickness as “dental plaque” rather than pellicle.

Micro-organisms living in dental plaque have many advantages over oral bacteria living outside such an environment. [23] Molecules that cannot be broken down by individual species of bacteria may be catabolised by the combination of micro-organisms living in dental plaque. A pathogenic synergism may even occasionally occur, which results in a combination of organisms being more pathogenic than the individual micro-organisms. Additionally, the micro-organisms in a microbial consortium can be more resistant to antimicrobial therapeutics, environmental stress and host defences than oral bacteria not living in a microbial plaque community because of the collaboration and gene transfer likely to occur between these bacteria [23] as discussed later.

A survival strategy that oral bacteria can use during nutrient deprivation is a dormancy state during which they survive in a state of metabolic arrest without any cell division or growth. This state is also known as a viable but nonculturable (VBNC) state [24] during which bacteria are less sensitive to antimicrobial agents, to changes in temperature and pH. If nutrients become available again to bacteria in a dormancy state, they can return to their higher metabolic rates

(metabolic reactivation), resuming cell growth and division. Chávez de Paz et al. [19] investigated the metabolic reactivation of two oral bacteria (*Streptococcus anginosus* and *Lactobacillus salivarius*) found in oral biofilms. The low reactivity of these nutrient-deprived oral bacteria after the introduction of nutrients was suggested to be part of their survival strategy. Additionally, the enhanced synthesis of certain proteins that could be regarded as stress proteins, by oral bacteria such as *Streptococcus mutans* also help bacteria to survive different suboptimal conditions. [25]

Supragingival plaque can have a structured architecture with channels (pores) connecting the dental surface to the oral cavity. [23, 26] Viable micro-organisms in supragingival plaque can have an uneven spatial arrangement. [26] The most viable bacteria occur in the central part of the plaque and lining the channels, where more effective diffusion of nutrients occurs. Dead bacteria surrounded the viable bacteria and lie closest to the dental surface and to the oral cavity. The function of these layers of dead biological material may be to protect the underlying viable micro-organisms.[26]

Some species play key roles in plaque formation. For example, certain strains of *Fusobacteria nucleatum* can co-aggregate with both early and late oral biofilms and thus play a bridging role in the formation of dental plaque in humans.[27] As noted, the biofilm present in the oral cavity is highly intricate with dynamic microbial interactions occurring such as complex cell signalling between different species, and even different genera of bacteria, as well as inter-bacterial transfer of DNA. Conjugative transposons which facilitate transfer of DNA between bacteria have been detected in many genera of oral bacteria including *Fusobacteria*, *Streptococcus* and *Veillonella.*[28]

The biofilm matrix not only protects oral bacteria from mechanical challenges such as by saliva and food, it also offers some protection from the host's innate and adaptive immune response and can also reduce exposure to administered antimicrobial drugs. Bacteria within a biofilm matrix cannot be readily engulfed by macrophages and so host front-line immune responses such as phagocytosis are much less effective.[29] Walker et al. [30] also suggested that infiltration of neutrophils into dental plaque was not only ineffective against the bacteria it contains, but may even provide additional matrix material for bacterial attachment. Mechanical removal of dental plaque is performed when possible in treating brachydont periodontal disease. Presumably due to the prolonged mastication of forage and resulting high salivary flow in hypsodont species, plaque formation in the healthy equine oral cavity is uncommon except in

the interdental (interproximal) spaces of cheek teeth and on canine teeth where the plaque becomes calcified (calculus) (Figure 8.1).

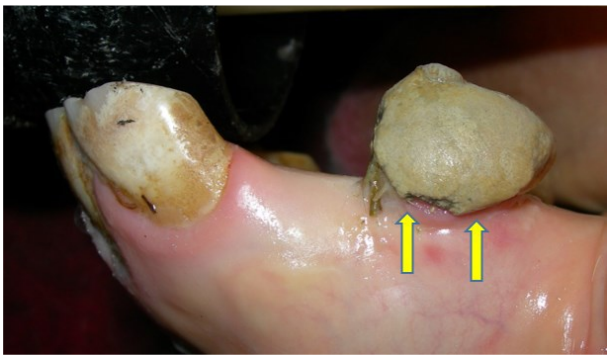

Figure 8.1. Calculus on a mandibular canine tooth with local gingivitis (arrows)
Photograph courtesy of P.M. Dixon.

The role of bacteria in oral health and disease

Our understanding of the role of bacteria in disease is changing, with traditional principles such as Koch's postulates [31] becoming increasingly irrelevant in modern microbiology. Koch's postulates were designed to establish prerequisites to assess whether a specific microorganism causes a particular disease. Koch's postulates state that: (1) The microorganism must be found in abundance in all organisms with the disease, but should be absent in healthy organisms. (2) The microorganism must be isolated from an organism with the disease and grown in pure cell culture (3) The disease should be caused after introducing the cultured microorganism into a healthy organism. (4) The microorganism causing disease (which is identical to the original microorganism) must be re-isolated from the experimentally diseased organism.

Many species of bacteria prove challenging to culture under standard laboratory condition, especially the fastidious anaerobes and spirochetes implicated in dental disease. This means that it cannot be stated that an organism does not play a role in a particular disease purely based on the lack of culturing a particular organism from clinical cases. In addition, there is marked variation in host-pathogen interaction between individuals and so to imply that an organism is not involved in disease becasue it is found in healthy individuals, is also now known to be incorrect.

It was experimentally shown that *Streptococcus mutans* inoculation could cause caries in gnotobiotic rats [32, 33] and conventional hamsters, [34, 35] showing that *S mutans* causes a transmissible infectious disease. However, Koch's postulates are not fully applicable to dental caries because one of the postulates states that the disease-causing micro-organism must be present in abundance in all subjects affected by the disease, and this micro-organism should not be present in subjects without the disease. Several bacteria have been associated with dental caries, but these bacteria are also often found in subjects without caries. Moreover, although *S. mutans* is still thought to be one of the most important cariogenic (causing dental caries) bacteria in human, caries can develop in its absence.[36-39] This supports the *ecological plaque hypothesis* which states that dental caries is a complex disease and is thought to be caused by a *dysbiosis*, i.e., an imbalance of the resident oral bacteria community following a change in the local environment.[40-43]

Modern molecular microbiology methods which do not rely on bacterial culture have been extensively used in recent years in order to gain a more accurate and detailed overview of the oral microbiome.[1] Wang et al. [44] and many others have noted a marked population shift in the human oral microbiome between orally healthy individuals and those with periodontitis. This finding has been echoed in horses with periodontal disease.[3] It is possible to interpret the substantial shift in the microbial population of in these studies in several ways. The traditional (Koch's) hypothesis of one (or more) pathogenic species being present in high numbers and not being detected in healthy individuals is as noted, overly simplistic. In fact, many complex interactions between host and oral microbiome occur, leading to an apparent dysbiosis or dysregulation of commensal bacteria.

Firstly, local environmental changes within the oral cavity may occur during the development of diseases such as periodontitis including the formation of periodontal pockets, which can be especially deep in the horse.[45] The availability of oxygen to bacteria is greatly reduced in these pockets. This encourages the invasion and proliferation of different subsets of bacteria such as anaerobes, spirochetes and microaerophilic species which are generally not well supported within the healthy oral cavity.[46]

Secondly, the dysbiosis found in oral disease may also be a result of the *keystone pathogen hypothesis* which suggests that certain pathogens, which exist at a low abundance in the healthy oral cavity may under specific conditions be able to modulate their environment through a variety of host-pathogen interactions. These interactions disturb the normally symbiotic relationship between the host and commensal oral bacteria. In man, *Porphyromonas gingvalis*

is a well-studied keystone pathogen which has been recognised to be able to modulates the host's immune response to the entire oral biofilm, thus creating a dysbiosis amongst constituents of a previously stable biofilm.[47]

Innate immunity in the oral cavity

Although the host immune system is ineffective in removing the bacterial biofilm, it has a significant effect on the surrounding host tissues and may stimulate severe inflammation and even cause destruction of the periodontium. Such marked inflammation is well described in brachydont periodontitis and has recently been recorded in equine periodontitis, such as the massive infiltration of inflammatory cells into the equine subgingival connective tissue shown in Figure 8.2. Pathogens encountered by the vertebrate immune system evoke two types of immune response; the innate immune response and the acquired immune response. Acquired immunity includes the production of antibodies specific to encountered pathogens. Innate immunity is less specific but quicker to respond and can be termed the first line of defence against invading microorganisms. Innate immunity is sometimes described as a broad spectrum, non-specific response, whereas it can actually distinguish between some groups of microorganisms.

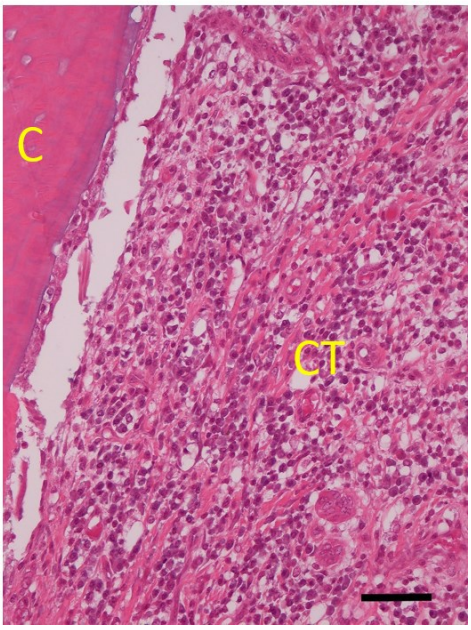

Figure 8.2 Histological image showing massive infiltration of inflammatory cells into the interdental subgingival connective tissue (CT) of a horse with periodontal disease. The separation of connective tissue from cementum (C) is artefactual during histological preparation. (bar =50µm). Image courtesy of Dr A. Cox.

The two main functions of innate immunity are firstly to provide the initial host response to a pathogen by containing and reducing numbers of the invading organism. Secondly, the innate immunity induces a more specific response, i.e. by stimulating the acquired immune response through a number of receptor molecules and activation pathways.[48] Invading pathogens are recognised by pathogen-receptor molecules (PRRs), a family of receptors which respond to highly conserved microbial molecules known as pathogen associated molecular patterns. [49] Specific cell signalling pathways are activated following binding and recognition of these molecules (such as lipopolysaccharides, bacterial DNA or lipoproteins) causing up- or down-regulation of expression of genes that encode cytokines, chemokines and other inflammatory proteins.

Toll-like receptors (TLRs) are a family of PRRs that have been highly effective components of innate immunity throughout evolution and are highly conserved.[49] The involvement of Toll-like receptors in periodontitis and their interaction with periodonto-pathogenic bacteria has been well recognised in other species.[50-53] In brachydont periodontal disease, the recognition of periodontal pathogenic bacteria in gingival and periodontal tissue by host Toll-like receptor initiates cytokine production. This can lead to a destructive inflammatory response thus promoting disease progression. Similarities in the expression of certain TLRs and pro-inflammatory cytokines has recently been noted between those expressed in brachydont and equine periodontitis [3]. The importance of the innate immune system in oral disease has been well recognised in other species, and its interaction with the equine oral microbiome requires further attention.

Culture independent molecular microbiology

During characterisation of any microbial community, the method of analysis has great effects upon the number and diversity of the species detected. Studies of oral microbiota in other species have estimated that around 50% of oral bacteria cannot be cultured by traditional techniques.[54] Culture and biochemical identification of bacteria isolated from the oral cavity has also often proved to be inaccurate in comparison to molecular methods.[55] Consequently, previous, culture-based studies have greatly underestimated both the number and variety of oral bacterial species. Organisms which are notoriously difficult to culture including some anaerobes, are even more likely to be vastly underrepresented when using such conventional methods. As the equine oral cavity is a previously understudied area, it continues to be shown to contain many novel and previously uncharacterised species.

Culture-independent molecular microbiological methods are increasingly used to characterise the equine oral microbiome and investigate microbial population shift between oral health and disease. One such method involves high throughput sequencing of the gene encoding the 16s subunit of the bacterial ribosome (16s rRNA). This technique can be used to assess composition of microbial communities directly from clinical samples and can also identify novel and uncultivable species.[56]

The 16s gene is universal in bacteria and highly conserved between phyla due to its importance in basic cell functions. This gene is an excellent candidate for bacteriological studies because it is not found in mammalian cells and its use has allowed a more complete analysis of the equine oral microbiome[3-5] by identifying cultivable, non-cultivable, including novel bacterial species.

In particular, high throughput 16s rRNA sequencing is particularly useful in disorders where anaerobic organisms are likely to play a role in disease, such as equine periodontitis.

In a study investigating the oral microbiome of 14 donkeys before and 20 days after basic dental treatment, it was shown that the relative abundance of several bacterial taxa before dental treatment differed significantly from 20 days after basic dental treatment at the phylum and genus levels, but there was no statistically significant difference observed in richness and diversity when comparison was made at the two time points.[6]

The equine oral microbiome in oral health

Early culture-based studies by Baker [57] found high counts of *Streptococci* sp., *Micrococci* sp. and starch hydrolysing bacteria in the healthy equine oral cavity. Baker also noted intermediate counts of anaerobic organisms such as *Veillonella* sp. and low counts of *Lactobacilli, Fusobacteria* and coliforms. [57] Since Baker's early work, many other bacterial species have been identified as normal inhabitants of the equine oral cavity and these are summarised in Table 1. Gao et al investigated the healthy oral equine microbiome by using pyrosequencing to analyse gingival sulcus sites. [4] Twelve phyla were identified, with the most prevalent being *Gammaprotebacteria* (28%), *Firmicutes* (27.6%) and *Bacteroidetes* (25.11%). These authors suggested that many similarities exist between equine, human, canine, and feline subgingival microbiota, despite obvious differences in diet and dentition. Many bacteria identified in this study were not closely related to known bacteria and the authors suggest these may represent unique equine specific bacterial taxa.

Gemella and *Actinobacillus* were the two genera most associated with the control group when linear discriminant analysis (LDA) effect size (a data analysis method to analyse microbial populations) was performed at genus or higher level.[3, 5] In the former study, the control group reflected gingival samples of periodontally healthy horses (LDA score>3, $p<0.05$). In the latter study, the control group consisted of samples of the palatal aspect of maxillary cheek teeth without peripheral caries (LDA score>2, $p<0.05$). Gao et al. [4] also found *Actinobacillus* species to be an abundant taxa present in healthy subgingival plaque along with together with an unclassified *Pasteurellaceae*. Additionally, a high abundance of *Moraxella* species was found in one horse. *Moraxella* was also associated with healthy periodontium by Kennedy et al. [3] and with teeth unaffected by PC.

Bacteria identified in the healthy equine oral cavity

Actinobacillus sp. (Gao et al. 2016)	Porphyromonas catoniae
Actinobacillus equuli (Bisgaard et al. 2009;	Porphyromonas circumdentaria
Platt 1973; Sternberg 1998 and 1999 and	Porphyromonas gulae
Sternberg and Brändström, 1999)	Porphyromonas macacae
Actinobacteria (Gao et al. 2016)	Porphyromonas sp. (all from Gao et al. 2016)
Arcobacter sp. (Gao et al. 2016)	Prevotella sp. (Gao et al. 2016)
Atopobium (Gao et al. 2016)	Proprionbacterium sp. (Gao et al. 2016)
Bacteroides (Bailey and Love 1990)	Proteobacteria (Gao et al. 2016)
Bacteroidetes (Gao et al. 2016)	Pyramidobacter (Gao et al. 2016)
Campylobacter gracilus (Gao et al. 2016)	Spirochaetes (Cox et al. 2012; Gao et al. 2016)
Campylobacter sp. (Baker 1979)	SR1 (Gao et al. 2016)
Cardiobacterium sp.	Staphylococcus sp. (dos Santos et al. 2014)
Chloroflexi (Gao et al. 2016)	Streptococcus sp. (Baker 1979; dos Santos et al. 2014; Gao et al. 2016)
Clostridium (Bailey and Love 1990)	Streptococcus minor (Gao et al. 2016)
Eubacterium fossor (Bailey and Love 1986)	Streptococcus suis (Gao et al. 2016)
Firmicutes (Gao et al. 2016)	Suttonella sp. (Gao et al. 2016)
Fretibacterium fastidiosum (Gao et al. 2016)	Synergistetes (Gao et al. 2016)
Fusobacterium necrophorum (Gao et al. 2016)	Tannerella sp. (Sykora et al. 2014; Gao et al. 2016)
Fusobacterium sp. (Baker 1979; Bailey and Love 1990; Gao et al. 2016)	Tannerella forsythia (Sykora et al. 2014; Gao
GN02 (Gao et al. 2016)	et al. 2016)
Lactobacillus sp. (Baker 1979)	Tenericutes (Gao et al. 2016)
Lautropia sp. (Gao et al. 2016)	TM7 (Gao et al. 2016)
Leptotrichia sp.	Treponema sp. (Sykora et al. 2014; Gao et al.
Leptotrichia hongkongensis (Gao et al. 2016)	2016)
Megasphaera (Bailey and Love 1990)	Treponema denticola
Moraxella sp. (dos Santos et al. 2014; Gao et al. 2016)	Treponema medium
Neisseria sp.	Treponema pectinovorum
Neisseria shayganii (Gao et al. 2016)	Trepomena vincentii
Nocardia sp. (dos Santos et al. 2014)	Treponema porcinum
Olsenella sp. (Gao et al. 2016)	Treponema lecithinolyticum (all Sykora et al.2014
Pasteurellaceae (Gao et al. 2016)	Veillonella sp. (Baker 1979; Bailey and Love1990; Gao et al. 2016)
Peptostreptococcus (Bailey and Love 1990)	Veillonella parvula (Gao et al. 2016)

The equine oral microbiome in disease

Bacteria detected in apical infections

Early equine oral microbiological studies were mostly on bacteria involved in equine apical infections. Bacteria identified from such cases include *Peptostreptococcus sp.* [58, 59], *Bacteroides fragilis*, *Bacteroides melaninogenicus, Bacteroides oralis*, *Peptostreptococcus anaerobis*, *Fusobacterium mortiferum* and unidentified *Fusobacterium.*[59] Bienert et al. [60] also detected a predominantly anaerobic bacterial population, with *Prevotella sp.* isolated from 80% and *Fusobacterium* sp. from 75% of apical infection cases. Culture of swabs from extracted apically infected cheek teeth by Kern et al. [61] revealed *Actinobacillus* spp., *Streptococcus* spp., *Actinomyces* spp. and *Staphylococcus* spp. to be the most commonly isolated aerobic organisms whilst *Fusobacterium* spp., *Prevotella* spp. and *Peptostreptococcus* spp. were the most commonly isolated anaerobes. These authors also showed (that similar to brachydont species), horses can develop transient bacteraemia during extraction of apically infected cheek teeth. The organisms isolated from such blood cultures generally correspond to those identified on culture of extracted teeth. [61]

The equine oral microbiome associated with periodontal disease

Although there are some similarities in the aetiopathogenesis of periodontal disease in brachydont and hypsodont dentition, the initiating factors differ. In brachydont species, the inflammatory response within the gingiva is provoked by an accumulation of plaque in the gingival sulcus leading to the first stage of periodontal disease i.e., gingivitis. Production of both pro- and anti-inflammatory cytokines are induced by recognition of microbial proteins by Toll-like receptors. As noted, the inflammatory response to the periodontopathogenic bacteria present in the biofilm may become destructive with time, leading to gingival recession and the development of deep periodontal pockets. The reduced oxygen tension present in these periodontal pockets, promotes the proliferation of anaerobic bacteria which would otherwise be present in very low numbers. These anaerobes further propagate the destructive inflammatory response.

In horses, periodontitis most commonly occurs following trapping and subsequent decomposition of feed material secondary to anatomical defects between adjacent teeth such as diastemata (Figure 8.3) as discussed further in Chapters 10 and 26. Periodontal disease was noted in just 0.9% of 349 horses in absence of concurrent dental disease.[62, 63] Walker et al. [64]

noted that 34% of diastemata had associated gingivitis with 44% having associated periodontal pocketing.

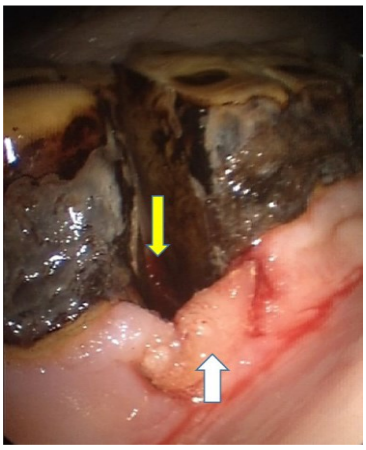

Figure 8.3. Oral endoscopic view of severe equine cheek teeth periodontal disease caused by a diastema. The teeth adjacent to the diastema have caries of the peripheral cementum and are covered in a grey-coloured dental plaque. There is marked loss of periodontal tissues in the interdental space (yellow arrow) and markedly hyperplastic remodelled gingiva (white arrow). Photograph courtesy of P.M. Dixon.

The trapped feed material causes abrasion to the sensitive gingiva and mechanical damage to surrounding tissue in addition to acting as a nidus for bacterial growth, thus provoking the host's inflammatory response to bacteria and decomposing plant material within the periodontal pocket. This destructive local inflammatory response results in further breakdown of periodontal tissue, deeper periodontal pockets and further proliferation of anaerobic organisms. In man, a number of periodontopathogenic bacteria are implicated in induction of this severe inflammatory response, which leads to periodontal ligament destruction and alveolar bone loss. Prolonged cytokine production in the affected periodontal tissues leads to protease production to destroy invading microorganisms but these proteases can also, unfortunately damage host periodontal tissue.[65] In humans this inflammatory response can show individual variation which includes a genetic component[66] The continued cycle of inflammation eventually results in such severe loss of attachment that the tooth itself is lost. A proposed model for the aetiopathogenesis of equine periodontal disease is shown in Figure 8.4.

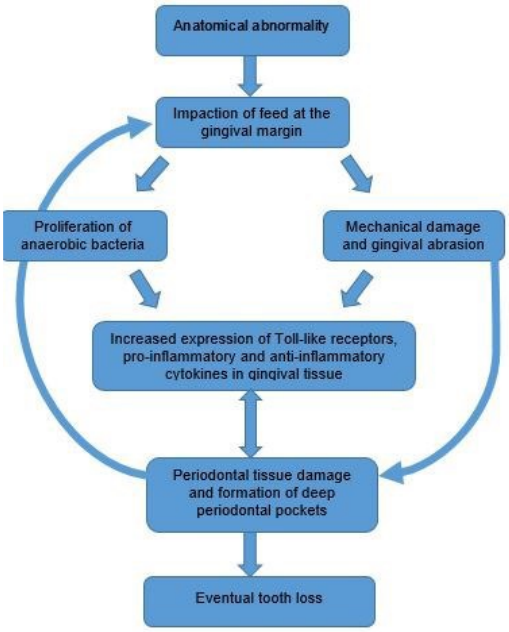

Figure 8.4. Proposed model for the aetiopathogenesis of equine periodontal disease

Histological studies of equine periodontal pockets have shown hyperplastic mucosa, with disruption of the epithelium and the presence of large numbers of inflammatory cells, especially neutrophils infiltrating the lamina propria and adjacent connective tissues. Large numbers of bacteria have been noted alongside feed material and the use of Modified Young's stain had revealed spirochetes to be present within the gingival epithelium in diseased periodontal pockets (Figures 8.5 and 8.6).

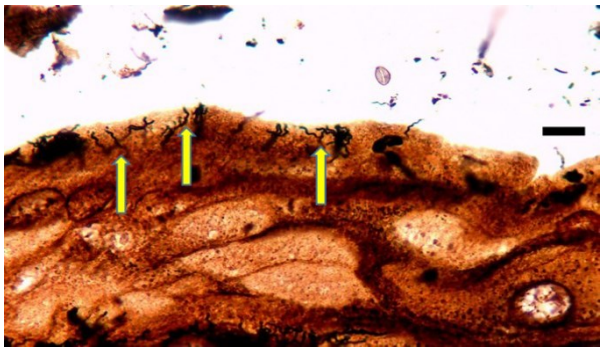

Figure 8.5. Spirochaetal bacteria in gingival epithelium of a diseased equine periodontal pocket. Modified Young's silver stain bar= 10µm. Image courtesy of Alistair Cox

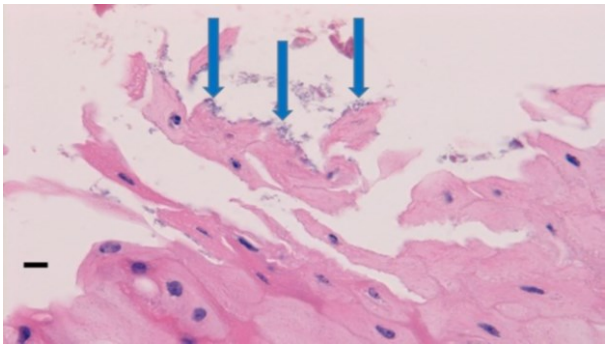

Figure 8.6. Cocci on the gingival epithelial surface of a periodontitis affected horse. Bar= 10µm. Image courtesy of Alistair Cox.

Early culture-based studies of equine periodontal pockets by Baker[57] revealed a significant shift in bacterial population between horses with and without periodontal disease. High counts of *Streptococci* and *Micrococci* were detected in orally healthy samples, with intermediate counts of *Veillonella* sp. and low counts of *Lactobacillus* sp., *Fusobacteria* sp. and coliforms also found. In horses with periodontal disease, *Streptococcus*, *Fusobacterium* and coliforms were the predominant genera present. *Campylobacter* sp. and spirochetes were also detected from direct smears from periodontal pockets. *Porphyromonas gingvalis*, *Tannerella sp*. and *Treponema sp.* ('red-complex' pathogens) have long been implicated in human periodontal disease. They have also been isolated more frequently in gingival crevicular fluid samples from

horses with clinical Equine Odontoclastic Tooth Resorption and Hypercementosis (EOTRH) than from control horses.[67]

To further study the microbiota involved in equine periodontitis, DNA extraction was performed on subgingival plaque samples from 24 horses with periodontal disease and gingival swabs from 24 orally healthy horses) and bacterial 16srRNA gene sequencing was performed on these samples.[3] In total, 1308 operational taxonomic units (OTUs), (distinct organisms grouped by DNA sequence) were identified from all samples. Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify population differences (at the genus level or higher) between the two sample groups. Bacterial populations in healthy horses were clustered together and showed lower variability in comparison to those from horses with periodontitis when principal component analysis was performed. In addition, samples from healthy horses were significantly less diverse (161 OTUs; SD 116, range 64-568) than samples from horses with periodontitis (252 OTUs; SD 81 range 85-380.

From 1308 OTUs, 266 were significantly different between the healthy and periodontitis groups. In addition, at genus level 107 out of 356 identified genera were significantly different between the diseased and healthy groups (Figure 8.7 and 8.8). The presence of *Gemella* spp. and *Actinobacillus* spp. was most discriminative of all genera for equine oral health indicating that these genera comprise part of the normal equine oral flora. In humans, *Gemella* species comprise a high proportion of the normal microbiota of the dorsal surface of the tongue.[68] The presence of *Prevotella* spp. and *Veillonella* spp. were most discriminative for periodontitis with the abundance of these genera statistically significantly higher in samples from abnormal periodontal pockets than from orally healthy samples. *Prevotella intermedia* and *Prevotella melaninogenica* have also been implicated in human periodontal disease.[69]

Several species of *Veillonella* have been isolated from both periodontitis affected and healthy humans, however *Veillonella parvula* has been associated with chronic human periodontitis.[70] *Prevotella intermedia*, *Prevotella nigrescens* and *V. parvula* have all been shown to stimulate cytokine production via the Toll-like receptor 2 and *V. Parvula* is also recognised by TLR 4.[51] This may be of potential importance as dysbiosis followed by stimulation of innate immunity by periodontopathogenic bacteria. Subsequent cytokine and chemokine production plays a central role in the aetiopathogenesis of human periodontitis.

Whole genome sequencing of plaque samples from five horses with periodontitis and gingival swabs from three orally healthy horses (identified bacteria belonging to 63 phyla, 136 classes,

257 orders, 414 families, 757 genera and 2001 species. A total of 75 species of *Prevotella* were detected as well as several known human periodontopathogenic bacteria including significantly higher levels in periodontitis samples for *Peptostreptococcus anaerobius*, *Prevotella bivia*, *Prevotella dentalis*, *Prevotella denticola*, *Prevotella intermedia*, *Prevotella melaninogenica* , and *Prevotella nigrescens* . Again, samples taken from horses with periodontitis were shown to be more diverse than orally healthy samples. However, this whole genome pilot study contained low sample numbers and a much larger study would help build a more complete picture.

Then identification of a predominance of anaerobic organisms such as *Prevotella* spp. and *Veillonella* spp. (including human putative periodontal pathogens) in equine periodontal pockets in comparison to a predominance of aerobic organisms such as *Gemella* at healthy equine gingival margins demonstrates the significant difference in microbial populations between periodontal health and disease. Some of the species detected in this study are known to be periodontal pathogens in man. However, it is more likely in the horse, that these anaerobes simply flourish in the environment created by an anatomical abnormality such as diastemata or displacement of cheek teeth with subsequent feed impaction and decomposition. This environment allows a local dysbiosis to occur, promoting growth of potential equine periodontal pathogens which subsequently provide additional stimulation of the innate immune system.

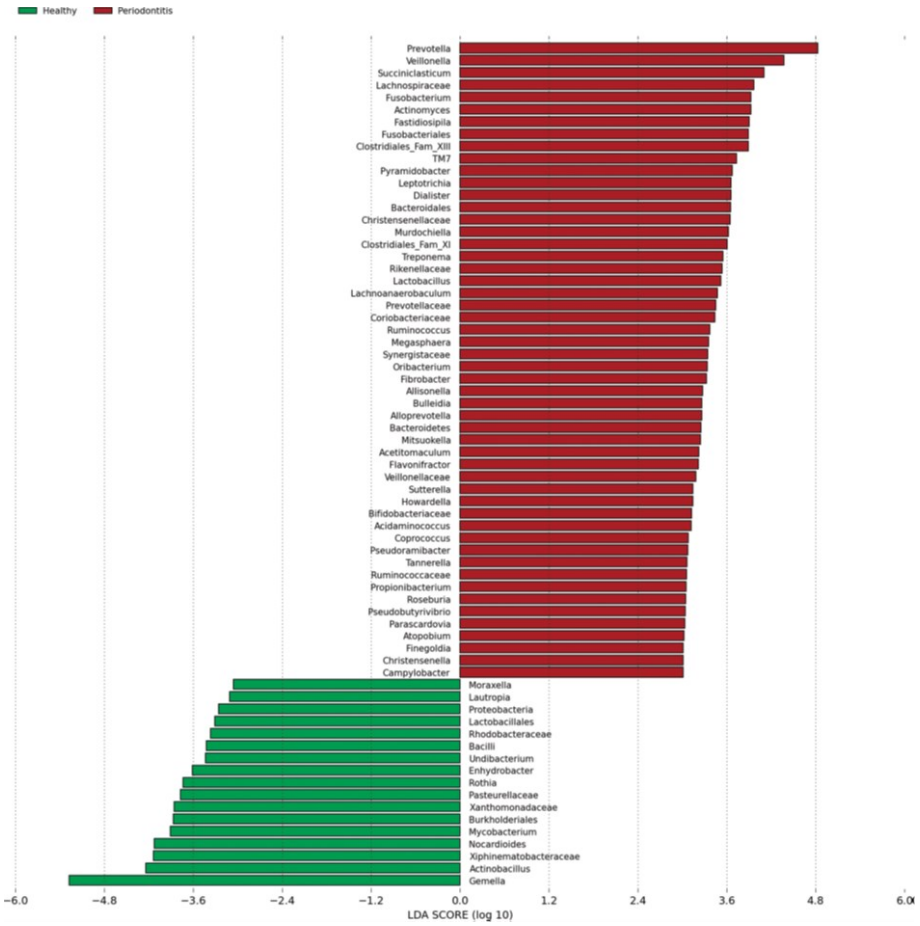

Figure 8.7. Visualisation of most significant taxa (genus or higher level) that differentiate between health and periodontal disease in equine oral microbiomes.[3]

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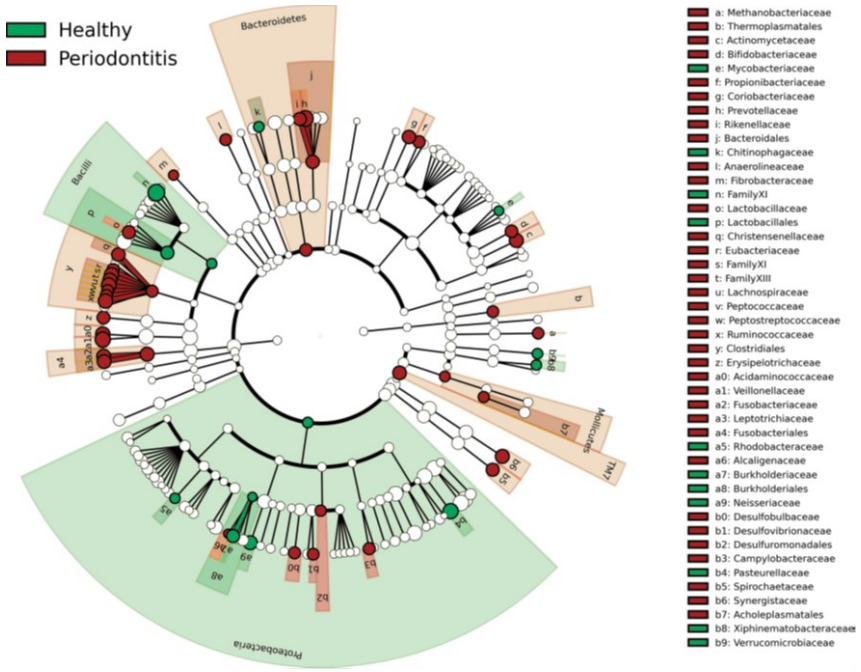

Figure 8.8. Taxonomic representation of statistically significant differences between the healthy and periodontitis samples at family or higher taxonomic level. Differences are represented in colour (shades of red periodontal disease, green health).[3]

The equine oral microbiome associated with peripheral caries

The prerequisites for dental caries to develop are: tooth, substrate (monosaccharides, disaccharides or other fermentable carbohydrates), plaque and bacteria.[35] Acidogenic oral microorganisms which convert fermentable carbohydrates to acids cause dental caries, by causing a demineralisation and disintegration of the inorganic and organic substances of the tooth, respectively.[18] Factors influencing the characteristics of the plaque microbiota include (1) oxygen concentration, (2) nutrient availability and (3) pH.[71] Because of close cell-to-cell contact there is a synergism between oxygen-consuming and oxygen-sensitive bacteria.[72] Most (72%) of the bacteria associated with human dental caries are facultative anaerobes and 28% are obligate anaerobes.[73] Because the environment on the tooth surface beneath plaque is largely anaerobic, the subsequent anaerobic metabolism of carbohydrates by plaque bacteria will preferentially produce acids.[3] More nutrients become available when bacteria collaborate and

thus benefit by sharing each other's enzymes for the catabolism of certain molecules.[3] Cariogenic and acidogenic bacteria can also be present in a healthy oral bacterial community at neutral pH, but only in small numbers as these bacteria are not competitive in non-acidic environments.[72] Frequent access to dietary fermentable carbohydrates or a decreased clearance of ingested carbohydrates by saliva (due to a lower saliva secretion rate), leads to more acid being produced with subsequent demineralisation of dental substance.[40, 72] A low pH is beneficial for the growth of acidogenic and aciduric bacteria, thus enhancing their acidifying effect and predisposing the site of the tooth beneath the plaque to develop caries.[71]

As noted, two variants of equine dental caries are recognised: equine dental peripheral caries (PC), i.e. caries at the peripheral sites of the teeth, and infundibular caries (IC), i.e. caries of the infundibulum of maxillary cheek teeth.

Using histological and ultrastructural techniques (Figures 8.9 - 8.11), dental plaque including a complex network of micro-organisms of different shapes and sizes was observed in PC lesions.[74] In order to assess for differences in microbiota between PC and a control group (no PC), and to identify which bacteria are associated with PC versus the control group, bacterial DNA was isolated from dental plaque samples on the palatal aspects of maxillary cheek teeth. PCR and high-throughput sequencing (Next Generation Sequencing) were performed on these samples, targeting the V4 region of the 16S rRNA gene.[5] Because PC is more common in the caudal cheek teeth (Triadan 09-11) compared to the rostral teeth (Triadan 06-08) [75-79], differences in microbiota between these sites were also assessed.

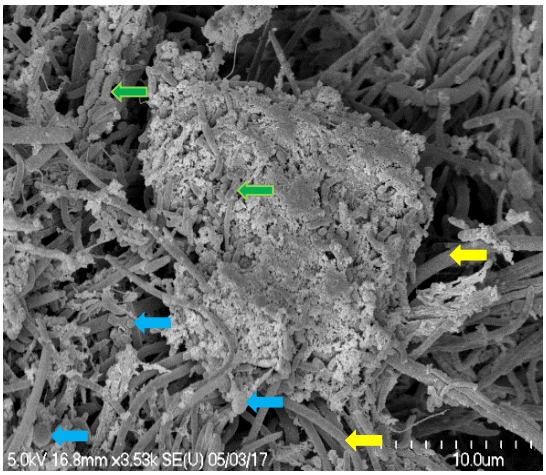

Figure 8.9. Scanning electron microscopy (SEM) image of an undecalcified peripheral section of the palatal aspect of a maxillary cheek tooth (110) with peripheral caries lesions (grade 1.1) in its lines of arrested growth that are covered by a layer of dental plaque. Pictured is the dental plaque with its network of micro-organisms of different shapes and sizes. Yellow arrows = filamentous micro-organisms; blue arrows = large and small cocci; green arrows = bacilli (rods) [74] (With permission of Editor of Equine Veterinary Journal)

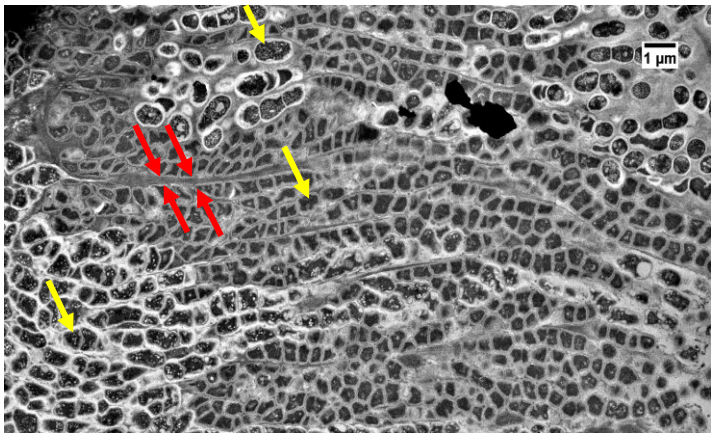

Figure 8.10. Transmission electron microscopy (TEM) image of the (lingual) peripheral aspect of a mandibular cheek tooth with grade 1.1 peripheral caries lesion covered with a thick layer of supragingival dental plaque that contains micro-organisms. These micro-organisms are tightly arranged in rows (yellow arrows) with layers of fibrillar material (red arrows) present between them. [74] (With permission of Editor of Equine Veterinary Journal)

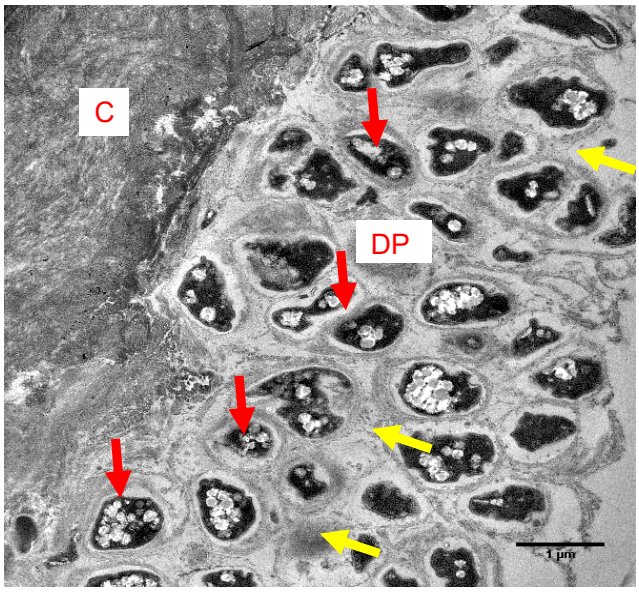

Figure 8.11. TEM image of peripheral cementum (C) with grade 1.1 peripheral caries lesion at the lingual aspect of a mandibular cheek tooth (406) that is covered by a layer of dental plaque (DP) containing micro-organisms (red arrow) embedded in a honeycomb of fibrillar matrix (yellow arrows).[74]

There was no statistically significant difference between the microbial profiles of the PC and control groups (p = 0.371, F = 1.0482, PERMANOVA). The dissimilarity percentage found between the PC and control group was 57%.

Firmicutes, Actinobacteria, Proteobacteria, Bacteriodetes and *Fusobacteria* were the most common phyla (>1%) present in supragingival plaque in both PC and control groups. The

relative abundance of *Firmicutes* in the PC group was higher than in the control group. In contrast, the relative abundance of *Proteobacteria* was lower in the PC than in the control group. From 161 taxa at the family or higher level, 29 taxa were found to be significantly different between the PC (n=10) and the control group (n=23) using LEfSe (Figure 8.12).

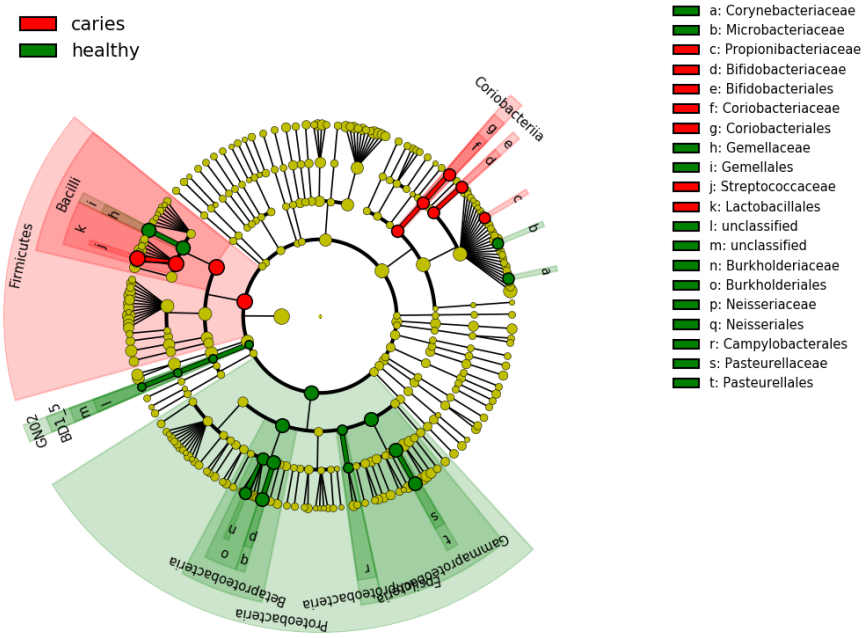

Figure 8.12. This cladogram depicts the results of linear discriminant analysis effect size (LEfSe) at the family or higher level, showing which taxa are statistically significantly more associated with the PC or control group (LDA score>2, $p<0.05$). The size of each circle is proportional to the abundance of the taxon it represents.[5]

Using LEfSe, 51 of the 303 taxa identified at the genus or higher level were discriminant taxa between the PC and control group (Figure 8.13). More taxa were associated with the control group (37 taxa) than with the PC group (14 taxa). As mentioned above, *Gemella* and

Actinobacillus were the genera most associated with the control group. *Streptococcus*, *Olsenella* and *Scardovia* were the genera most associated with the PC group. If LEfSe was performed at genus level only, an additional genus *Mitsuokella* was shown to be associated with the PC group.

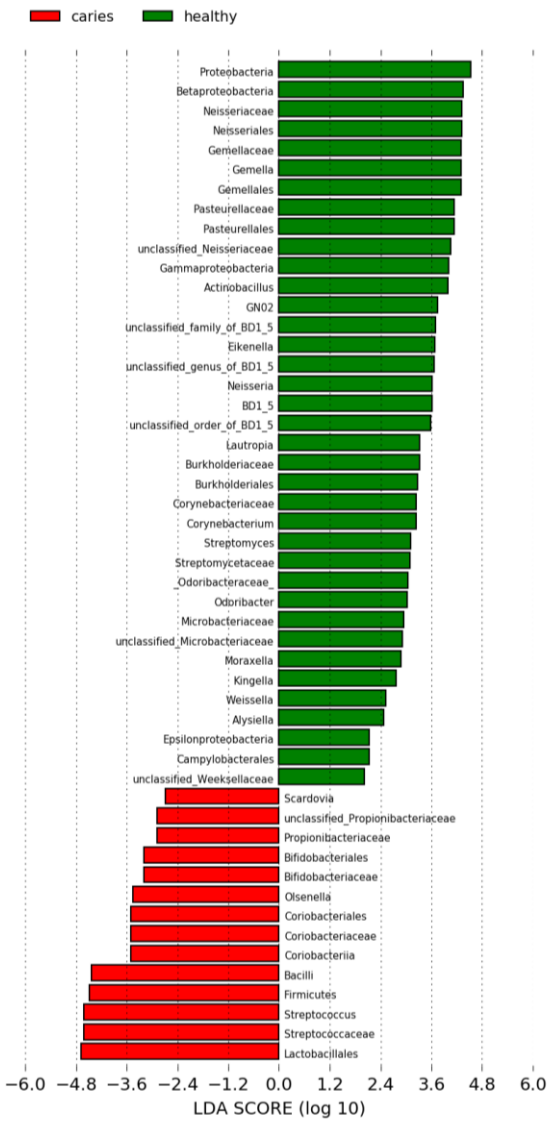

Figure 8.13. Results of linear discriminant analysis effect size (LEfSe) at genus or higher level, showing which taxa are statistically significantly more associated with the PC or control group (LDA score>2, $p<0.05$).[5]

A comparison of bacterial composition of rostral and caudal maxillary in cheek teeth peripheral caries and control group was performed by Borkent et al in 38 horses |[5].Forty-two of the 262 taxa identified at the genus or higher level were discriminant taxa between the rostral cheek teeth PC group, caudal cheek teeth PC group and control group (LDA score >2, p-value<0.05). Most taxa were associated with the control group (29 taxa), only three taxa were associated with rostral cheek teeth PC and ten taxa with caudal cheek teeth PC. [5]

Bacterial genera most associated with each group were *Veillonella* for the rostral cheek teeth PC group, *Streptococcus* for the caudal cheek teeth PC group and *Corynebacterium* for the control group. The bacterial genera most associated with equine PC (*Streptococcus*, *Olsenella*, *Veillonella*) also have been linked to human dental caries. Some features of these bacteria are displayed in table 4.2.

Streptococcus species (mainly members of the group of *mutans Streptococci,* especially *S. mutans*) are still considered to be the most important cariogenic bacteria in humans and are usually the initiators of caries. Members of the mutans Streptococci group include *S. mutans*, *S. sobrinus*, *S. criceti*, *S. ferus*, *S. ratti*, *S. macacae, S. downei* and *S. devriesei*. These bacteria can produce extracellular sticky glucans which allows them to adhere to the tooth surface. Additionally, they can produce intracellular bacterial polysaccharides if there is an excess of dietary sugar, which can be converted to acidic end-products, even when dietary sugar is unavailable in the oral cavity.[41, 80, 81]—*Olsenella* spp, such as *O. profusa* are associated with human caries affecting dentine [82, 83], as well as root (i.e. cemental) caries.[84]

Veillonella has been highly correlated with total acid-producing species in human dental caries.[38, 39] Because *Veillonella* can convert lactic acid into weaker (acetic and propionic) acids, actually causing the dental plaque pH to rise [85, 86], this bacterium has been described as an *acid sink*. This feature allows other fermentative bacteria to survive and remain metabolically active. Some acidogenic *Streptococcus* and *Granulicatella* bacteria possess the L-lactate-dehydrogenase gene, which may also enable these bacteria to cause a reduction of the acidity of their environment.[86, 87]

The equine oral microbiome associated with infundibular caries

Cultures of infundibular hypoplasia/caries lesions by Baker[57] were unrewarding with only a few *Streptococci* or *Micrococci* identified or, alternatively, bacterial overgrowth on the plates,

despite the preparation of serial dilutions. Lundström et al. [88] isolated *Streptococcus devriesei* from infundibular caries lesions of 50 teeth and also (in lower numbers) from 4 control teeth.

Next generation sequencing (NGS) of eight infundibular caries dental plaque samples and seven control samples which were collected from maxillary cheek teeth infundibula was performed by Borkent [89]. From 205 taxa identified at the genus or higher level, 15 discriminant taxa were found between the IC and control group using LEfSe.One taxon was associated with IC (*Acidaminococcus*) and 14 taxa with the control group (*Actinomycetales, Actinobacteria, Actinomycetaceae, Bacillus, Campylobacter*, unclassified family of *Bacteroidales,* unclassified genus of *Bacteroidales, Tissierellaceae, Parvimonas, Peptostreptococcus, Peptostreptococcaceae, Campylobacteraceae, Campylobacterales, Epsilonproteobacteria*). The genus most associated with IC using LEfSe at genus or higher level was *Acidaminococcus*, while *Bacillus* was the genus most associated with the IC study control group (no IC).

Table 8.2 Characteristics and features of bacterial genera which were most associated with equine peripheral caries (*) and infundibular caries (**)

Bacteria	Characteristics	Features
*Streptococcus**	facultative anaerobic, Gram positive coccus	The mutans group: highly acidogenic (lactic acid) and aciduric, can produce intracellular polysaccharides and can metabolise sucrose to form extracellular polysaccharides
*Olsenella**	small, Gram positive rod-shaped micro-aerotolerant (moderately obligate) anaerobic bacterium	Can ferment carbohydrates, predominantly to lactic acid but also to formic and acetic acids
*Veillonella**	small, Gram negative, anaerobic coccus	Is asaccharolytic and can convert lactic acid into weaker (acetic and propionic) acids

*Acidaminococcus***	Gram negative, anaerobic coccus	Utilises amino acids (mainly glutamic acid) for its growth, degrading them to acetate and butyrate; Can use citrate as a source of energy, producing hydrogen and hydrogen sulphide as metabolites

Conclusions

The bacterial genera found to be most associated with equine dental caries and equine periodontal disease using molecular microbiological techniques have also been found in humans affected by these diseases.[36-38, 41, 69, 70, 80-84] Although some bacteria were more associated with healthy horses and some more associated with disease, equine periodontal disease and dental caries (peripheral or infundibular) could not simply be ascribed to certain bacteria which were only present in disease but not in health. Similar to human caries, these diseases more likely reflect a dysbiosis which could be initiated by an environmental change that disturbed the previously existing balance between the different micro-organisms and tilted the balance towards a disease-producing microbial community.

Despite multiple recent molecular microbiological studies of both health and disease equine oral cavity, it is clear that researchers have only scratched the surface of this complex area. This is a field of equine dentistry that requires much additional research and investigation in order to fully characterise the equine oral microbiome in health and disease and to more fully interpret the role of complex host-pathogen interactions in the diseased equine oral cavity. Such studies will lead to a better understanding of oral disease in the horse, and hopefully aid development of novel therapeutics and promote equine health.

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