Microbiological and immunological aspects of equine periodontal disease

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Abstract

Periodontal disease is a common and painful condition in the horse. Although awareness of the condition is growing amongst the veterinary profession and horse owners, the presence of the disease is often overlooked and treatment can be difficult. Despite this, there have been few recent studies of the aetiopathogenesis of the condition. Certain species of bacteria may act as periodontal pathogens, stimulating a destructive inflammatory response in periodontal tissues and this has been well recognised as being important to the aetiopathogenesis of the disease in man. However few equine studies on this aspect of the disease have been carried out. The main aims of this study were: - 1) to identify the bacteria associated with a healthy oral cavity and periodontitis in horses using culture dependent and independent methods; 2) to assess the differences in bacterial populations between the healthy and periodontitis groups and identify putative pathogens; 3) to quantify the expression patterns of TLRs 2, 4 and 9, the pro-inflammatory cytokines IL-1ß and TNFα, anti-inflammatory cytokine IL-10 and Th1/Th2/Th17 cytokines IL-4, IL-6/ IL-12, IFNɣ/ IL-17, within gingival tissue from each sample group; 4) to use matched data to establish if associations exist between the presence and quantity of bacterial species present and TLR expression and 5) to determine activation of TLRs 2, 4 and 9 by putative pathogens using specific *in- vitro* TLR assays.

Swabs were taken from the gingival sulcus of 42 orally healthy horses and plaque samples were taken from the periodontal pockets of 61 horses with periodontal disease. The location and grade of the lesion was noted and an equine dental chart completed for each case. Bacteria were identified using high throughput 16S rRNA gene sequencing, QPCR, whole genome sequencing and conventional culture followed by 16S gene sequencing. Gingival biopsies were taken from 13 orally healthy horses and 20 horses with periodontitis and gene expression of TLR 2, TLR 4, TLR 9, IL-1ß, IL-4, IL-6, IL-10, IL-12, IL-17, TNFα and IFNɣ was measured. THP-1X Blue, MyD88 THP-1X Blue, HEK hTLR 2 Blue and HEK hTLR 4 Blue human cell lines were co-cultured with putative periodontal pathogens and their response measured via level of secreted embryonic alkaline phosphatase. Clinical, microbiological and immunological data underwent cross-matching analysis.

Microbial populations showed 89% dissimilarly between oral health and periodontitis with a less diverse population present in diseased equine periodontal pockets. The most discriminative bacteria between health and disease identified at genus level were *Fusobacteria* and *Acinetobacter* in health and *Pseudomonas* and *Prevotella* in periodontitis. The most abundant genera were *Gemella* (36.5%), *Pseudomonas* (14%) and *Acinetobacter* (8%) in orally healthy samples and *Pseudomonas* (25%), *Prevotella* (14%) and *Acinetobacter* (9.4%) in periodontitis samples. Whole genome sequencing revealed the presence of 75 species of *Prevotella* in the equine oral cavity and a significantly higher number of reads corresponding to *Prevotella bivia*, *Prevotella dentalis*, *Prevotella denticola*, *Prevotella intermedia*, *Prevotella melaninogenica*, *Prevotella nigrescens* were noted in diseased samples. Significant increases in expression of TLR 4 mRNA, TLR 9 mRNA and, in particular TLR 2, mRNA were noted in diseased equine gingival tissue in addition to increased pro-inflammatory and anti-inflammatory cytokine mRNA expression. Presence of *P. intermedia* was significantly positively correlated with expression of TLR 2 in equine periodontitis. In addition, the presence of *Aggregatibacter actinomycetemcomitans* was positively associated with disease severity and expression of TLR 4 mRNA in the horse. Co-culture of periodontal pathogens with human cell lines revealed that the innate immune response to the presence of these bacteria is mainly mediated through TLR 2 activation.

The use of both culture dependent and culture independent methods to investigate the equine oral microbiome has provided significant breadth and depth of information on the microbiology of equine periodontal disease. Microbial populations are significantly different as expected and bacteria belonging to the *Prevotella* genus have been strongly implicated in the aetiopathogenesis of the condition. The innate immune response produced in periodontally diseased equine gingival tissue has been characterised for the first time in the horse.

Table of Contents

List of tables

List of figures

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Declaration

This thesis is the original work of the author, except where explicit reference is made to the contribution of others. The thesis or parts from it have not been submitted for any other degree at the University of Glasgow or any other institution.

Abbreviations

Abbreviation	Meaning
ACTA	Academisch Centrum Tandheelkunde Amsterdam
ANOVA	analysis of variance
APC	antigen presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B-cell activating factor
BEVA	british equine veterinary association
BLAST	basic local alignment search tool
BMP	bone morphogenetic proteins
BRAK	breast and Kidney Expressed Chemokine
C	Canine
CBA	columbia blood agar
CD14	cluster of differentiation antigen 14
Cdna	complementary deoxyribonucleic acid
CFU	colony forming units
CLR	C-type lectin receptor
CpG	5'—C—phosphate—G—3
Ct	critical threshold
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
ENA	extractable nuclear antigens
EOTRH	equine odontoclastic tooth resorption and hypercementosis
FAA	fastidious anaerobe agar
FAB	fastidious anaerobic broth
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte colony stimulation factor
GDF	growth differentiation factor
GM-CSF	granulocyte macrophage colony stimulation factor
GRO	growth-related oncogene
H	Horse
HT	high throughput
I	Incisor
IFN	Interferon
IL	interleukin
IP-10	interferon gamma-induced protein 10
IRAK	interleukin 1 associated kinases
LDA	linear discriminate analysis
LEfSe	linear discriminate analysis effect size
LGP2	laboratory of genetics and physiology 2 receptor
LIF	leukaemia inhibitory factor
LPS	Lipopolysaccharide
LTA	lipotechioic acid
M	Molar
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony stimulation factor
MDA5	melanoma differentiation-associated protein 5 receptor

MDC	macrophage derived cytokine
MEC	mucosa-associated epithelial chemokine
MHC	major histocompatibility complex
MIG	membrane immunoglobulin
MINCLE	macrophage inducible Ca^{2+}-dependent lectin
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response protein 88
NAP	neutrophil-activating protein
NF-κb	nuclear factor kappa B
NK	natural killer cell
NLR	NOD-like receptors
NO	nitrous oxide
NOD	nucleotide-binding oligomerization domain-containing protein receptor
OSM	oncostatin M
OTU	operational taxonomic unit
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PCA	principle component analysis
PCoA	principle co-ordinate analysis
PCR	polymerase chain reaction
PF	platelet factor
PM	Premolar
PRR	pattern recognition receptor
QPCR	quantitative polymerase chain reaction
RANKL	receptor activator nuclear factor-kappaB ligand
RIG	retinoic acid acid inducible gene
RLR	RIG-1 -like receptor
RNA	ribonucleic acid
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
RSV	respiratory syncytial virus
RT PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SEAP	secreted embryonic alkaline phosphatase
TAK1	transforming growth factor-beta activated kinase
TB	Thoroughbred
TECK	thymus expressed chemokine
TGF	transforming growth factor
Th	T helper
TIRAP	TIR domain containing adaptor protein
TLR	toll-like receptor
TNF	tissue necrosis factor
TRAF9	TNF receptor-associated factor 9
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TWEAK	TNF-related weak inducer of apoptosis

UPGMA	unweighted pair group method with arithmetic mean
WB	Warmblood
WGS	whole genome sequencing
WNV	west Nile virus

Chapter 1 Introduction

Periodontal disease is increasingly recognised as a common and painful equine oral disorder. The earliest stage of the condition is inflammation of the gingival tissue (gingivitis) which with time progresses to inflammation of deeper periodontal tissues (periodontitis). With progression of this disease, the other periodontal tissues surrounding and supporting the tooth including the periodontal ligament and adjacent peripheral cementum and alveolus are progressively destroyed until eventually the tooth itself is lost. The earliest recorded observations of equine periodontal disease by Aristotle date back to 333BC (Carmalt 2007). In the early 1900s, several historical reports described its clinical features and high prevalence, especially in urban horses (Colyer 1906; Little 1913; Harvey 1920). Colyer (1906) also acknowledged its substantial welfare impact when describing periodontal disease as 'the scourge of the horse.' More recent studies have shown periodontitis in up to 75% of horses (Baker 1970; Ireland *et al*. 2012a) with its prevalence increasing with advancing age. This disorder is very painful, causing quidding (dropping of feed during mastication) and weight loss (Dixon *et al*. 2008a, 2014), but these clinical signs may be subtle or even absent in some horses and so the disease can go unnoticed for some time (Dixon *et al*. 2008a). There has been increased recognition of the importance of equine periodontal disease in recent years, however very few scientific papers on the aetiopathogenesis of this disorder have been published.

Bacteria have been shown to be the causative agents in feline, canine and human periodontal disease and so it is highly likely they play a crucial role in the pathogenesis of the equine condition. Involvement of bacteria in equine periodontal disease was recently acknowledged (Cox *et al*. 2012; Sykora *et al*. 2014). However, our current understanding of the equine oral microbiome is limited and little is known about the role bacteria play in equine periodontitis (Cox *et al*. 2012). Studies in other species have estimated that around 70% of oral bacteria cannot be cultured by conventional means (Dewhirst et al. 2010) and thus it is certain that the number and variety of bacterial species present in the oral microbiome has been greatly underestimated to date.

Bacteria present in the oral cavity, particularly those involved in the dental biofilm, are capable of stimulating an inflammatory response in periodontal tissues. This response can be destructive and can eventually result in degradation of periodontal tissue and loss of alveolar bone and cementum as seen in human (Graves and Cochran 2003) and more recently equine (Cox *et al*. 2012) periodontitis cases. Loss of support around the tooth results in deep periodontal pockets which support growth of anaerobes and Gram-negative spirochaetes, further stimulating an inflammatory response and worsening the condition. The interaction between oral bacteria and the innate immune system is well recognised to play a pivotal role in the aetiopathogenesis of periodontitis in other species, however this aspect of equine periodontal disease has not previously been investigated.

With few studies investigating the complex aetiopathogenesis of equine periodontal disease there is a real need to investigate the role of bacteria in the oral cavity of the horse in both periodontal health and disease; the immune response in equine periodontitis and, crucially, the interaction between them. In order to achieve these aims, the current study will employ a variety of microbiological and immunological techniques. Conventional culture and modern molecular microbiological methods will be used to characterise the oral microbiome of the horse in both oral health and periodontitis hopefully allowing putative pathogens to be identified. Combining bacteriological methods will allow detection of cultivable, and highly fastidious bacteria. Innate immunity in equine oral health and periodontitis will be investigated by determining expression of genes encoding Toll-like receptors and cytokines in gingival tissue and the ability of putative pathogens to stimulate an immune response will be assessed using cell-based assays. By investigating these aspects novel information will be gained on the aetiopathogenesis of periodontitis in the horse.

1.1 Equine dental anatomy and physiology

1.1.1 The Equine oral cavity

1.1.1.1 Equine Dentition

Equine dentition consists of incisor (I), canine (C) premolar (PM) and molar (M) teeth and formulae for both deciduous and permanent dentition are shown below. In mares, canine teeth may be rudimentary or absent. First premolars, known as 'wolf teeth' are often absent, especially in the mandible.

- Deciduous equine dentition - I_D 3/3, C_D 0/0, PM_D 3/3 = 24

- Permanent equine dentition - I 3/3, C1/1 or 0/0, PM 3/3 or 4/4, M3/3 = 36 to 44 teeth

The Triadan System of dental nomenclature (Floyd 1991) has been used throughout this thesis to identify each tooth. The anatomy of the equine oral cavity is shown in Fig 1.1.

1.1.1.2 Equine dental evolution

Unlike humans, cats, dogs and other brachydont species, the horse possesses hypsodont dentition teeth ('high crowned' with short roots) as a result of millions of years of complex environmental pressures. Early ancestors of the modern equine such as *Hyracotherium* were browsing animals with brachydont dentition; however, as the environment changed with spread of grasslands they evolved to become mostly grazers (MacFadden 2011), spending a large portion of the day masticating grassland high in abrasive silicates, causing a high degree of dental attrition. In order to cope with this and maximise the life of the tooth, the ancestors of today's horse (*Equus caballus*) developed molarisation of the premolars (excluding premolar 1) which are morphologically identical to the molars in the modern horse, forming a tight row of six 'cheek teeth' (Dixon and du Toit 2011) as well as hypsodont dentition. Hypsodont teeth normally erupt continuously throughout the life of the horse at a rate of around 2-3mm per year which is similar to the rate of attrition on the occlusal surface of teeth of a pasture fed horse (Dixon and du Toit 2011). Rates of attrition may vary due to differences in diet, abnormalities of anatomy or presence of oral pathology. As

the equine tooth must continuously erupt to compensate for dietary attrition, it is essential for the equine periodontium to constantly remodel in order to support and erupt the tooth effectively and it is this prolonged eruption which means that mild periodontitis is not irreversible in the horse (Warhonowicz *et al*. 2006).

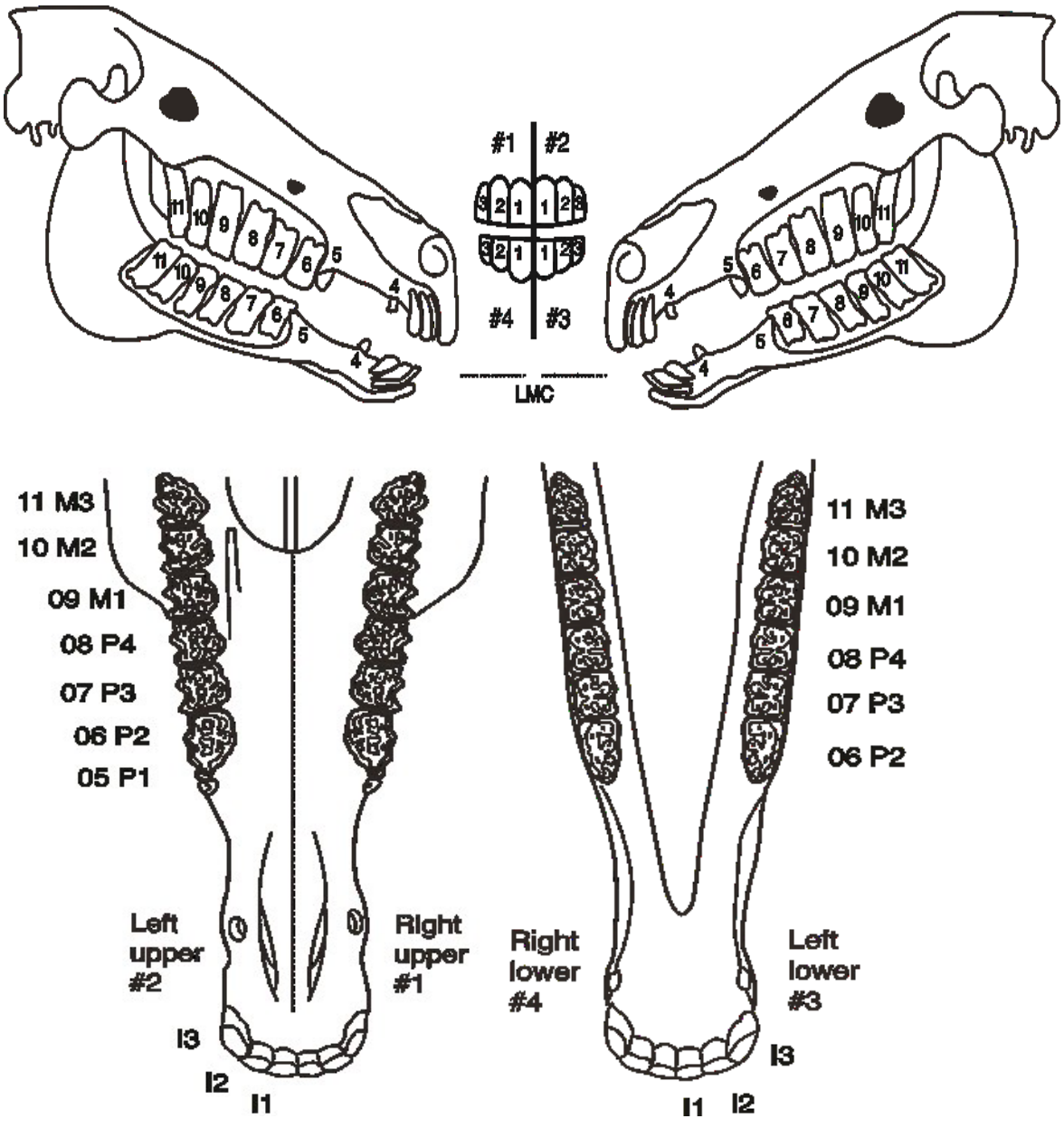

Figure 1.1 Equine dental anatomy.

Adapted from the British Equine Veterinary Association Dental chart (2015).

I, incisor; P, premolar; M, molar; LMC, lateral molar contact

1.1.1.3 Equine dental anatomy

Equine teeth are composed of layers of cement, enamel and dentine, surrounding a vital pulp or pulps (Fig 1.2, 1.3, 1.4), and the arrangement of these layers differs from that of brachydont teeth due to adaptations required to cope with continuous attrition of the occlusal surface and the need for continuous eruption and longevity of equine teeth.

In the horse a large proportion of the anatomical crown lies below the gingiva in the alveolus and is known as the reserve crown, while the erupted crown is also termed the clinical crown. Hypsodont teeth have no true roots (i.e. enamel-free zone) when they initially erupt, and this area, at the very base of the tooth may be better described as the periapical region. After a few years true roots develop and continue elongating for the life of the tooth. Equine teeth taper towards their apices which along with loss of cheek teeth angulation with age can cause formation of senile diastemata with associated periodontitis in older horses (Dixon *et al*. 2008a).

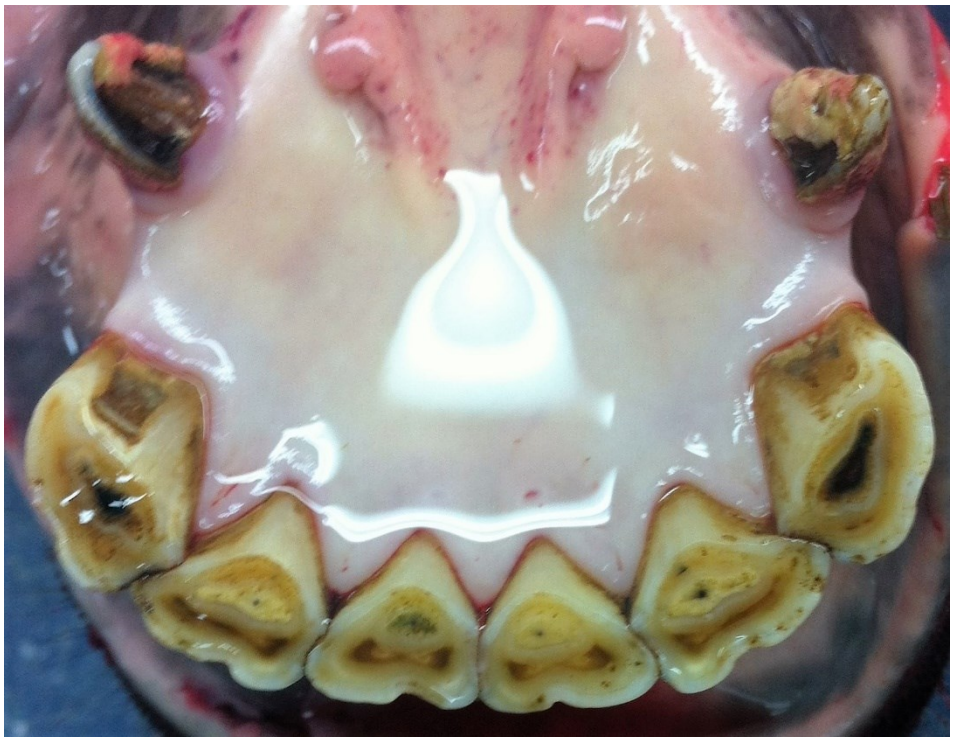

Figure 1.2 Equine mandibular incisors and canines

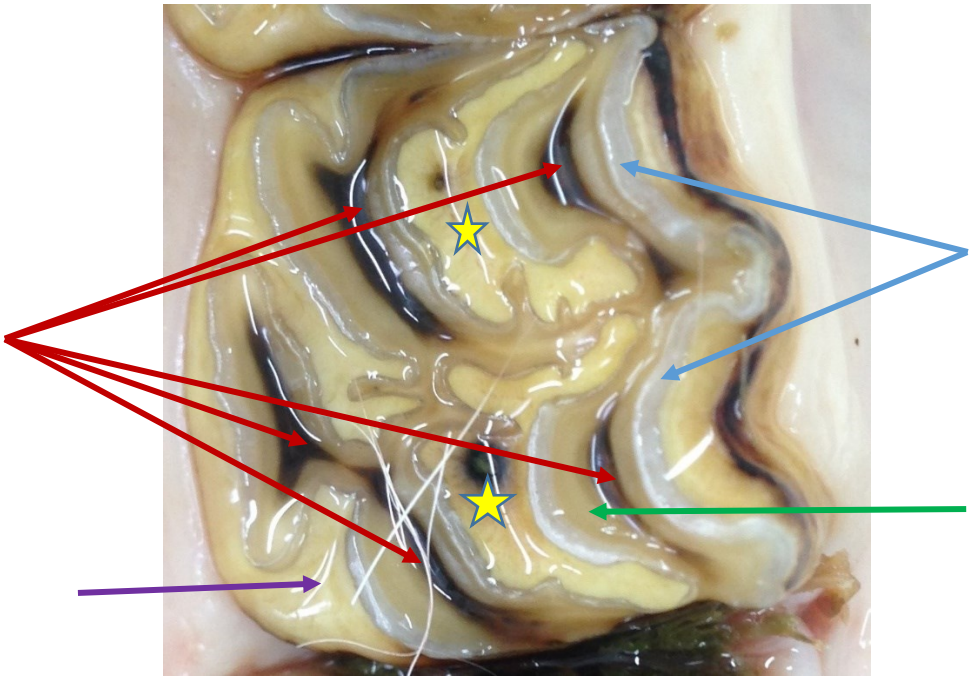

Figure 1.3 Maxillary equine cheek tooth 209

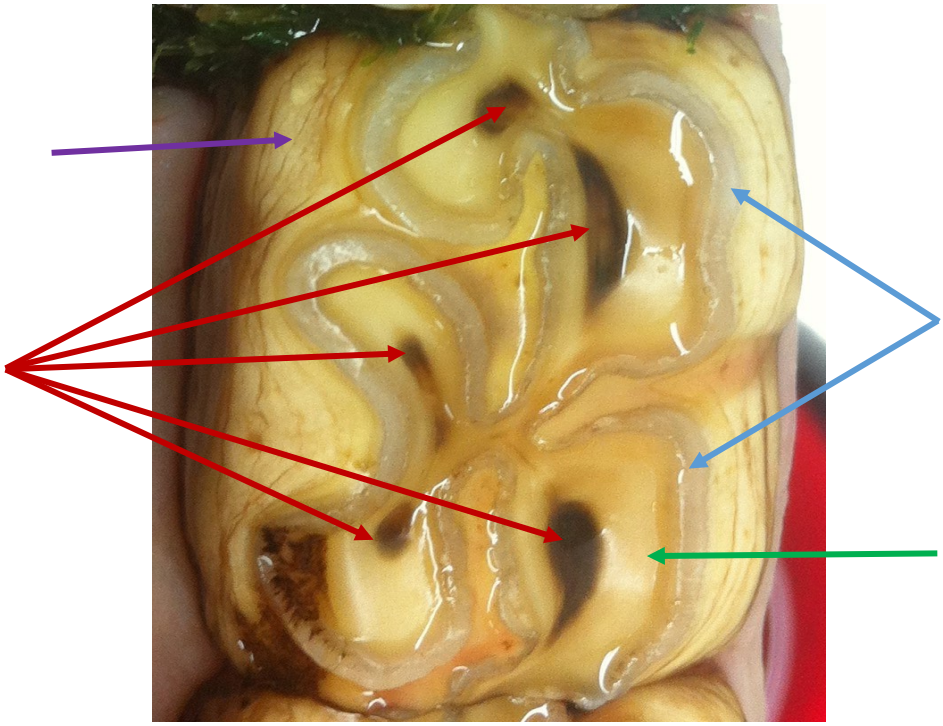

Figure 1.4 Mandibular equine cheek tooth 309

Peripheral Cementum Primary dentine Enamel

Secondary dentine overlying pulp horn Infundibulae

1.1.2 The equine periodontium

1.1.2.1 Equine periodontal anatomy and physiology

The periodontium is a complex, dynamic structure comprised of four separate tissues, i.e. the gingiva, peripheral cementum, periodontal ligament and alveolar bone, interacting to protect and support the tooth. Staszyk and Gasse (2005) described the equine periodontium as having three major supportive functions: 1). To secure the tooth in the alveolus; 2). To accept a variety of masticatory forces; 3). To restore the tooth to its original position after temporary displacement during mastication. The equine periodontium must also adapt to allow for the prolonged eruption of hypsodont dentition and cope with massive masticatory forces of over 1550 Newtons exerted on the caudal cheek teeth for up to 18 hours per day (Huthmann *et al*. 2009). Gingiva also acts to physically protect the periodontal ligament from oral bacteria.

Although it is intuitive to think of peripheral cementum as part of the tooth itself, especially in hypsodont teeth where it comprises a substantial part of the clinical crown (Mitchell *et al*. 2003) - it can also be considered as a component of the periodontium due to its distinctive odontogenic development (Stasyzk *et al*. 2015). Throughout the life of the tooth peripheral cementum is continually produced by cementoblasts and deposited at the apex and around the periphery of the reserve crown, a feature unique to the equine periodontium (Stasyzk and Gasse 2007). Cementum has an organic content of around 50% with small collagen fibrils being produced by cementoblasts and nourished by vessels within the periodontal ligament (Hands 2008). Larger fibrils known as Sharpey's fibres originate from the periodontal ligament and are incorporated into both the peripheral cementum and alveolar bone, flexibly anchoring the tooth into the alveolus (Grant and Bernick 1972).

The periodontal ligament is a highly vascular and cellular structure which largely consists of collagen fibres, fibroblasts and ground substance interspersed with blood and lymphatic vessels (Staszyk and Gasse 2005). The equine periodontal ligament contains unique vasculature which both nourishes and supports the tooth during mastication and prolonged eruption. Periodontal blood vessels are integrated into the equine periodontal ligament in three distinct ways (Staszyk

and Gasse 2004). The Type 1 arrangement of blood vessel groups are protected from masticatory forces by a sheath of 'veil cells' and connective tissue which is thought to protect blood flow during mastication. An elaborate collagen network anchors blood vessels in the Type 2 arrangement, resulting in a functional fibro-vascular unit able to resist forces of traction during mastication. In the Type 3 vascular arrangement, dilated ballooned venules running parallel to the tooth surface between collagen fibre bundles have a cushioning effect, absorbing the substantial forces of mastication. Staszyk and Gasse (2004) also noted the presence of elastic oxytalan fibres in the equine periodontal ligament, which allow regeneration and remodelling, whilst improving periodontal blood vessel stability.

Matrix metalloproteinase-1 which initiates collagen breakdown to allow remodelling has been detected in the equine periodontal ligament (Warhonowicz *et al*. 2007). As eruption progresses, equine teeth reduce in size and shape (narrowing towards the apex), and in turn the surrounding alveolar bone must constantly remodel in order to provide sufficient support to the tooth. Sharpey's fibres in the periodontal ligament insert into a thin, compact layer of "cortical" alveolar bone which lines the alveolus and is radiographically known as the *lamina dura denta*. The alveolar bone surrounding this superficial compact layer is more porous which may reflect its constant remodelling (Dixon and du Toit 2011).

The gingiva is a firm, keratinised epithelium covering the underlying alveolar bone, periodontal ligament and reserve crown and acts as a physical barrier against oral microbial invasion of the periodontal tissues. It is possible to further classify gingival tissue by its location within the oral cavity and with regards to its position relative to the tooth. Stasyzk *et al*. (2015) describe two distinct zones of gingiva: the interdental gingiva also known as the interdental papilla and the remaining bulk of the gingiva located on the buccal, labial, palatal and lingual aspects of the teeth. The free gingiva is the most occlusal and mobile aspect of the gingiva and acts as an interface between the epithelium of the gingival (crevicular) sulcus which is a shallow pocket between the tooth and adjacent sulcular epithelium. Junctional epithelium at the base of the sulcus adheres tightly to the peripheral cementum on the tooth surface. Gingival sulcus

depth is between 1 to 4mm in periodontally healthy horses (Cox *et al*. 2012) and an increase in sulcular depth indicates the presence of periodontal disease. Gingival crevicular fluid containing antibodies, enzymes and other inflammatory mediators and immune components is secreted into the sulcus and together with gingival tissue plays an important role in responding to immunological challenges posed by oral bacterial communities. In periodontal health, the gingiva function to provide a tight seal around the erupted crown of the tooth protecting underlying structures by forming a mechanical barrier. However, in periodontal disease, this barrier is damaged, leaving underlying sensitive tissues exposed and open to both mechanical damage and bacterial colonisation.

1.2 Equine periodontal disease

1.2.1 Clinical signs

Despite the painful nature of the condition, periodontal disease may go unnoticed for a prolonged period of time as the clinical signs associated with it are often subtle or even absent in some horses (Dixon *et al*. 2008a). Quidding (dropping partly masticated food) and difficulty eating due to pain upon mastication are the most common signs associated with the disease (Little 1913; Lane 1994; Klugh 2005; Tremaine 2010 and Dixon *et al*. 2008a; 2014). A gradual loss of condition due to inefficient feeding may also be evident and halitosis, hypersalivation, reluctance to eat and buccal food pocketing may also be present, especially when the disease is associated with diastemata (Dixon *et al*. 2014). Behavioural problems associated with oral pain may be noticed by owners including head shyness (Klugh 2005) or poor performance such as abnormal reactions to the bit and head shaking when exercised (Lane 1994). Dixon *et al*. (2014) reported biting problems in 20% of horses with diastemata (abnormal space between adjacent teeth). Severe secondary problems to periodontal disease as a result of deep tracking infection through the periodontium have been reported, such as formation of apical abscesses and sinusitis (Harvey 1920). Severe mandibular osteomyelitis, malignant oedema and tetanus have also been reported as rare secondary consequences of the disease (Harvey 1920). Hawkes *et al*. (2008) reported oromaxillary fistulae with secondary sinusitis secondary to cheek teeth diastemata and periodontal disease.

1.2.2 Prevalence

Equine dental disease is common with prevalence increasing with advancing age (Baker 1970). Despite this, dental abnormalities often go unnoticed by horse owners. Ireland *et al*. (2012b) reported that 95.4% of horses aged 30 years and older had at least one dental abnormality however only 24.5% of owners were aware their horse had a dental abnormality. This is especially worrying with regards to periodontal disease as it has been described as one of the most painful oral conditions of the horse when associated with diastemata (Collins and Dixon 2005).

Several authors have studied the prevalence of periodontitis in domesticated horse populations. Colyer (1906), described periodontal disease as 'the scourge of the horse' after examining 484 skulls of London's working horses, discovering that 33% were affected by periodontal disease. Voss (1947) conducted a study of 647 horses of all ages and found 34% to be suffering from the disease. When only horses aged 13 years and older were considered this figure rose to 61%. Other studies have agreed with this figure, finding that around 60% of horses over the ages of 15 (Baker 1970) and 20 years old (Wafa 1988) were affected. Prevalence is known to increase with advancing age with Ireland *et al*. (2012a), reporting the presence of periodontal disease in 75% of horses aged 30 and older. The disease has also been described in donkeys. du Toit *et al*. (2009) recorded a 14% prevalence in donkeys of all ages, increasing to 28% in the 21-25 year old group. Age-related increases in number of teeth affected and the severity of periodontitis has also been described in donkeys (Rodriguez *et al*. 2013). Periodontitis has also been documented in wild zebra (Penzhorn 1984).

1.2.3 Aetiopathogenesis

1.2.3.1 Plaque-induced periodontal disease in brachydont species

Periodontal disease is also of major importance in humans and brachydont domestic animals and consequently has been extensively studied in many brachydont species, often as a model for human disease. In brachydont dentition the initiating factor for gingivitis, which is the earliest and often reversible stage of periodontal disease, is the accumulation of dental plaque in the gingival sulcus, which may eventually become calcified (calculus). More specifically, the presence of plaque in the gingival sulcus initiates an inflammatory reaction (gingivitis) that may or may not proceed to involve the deeper periodontal tissues. More severe periodontal disease is most frequently due to the host's response to bacterial invasion (Kikkert *et al*. 2007).

1.2.3.2 Periodontitis induced by trapped feed in hypsodont species

Plaque induced periodontitis does not appear to be a common problem in horses, unlike in brachydont species (Dixon *et al*. 2002). One exception is the canine teeth, especially the lower canines, where the presence of calculus can cause gingivitis and, rarely, more severe periodontitis. (Fig 1.5). In equidae, food trapping in anatomical defects between adjacent cheek teeth is the usual instigator of periodontitis. In a study of referred equine cases with cheek teeth disorders, Dixon *et al*. (1999, 2000), found periodontal disease in the absence of concurrent dental disorders and without the presence of plaque in just 3/349 (0.9%) cases.

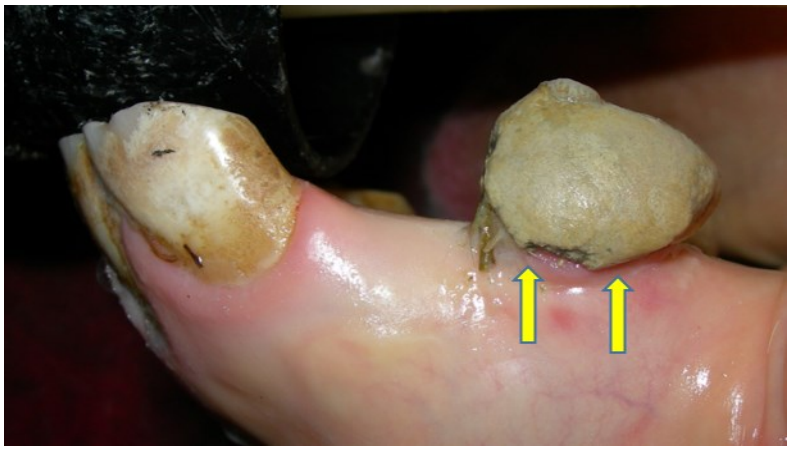

Figure 1.5 Calculus on a canine tooth with local gingivitis (arrows)
Photographgraph courtesy of P.M. Dixon.

Equine periodontitis is particularly associated with diastemata which can be described as abnormal spaces between adjacent teeth - which should normally be in tight occlusal apposition. Food material becomes impacted into this abnormal space during mastication, often becoming tightly entrapped and initiating inflammation of the underlying gingiva, that invariably progresses to the deeper periodontal tissues (Fig. 1.6 and 1.7). Colyer (1906) noted the high prevalence and importance of equine periodontal disease and attributed it to a coarse diet, but Dixon *et al*. (2000) noted that the illustrated specimens of cheek teeth periodontal disease from Colyer's study (Miles and Grigson 1990) all had diastemata, and that the reported periodontal disease appears to have been initiated by interproximal food trapping. Little (1913) had previously attributed diastemata as the cause of equine periodontal disease, especially periodontal disease in the interdental (interproximal) spaces adjacent to the mandibular 10s. Although diastemata and associated periodontitis may be present between incisor teeth, they may be easily managed by regular cleaning; however, cheek teeth diastemata pose a more significant problem (Collins and Dixon 2005).

Diastemata may be congenital or acquired. Dixon *et al*. (2008a, 2014) classified equine diastemata as primary diastemata (where the teeth developed too far apart and/or with insufficient angulation of the peripheral teeth to compress them) (Fig. 1.7) and as secondary diastemata such as those caused by displaced (Fig. 1.8) or rotated (Fig. 1.9) cheek teeth, dental overgrowths or supernumerary cheek teeth. In addition, senile diastemata may form due to the tapering of equine cheek teeth towards their apices resulting in decreased surface area at the occlusal surface and age-related loss of angulation of the 06s and 11s (Fig. 1.10). Diastemata have been documented in up to 50% of horses in a UK equine practice survey, with feed material becoming trapped in 91.4% of diastemata (Walker *et al*. 2012). In addition, 34% of diastemata had associated gingivitis and 44% were accompanied by periodontal pockets. Impaction of feed material into the periodontal pocket has also been recorded in 76% of cheek teeth diastemata in donkeys (du Toit *et al*. 2009), with 71% of these donkeys also having concurrent dental disorders such as displaced cheek teeth, which likely initiated or contributed to diastemata formation.

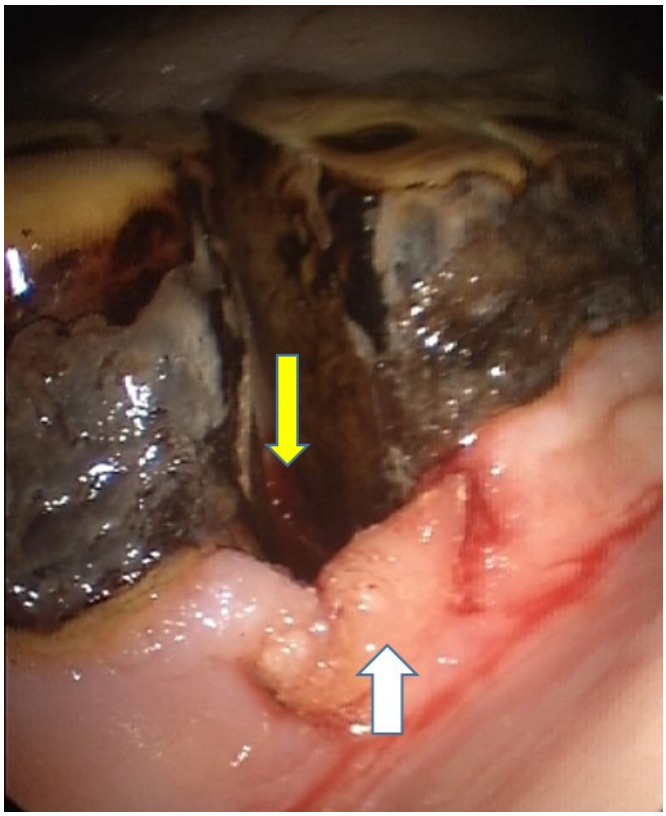

Figure 1.6 Oral endoscopic view of severe equine cheek teeth periodontal disease caused by a diastema

The teeth adjacent to the diastema have caries of the peripheral cementum and are covered in plaque. There is marked loss of periodontal tissues in the interdental space (yellow arrow) and markedly hyperplastic gingiva (white arrow).

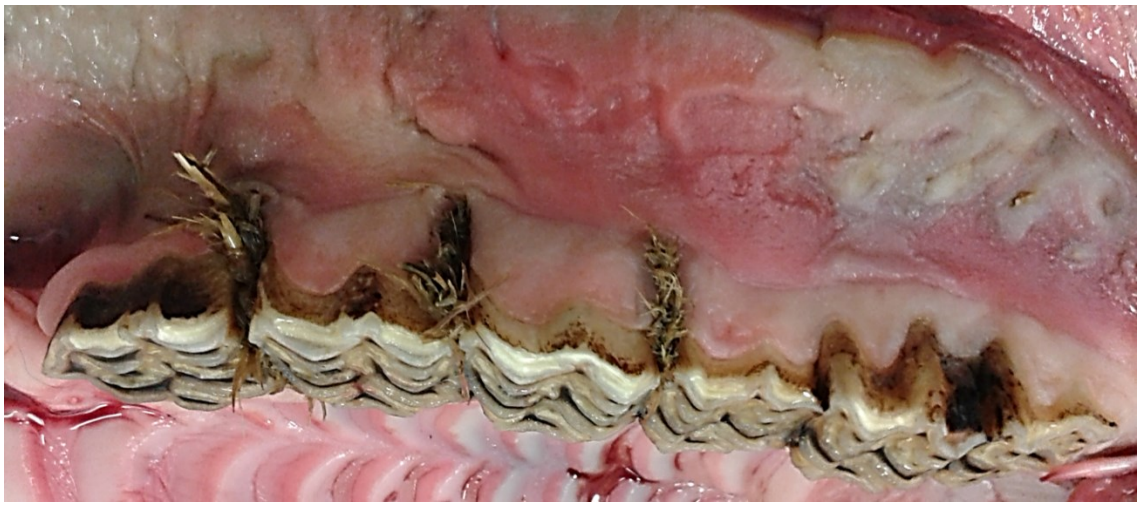

Figure 1.7 Post-mortem image of left maxillary cheek teeth showing deep periodontal pockets between the rostral four cheek teeth with deep periodontal pocketing of food.

Photograph courtesy of P.M. Dixon.

Disorders of cheek teeth eruption are also often associated with periodontitis. Dixon *et al*. (1999) found periodontitis to be associated with 87% of cases with cheek teeth displacements, 30% of cases with rostral/caudal cheek teeth overgrowths and 90% of cases with supernumerary cheek teeth. Rotated maxillary cheek teeth have also been described as the primary cause of diastemata formation and associated severe periodontitis (Casey and Tremaine 2010). Voss (1937) suggested that irregular feeding times interrupted salivary flow in horses, which in turn could contribute to the development of periodontal disease, but no evidence for this hypothesis has been presented.

1.2.3.3 Feed stasis and bacterial proliferation

The above described trapping, stasis and subsequent decomposition and fermentation of food material in interdental spaces can abrade the sensitive gingiva, causing mechanical damage and gingival inflammation (Little 1913; Baker 1979; Cox *et al*. 2012). Over time, impacted feed, (a porous foreign body) acts as a bacterial nidus, supporting the proliferation of bacteria which ferment the trapped feed. The *Lactobacillales* order of bacteria which include *Lactobacilli*, *Streptococci* and *Enterococci* ferment plant material by anaerobically metabolising carbohydrates to produce lactic acid (Gänzle 2015).

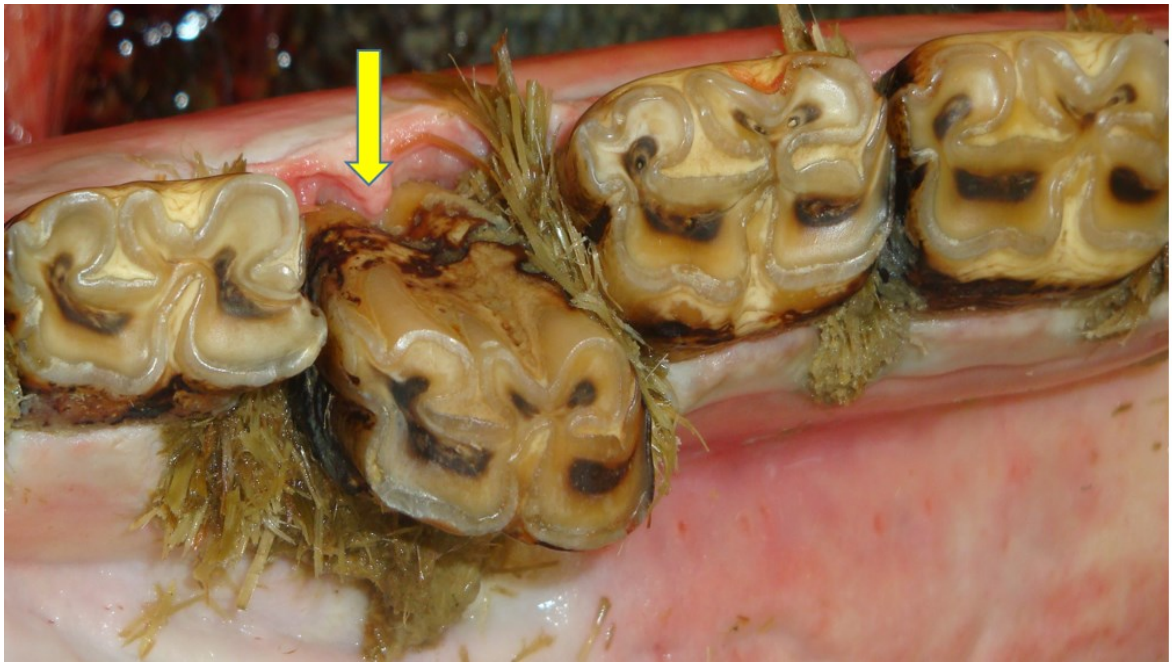

Figure 1.8 Secondary diastema

This post-mortem image shows a developmentally displaced (and also curved) cheek tooth with secondary diastemata rostral and caudal to it and a deep periodontal pocket on its lingual aspect (arrow). There are also “primary” diastemata between other adjacent teeth. Photograph courtesy of P.M. Dixon.

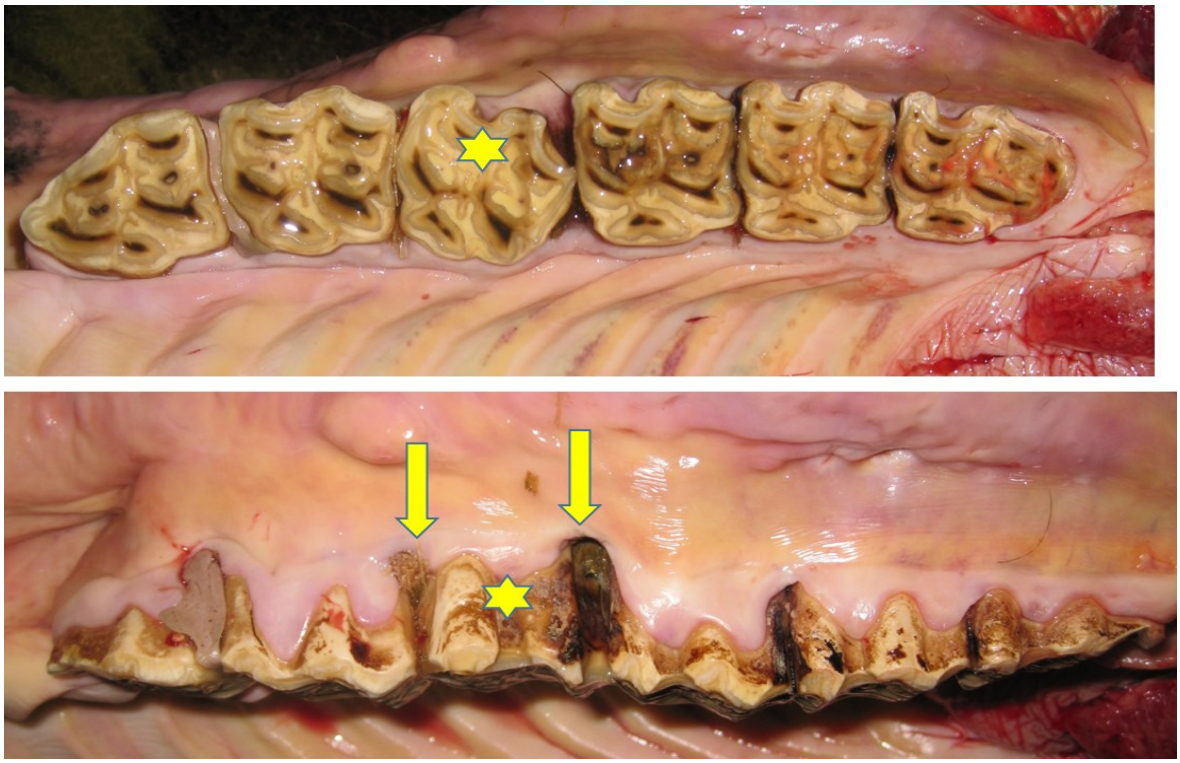

Figure 1.9 Diastemata secondary to rotated 208

These post-mortem images (occlusal aspect –top image: buccal aspect –bottom image) show a developmentally rotated 208 tooth (yellow star) with a narrow diastema rostrally and wide diastema caudally (yellow arrows).

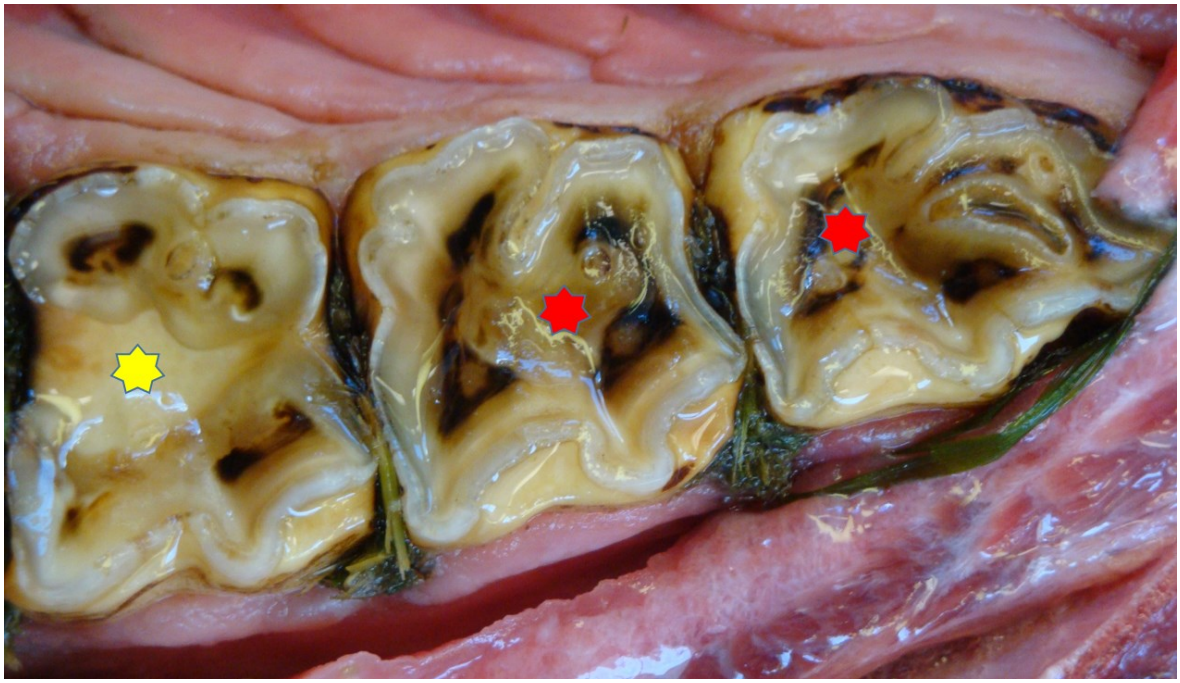

Figure 1.10 Post mortem images of the caudal cheek teeth of a horse with senile diastemata

Note the 209 (yellow star) worn down to the roots with compensatory cementum deposition, and loss of some infundibulae and senile excavation in the 210 and 211 (red stars). Photograph courtesy of P.M. Dixon.

1.2.3.4 Inflammation of the periodontium

The initial insult provokes a substantial inflammatory response within gingival tissue due to both mechanical abrasion of sensitive gingival epithelium and bacterial proliferation. This is apparent clinically as gingivitis with redness, hyperaemia and bleeding upon gingival probing. In man, a number of different pathogenic bacteria are implicated in the induction of a marked inflammatory response (Kikkert *et al*. 2007) which then leads to destruction of the periodontal ligament and resorption of alveolar bone and cementum which inevitably leads to end stage disease i.e. loss of the tooth. This exaggerated inflammatory response is the result of prolonged cytokine production in gingival tissue leading to increased production of proteases which can destroy invading microbes but can also damage the host's periodontal tissues (Teng 2003). This response may show individual variation, which includes a genetic component in humans (Yoshie *et al*. 2007).

Apart from the study by Cox *et al*. (2012) there appears to be no published work on equine periodontal histology. These authors showed the mucosal surface of equine periodontal pockets to be hyperplastic, with epithelial disruption and

presence of large numbers of inflammatory cells such as neutrophils in the *lamina propria* (Fig 1.11) and adjacent connective tissues (Fig. 1.12) and destruction of the periodontal tissues, including peripheral cementum in which the periodontal membranes were once attached (Fig.1.12) Accumulations of food material, which may be obvious clinically, has also been confirmed on histopathology alongside large numbers of bacteria, including spirochaetes and micro-abscesses in the submucosa of periodontal pockets (Cox *et al*. 2012). In the absence of clinical intervention, the disease progresses and inflammation spreads to the periodontal ligament with infiltration of mononuclear cells (Figs. 1.11, 1.12). The ligament is gradually destroyed over time, as is the surrounding alveolar bone and cementum (Fig. 1.13), decreasing tooth support and further deepening periodontal pockets. Teeth may become increasingly mobile at this stage. The increasing depth of the periodontal pocket provides the ideal environment for further invasion and proliferation of anaerobic bacteria and the cycle of inflammation and tissue degradation continues until tooth loss occurs.

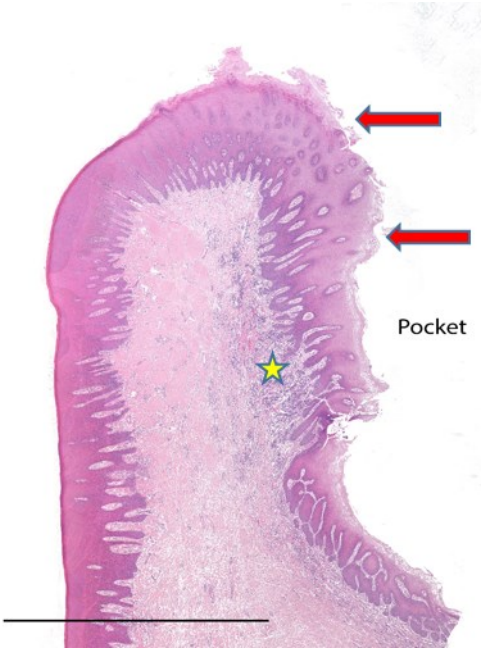

Figure 1.11 Histological section of periodontium from a horse with periodontitis.

This shows a periodontal pocket circa 5mm deep. The calcified dental tissue (i.e., tooth) on right side of periodontal pocket has been lost during decalcification. Moderate gingival hyperplasia (red arrows) is present on the gingiva facing the periodontal pocket and at the free gingival margin. There is also modest infiltration of inflammatory cells into the lamina propria (yellow star). (bar = 2mm). Image courtesy of Dr. A. Cox.

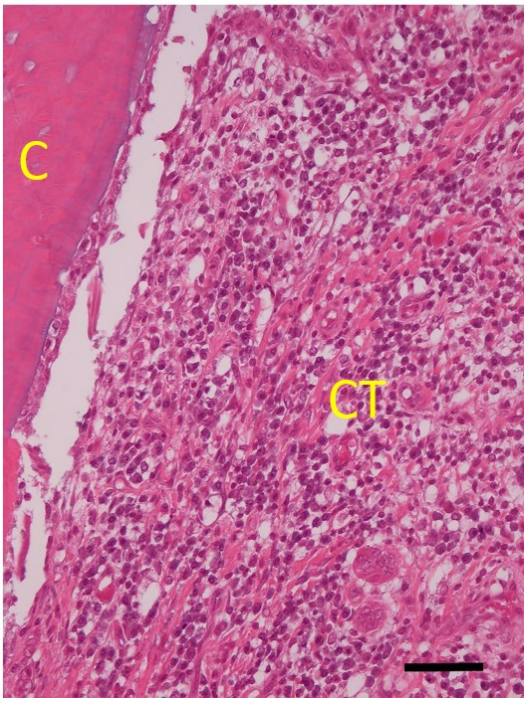

Figure 1.12 Massive infiltration of inflammatory cells into interdental subgingival connective tissue (CT).

The separation of this connective tissue from cementum (C) is artefactual during histological preparation. (bar =50μm). Image courtesy of Dr A. Cox.

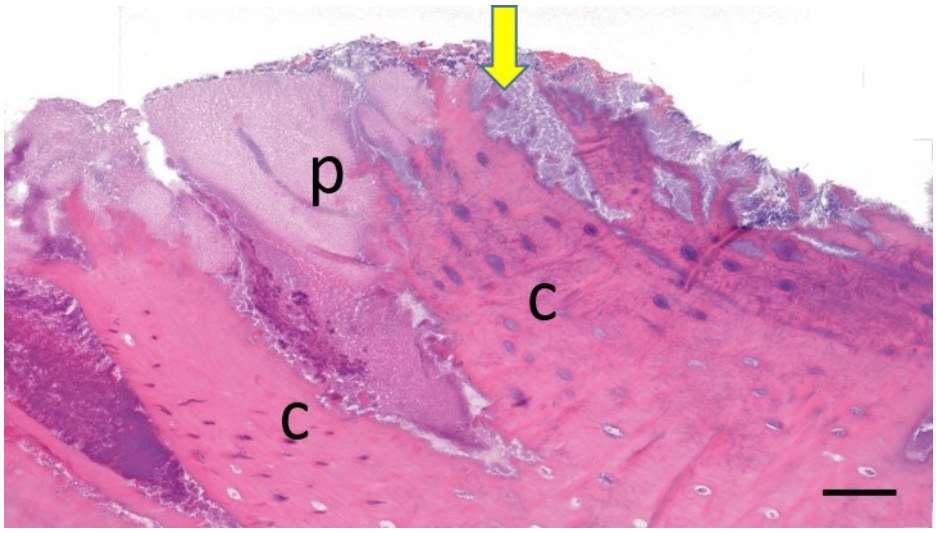

Figure 1.13 Disintegration of peripheral cementum (C) of the periodontium due to advanced periodontal disease.

Includes the development of cemental clefts (yellow arrow) that become infilled with plaque (P). Image at x40 magnification. Image courtesy of Dr. A. Cox.

1.2.3.5 Equine Odontoclastic Tooth Resorption and Hypercementosis (EOTRH)

Equine Odontoclastic Tooth Resorption and Hypercementosis (EOTRH) is a recently described condition affecting older horses and in some cases is associated with gingival ulceration, purulent periodontitis and oral pain affecting the incisors and canine teeth (Staszyk *et al*. 2008). Bulbous cemental apical enlargements can form alongside resorptive lesions that may extend to the tooth surface which may ultimately lead to pulp exposure (Dixon *et al*. 2008b). Histopathological examination of affected periodontal tissue shows severe inflammation with infiltration of neutrophils, lymphocytes and plasma cells (Caldwell 2007, Staszyk *et al*. 2008), with resorptive lesions causing total loss of dental architecture alongside areas of cemental hypertrophy (Dixon *et al*. 2008b). The aetiology of EOTRH is currently unknown although mechanical stresses on the periodontal ligament have been suggested (Dixon *et al*. 2008b). While bacteria are not always seen upon histological examination (Baratt 2007) and their presence is thought to be secondary (Staszyk *et al*. 2008), Sykora *et al*. (2014) discovered a higher prevalence of 'red complex' bacteria (*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola)* commonly associated with human periodontitis in horses with clinically overt EORTH compared to unaffected horses. As EOTRH appears to be a clinically and histologically distinct form of periodontal disease it is most likely that its aetiopathogenesis which involves abnormal activation of odontoclasts is distinct from classic equine periodontitis.

1.2.3.6 Periodontitis secondary to *Gasterophilus* migration

*Gasterophilu*s larvae, more commonly known as bot fly larvae, have also been associated with periodontal disease in the horse (Cogley 1989; Osterman *et al*. 2012). The adult fly lays its eggs on the horse's coat, typically sticking to the hair of the forelegs or around the nose and mouth. During grooming, stimulated by warmth and moisture from the tongue, the eggs hatch into larvae and migrate into the oral cavity where they spend approximately three weeks in the soft tissues of the gingiva, tongue and lips. After this time larvae migrate to the stomach where they spend the winter attached to the stomach mucosa before being passed through the digestive tract, finally burying into the soil to complete the life cycle and become adult flies (Cogley *et al*. 1982). The

presence of *Gasterophilus* larvae in equine periodontal pockets is well documented (Cogley 1989; Griss and Simhofer 2004; Osterman 2012). Cogley (1989) noted gingival recession and ulceration upon histopathological examination of periodontal pockets created by migrating *Gasterophilus* larvae. More recently, a *post-mortem* dental pathology study of 40 South African horses showed the presence of *Gasterophilus* larvae (identified as *G. nasalis* and *G. pecorum*) in periodontal pockets of 20% of horses although this prevalence increased to 80% when only those horses examined in winter months were taken into consideration (Vemming *et al*. 2015).

1.2.4 Diagnosis and staging of equine periodontal disease

1.2.4.1 Oral Examination

In order to diagnose and appropriately stage equine periodontal disease, a full oral examination must be performed with the use of an appropriate full mouth speculum and strong light source or an oral endoscope. Due to equine oral anatomy with limited jaw opening and a long oral cavity, visualisation of the caudal cheek teeth can be difficult and subtle changes such as diastemata may go undetected (Simhofer *et al*. 2008). The use of dental mirrors facilitates the examination although the use of oral endoscopy to evaluate the caudal cheek teeth is regarded as superior to simple clinical examination alone (Simhofer *et al*. 2008; Tremaine 2005). All dental abnormalities should be recorded on an equine dental chart (Fig 1.1). Gingiva should be assessed for presence of inflammation and all periodontal pockets should be measured using an equine periodontal probe. Affected teeth should be assessed for mobility and presence of peripheral cemental caries (Klugh 2005). Radiography may be useful to assess periodontal attachment loss (Klugh 2005) and further diagnostic imaging may also assist in evaluation of dental disorders associated with secondary periodontitis such as displaced, rotated or supernumerary cheek teeth (Collins and Dixon 2005) however these disorders are best assessed visually.

1.2.4.2 Staging of equine periodontal disease

The Periodontal Disease Index has been adapted for veterinary use (Wiggs and Loprise 1997) and to the equine anatomy by Klugh (2005) **(**Fig 1.14).

Stage 0 – Normal

- No attachment loss.
- Probing depth <5mm.

Stage 1 – Gingivitis

- Gingiva swollen and reddened.
- No attachment loss.
- Probing depth <5mm.
- Bleeding may occur on probing.
- Cemental decay may be present but if so remains supragingival.

Stage 2 – Early Periodontal Disease

- Up to 25% attachment loss.
- Periodontal pockets and feed impaction present.
- Gingiva ulcerated and receded.
- Necrosis of sulcular epithelium may be present.
- Sub-gingival cementum may be decayed.
- Tooth mobility slight if present (stage 0 or 1)
- Probing depth > 5mm.

Stage 3 – Moderate Periodontal Disease

- 25-50% attachment loss or bone loss.
- Probing depth often exceeds length of probe.

Stage 4 – Advanced Periodontal Disease

- >50% attachment loss or bone loss.
- Severe gingival disease and purulent exudate may be present.
- Tooth mobility is stage 2-3.
- Stage 4 immediately proceeds loss of the tooth.

Figure 1.1 Staging of equine periodontal disease as described by Klugh (2005).

Veterinary Periodontal Disease Index uses radiography to assess periodontal attachment loss and this may not always be practical in the field. As an alternative, a grading system using periodontal pocket depth was used by Cox *et*

al. (2012) (Table 1.1) and this system has been used throughout the current study.

Grade of periodontal disease	Descriptive Grade	Sulcus/periodontal pocket depth (mm)
0	No disease	0-4
1	Mild disease	5-9
2	Moderate disease	10-14
3	Severe disease	>15

Table 1.1 Staging of equine periodontitis based on pocket depth as described by Cox *et al*. (2012)

The tooth mobility index has been modified for use in equine dentistry by Klugh (2005), as shown in Table 1.2.

Tooth Mobility Stage	Descriptive Stage	Mobility
0	Normal	None
1	Mild	First distinguishable movement greater than normal
2	Moderate	Movement of up to 3mm
3	Severe	Movement over 3mm in any direction and/or is depressible

Table 1.2 The index of tooth mobility modified for equine dentistry by Klugh (2005).

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1.2.5 Treatment and management

1.2.5.1 Management of concurrent oral disorders

As equine periodontitis is most often a secondary consequence of pre-existing oral disorders (Dixon *et al*. 2000) treatment often involves addressing of these conditions. In addition, equine periodontal disease may also result in development of other oral disease such as focal overgrowths and disorders of wear due to altered masticatory patterns caused by associated oral pain.

1.2.5.2 Treatment of diastemata

Incisor diastemata are easily managed by cleaning, but cheek teeth diastemata are notoriously difficult to resolve (Collins and Dixon 2005) and there has been little consensus regarding recommended treatment with no one single treatment suitable for every case (Dixon *et al*. 2014; Jackson *et al*. 2016). Traditional treatment of periodontitis secondary to diastemata is extraction of affected teeth or removal of the clinical crown (Little 1913). In cases with widespread and severe periodontitis extraction may be necessary but recently, more conservative treatments have been used. It is important to firstly clean the periodontal pocket, removing all impacted feed material. This may be performed with a combination of hand tools and high pressure water/air flushing (Klugh 2005). Some authors then advise the packing of the diastema with dental impression material (e.g. Polyvinyl siloxane) overlying a layer of doxycycline gel to reduce bacterial load and prevent further impaction of food which then provides relief from periodontal pain (Collins and Dixon 2005).

Recently, mechanical widening of cheek teeth diastemata has been used (Dixon *et al*. 2008a, 2014). Mechanical widening allows feed to move freely, preventing trapping and subsequent periodontitis and is particularly useful in management of valve diastemata. This treatment has been found to be successful but repeat treatments may be required due to orthodontic movement (Dixon *et al*. 2014). It is also important to note the risk of iatrogenic damage to the pulp during treatment and it is recommended widening should only be carried out by experienced personnel (Dixon *et al*. 2008a, 2014).

It is imperative that any dental overgrowths such as those forming opposite to the diastema are removed as if left untreated overgrowths will result in abnormal forces during mastication and encourage feed packing and further diastemata formation. Removing such abnormally large stresses on the diseased periodontium may promote healing (Klugh 2005). Dietary modification is also important in the management of certain cases of diastemata such as senile diastemata, with substitution of long-fibre feeds such as haylage with short-fibre feeds such as fibre cubes which are less likely to become trapped (Collins and Dixon 2005).

1.2.5.3 Treatment of disorders of eruption

Periodontitis induced by feed trapping is often a consequence of disorders of eruption such as rotated cheek teeth (Casey and Tremaine 2010), supernumerary cheek teeth and displacement of cheek teeth (Dixon *et al*. 1999). The best course of treatment for severely affected cases with deep secondary periodontitis is often extraction of the affected teeth in conjunction with reduction of associated overgrowths, thus removing any abnormal forces acting during mastication (Dixon *et al*. 2010).

1.2.5.4 Antimicrobial therapy

Systemic antimicrobials, used in combination with anti-inflammatories may result in temporary or partial improvements but are not recommended as the main treatment modality (Collins and Dixon 2005). Topical antimicrobial treatment has been described by Klugh (2005) by placing doxycycline gel into the periodontal pocket in addition to using chlorhexidine mouthwashes however widespread clinical trials on the efficacy of these treatments in equine periodontitis have not yet been performed.

1.2.5.5 Analgesia and anaesthesia

Periodontal disease is painful, particularly when associated with diastemata (Dixon *et al*. 2000). Non-steroidal anti-inflammatory drugs should be used alongside the appropriate use of sedatives such as alpha-2 agonists to provide pain relief and facilitate treatment (Dixon *et al*. 2014).

1.3 Equine oral microbiology

1.3.1 Bacteria in the oral cavity

1.3.1.1 The oral microbiome

As previously noted, bacteria play a major role in the aetiopathogenesis of periodontal disease in human, canine and feline periodontal disease and it is easy to appreciate the potential importance of bacteria in equine periodontitis. This role was recently supported by the histopathological finding of spirochaetes in the sulcar epithelium (Fig 1.15) of diseased equine periodontal pockets, which

also had cocci on the epithelial surface (Fig 1.16) (Cox *et al*. 2012). The ecological community of bacteria both commensal and pathogenic inhabiting the oral cavity is known as the oral microbiome. There are approximately 700 different species identified to date in the human oral cavity (Dewhirst *et al*. 2010; Liu *et al*. 2012; Wade 2013) even though the human oral microbiome has not yet been fully characterised. This bacterial community is incredibly complex and dynamic, with species interacting with each other and also with the host immune system. In order to survive in an environment being constantly washed with host saliva and in addition being challenged with mechanical abrasion from masticatory movements, bacteria have found a method of adhering to the surface of oral tissue and so exist in a biofilm.

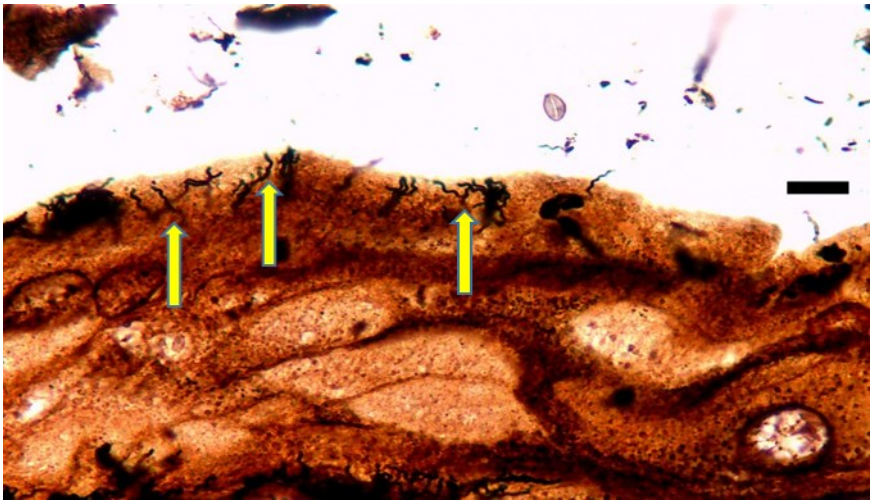


Figure 1.15 Spirochaetal bacteria in gingival epithelium of a diseased equine periodontal pocket.

Modified Young's silver stain bar= 10µm. Image courtesy of Dr. A. Cox

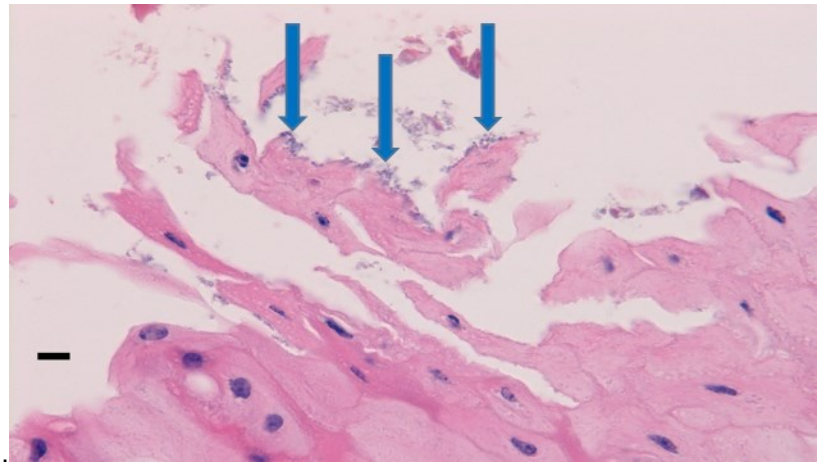


Figure 1.16 Cocci on the gingival epithelial surface of a periodontitis affected horse.

Bar= 10µm. Image courtesy of Dr. A. Cox.

1.3.1.2 Oral biofilms

A biofilm is defined as 'a biopolymer matrix-enclosed bacterial population, adherent to each other and surfaces' (Costerton *et al*. 1999). Multispecies bacterial communities existing within the oral biofilm are supported and protected by the surrounding matrix. The composition of an oral biofilm is very much dependent upon its location within the oral cavity, and thus tooth-associated biofilms can be divided into two categories. Supragingival biofilms adhere to the surface of the clinical crown and subgingival biofilms adhere below the gum line, within the (normal) gingival sulcus or (abnormal) periodontal pocket (Kolenbrander *et al*. 2010). Early bacterial colonisers which are well adapted to community formation and multispecies growth (Kolenbrander *et al*. 2010) initially adhere to the salivary pellicle, a layer of proteins and glycoproteins which permanently coats all normal oral surfaces. Adhesion and subsequent proliferation of early colonisers is followed by co-adhesion of genetically distinct bacteria on to the existing attached population. In addition to co-adhesion, distinct bacterial species also interact via cell surface components when both are suspended in fluid, which is termed co-aggregation. For example, strains of *Fusobacterium nucleatum* are able to co-aggregate with early and late oral biofilms and may play a bridging role in the development of human dental plaque (Kolenbrander and London 1993; Kolenbrander *et al*. 2010). Over time, the biofilm becomes increasingly complex and matures into dental plaque. The oral biofilm is highly intricate with dynamic microbial interactions including complex cell signalling between bacteria of differing genera as well as transfer of DNA between bacteria. Conjugative transposons which facilitate DNA transfer between bacteria have been detected in many genera of oral bacteria including *Fusobacterium*, *Streptococcus* and *Veillonella* (Rice 1998).

Existing within the biofilm matrix, bacteria are protected from exposure to host innate and adaptive immune mechanisms as well as to administered antimicrobial compounds. Front-line immune responses such as phagocytosis are ineffective in the biofilm matrix as bacterial cells cannot be readily engulfed at this site (Kharazmi 1991) and infiltration of neutrophils into the plaque may even provide an additional matrix for bacterial attachment (Walker *et al*. 2005).

Although ineffective in removing the bacterial biofilm, the immune response has a significant side-effect on surrounding tissue, stimulating inflammation and even destruction of the periodontium (Teng 2003). Mechanical removal of dental plaque is recommended where possible in brachydont periodontal disease.

1.3.2 The equine oral microbiome

1.3.2.1 The equine oral microbiome in health

It has been acknowledged that the equine oral microbiome in both health and disease has been a neglected field of research until recently (Dacre *et al*. 2008; Cox *et al*. 2012; Sykora *et al*. 2014).

Previously, culture-based studies with biochemical identification of isolates were performed by Baker (1979), who observed high counts of *Streptococci* sp., *Micrococci* sp., and starch hydrolysing bacteria. Intermediate counts of anaerobes, *Veillonella* sp. and hydrogen sulphide producers and low counts of *Lactobacillus* sp., *Fusobacteria* sp. and coliforms were also found. *Actinobacillus equuli* has been shown to be a commensal of the healthy oral cavity of adult horses (Platt 1973; Sternberg 1998; Sternberg and Brändström 1999; Bisgaard *et al*. 2009). Bailey and Love (1990) studied the flora of the oral cavity and pharynx of twelve normal horses and detected bacteria from seven genera: *Peptostreptococcus* (1 isolate); *Eubacterium* (9); *Clostridium* (6); *Veillonella* (6); *Megaphera* (1); *Bacteroides* (28) and *Fusobacterium* (6). *Eubacterium fossor* was reported as an inhabitant of the healthy pharynx of seven horses, however it was also isolated from apical abscesses and pleuropneumonia lesions (Bailey and Love 1986). Limitations of this study include the classification of 'normal', which is based on the normal appearance of the pharynx and normal histopathology of the tonsillar epithelium, with no description of an examination of the oral cavity itself, especially dental tissues, which may well have possessed some level of disease. The study is also limited because of its focus on obligate anaerobes, and only those which could be cultured and so, while useful, this study, like the other culture-dependent studies, is not truly representative of the equine oral microbiome.
Recently, Gao *et al*. (2016) investigated the microbiome of the equine gingival sulcus by pyrosequencing pooled samples from 200 sulcus sites in two orally

healthy horses identifying 12 phyla, the most prevalent being *Gammaprotebacteria* (28.8%), *Firmicutes* (27.57%) and *Bacteroidetes* (25.11%). A summary of identified taxa can found in Table 1.3. The study suggested that there are many similarities between equine subgingival microbiota and the subgingival microbiota detected in human, feline and canine studies. In order to more thoroughly investigate presence of putative equine periodontal pathogens from the Synergistetes and Spirochaetes phyla, Gao et al. (2016) also used phylum specific clone-based DNA sequencing and found the majority of spirochaetes corresponded to *Treponema* species. In addition, other putative periodontal pathogens such as *Tannerella* and *Porphyromonas* species were detected at low levels in these samples, which the authors suggest indicates periodontal health in these two animals. Gao et al. (2016) also discovered that the oral microbiota harboured by both horses was notably different, however the more abundant phyla were generally common between animals. Many bacteria identified were not closely related to other known bacteria and the authors suggest these may represent 'equine-specific' taxa. As few previous studies have been performed investigating the equine oral microbiome, it is highly likely that novel, previously undetected bacteria will be identified when using modern, culture independent techniques.

1.3.2.2 The equine oral microbiome in disease

There have been few investigations into the bacteria present in equine periodontitis lesions, with most microbiological studies of the diseased equine oral cavity being based on apical infections. Bacteria isolated from these cases have included unidentified *Peptostreptococcus* sp. (Scott *et al*. 1977; Mackintosh and Colles 1987*), Bacteroides fragilis, Bacteroides melaninogenicus, Bacteroides oralis, Peptostreptococcus anaerobis, Fusobacterium mortiferum* and unidentified *Fusobacterium* (Mackintosh and Colles 1987). In addition, Bienert *et al*. (2003) detected a bacterial population which was predominantly compromised of anaerobes with *Prevotella* sp. isolated from 80% and *Fusobacterium* sp. isolated from 75% of apical infection cases. Novel bacterial species have been identified more recently in the equine oral cavity by Collins *et al*. (2004) who discovered a novel species of *Streptococcus*, closely related to *Streptococcus mutans* named *Streptococcus devriesei* which was found to be more commonly present in equine infundibular caries lesions than in healthy

teeth (Lundström *et al*. 2007). In a study examining bacteria present in the normal and diseased equine oral cavity and equine lower respiratory infections, Dorsch *et al*. (2001) identified a novel species of *Fusobacterium* named *Fusobacterium equinum*.

Baker (1979) performed traditional bacterial culture and biochemical identification of organisms from orally healthy and periodontitis horses and noticed a significant bacterial population shift between oral health and periodontitis. 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. In periodontitis, the predominant genera present were *Streptococcus, Fusobacterium* and coliforms. In addition, *Campylobacter* sp. and spirochaetes were detected in direct smears of periodontally diseased samples (Baker 1979). More recently, after histological examination of diseased tissue sections, Cox *et al*. (2012) described the presence of spirochaetes in gingival epithelium (Fig. 1.15) and cocci on the gingival epithelium (Fig.1.16). In addition, Sykora *et al*. (2014), reported that *Porphyromonas gingivalis, Tannerella* and *Treponema* species were more commonly detected in crevicular fluid samples from horses with clinically overt equine odontoclastic tooth resorption and hypercementosis (EOTRH) than in orally healthy horses. It was suggested however by Gao *et al*. (2016) that use of primers targeting the outer membrane protein lipid (omp28) by Sykora *et al*. (2014) may have resulted in other *Porphyromonas* being detected aside from *P. gingivalis*. The types of bacteria detected to date in the equine oral cavity in both health and periodontitis is summarised in Table 1.3.

Bacteria isolated from the equine oral cavity	
Oral Health	**Periodontitis**
Actinobacillus sp. (Gao *et al*. 2016)	
Actinobacillus equuli (Bisgaard *et al*. 2009; Platt 1973; Sternberg 1998 and 1999 and Sternberg and Brändström, 1999)	
Actinobacteria (Gao *et al*. 2016)	
Arcobacter sp. (Gao *et al*. 2016)	
Atopobium (Gao *et al*. 2016)	
Bacteroides (Bailey and Love 1990)	
Bacteroidetes (Gao *et al*. 2016)	
Campylobacter sp.	*Campylobacter* sp. (Baker 1979)
Campylobacter gracilus (Gao *et al*. 2016)	
Cardiobacterium sp.	
Chloroflexi (Gao *et al*. 2016)	
Clostridium (Bailey and Love 1990)	
Eubacterium fossor (Bailey and Love 1986)	
Firmicutes (Gao *et al*. 2016)	
Fretibacterium fastidiosum (Gao *et al*. 2016)	
Fusobacterium necrophorum (Gao *et al*. 2016)	*Fusobacterium* sp. (Baker 1979)
Fusobacterium sp. (Baker 1979; Bailey and Love 1990; Gao *et al*. 2016)	
*Gammaproteobacteria (*Gao *et al*. 2016*)*	
GN02 (Gao *et al*. 2016)	
Lactobacillus sp. (Baker 1979)	
Lautropia sp. (Gao *et al*. 2016)	
Leptotrichia sp.	
Leptotrichia hongkongensis (Gao *et al*. 2016)	
Megasphaera (Bailey and Love 1990)	
Moraxella sp. (dos Santos *et al*. 2014; Gao et al. 2016)	
Nesseria sp.	
Nesseria shayganii (Gao *et al*. 2016)	
Nocardia sp. (dos Santos *et al*. 2014)	
Olsenella sp. (Gao *et al*. 2016)	
Pasteurellaceae (Gao *et al*. 2016)	
Peptostreptococcus (Bailey and Love 1990)	

Table 1.3 Bacteria isolated from the healthy equine oral cavity and equine periodontitis

Porphyromonas catoniae *Porphyromonas circumdentaria* *Porphyromonas gulae* *Porphyromonas macacae* *Porphyromonas* sp. (all from Gao *et al*. 2016)	*Porphyromonas gingivalis* (Sykora *et al*. 2014)
Prevotella sp. (Gao *et al*. 2016)	
Proprionbacterium sp. (Gao *et al*. 2016)	
Proteobacteria (Gao *et al*. 2016)	
Pyramidobacter (Gao *et al*. 2016)	
Spirochaetes (Cox *et al*. 2012; Gao *et al*. 2016)	
SR1 (Gao *et al*. 2016)	
Staphylococcus sp. (dos Santos *et al*. 2014)	
Streptococcus sp. (Baker 1979; dos Santos *et al*. 2014; Gao *et al*. 2016) *Streptococcus minor* (Gao *et al*. 2016) *Streptococcus suis* (Gao *et al*. 2016)	*Streptococcus* sp. (Baker 1979)
Suttonella sp. (Gao *et al*. 2016)	
Synergistetes (Gao *et al*. 2016)	
Tannerella sp. (Sykora *et al*. 2014; Gao *et al*. 2016) *Tannerella forsythia* (Sykora *et al*. 2014; Gao *et al*. 2016)	*Tannerella* sp. (Sykora *et al*. 2014) *Tannerella forsythia* (Sykora *et al*. 2014)
Tenericutes (Gao *et al*. 2016)	
TM7 (Gao *et al*. 2016)	
Treponema sp. (Sykora *et al*. 2014; Gao *et al*. 2016) *Treponema denticola* *Treponema medium* *Treponema pectinovorum* *Trepomena vincentii* *Treponema porcinum* *Treponema lecithinolyticum (*all Sykora *et al*. *2014)*	*Treponema* sp. *Treponema medium* *Treponema denticola* *Treponema pectinovorum* *Treponema putidium (All Sykora et al. 2014)*
Veillonella sp. (Baker 1979; Bailey and Love 1990; Gao *et al*. 2016) *Veillonella parvula* (Gao *et al*. 2016)	

Table 1.3 (Continued) Bacteria isolated from the healthy equine oral cavity and equine periodontitis

1.3.2.3 Exploring the equine oral microbiome

When analysing any microbiological community, the number and diversity of bacterial species detected is dependent on the method of analysis used (Lozupone *et al*. 2008). Studies in humans have estimated that approximately 70% of oral bacteria cannot be cultured by conventional means (Dewhirst et al. 2010). It is therefore likely that previous culture studies have vastly underestimated the number and variety of bacterial species present in the equine oral cavity, since novel species and bacteria difficult to culture would have gone undetected. It is now possible to characterise the oral microbiome using methods which do not rely on culture and so can detect novel and previously uncultured bacteria. One such method involves high-throughput sequencing of the gene encoding the 16S rRNA sub-unit of the bacterial ribosome that is useful in assessing the composition of complex microbial communities directly from clinical samples (Song *et al*. 2013). The 16S rRNA gene is universal in bacteria but not found in mammalian cells. It is approximately 1550 base pairs in length and consists of nine hypervariable regions (V1-V9) which are interspersed between constant regions (Song *et al*. 2013). These constant regions are highly conserved between phyla and are thought to be so due to the critical importance of the ribosome to basic cell function (Clarridge 2004). Sequencing of hypervariable regions may allow for differentiation of bacteria to species level (Chakravoty *et al*. 2008). Most hypervariable regions occur within the first 500 base pairs of the 16S rRNA gene (Keller 2010) and so sequencing of the whole gene is not required, with read lengths of 500-700 base pairs being sufficient for identification at species level (Paster *et al*. 2001; Clarridge 2004; Song 2013). However, whole 16S rRNA gene sequencing is desirable for identifying previously unknown species (Clarridge 2004). It is highly likely that the equine oral microbiome in both health and disease contains many novel and previously uncharacterised species as few prior studies have been published using this approach (Gao *et al*. 2016). In addition to high throughput 16S rRNA sequencing to uncover the oral microbiome, the 16S rRNA gene can also be used as a target in PCR reactions, to screen samples for specific bacteria. This technique was used by Sykora *et al*. (2014) who screened DNA extracted from gingival crevicular fluid samples from horses with and without EOTRH for known human periodontal pathogens. The products of the PCR reactions were then sequenced and identified by BLAST (The Basic Local Alignment Search Tool)

analysis, with both known and novel *Tannerella* and *Treponema* species being detected in association with EOTRH.

Non-culture dependent methods allow more complete analysis of the oral microbiome by identifying both cultivable and non-cultivable genera. This technique is of particular use in investigating periodontitis as the bacteria known to be involved in periodontal disease pathogenesis such as spirochaetes (Dewhirst *et al*. 2000) are fastidious and difficult to culture. In addition to the resident population of commensal oral bacteria and those which may be involved in periodontitis, is is important to acknowledge the influx of bacteria which may be brought into the oral cavity as the horse naturally spends up to 18 hours per day grazing. Whilst bacteria residing in soil and on grasses and other feedstuffs may or may not be able to colonise the oral cavity, it is possible bacterial DNA from these sources may be present and identified in the equine oral cavity.

1.4 Innate immunity in the oral cavity

1.4.1 The innate immune response

Pathogens encountered by the vertebrate immune system evoke two types of immune response: the innate immune response and the acquired immune response. While acquired immunity involves the development and production of specific antibodies to encountered pathogens, innate immunity is less specific but much quicker to respond and is often known as the 'first line of defence' against invading microorganisms (Akira and Takeda, 2004). Although innate immunity is a broad spectrum immune response, previously described as being 'non-specific' it is able to distinguish between self and a variety of groups of micro-organism (Akira 2006). The need for quick, broad spectrum responses against invading pathogens is obvious and consequently, the innate immune system is present throughout lower (invertebrates and plants) and higher organisms, having developed early on in evolutionary history before separation of vertebrates and invertebrates (Kimbrell and Beutler 2001). Integral components of the system such as pathogen recognition receptors are present in early, primitive organisms and have been highly conserved (Yuen *et al*. 2014).

The innate immune system has two main functions, the first being the initial host response to a pathogen by containing and reducing numbers of the invading

microorganism by nonspecific mechanisms such as phagocytosis. Secondly, through a number of receptor molecules and activation pathways which respond to the infecting microbes, the innate immune system is able to induce the acquired immune response resulting in a more specific and effective combined response (Medzhitov and Janeway 2000). After activation of innate immunity, subsequent cell signalling via cytokine and chemokine release attracts a variety of other immune cells, such as antigen presenting cells to the source of infection and this provides a vital link between innate and adaptive immunity. Antigen presenting cells (APCs) such as dendritic cells, macrophages and B-cells uptake and process antigens such as peptides, toxins and intracellular pathogens and display fragments of these antigens on their cell surface together with major histocompatibility complexes and other molecules required for lymphocyte activation. In addition to subsequent cytokine release by the APC, naïve T-cells are then stimulated to become pathogen specific and mediate the adaptive immune response (Janeway *et al*. 2010).

1.4.1.1 Pathogen recognition in the oral cavity

Invading pathogens are recognised by pattern recognition receptors (PRRs), a family of receptors which respond to highly conserved microbial molecules known as pathogen-associated molecular patterns (PAMPs) (Akira *et al*. 2006). PAMPs are often molecules essential for survival or important in the pathogenicity of the organism (such as LPS) and because they are only produced by microbes they can be differentiated from host cells by the host immune system. Although there are several groups of PRRs, all recognise specific PAMPs and following ligand binding, specific signalling pathways are activated in order to produce a distinct response (Akira *et al*. 2006). Four families of PRRs have been identified and include transmembrane proteins Toll-like receptors and C-type lectin receptors (CLRs) as well as cytoplasmic proteins, retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi and Akira 2010). Activation of PRRs by their binding ligand then begins a cell signalling cascade, up or downregulating expression of genes encoding cytokines, chemokines and other proteins involved in the inflammatory and immune responses. A summary of pattern recognition receptors and their ligands is provided in Table 1.4 with special attention given

to Toll-like receptors in Table 1.5

Pattern Recognition Receptor	Ligand(s) (Origin)	Reference
RLRs		
RIG-1	Short dsRNA, 5'triphospahte dsRNA (Viral)	Takeuchi and Akira 2010
MDA5	Long dsRNA (Viral)	Takeuchi and Akira 2010
LGP2	ds RNA (Viral)	Li *et al*. 2009
NRLs		
NOD1	Bacterial peptidoglycans, g-D-glutamyl-meso- diaminopimelic acid (Bacterial)	Takeuchi and Akira 2010
NOD2	Muramyl dipeptide (Bacterial)	Takeuchi and Akira 2010
CLRs		
Dectin-1	ß-glucan (Fungi)	Takeuchi and Akira 2010
Dectin-2	ß-glucan (Fungi)	Takeuchi and Akira 2010
MINCLE	spliceosome-associated protein 130 (SAP130) (Necrotic host cells; fungi)	Yamasaki *et al*. 2008; 2009
TLRs	See table 1.5	

Table 1.4 Pattern recognition receptors and their ligands

1.4.1.2 Toll-like receptors

A major group of PRRs are known as the Toll-like receptors (TLRs) (Table 1.5) which are highly important receptors involved in both the initial immune response and also activation of the adaptive immune response, thus providing the link between the two branches of the immune system (Akira *et al*. 2001; Akira and Takeda 2004). TLRs have been extensively studied and are thus one of the best characterised families of pattern recognition receptor.

As PAMPs are highly conserved between different bacterial and viral classes and different TLRs respond to each of these classes, the host is able to gather information on the nature of the pathogen from detected PAMPs (Akira and Hoshino, 2003). These pathways are also very useful for the researcher since by measuring the levels of expression of different TLRs in diseased tissues it is possible to gain insight into which type of putative pathogens may be important

in the disease process itself. A summary of Toll like receptors and their ligands is provided in Table 1.5.

Toll like receptors play an integral role in 'first-line' pathogen recognition and have been effective components of innate immunity throughout evolution and have thus been highly conserved (Akira *et al*. 2006). As TLRs are also highly conserved across species (Akira *et al*. 2006) this may allow comparison of innate immune responses between species as it is possible the mechanisms behind each highly conserved TLR's activation and response is very similar.

In addition, the role of TLRs in periodontitis in other species, especially the human has been extensively studied (Lappin *et al*. 2001; Mahanonda and Sarah *et al*. 2006; Pichyangkul 2007; Chen *et al*. 2012). The interaction between periodontopathogenic bacteria and TLRs is well recognised (Kikkert *et al*. 2007; Wara-aswapati *et al*. 2013). The highly conserved nature of TLRs both throughout evolution and across species, in addition to the wealth of information regarding their role in human periodontitis makes this family of pattern recognition receptors suitable for study in this thesis. Although few studies exist investigating oral TLRs in the horse, this family of receptors is well-studied in other equine diseases (Berndt *et al*. 2009; Stokes *et al*. 2010; Fossum *et al*. 2012) leading to good availability of reagents and primers.

TLR	Ligand(s) (Origin)	Reference
TLR 1/2	Triacyl lipoprotein	Kumar *et al*. 2009
TLR 2	Lipotechioic acid (LTA)(Gram +ve bacteria)	Takeuchi *et al*. 1999
	Lipoproteins (Gram +ve bacteria)	
	Lipopeptides ((Gram +ve bacteria)	
	Lipopolysaccheride (LPS) (Gram –ve bacteria)	Lappin *et al*. 2007
	Haemagglutanin protein (Viral)	Akira *et al*. 2006
	tGPI-mutin (Parasitic)	Akira *et al*. 2006
	Glucuronoxylomannan (Fungal)	Akira *et al*. 2006
	Phospholipomannan (Fungal)	Akira *et al*. 2006
TLR 3	ssRNA virus (WNV), dsRNA virus(Reovirus)	Kumar *et al*. 2009; Takeuchi
	RSV, MCMV	and Akira 2010
TLR 4	LPS (Gram –ve bacteria)	Takeuchi and Akira 2010
	Mannan (Fungal)	Kumar *et al*. 2009
	Glucuronoxylomannan (Fungal)	Akira *et al*. 2009
	Glycoinositolphospholipids (Parasitic)	Kumar *et al*. 2009
	Envelope proteins (Viral)	Akira *et al*. 2006
	Fibrinogen (Host)	Akira *et al*. 2006
	Heat-shock protein (Host)	
TLR 5	Flagellin (Bacterial)	Underhill and Ozinsky 2002
TLR 6/2	Diacyl lipoprotein	Kumar *et al*. 2009
	Zymosan (Fungal)	Akira *et al*. 2006
	LTA	Akira *et al*. 2006
TLR 7	ssRNA (Viral)	Takeuchi and Akira 2010
TLR 8 (human)	ssRNA (Viral)	Kumar *et al*. 2009
TLR 9	unmethylated cytosine-phosphate-guanine DNA (Bacterial)	Hemmi *et al*. 2000
	ds DNA (Viral)	Kumar *et al*. 2009
	Hemozoin (Parasitic)	Akira *et al*. 2006
TLR10	Unknown	Takeuchi and Akira 2010
	Potential *Listeria monocytogenes* ligand source	Regan *et al*. 2013
	Potential viral associated ligand	Lee *et al*. 2014
TLR 11 (mice)	Profilin-like molecule (Protozoa)	Takeuchi and Akira 2010

Table 1.5 Toll-like receptors and their ligands

1.4.1.2.1 TLR 2 with TLR 1 and TLR 6 heterodimers

TLR 2 is found on the plasma membrane of monocytes, dendritic cells, mast cells, eosinophils and basophils and responds to a wide variety of molecules from fungal, bacterial and mycobacterial pathogens. While TLR 2 is mainly activated by lipotechioic acid, lipoproteins and lipopeptides from Gram-positive bacterial species (Takeuchi *et al*. 1999), many Gram-negative periodontopathogenic micro-organisms, which express LPS, also activate TLR 2 efficiently (Lappin *et al*. 2011). TLR 2 also associates with other TLR molecules which allows for recognition of a wide variety of molecules and has been shown to form functional heterodimers with TLR 1 and TLR 6 (Ozinsky *et al*. 2000), in addition to being able to function alone (Buwitt-Beckmann *et al*. 2006). The TLR1/2 heterodimer can recognise triacyl lipopeptides whereas the TLR6/2 heterodimer can recognise diacyl lipopeptides (Kumar *et al*. 2009). Expression of TLR2 has also been shown to be upregulated after LPS stimulation of TLR4 (Fan *et al*. 2003). The known periodontal pathogens *Prevotella intermedia*, *Prevotella nigrescens and Veillonella parvula* have been shown to stimulate cytokine expression by activation of TLR 2 (Kikkert *et al*. 2007).

1.4.1.2.2 TLR 4

TLR 4 is located within the plasma membrane of macrophages, dendritic cells, mast cells and eosinophils and mainly detects the presence of Gram-negative bacteria by binding with LPS found on the bacterial cell surface (Akira *et al*. 2006). There are also two accessory proteins known as MD2 and CD14 which assist in detection of LPS; MD2 binds to TLR 4 within the cell and is important in ensuring correct transport of TLR 4 to the cell surface and recognition of surrounding LPS. In the bloodstream, free LPS is bound by LPS-binding protein and transferred to CD14 which is found on the surface of macrophages, dendritic cells and neutrophils. CD14 associates with TLR 4 and when LPS is bound to CD14, TLR 4 is activated resulting in cytokine release. *Veillonella parvula* a known periodontopathogenic bacterium has been shown to induce cytokine expression by activation of TLR4 (Kikkert *et al*. 2007).

1.4.1.2.3 TLR 5

TLR5 recognises the flagellin protein of flagellated bacteria and is located on the plasma membrane (Underhill and Ozinsky 2002). Following recognition of flagellin by TLR5, the myeloid differentiation primary-response protein 88 (MyD88) pathway is activated and inflammatory cytokines such as TNFα, IL-1β and IL-6 are released (Kumar *et al*. 2005; Beklen *et al*. 2009). Using immunohistochemistry to localise TLR 5 in periodontally diseased human gingival tissue samples, Beklen *et al*. (2008, 2009) reported a significant increase in TLR 5 in diseased samples.

1.4.1.2.4 TLR 9

TLR 9 recognises and binds to unmethylated cytosine-phosphate-guanine bacterial, fungal and viral DNA (Hemmi *et al*. 2000). The binding of unmethylated CpG DNA to TLR9 induces signalling within the cell via transcription factor NF-κB, resulting in activation of antigen presenting cells and subsequent release of pro-inflammatory cytokines in a Th1 type response to the pathogen. In contrast to TLRs 2 and 4 which are found on the plasma membrane, TLR9 is found within the cell, specifically in the endosome of many different cell types such as dendritic cells (Demedts *et al*. 2006), eosinophils (Wong *et al*. 2007) and B cells (Krieg *et al*. 1995). O' Mahony *et al*. (2008) also detected the presence of TLR 9 in human neutrophils and macrophages. Increased levels of TLR 9 mRNA have been detected in periodontally diseased human gingival tissue (Beklen *et al*. 2008).

1.4.1.3 Signalling Pathways

Toll-like receptors belong to the Toll/IL-1R (TIR) superfamily which is comprised of the Ig domain family (IL-1 and IL-18 receptors as well as IL-1R-like receptors), the leucine-rich domain family (TLRs and similar receptors), and a series of TIR domain-containing intracellular adapter molecules. It is the intracellular TIR domain, characterized by the presence of three highly homologous regions which is important in initiating the cell signalling cascade following ligand binding to the Toll like-receptor (Boraschi and Tagliabue 2006). The signalling pathway is then activated leading to recruitment of TIR domain-containing adaptors such as

MyD88, TIR domain–containing adapter protein (TIRAP), domain–containing adapter protein inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) (Kumar *et al*. 2009). These adaptor molecules act as a midpoint between initial ligand binding to the TLR and the later stages of the signalling pathway, most often a release of transcription factor nuclear factor kappa B (NF-κB) and subsequent induction of gene expression within the cell nucleus. In addition to adaptor molecules, other downstream signalling molecules such as IL-1R-associated kinases (IRAKs), transforming growth factor-beta activated kinase (TAK1), TAK1-binding proteins 1 and 2 and tumour-necrosis factor receptor-associated factor 6 (TRAF6) are also recruited following ligand binding (Akira and Takeda 2004) (Fig 1.17).

Pro-inflammatory cytokines and Type 1 IFNs are produced after activation of signalling pathways by the MyD88 and TRIF adaptors respectively. MyD88 is recruited by almost all TLRs except TLR3 (Kumar *et al*. 2006) and is required for induction of inflammatory cytokines, including IL-6 and IL-12p40 (Kaisho and Akira 2006). As shown in Figure 1.17, the TLR1/2 and TLR2/6 heterodimers use MyD88 and TIRAP/MAL (MyD88-adaptor-like) adaptors, TLR4 uses MyD88, TIRAP/MAL, TRAM and TRIF. TLR 7/8, TLR9 and TLR11 only use MyD88 and TLR3 is MyD88 independent, only using the TRIF adaptor (Kawai and Akira 2006). TLR2 and 4 require an additional adaptor, the TIRAP/MAL adaptor, in order to induce inflammation through MyD88 signalling (Kawai and Akira 2006). Whereas the MyD88-dependant pathway controls inflammatory responses, the TRIF pathway mainly controls Type 1 IFN responses, however in plasmacytoid dendritic cells, TLR7/8 and TLR9 are able to induce Type 1 IFN independent of MyD88 (Kawai and Akira 2006).

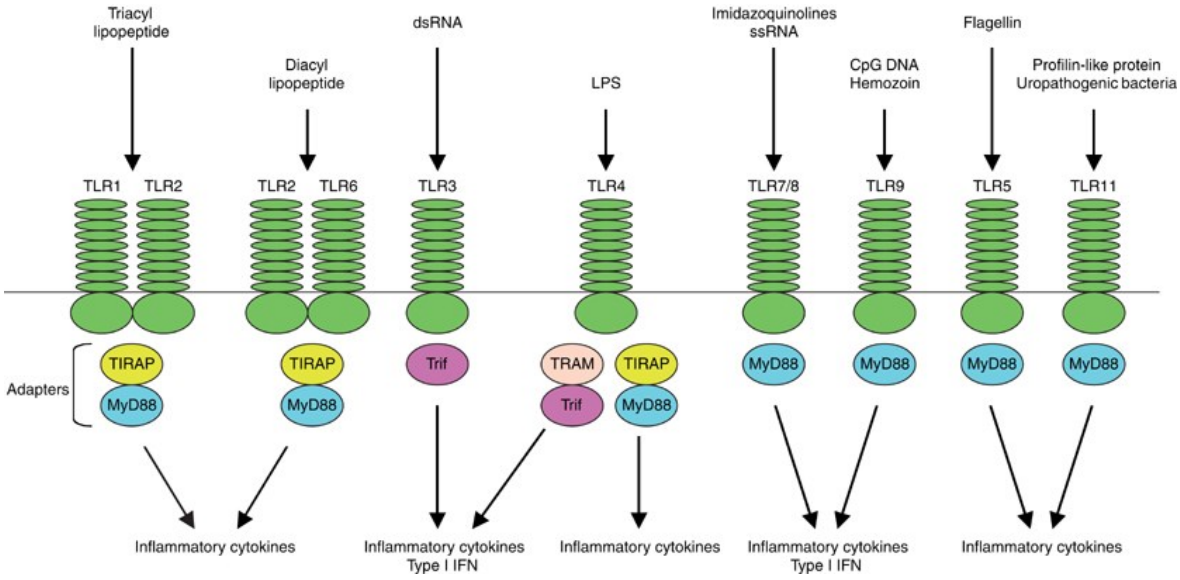


Figure 1.17 Toll-like receptor signalling pathways
(Reproduced from Kawai and Akira 2006)

1.4.1.4 Chemokines

Chemokines are small, heparin binding proteins which are produced primarily by monocytes and macrophages as a response to infection or cell damage and also direct cell migration. In response to chemokine production, lymphocytes, monocytes and neutrophils are attracted to areas of damaged or infected tissue (Lydyard *et al*. 2011) and so these small proteins have a central role in the inflammatory response (Janeway *et al*. 1999). Four groups of chemokines exist based on their amino acid sequences, namely, the CXCL group, the ELR group, the CCL group and the CX_3C group. A summary of selected chemokines has been provided in Table 1.6 (adapted from Murphy *et al*. 2008).

Chemokine Systematic name	Common Name
CXCL (ELR+)	
1	**GRO-α**
2	**GRO-ß**
3	**GRO-γ**
5	**ENA-78**
6	**GCP-2**
7	**NAP-2**
8	**IL-8**
14	**BRAK**
15	Lungkine/WECHE
ELR$^-$	
4	**PF4**
9	**Mig**
10	**IP-10**
11	**I-TAC**
12	**SDF-1α/ß**
13	**BLC/BCA-1**
16	**No common name**
CCL	
1	**I-309**
2	**MCP-1**
3	**MIP-1α**
4	**MIP-1ß**
5	**RANTES**
6	**C10/MRP1**
7	**MCP-3**
8	**MCP-4**
9	**MRP-2/MIP-1γ**
11	**Eotaxin**
12	**MCP-5**
13	**MCP-4**
14a	**HCC-1**
14b	**HCC-3**
15	**MIP-5/HCC-2**
16	**HCC-4/LEC**
17	**TARC**
18	**DC-CK1/PARC**
19	**MIP-3ß/ELC**
20	**MIP-3α/LARC**
21	**6Ckine/SLC**

22	**MDC**
23	**MPIF-1/CK-β**
24	**Eotaxin-2MPIF**
25	**TECK**
26	**Eotaxin-3**
27	**CTACK**
28	**MEC**
CX_3C	
XCL1	**Lymphotacin**
XCL2	**scm-1β**
CX3CL1	**Fractalkine**

Table 1.6 Chemokines (adapted from Murphy 2008)

1.4.1.5 Cytokines

Cytokines are small proteins which are released in response to activating stimuli such as TLR activation by PAMPs and subsequently induce further responses by binding to specific molecules (Janeway *et al*. 2010). Cytokines can be grouped into families for example: colony stimulating factors, the interferons, several interleukin families (IL-1, IL-6, IL-10, IL-12, etc.), the Tumour Necrosis factor (TNF) family members and the unassigned. A summary of the cytokines belonging to each family is provided in Table 1.7 (Adapted from Murphy 2008), although the interleukins are listed collectively.

Cytokine family	Cytokine
Colony Stimulating factors	G-CSF
	GM-CSF
	M-CSF
Interferons	IFN-α,β,γ
Interleukins	IL-1α, IL-1β, IL-1RA
	IL-2
	IL-3
	IL-4
	IL-5

	IL-6
	IL-7
	IL-9
	IL-10
	IL-11
	IL-12
	IL-13
	IL-15
	IL-16
	IL-17A,B,C,D,E,F
	IL-18
	IL-19
	IL-20
	IL-21
	IL-22
	IL-23
	IL-24
	IL-25 (IL-17E)
	IL-26
	IL-27
	IL-28A, IL-28B
	IL-29
	IL-33
	IL-36α,β,γ
	LIF
	OSM
TNF family	TNF-α,β LT-α, LT-β CD 40 ligand FAS ligand CD27 ligand CD 30 ligand 4-1BBL Trail OPG-L (RANK-L) APRIL LIGHT TWEAK BAFF (CD257)

Cystine knot cytokines (Rider and Mulloy 2010)	TGF-β1 Nodal GDF-1 GDF-3 GDF-8/Myostatin GDF-9 GDF-15 BMP-1,2,3, 3B,4,5,6,7 BMP-8a BMP-9/GDF-2 BMP-10 BMP-11/GDF-11 BMP-12/GDF-7 BMP-13/GDF-6 BMP-14/GDF-5 BMP-15
Unassigned	MIF

Table 1.7 Cytokine families (adapted from Murphy 2007)

Cytokines have been well studied in health and disease across different species including in equine disease (Horov 2003). However, there have been, to date, no studies on the role of cytokines in equine periodontitis, in contrast their involvement in human periodontal disease has been extensively studied previously giving a vast body of literature.

In particular, the Th1, Th2 and Th17 cytokines have received particular focus in human periodontitis studies (Gemmel and Seymour 2004). T-helper (Th) cells can be split into groups which produce a distinct functional pattern of cytokines (Cruse and Lewis, 2010). For the purpose of this study, Th1 cytokines which tend to produce a pro-inflammatory response, Th2 cytokines which tend to produce an anti-inflammatory response and Th17 cytokines will be investigated. The eight cytokines investigated in this study can be split into three groups; Th1 cytokines IL-1β, TNFα, IFN-ɣ and IL-12, the Th2 response cytokines including IL-4, IL-6 and anti-inflammatory cytokine IL-10 and lastly the Th17 cytokine IL-17. The Th2 response regulates the inflammatory response and counteracts the effects of the pro-inflammatory Th1 response. As these cytokines are already well studied and associated with periodontitis in other species, including humans, they make good candidates for use in equine periodontitis studies. The

selected cytokines will allow for comparisons to be drawn between immunological mechanisms at work in periodontally diseased equine tissue and tissue from other species and allow similarities and differences in aetiopathogenesis of periodontitis across species to be recognised. Although it is possible other cytokines and also chemokines play important roles in the aetiopathogenesis of equine periodontitis, focus remained on the selected cytokines for the following reasons: the availability of reagents, the need to keep within the planned scope of the study and time restrictions of a PhD project.

1.4.1.5.1 Pro-inflammatory cytokines

1.4.1.5.1.1 IL-1ß

IL-1ß is an IL-1 family member, a pro-inflammatory cytokine produced by macrophages, monocytes, lymphocytes, fibroblasts and epithelial cells (Okada 1998) and plays an important role in supporting the proliferation of fibroblasts, endothelial cells and keratinocytes and production of collagenase, prostaglandin E2, fibronectin, procollagen and hyaluronase (Janeway *et al*. 2010).

It has been established that IL-1ß plays a pivotal role in periodontal tissue destruction (Liu *et al*. 1996) by enhancing bone resorption and inducing synthesis of matrix metalloproteinases (MMPs) which may lead to further tissue destruction. Increased levels of IL-1ß in periodontitis cases were also found by Beklen *et al*. (1997) who also discovered higher levels of MMPs 1 and 3. This is further supported by Mogi *et al*. (1999) who reported elevated levels of IL-1ß in patients with periodontitis when compared to controls. IL-1ß has been shown to increase with severity of periodontitis lesions (Sanchez *et al*. 2013; Oh *et al*. 2015) and decrease following clinical intervention in humans (Reis *et al*. 2014; Oh *et al*. 2015). Gingival epithelial cell infection with *Fusobacterium nucleatum*, a known periodontal pathogen in humans, has been shown to result in production of IL-1ß (Bui *et al*. 2015).

1.4.1.5.1.2 TNF-α

TNF-α (the prototype TNF family cytokine) also promotes inflammation and is an important mediator of endotoxaemia (Su *et al*. 1991). It supports production of collagenase by fibroblasts and can also induce IL-1 and prostaglandin E2

production, as well as activating osteoclasts (Okada 1997). It has been suggested that expression of TNFα in gingival tissue, together with IL-1ß, plays an instrumental role in periodontal tissue destruction by stimulating osteoclast development and loss of alveolar bone (Graves and Cochran 2003), in addition to enhancing synthesis of collagenase by fibroblasts (Meikle *et al*. 1989 and Okada and Murakami 1998). Since these early observations, levels of TNF-α have been shown to increase in gingival crevicular fluid of human periodontitis cases (Gokul 2012) and in rodents (Liao *et al*. 2014). In addition, Koppolu *et al*. (2014) has shown a decrease in TNF-α following periodontal treatment. Yücel *et al*. (2015) associated a genetic polymorphism of the TNFα gene with aggressive human periodontitis, in agreement with earlier studies by Erciyas *et al*. (2010).

1.4.1.5.2 Th1 cytokines

1.4.1.5.2.1 IL-12

IL-12 (NK-stimulating factor and IL-12 family cytokine) is key in the development of cell-mediated immune responses and is produced by macrophages and dendritic cells; it activates NK cells and induces CD4 T cell differentiation into Th1-like cells (Honda *et al*. 2008). This heterodimeric cytokine, comprised of p35 and p40 subunits, is important in developing Th1 like immunity to related bacterial species and has been previously explored as a potential equine vaccine adjuvant due to its immunomodulatory effects, especially in neonates (Measley *et al*. 2007). Honda *et al*. (2008) found higher expression levels of IL-12p35 in chronic periodontitis lesions compared with gingivitis in humans.

1.4.1.5.2.2 IFN-ɣ

IFN-ɣ (Interferon family member) is produced by Th1 cells, stimulates phagocytosis by macrophages and also acts as a positive regulator of the Th1 response, thereby increasing production of cytokines such as IL-1 α, IL-1ß (O'Gara, 1989; Trinchineri *et al*. 1993). IFN-ɣ increases IL-12 responsiveness (Mullen *et al*. 2001) and suppresses the Th2 responses (Huber *et al*. 2010) and so is also regarded as a pro-inflammatory cytokine.

Significantly higher levels of IFN-ɣ expression have been noted in active human periodontitis lesions (Aggarwal *et al*. 2003) and positive correlations between IFN-ɣ levels in diseased tissue and periodontal disease severity have been made (Ebersole and Taubman 1994; Tsia *et al*. 2007; Ukai *et al*. 2001 and Papathanasiou *et al*. 2013).

1.4.1.5.3 Th2 response cytokines

1.4.1.5.3.1 IL-4

IL-4 is thought to stimulate B-cell proliferation and subsequent immunoglobulin production (Dohnmann *et al*. 2000), whilst inhibiting phagocytosis and production of periodontally destructive Th1 cytokines such as IL-1ß and TNF-α (Gemmel *et al*. 1997). Both human and murine IL-4 have been shown to induce immunoglobulin class switch to IgE in B cells stimulated by LPS (Coffman and Carty 1986 and Coffman *et al*. 1986). IL-4, together with IL-13, is required for inducing differentiation of T cells in to Th2 cells and is important in the production of an allergic inflammatory response (Cordeu *et al*. 2003).

A lack of IL-4 in gingival tissue can lead to excessive production of IL-1ß, TNFα and CD-14 and may predispose patients with gingivitis to develop more severe periodontitis (Shapira *et al*. 1992; Graves *et al*. 2011). It has also been found that the ratio of gingival cells expressing IL-4 to gingival cells expressing IFN-ɣ is significantly lower in severe periodontal lesions compared with moderate lesions, supporting the hypothesis that a lack of IL-4 may be linked with more severe periodontal disease. In addition to these findings, an IL-4 genetic polymorphism was discovered by Anovazzi *et al*. (2010), which resulted in the affected individual being more susceptible to developing chronic periodontitis.

1.4.1.5.4 IL-6

IL-6 is a (IL-6 family) cytokine produced by monocytes, endothelial cells, fibroblasts, macrophages, B cells and T cells (Okado and Murakam 1998) and is strongly induced in endotoxaemia. IL-6 also promotes maturation of B cells into plasma cells which produce immunoglobulins (Hirano 1991).

Levels of IL-6 have been found to be higher in gingival tissue from patients with gingivitis than in healthy controls (Bartold and Hayes 1991) and inflamed periodontal tissues have shown to have an increase in B cells and plasma cells which Fujihashi *et al*. (1993) demonstrated was due to an increase in expression of IL-6 in periodontitis cases. Human periodontal pathogens (*Treponema denticola*, *Treponema vincentii* and *Treponema medium)* have been shown to induce expression of significant levels of both IL-6 mRNA and IL-8 mRNA in human gingival epitheial cells (Asai *et al*. 2003). T helper cells from patients with aggressive periodontitis activated with *P. gingivalis* outer membrane protein have been shown to produce signifigantly higher levels of IL-6 than cells from healthy controls (Gonzales *et al*. 2014) and Matsuda *et al*. (2015) detected higher serum levels of IL-6 in mice with experimentally induced periodontitis.

1.4.1.5.5 Anti-inflammatory cytokine IL-10

IL-10 (IL-10 family) is regarded as an anti-inflammatory cytokine due to its ability to potently suppress macrophage and monocyte function, thus inhibiting the synthesis of pro-inflammatory cytokines. In addition, it has been shown that IL-10 can inhibit production of monokines, nitrous oxide (NO) and co-stimulatory molecules as well as reducing expression of Class II MHC molecules (Moore *et al*. 2001). IL-10 was first described as cytokine synthesis inhibitory factor (CSIF), produced by mouse Th2 cells, inhibiting activation and cytokine production by Th1 cells and promoting a shift towards a Th2 response (Fiorentino *et al*. 1989). It has a wide immunoregulatory role in that it can regulate differentiation of B and T cells, NK cells, mast cells and dendritic cells amongst others (Moore *et al*. 2001).

IL-10 down regulates the pro-inflammatory response, having a protective role against disease progression in human periodontitis (Bozkurt *et al*. 2006) and IL-10-producing cells are widely distributed in periodontitis granulation tissue (Lappin *et al*. 2001). The inhibitory effects of IL-10 on bone resorption in periodontitis has been recently reviewed by Zhang *et al*. (2014).

1.4.1.5.6 Th17 response cytokine – IL-17

The IL-17 family is a group of pro-inflammatory cytokines produced by a subset of T helper cells known as Th17 cells and comprises six members, IL-17A to Il-17F. CD8+ T cells, CD4+ T cells, memory CD4+ cells, NK cells and neutrophils are also able to produce IL-17 (Korn *et al*. 2007). Release of IL-17 induces an inflammatory response via further production of cytokines and chemokines such as IL-6, IL-8, CXCL1 and CXCL10 (Korn *et al*. 2007). IL-17 also plays a key role in recruiting and activating neutrophils at the site of the inflammatory response, further enhancing the immune response against invading pathogens (Aggarwal *et al*. 2002).

Significant increases in IL-17A expression in patients with chronic periodontitis have been noted in comparison with patients with gingivitis (Honda *et al*. 2008). Beklen *et al*. (2007) used immunohistochemistry of periodontal tissue to study IL-17A levels (in addition to IL-1β and TNF-α which can be upregulated by IL-17A) and found increased levels of all three cytokines in periodontitis cases. Both IL-17A protein and mRNA have also been detected in diseased periodontal tissues (Takahashi *et al*. 2005). In addition, Awang *et al*. (2014) found that serum, saliva and gingival crevicular fluid levels of IL-17A were higher in periodontitis patients, positively correlating with disease severity. In contrast the authors found that levels of IL-17E were lower in periodontitis patients and that IL-17E inhibited *P. gingivalis* and IL-17A induced expression of cytokines *in vitro*.

1.4.1.6 Neutrophils in periodontal inflammation

Neutrophils are abundant in the gingival crevicular epithelium and are known to play a key role in the inflammatory response in periodontal disease in humans, linking the innate and adaptive immune responses (Scott and Krauss 2010). Upon histological examination of gingival tissue samples, Cox *et al*. (2012) noted neutrophil transmigration through the periodontal pocket epithelium in horses and that the median periodontal disease grade was statistically significantly higher in horses with neutrophil transmigration than in those without. Although a low level of neutrophil transmigration into the gingival sulcus has been described as a normal gingival defence mechanism in humans (Bulkacz and Carranza, 2006), neutrophil-mediated tissue destruction has been described following loss

of the normal homeostasis between the oral biofilm and the host immune system (Scott and Krauss 2010). Whilst genetic neutrophil defects have been linked to a predisposition to aggressive periodontitis some authors have described the role of 'hyper-active' neutrophils, 'primed' by inflammatory mediators such as TNF-α or microbial products such as LPS (Condliffe *et al*. 1998) in human patients with periodontitis. This results in elevated neutrophil chemotaxis which has been linked to cases of aggressive human periodontitis and increased tissue destruction (Ryder 2010). Matrix metalloproteinase 8 which is released by infiltrating neutrophils is known to play an important role in periodontal tissue destruction and alveolar bone loss (Nisengard *et al*. 2006). Although Cox et al. (2012) was the first to describe the potential role of neutrophils in equine periodontitis, their role in the equine disease is still unknown.

1.4.1.7 Complement in periodontal inflammation

The complement system is an important component in the 'first-line defence' against invading pathogens, promoting activation of the adaptive immune response and also playing a key role in maintaining the homestasis between host and microbial community such as the oral biofilm. Activation of complement drives recruitement of inflammatory cells, phagocytosis and regulation of additional immune components such as TLRs (Hajishengallis 2010). Complement provides an important bridge between the innate and adaptive immune system by its ability to activate B and T-cells through effects upon antigen presenting cells (Dunkelberger and Song. 2010). Following B-cell activation, antibody is secreted to bind pathogens. T-cell activation results in further activation of immune cells such as macrogphages and B-cells and additional cytokine activation. In addition, activation of cytotoxic T-cells results in destruction of infected cells (Cekici *et al*. 2014)

In periodontitis, there may be continuous activation of complement, due to chronic stimulation by the subgingival biofilm and this can have detrimental effects to the host, contributing to tissue destruction (Damgaard *et al*. 2014). Activated complement fragments have been noted to be present in high abundance in gingival crevicular fluid of human periodontitis patients in comparison to orally healthy individuals (Hajishengallis 2010) and genetic polymorphisms of some complement factors have been found more commonly in

periodontitis patients (Chai *et al*. 2010). Complement is known to activate neutrophils via complement receptors 3 and 5a and it has been suggested that complement may be invlolved in the 'hyper-activation' of neutrophils seen in periodontal tissues (Damgaard *et al*. 2014). The homeostatic relationship seen in oral health between the host immune system and the oral biofilm is complex and balance may be easily lost as is seen in periodontal disease. Components of the oral microbiome, for example, may disrupt the host-biofilm homeostasis and *P. gingivalis* is well known for its immunomodulatory effects, being able to subvert complement receptor 3 and 5a anaphylatoxin receptor signalling which may promote survival of the biofilm in face of challenge from the innate and adaptive immune systems.

A summary diagram of the inflammatory response involved in periodontitis is provided below in Figure 1.18.

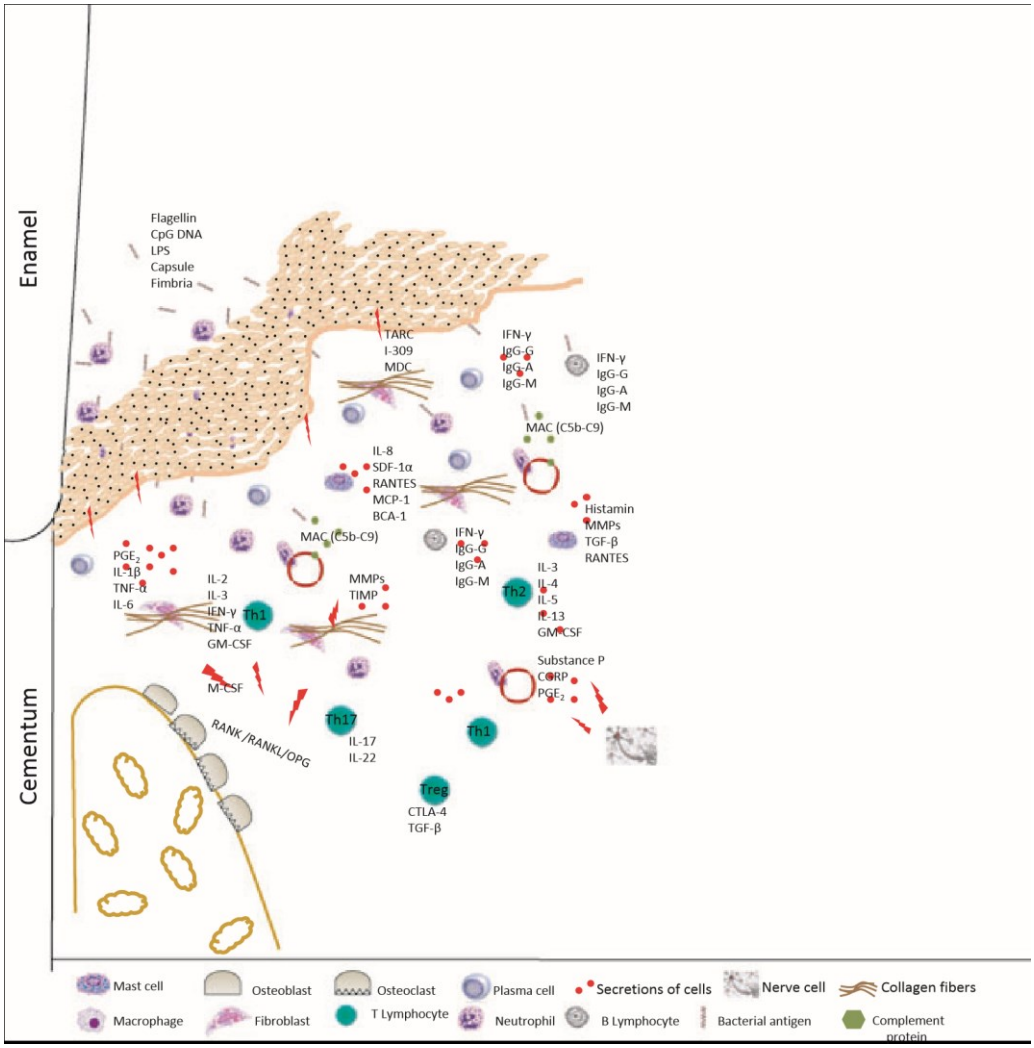


Figure 1.18 An overview of the complex inflammatory response in periodontal disease
(Reproduced from Cekici et al. (2014)

1.5 Summary

Periodontal disease is a common and painful equine oral condition that is usually induced by the mechanical impaction and subsequent bacterial fermentation of food material between and around cheek teeth. Further progression of the disease is dependent upon invasion of the periodontal tissues by the many and varied oral bacteria along with the host's immune response to these micro-organisms, with a severe host inflammatory response resulting in increased tissue breakdown and progression of the disease. In addition, key oral bacterial pathogens may alter the host's immune response to other components of the biofilm. Whilst it is clear that feed stasis and subsequent bacterial proliferation play an important role in the initiation and progression of periodontitis in the horse, there is a real need for further studies into the aetiopathogenesis of this disorder. Recent work has given an insight into which bacterial species are present in the periodontal pockets of horses with periodontitis but it is crucial to distinguish which species are important in disease pathogenesis and which simply flourish due to the change in oral environment. In particular, the interaction between bacteria of the diseased equine oral microbiome and the local immune system requires further investigation in order to provide additional insights into the aetiopathogenesis of equine periodontal disease.

1.6 Aims and Objectives

Bearing in mind the requirement for further investigation of the microbiological and immunological aspects of equine periodontitis, the aims and objectives of this study are as follows:

1. Identify the bacteria associated with a healthy oral cavity and periodontitis in horses using conventional aerobic and anaerobic microbiological culture.

2. Identify the bacteria associated with a healthy oral cavity and periodontitis in horses using high throughput bacterial 16S rRNA gene sequencing.

3. Determine which previously uncultured and novel species in addition to known cultivable species are present in oral health and periodontitis.

4. Assess the differences in bacterial populations between the healthy and periodontitis groups and identify putative pathogens.

5. Quantify the expression patterns of TLRs 2, 4 and 9, the pro-inflammatory cytokines IL-1ß and TNFα, anti-inflammatory cytokine IL-10 and Th1/Th2/Th17 cytokines IL-4, IL-6/ IL-12, IFNɣ/ IL-17/ within gingival tissue from each sample group using quantitative real time PCR.

6. Using matched data arising from Aims 1-5, establish if associations exist between the presence and quantity of bacterial species present and TLR expression.

7. Determine activation of TLRs 2, 4 and 9 by putative pathogens using specific *in-vitro* TLR assays.

8. Identify putative pathogens at species level by metagenomic (whole genome) sequencing of the microbiota from a subset of samples from equine periodontitis and healthy oral cavities.

Chapter 2

General materials and methods

2.1 Ethical approval

Ethical approval was granted prior to the start of the study by the University of Glasgow School of Veterinary Medicine Ethics and Research Committee and by the University of Edinburgh Veterinary Ethical Review Committee. All horses involved in the study presented either to the Weipers Centre Equine Hospital, University of Glasgow or the Dick Vet Equine Hospital, Royal (Dick) School of Veterinary Studies, University of Edinburgh for routine dental examination or for investigation of dental disease or had been humanely euthanatised for reasons unrelated to the oral cavity and sent for *post-mortem* examination.

2.2 Sample population

Following a thorough oral examination horses were categorised as either 'orally healthy' or 'periodontitis' and placed into two groups accordingly. The 'orally healthy' group had no evidence of gingival inflammation, no periodontal pockets and no evidence of any other oral pathology. The 'periodontitis' group had obvious gingival inflammation and periodontal pockets of over 5mm in depth (Cox *et al*. 2012). British Equine Veterinary Association (BEVA) dental charts (Figure 1.1) were completed for all horses, documenting any oral pathology present and periodontitis lesions were graded using the equine periodontal disease grading system (Table 1.1) as suggested by Cox *et al*. (2012). The Adapted Tooth Mobility Index (Klugh 2005; Wiggs and Loprise 1997) was also used to assess teeth affected by periodontitis. Horses were excluded from the study if any antibiotics had been given in the past eight weeks.

2.2.1 Sample Collection

2.2.1.1 Orally healthy horses

Oral swabs were taken from the buccal aspect of the gingival margin of teeth 307 to 308 (using the Modified Triadan Numbering System as described by Floyd

1991) from 39 orally healthy horses using Sterilin™ Amies Transport Swabs (Fisher Scientific Ltd, Loughborough, UK). A Sterilin™ Amies Transport Swab was also taken from the gingival margin of 6 orally healthy horses, washed in 0.5mLs RNA Later® for one minute and stored at -20ºC whilst awaiting transport to Academisch Centrum Tandheelkunde Amsterdam (ACTA) for further processing for use in the whole genome sequencing study. In addition to an oral swab, a 4mm x 4mm gingival biopsy was taken from the gingival margin of 307/308 from 13 horses at *post-mortem* examination and placed into 0.5mls of RNAlater® (Sigma Aldrich, Poole, UK). All tissue samples were collected within forty minutes of euthanasia.

2.2.1.2 Horses with periodontal disease

Subgingival plaque samples were taken from the periodontal pocket of 46 horses with periodontal disease using an equine dental scaler (Equine Blades Direct, Wedmore, UK.), placed into 0.5mLs FAB (Lab M Ltd, Bury, UK.) and mixed for use in microbiological culture and high throughput 16S rRNA sequencing. Subgingival plaque samples from 11 horses were placed into 0.5mL RNAlater® for use in the whole genome sequencing studies.

In addition, 5 horses with periodontal disease were sampled to compare the microbiome at three sites in the oral cavity using high throughput 16S rRNA sequencing (total of 15 samples). A sub gingival plaque sample was collected from within the periodontal pocket as previously described and placed into 0.5mL RNAlater®. Two Sterilin™ Amies Transport Swabs (Fisher Scientific Ltd., Loughborough, Leicestershire, UK) were also collected, one from the gingival margin directly above the periodontal pocket and one from the gingival margin at a site in the oral cavity unaffected by periodontitis. The corresponding site on the opposite side of the oral cavity to the lesion was used where available, for example if periodontitis affected 308/9, then 408/9 would be used as a healthy control site assuming it was disease free.

A 4mm x 4mm gingival biopsy was taken from the gingival margin of the area affected by periodontitis in 20 horses at *post-mortem* examination and placed into 0.5mL RNAlater. Control gingival biopsies from unaffected sites were also taken from eight horses at *post-mortem* examination. All gingival biopsies were

taken no longer than 40 minutes after humane euthanasia. The particular samples taken from each horse are shown in Tables 2.1 and 2.2 and information regarding particular subsets of samples used in different parts of this project is given in Tables 2.3 and 2.4.

2.2.2 Sample Processing

2.2.2.1 Microbiological culture and 16S rRNA high throughput sequencing samples

Plaque samples in Fastideous anaerobic broth (FAB) were each vortex mixed for one minute and Amies transport swabs were immersed in 0.5 mL FAB and mixed to remove bacteria. 100µL of each sample was then used to inoculate aerobic and anaerobic culture plates (described in detail in Section 4.2.2) and a crude DNA extract was prepared from the remainder of each sample by digestion with proteinase K (100 µg/mL) at 60ºC for 60 min, followed by boiling for 10 min. Following initial crude bacterial DNA extraction, samples were stored at -20ºC until transported to ACTA for analysis.

2.2.2.2 Microbiome site comparison samples

Sub-gingival plaque samples in 0.5mL RNAlater underwent crude DNA extraction as described in Section 2.2.2.1. Sterilin™ Amies Transport Swabs (Fisher Scientific Ltd., Loughborough, Leicestershire, UK) were washed in 0.5mL RNAlater® for one minute to remove bacteria. All samples were stored at -20ºC until transported to ACTA for further analysis.

2.2.2.3 Whole genome sequencing samples

Amies transport swabs from ‘orally healthy’ horses were washed in 0.5mL RNAlater as previously described then stored at -20ºC until transport to ACTA. Subgingival plaque samples in 0.5mL RNA Later from horses with periodontitis were also stored at -20ºC until transported to ACTA for analysis.

2.2.2.4 Gingival biopsies

The gingival biopsies were refrigerated overnight in RNAlater following collection and then stored at -20ºC until required for RNA extraction and cDNA synthesis.

	Orally Healthy Horses			
Horse	Swab	Plaque Sample	Gingival Biopsy	Dental Chart
H1				
H2				
H11				
H12				
H13				
H14				
H15				
H16				
H17				
H18				
H21				
H26				
H28				
H29				
H30				
H31				
H32				
H33				
H34				
H36				
H37				
H38				
H39				
H41				
H50				
H51				
H53				
H66				
H68				
h72				
H77				
H78				
H79				
H80				
h87				
H89				
BEVA 1				
BEVA 2				
BEVA 3				

Table 2.1 Orally healthy samples

	Horses with Periodontal Disease			
Horse	**Swab**	**Plaque Sample**	**Gingival Biopsy**	**Dental Chart**
Molly				
H3				
H4				
H5				
H6				
H7				
H8				
H9				
H10				
h19				
H20				
H22				
H23				
H24				
H25				
H27				
H35				
H40				
H42				
H43				
H44				
H45				
H46				
H47				
H48				
h49				
H52				
H54				
H55				
H56				
H57				
H58				
H59				
H60				
H61				
H62				
H63				
H64				
H65				
H67				
H69				
H70				
H71				
h73				
H74				
H75				
H76				
H81				
H82				
H83				
H84				
H85				
H86				

H88				
1ABC				
2ABC				
3ABC				
4ABC				
5ABC				

Table 2.2 Periodontitis samples

	Orally Healthy Samples				
Horse	**Microbiological culture**	**16S QPCR**	**High throughput 16S rRNA sequencing**	**TLR and cytokine expression analysis**	**Whole genome sequencing**
H1					
H2					
H11					
H12					
H13					
H14					
H15					
H16					
H17					
H18					
H21					
H26					
H28					
H29					
H30					
H31					
H32					
H33					
H34					
H36					
H37					
H38					
H39					
H41					
H50					
H51					
H53					
H66					
H68					
h72					
H77					
H78					
H79					
H80					
h87					
H89					
B1					
B2					
B3					

Table 2.3 Orally healthy sample subsets used in each study

	Periodontitis Samples					
	Microbiological Culture	16S QPCR	High throughput 16S rRNA sequencing	Gingival Biopsy	Whole genome sequencing	Microbiome site comparison
H0						
H3						
H4						
H5						
H6						
H7						
H8						
H9						
H10						
h19						
H20						
H22						
H23						
H24						
H25						
H27						
H35						
H40						
H42						
H43						
H44						
H45						
H46						
H47						
H48						
h49						
H52						
H54						
H55						
H56						
H57						
H58						
H59						
H60						
H61						
H62						
H63						
H64						
H65						
H67						
H69						
H70						
H71						
H73						
H74						
H75						
H76						
H81						
H82						

Table 2.4 Periodontitis sample subsets used in each study

	Periodontitis Samples					
	Microbiological Culture	**16S QPCR**	**High throughput 16S rRNA sequencing**	**Gingival Biopsy**	**Whole genome sequencing**	**Microbiome site comparison**
H83						
H84						
H85						
H86						
H88						
H90						
C1						
C2						
C3						
C4						
C5						

Table 2.4 (Continued) Periodontitis sample subsets used in each study

2.3 Individual Studies

Methods for each study will be discussed within the relevant chapter: microbiological culture (chapter 4); 16S QPCR and high throughput 16S rRNA sequencing and whole genome sequencing (chapter 5) and gingival tissue work (chapter 6)

2.4 Statistical Analysis

Statistical analysis will be described and discussed separately for each data set within their respective chapters.

2.5 List of Suppliers

Supplier	Product
Cambio Ltd., Cambridge, UK.	• Masterpure Gram positive DNA purification kit
E&O Laboratories Ltd, Bonnybridge, UK	• Defibrinated horse blood
E&O Laboratories Ltd, Burnhouse, UK	• Fastidious Anaerobe Agar (FAA)
GE Healthcare, Eindhoven, The Netherlands	• IllustraTM GFXTM PCR DNA and Gel Band Purification Kit
Illumina, Cambridge, UK	• MiSeq Reagent Kit v3
Invitrogen, Paisley, UK	• 27f (GGGCGGWGTGTACAAGGC) and 1387r (GGGCGGWGTGTACAAGGC) primers used in Chapter 4 • Agarose • QPCR primers used in Chapters 5 and 7 • SYBR® Green
Lab M Limited, Bury, UK	• Fastidious Anaerobic Broth (FAB)
Life Technologies, Paisley, UK.	• High Capacity cDNA Reverse Transcription Kit • UltraPure™ 10X TBE Buffer • Defined Keratinocyte Serum Free Medium • Dulbeccos PBS • Keratinocyte serum free medium • Antibiotics solution (penicillin [10000 U/mL]/streptomycin [10000 mg/mL]) • Amphoteracin B (25000 mg/mL) • 0.5% Trypsin EDTA

Table 2.5 List of suppliers

Promega, Southampton, UK	• 1.5mM $MgCl_2$ • 5x Go Taq® Green Buffer • Go Taq® Flexi DNA Polymerase
Qiagen, Manchester, UK	• Qiaquick PCR Purification Kit • RNeasy Fibrous Tissue Mini Kit
Sigma-Aldrich, Poole, UK.	• Columbia Agar • Ethidium bromide • Dulbecco's Modified Eagle's Medium • Lysozyme (lyophilised powder) • Motorised pellet pestle • Mutanolysin (lyophilised powder) • Proteinase K • RNA Later® • Fetal Bovine Serum
ThermoFisher Scientific, Loughborough, UK	• 1kb plus DNA Ladder • HyClone™ Ultrapure Water • Sterilin™ Amies Transport Swabs • PBS tablets

Table 2.5 (continued) List of suppliers

2.6 General Stock Solutions and Buffers

Stock solution/ Buffer	Preparation
Columbia blood agar	42 g powder dissolved in 1 litre H2O. Sterilise by autoclaving. Cool to 50°C and add 7.5% defribrinated horse blood.
Ethidium bromide (10 mg/mL)	10 g ethidium bromide, H2O to 1 litre. Store away from light.
Fastidious anaerobe blood agar (FAA)	46 g powder dissolved in 1 litre dH2O by swirling and mixing. Sterilise by autoclaving. Cool to 50°C and add 7.5% defribrinated horse blood.
Fastidious anaerobe broth (FAB)	29.7 g powder dissolved in 1 litre H2O. Sterilise by autoclaving.
Phosphate buffered saline (PBS)	Dissolve one tablet in 100 mL H2O. Sterilise by autoclaving and adjusted to pH 7.4

Table 2.6 General stock solutions and buffers

Chapter 3

Equine Dental Examination

3.1 Introduction

Periodontal disease is a common problem in the horse and its prevalence increases with advancing age, with up to 75% of horses over the age of 30 years being affected (Baker 1979; Ireland *et al*. 2012b). There is no evidence of any breed or sex predisposition to this disease.

This disorder is known to be one of the most painful oral diseases affecting the horse (Dixon *et al*. 2000) and the clinical signs of equine periodontitis are most often a result of this pain which is especially severe during mastication, consequently, dropping of food (quidding) and difficulty in masticating are the most common clinical signs present (Lane 1994; Klugh 2005; Dixon *et al*. 2008a, 2014). However chronic weight loss, halitosis, hypersalivation, buccal food pocketing (Dixon *et al*. 2014) and behavioural abnormalities (Lane 1994) may also occur in diseased horses. Despite the significant oral pain caused by the condition, the previously described clinical signs may be subtle or even absent in some cases (Dixon *et al*. 2008a; 2014) which means that the disease can go unnoticed.

In contrast to brachydont periodontal disease, primary periodontitis is rare in the horse (Collins and Dixon, 2000) and the condition is most often a secondary consequence of other oral disorders, especially formation of diastemata that allow food impaction between and beside cheek teeth (Dixon *et al*. 2008a). Displacements, overgrowths and supernumerary cheek teeth are also associated with secondary periodontitis by the same mechanism (Dixon *et al*. 1999; Casey and Tremaine 2010). Plaque-induced gingivitis, occasionally progressing to periodontitis as seen in brachydont species can, however, affect equine canine teeth, especially on the labial aspect.

Clinical evaluation of equine periodontal disease involves clearing any impacted feed in order to fully assess the periodontal pocket. The pocket depth measurement can be used to grade the severity of the lesion (Cox *et al*. 2012).

In addition, tooth mobility can be a useful indicator of periodontal disease severity (Klugh 2005). The Periodontal Disease Index used in human dentistry was adapted for veterinary use in brachydont species by Wiggs and Loprise (1997) and was further adapted for hypsodont equine teeth by Klugh (2005). This grading system can be used to stage periodontal disease in the horse and includes the use of radiography to assess the degree of periodontal attachment loss.

In this chapter, the signalment of the study population is described. The findings obtained from clinical examination of equine periodontitis lesions included in this study are also reported.

It is the hypothesis of this chapter that equine periodontal disease will be positively associated with advancing age. Positive relationships between age, tooth mobility, periodontal pocket depth and number of periodontitis lesions are also expected.

3.2 Materials and Methods

3.2.1 Signalment

Wherever possible, details of signalment were recorded for each horse. Details of any concurrent disease and treatment were also obtained to ensure no animal had received antibiotic therapy within 8 weeks of sample collection.

3.2.2 Oral and Clinical Examination

3.2.2.1 Oral examination

A complete oral examination was performed on every horse involved in the study. Oral examinations performed in live horses were completed using an Alpkit Prism 550 head torch (Alpkit, Nottingham, UK) to provide sufficient light and either an AlumiSpec™ equine mouth speculum (Veterinary Dental Products Europe, Wiltshire, UK) or a SE Speculum (Equine Blades Direct, North Somerset, UK) to provide sufficient access. To ensure thorough examination of the oral cavity in live horses, an oral endoscope or equine dental mirror (Equine Blades Direct) was used. An appropriate sedation protocol was used as required, depending on the animal's temperament and treatment(s) required following the dental examination. All horses examined *post-mortem* in the study were euthanatised humanely for reasons other than dental disease. Where dental examinations were performed *post-mortem* the mandible was disarticulated from the maxilla to allow sufficient access for examination of the whole oral cavity. This was undertaken no more than 40 minutes after humane euthanasia had been performed.

3.2.2.2 Recording of dental disorders and periodontal disease grading

Findings of each dental examination including all oral disorders were recorded on an equine dental chart (British Equine Veterinary Association, UK). After flushing the oral cavity to remove loose food material, areas of periodontal disease were identified. After clearing of impacted feed material from periodontal pockets, their depth was measured using a sterile calibrated periodontal probe (Figs 3.1 and 3.2) and depth and location were recorded on the dental chart. The severity of periodontal disease was graded according to

Cox *et al*. (2012) (Table 3.1). Mobility of teeth adjacent to any periodontitis lesion was also recorded (Table 3.2).

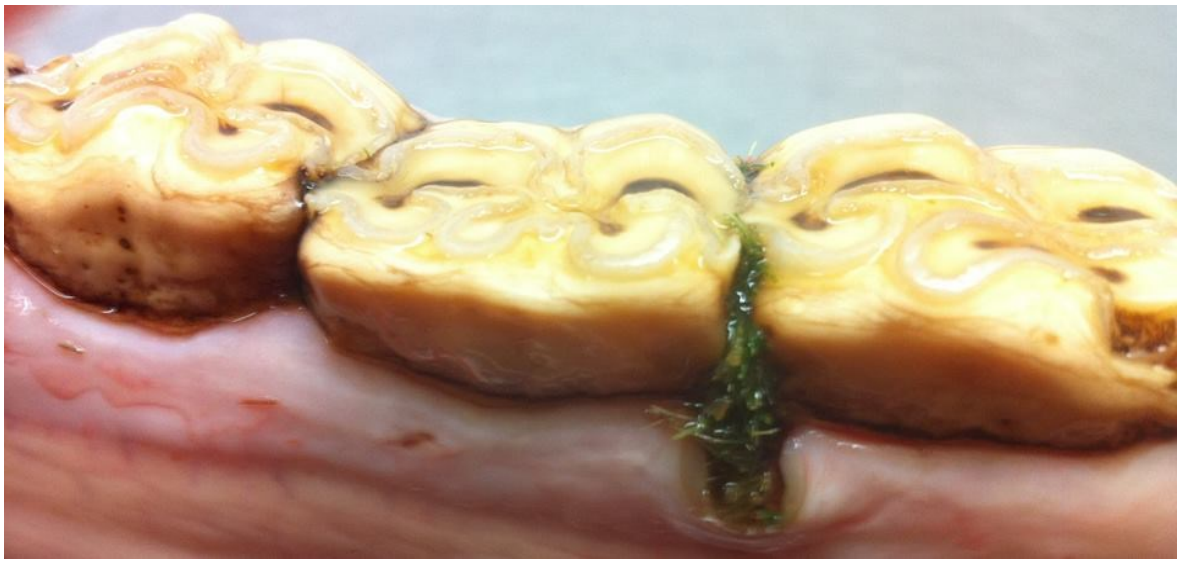


Figure 3.1 Periodontal disease secondary to diastema formation between teeth 308 and 309.

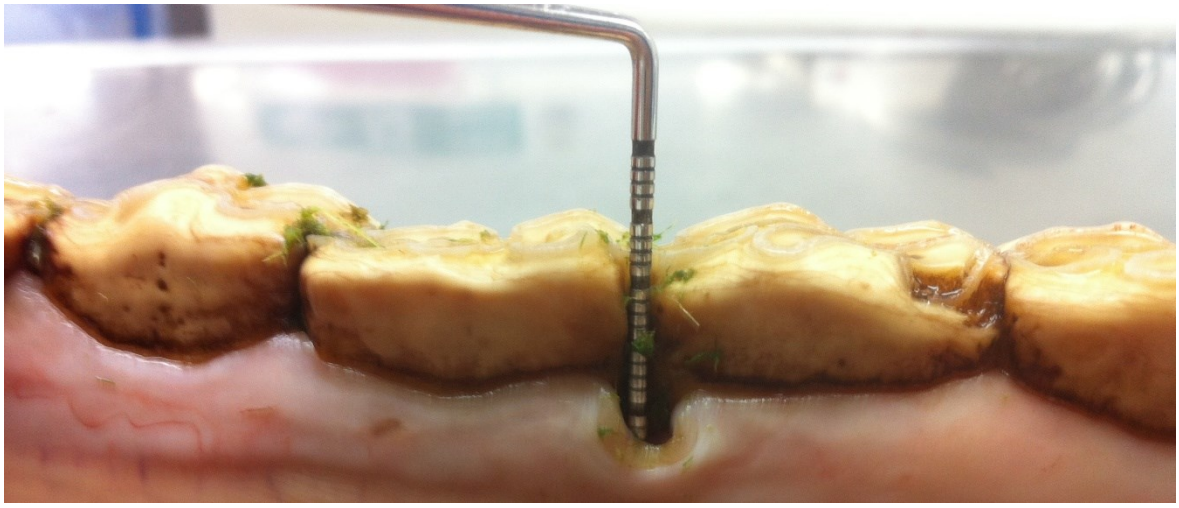


Figure 3.2 Measurement of a periodontal pocket using a calibrated periodontal probe following removal of impacted feed material.

Grade of periodontal disease	Descriptive Grade	Sulcus/periodontal pocket depth (mm)
0	No disease	0-4
1	Mild disease	5-9
2	Moderate disease	10-14
3	Severe disease	>15

Table 3.1 Staging of equine periodontitis based on pocket depth as described by Cox *et al*. (2012)

Tooth Mobility Stage	Descriptive Stage	Mobility
0	Normal	None
1	Mild	First distinguishable movement greater than normal
2	Moderate	Movement of up to 3mm
3	Severe	Movement over 3mm in any direction and/or is depressible

Table 3.2 The index of tooth mobility modified for equine dentistry by Klugh (2005).

3.2.3 Statistical analysis

Statistical analysis of results and construction of graphs were performed using SSPS (IBM Chicago IL, USA), Microsoft Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 5 (San Diego, CA, USA) for Windows. Chi-square tests were used in the Cross tabulation procedure to determine significant differences in the composition of the healthy and periodontitis groups in the study population concerning age, breed and sex. Mann-Whitney U-tests were used to determine whether there were significant differences between healthy horses and horses with periodontitis in age, breed and the clinical data, namely, number of lesions per animal, periodontal pocket depth and tooth mobility. The Kruskal-Wallis test was used to determine the level of significance for differences in clinical parameters between subgroups of horses, based on age or breed. Non-parametric correlation analysis was also performed on data arising from age of horses, number of lesions per animal, average periodontal pocket depth and average tooth mobility score using Spearman's Rho test.

3.3 Results

3.3.1 Study Population

In total, 99 horses were included in the study, comprising 39 orally healthy horses and 60 horses with periodontal disease. All horses presented to either the University of Glasgow School of Veterinary Medicine or the Royal (Dick) School of Veterinary Studies, Edinburgh UK. Twenty-four (24.2%) of horses had presented for a routine dental examination, thirty-seven (37.4%) had been referred for a dental examination and treatment and thirty-eight (38.4%) had been humanely euthanised for reasons other than dental disease as detailed below.

3.3.1.1 Reasons for euthanasia

Thirty-eight horses used in the study had been humanely euthanised for reasons other than dental disease and of these 21 had periodontal disease and 18 were orally healthy. Twenty-one of these horses were euthanised due to chronic disease: chronic musculoskeletal disease (n=11), poor condition/chronic weight loss (n=6), chronic ocular disease (n=2), chronic hepatic disease (n=1) and

chronic gastrointestinal disease (n=1). The reason for euthanasia was unknown in five horses; however, after dental examination, it was concluded that dental disease was unlikely to be the reason as all five horses were orally healthy. The remaining 13 horses were euthanised due to congenital disease (n=5), dangerous haviour (n = 4), neoplasia (n = 2), poor performance (n=1) and neurological disease (n = 1) (Figure 3.3).

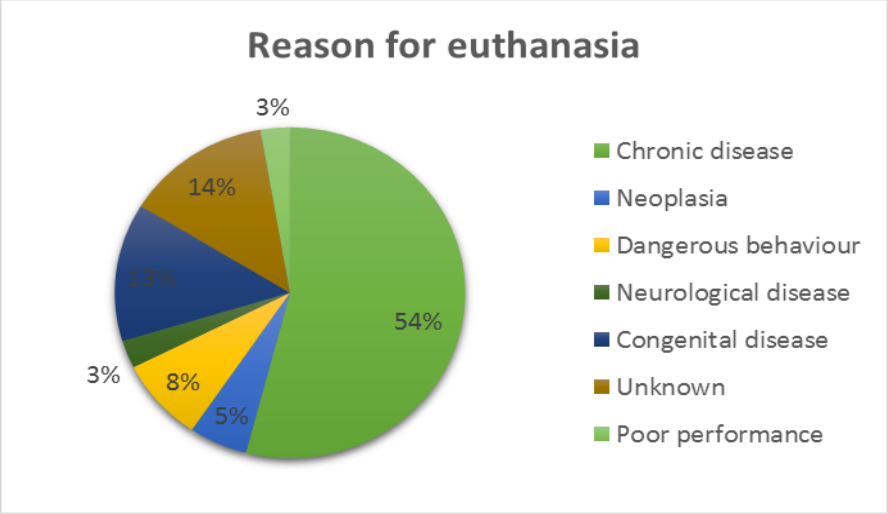


Figure 3.3 Reason for euthanasia of 38 horses included in the study population

3.3.2 Signalment

3.3.2.1 Age

The mean age of the orally healthy horses was 9 years (SD 6.1 years) with a range of 18 months to 22 years (Table 3.3). Thirty-eight percent (n = 15) of orally healthy horses were in the 5-9 years age group and a further 26% (n = 10) were in the 0 - 4 years age group (Figure 3.3). The mean age of horses affected by periodontitis was 13.8 years (SD 6.2 years) with a range of 18 months to 27 years of age (Table 3.4). Twenty-eight percent (n = 17) of horses with periodontal disease were in the 10-14 years age group and only 3% (n = 2) were in the 0-4 years age group (Figure 3.4).

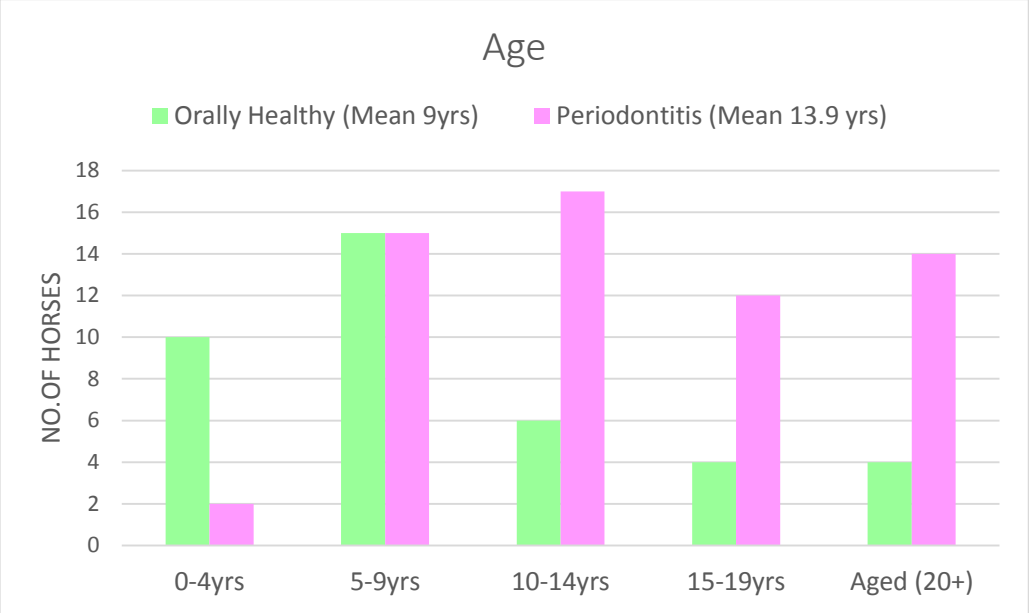


Figure 3.4 Age distribution of horses included in the study

When the horses were placed into age categories the Chi square test indicated that there were significant differences in prevalence of periodontitis between groups ($p = 0.001$).

A Kruskal-Wallis test on the age groups of horses with periodontitis showed no significant difference between age groups and periodontal pocket depth (Figure 3.5a) despite the mean pocket depth appearing to increase with advancing age in horses older than 9 years of age (Figure 3.5a). In contrast, the Kruskal-Wallis test indicated that significant age-related differences in the number of periodontitis lesions per horse ($p = 0.006$) and the average tooth mobility score ($p = 0.033$). Mann- Whitney U-test with Bonferroni correction was used in post-test analyses and indicated that, there was a significant increase in number of lesions per horse between horses aged 10 - 14 years and horses over 20 years of age ($p = 0.017$) (Figure 3.5b) and also in mean tooth mobility ($p = 0.04$) (Figure 3.5c) and in number of lesions ($p = 0.024$) between horses aged 10-14 years of age and those aged 15 to 19 years (Figure 3.5b).

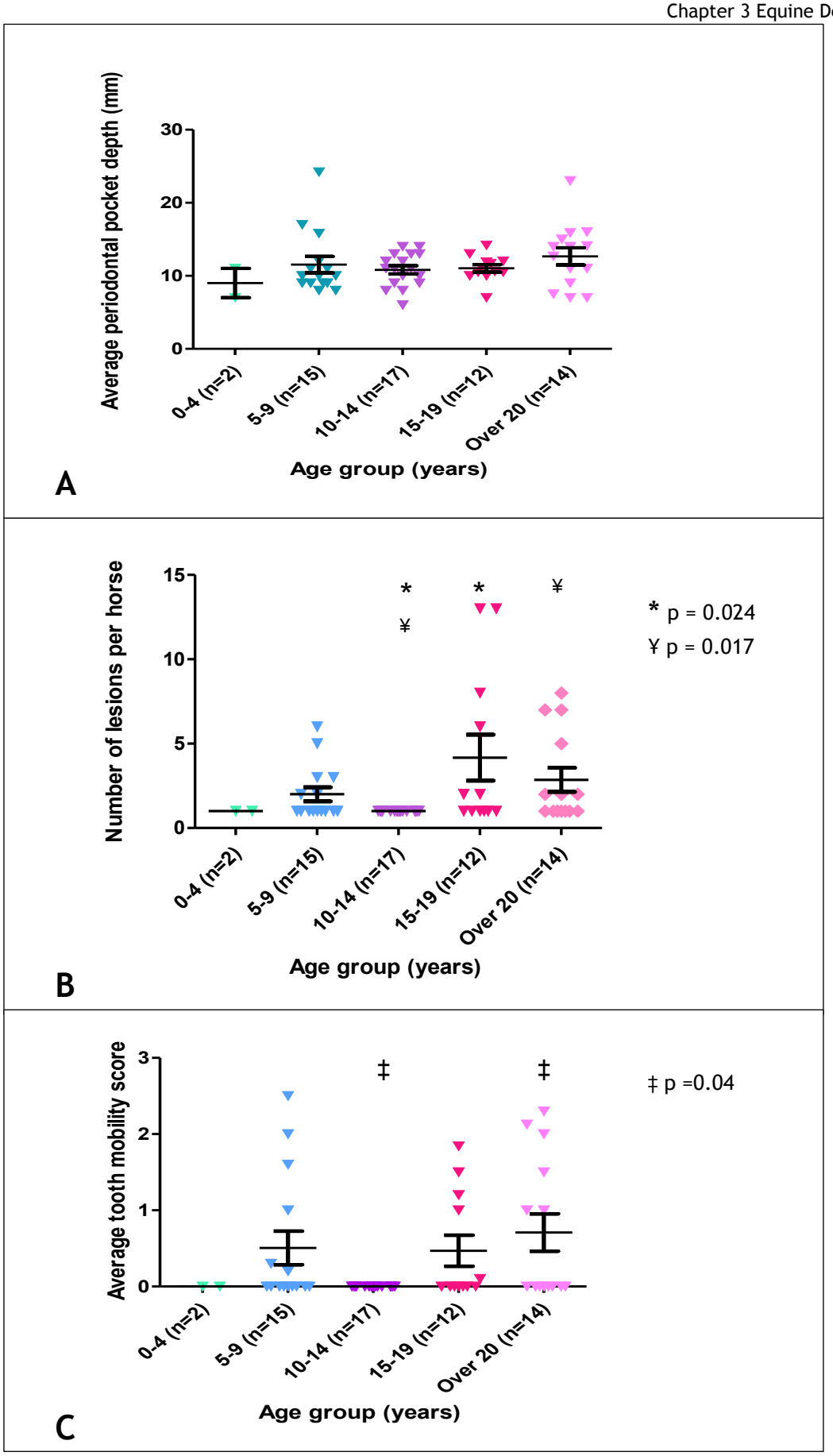


Figure 3.5 Age group of diseased horses and periodontitis clinical features. Error bars represent mean and standard error of the mean

A. Age group and average periodontal pocket depth. B. Age group number of lesions per horse. C. Age group and average tooth mobility. * p = 0.024; ¥ p = 0.017; ‡ p =0.04

3.3.2.2 Sex

Mares accounted for 30% (n = 12) of orally healthy horses and 53% (n = 32) of horses with periodontal disease (Tables 3.3, 3.4 and Figure 3.6). Geldings accounted for 56% (n = 22) of horses with a healthy oral cavity and 43% (n = 26) of horses affected by periodontitis (Tables 3.3, 3.4 and Figure 3.6). There were three stallions included in the study, two orally healthy and one with periodontitis and the sex of three orally healthy horses and one horse with periodontitis was unknown (Tables 3.3, 3.4 and Figure 3.6). A Chi-squared test indicated gender of the animal was not associated with presence of periodontal disease (p=0.08).

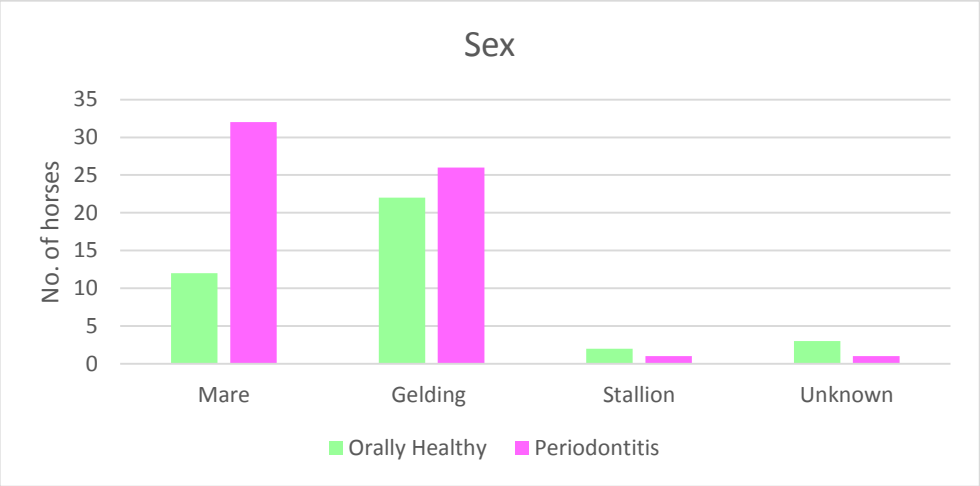


Figure 3.6 Sex and disease status of horses included in the study

3.3.2.3 Breed

There were 29 breeds (including cross-breeds) represented in the study (Tables 3.3 and 3.4), resulting in relatively low numbers in each group. To allow some meaningful analysis, the breeds were grouped into four categories with a fifth miscellaneous category for horses whose breed could not be categorised as either Native Pony/native cross, Cob/Cob cross, Thoroughbred/TB cross or Warmblood/WB cross (Figure 3.7). Native ponies accounted for 36.4% of horses included in the study, and represented 48.7% of orally healthy horses and 28.3% of horses with periodontitis. Cobs accounted for 15.1% of horses included in the study, representing 5.1% of orally healthy horses and 21.7% of horses with periodontitis. Fifteen percent of horses included in the study were Thoroughbreds/TB crosses and these horses accounted for 12.8% of the orally healthy group and 16.6% of the periodontitis group. Warmbloods/WB crosses comprised 6% of all horses involved and represented 2.5% of orally healthy horse and 8.3% of diseased horses. Twenty-seven percent of horses were grouped into the miscellaneous category and accounted for 30.7% of orally healthy horses and 25% of horses with periodontitis (Figure 3.7). Breed or groups of breeds were not significantly associated with the presence of periodontal disease (p= 0.09).

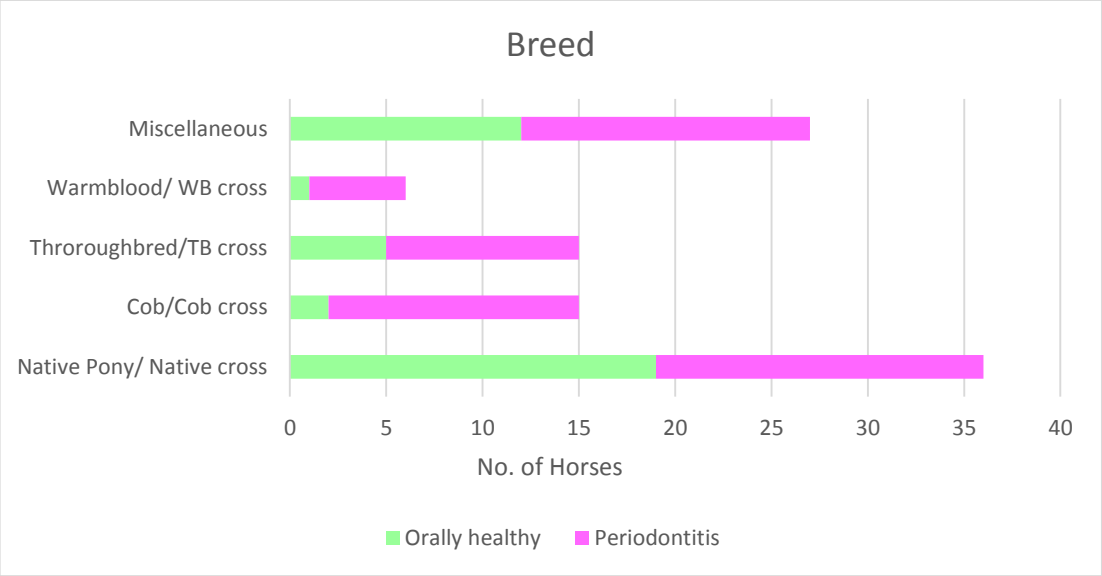


Figure 3.7 Breed and disease status of horses included in the study

HORSE	AGE	SEX	BREED
H1	22	mare	Connemara
H2	8	gelding	Cob
H11	7	mare	Dartmoor
H12	6	mare	Welsh Section A
H13	22	gelding	Fell
H14	6.5	gelding	Welsh Section A
H15	4	gelding	Clydesdale X
H16	4	gelding	Shetland X
H17	4	gelding	Connemara
H18	6	mare	Dartmoor
H21	16	gelding	Irish Sports Horse
H26	22	gelding	Trakenher
H28	6	mare	Exmoor
H29	8	mare	Exmoor
H30	6	gelding	Welsh X
H31	14	gelding	Clydesdale X
H32	18	gelding	Haflinger X
H33	9	gelding	Cob
H34	11	gelding	Warmblood
H36	7	gelding	Shire
H37	22	mare	Welsh Section D
H38	16	mare	Welsh Section D
H39	22	mare	Welsh Section A
H41	16	gelding	Thoroughbred
H50	5	gelding	Standardbred
H51	1	stallion	Welsh Section A
H53	1	stallion	Welsh Section A
H66	2	gelding	Fell
H68	7	mare	Irish Draft
H72	5	mare	Irish Draft
H77	10	gelding	Thoroughbred
H78	1.5	gelding	Welsh Section A
H79	1.5	gelding	Welsh Section A
H80	8	mare	Selle Francais
H87	11	mare	Thoroughbred X
H89	4	gelding	Thoroughbred X
B1	6	unknown	Shire x
B2	11	unknown	Thoroughbred X
B3	4	unknown	Welsh Section D

Table 3.3 Signalment of orally healthy horses

HORSE	AGE	SEX	BREED
H0	22	mare	Connemara
H3	15	gelding	Cob
H4	14	mare	Highland
H5	7	gelding	Cob
H6	5	mare	
H7	6.5	mare	Arab
H8	8	mare	Arab
H9	14	gelding	Cob
H10	16	gelding	Irish Sports Horse
H19	12	mare	Cob
H20	12	gelding	Thoroughbred X
H22	9	mare	Thoroughbred
H23	12	gelding	Welsh Section D
H24	15	mare	Thoroughbred
H25	11	gelding	Gerlander
H27	3	mare	Shetland X
H35	12	mare	Welsh Section C
H40	22	mare	Welsh Section A
H42	12	gelding	Irish Draft
H43	22	gelding	Icelandic
H44	22	mare	Icelandic
H45	16	gelding	Icelandic
H46	8	gelding	Warmblood
H47	14	mare	Arab
H48	24	mare	Irish Sports Horse
H49	22	mare	Thoroughbred
H52	1	stallion	Welsh Section A
H54	8	mare	Irish Sports Horse
H55	9	mare	Icelandic
H56	16	gelding	Cob
H57	22	gelding	Icelandic
H58	13	gelding	Connemara
H59	15	mare	Thoroughbred
H60	7	gelding	Cob
H61	22	gelding	Cob
H62	6	mare	Cob
H63	22	gelding	Irish Sports Horse
H64	18	mare	Cob
H65	10	mare	Cob
H67	18	mare	Warmblood
H69	9	mare	Shetland
H70	7	mare	Shetland
H71	5	Mare	Shetland
H73	17	mare	Shetland
H74	22	gelding	Cob

Table 3.4 Signalment of horses with periodontal disease

HORSE	AGE	SEX	BREED
H75	18	Gelding	Warmblood
H76	12	Unknown	Cob
H81	20	Mare	Welsh Cross
H82	24	Mare	Shetland
H83	17	Gelding	Welsh Section D
H84	18	Gelding	Thoroughbred X
H85	10	Gelding	Thoroughbred X
H86	24	Gelding	Shire x
H88	27	Mare	Shetland
H89	8	Gelding	Shetland
C1	12	Mare	Cob
C2	12	Gelding	New Forest Pony
C3	11	Mare	Warmblood
C4	5	Mare	Thoroughbred X
C5	10	Gelding	Thoroughbred X

Table 3.4 (continued) Signalment of horses with periodontal disease

3.3.3 Clinical Examination

3.3.3.1 Periodontal pocket number and location

In total, there were 139 separate periodontal pockets examined in the study giving a mean number of 2.3 pockets (SD 2.74) with a range of 1 to 13 pockets per diseased horse (Table 3.5). All periodontal pockets were seen with diastemata. Ninety-two percent of periodontal pockets were associated with diastemata (Fig.3.1) which were not further classified. Rotated cheek teeth (Figure 3.8) with associated diastemata accounted for a further 3% of periodontal pockets and the remaining 5% of periodontal pockets were associated with displaced cheek teeth and their adjacent diastemata (Fig 3.9). The majority (79.9%) of periodontal pockets were mandibular while the remaining 20.1% were located within the maxillary arcades. With regards to location within the 100 (upper right), 200 (upper left), 300 (lower left) and 400 (lower right) arcades, 10% of periodontal pockets were located in the 100 arcade, 10% in the 200 arcade, 42% in the 300 arcade and 38% in the 400 arcade. The most common locations for periodontal disease in the current study were between the teeth 308/309 (13.7%), 310/311 (8.6%), 406/407 (8.6%), 407/408 (8.6%) and 408/409 (7.9%) (Figure 3.10). The most prevalent maxillary periodontitis lesions were located at the Triadan 06/07 positions (28.5% of maxillary lesions) and 07/08 (28.5% of maxillary lesions). The most common

Triadan position for mandibular periodontitis lesions was 08/09 (27%). One periodontal pocket was located adjacent to a diastema between temporary dentition (807/808) but all other pockets were adjacent to permanent dentition. Periodontitis associated with the incisors accounted for 2.9% of lesions included in the study with the remaining 97.1% of lesions being associated with the cheek teeth.

Horse	**No. of lesions**	**Mean pocket depth** (mm)	**Median tooth mobility score**
H0	1	7	0
H3	1	12	0
H4	1	9	0
H5	1	9	0
H6	1	8	0
H7	6	11	0
H8	1	10	0
H9	1	8	0
H10	13	10.6	0
H19	1	13	0
H20	1	12	0
H22	1	10	0
H23	1	14	0
H24	1	10	0
H25	1	10	0
H27	1	11	0
H35	1	6	0
H40	1	7	0
H42	1	14	0
H43	1	15	0
H44	1	11	0
H45	1	7	0
H46	1	9	0
H47	1	11	0
H48	7	12.7	2
H49	2	7.5	0
H52	1	7	0

H54	2	11	0
H55	1	9	0
H56	1	11	0
H57	1	14	0
H58	1	8	0
H59	1	10	0
H60	1	12	0
H61	1	14	1
H62	1	8	0
H63	2	11	0
H64	2	10.5	1
H65	1	11	0
H67	2	10.5	0
H69	3	9	0
H70	2	17	2
H71	5	24.2	2
H73	13	11.7	2
H74	1	9	0
H75	1	13	0
H76	1	13	0
H81	5	23	2
H82	7	14.1	2
H83	8	11.9	2
H84	6	14.2	2
H85	1	10	0
H86	8	15.9	2
H88	2	16	1
H89	4	15.8	1
C1	1	12	0
C2	1	9	0
C3	1	11	0
C4	1	10	0
C5	1	13	0

Table 3.5 Results of oral clinical examination of periodontally diseased horses

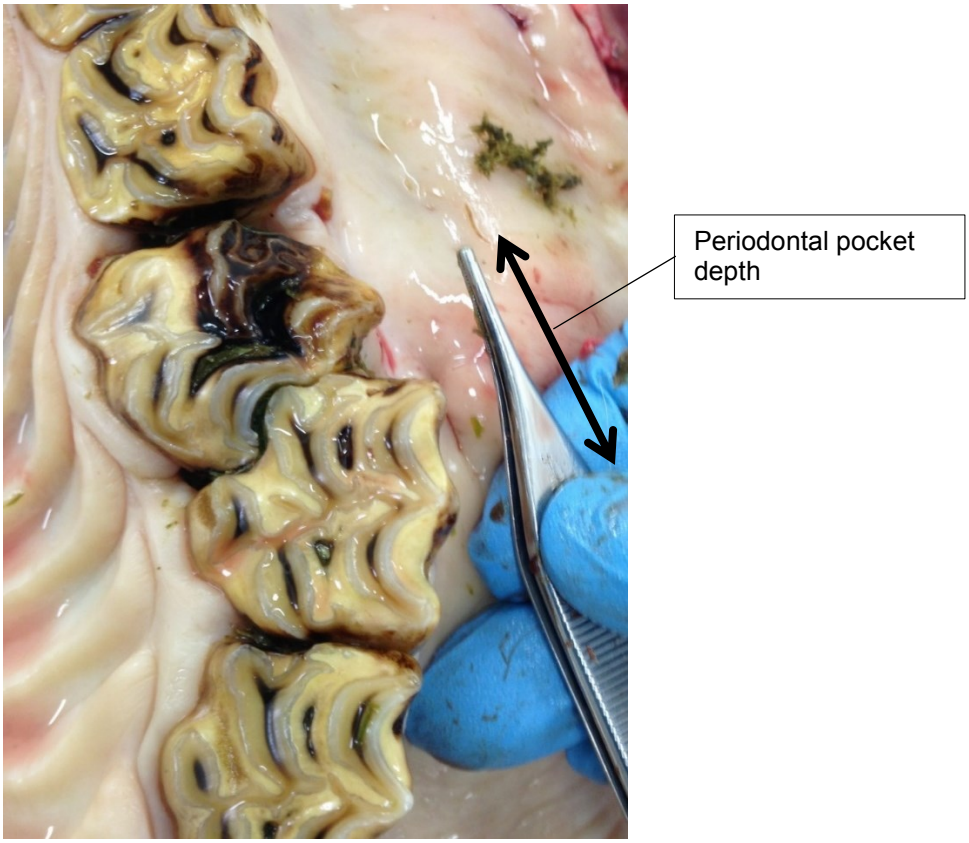


Figure 3.8 Rostro-palatally rotated 108 with associated 108/109 diastema and secondary periodontal disease (and extensive caries).

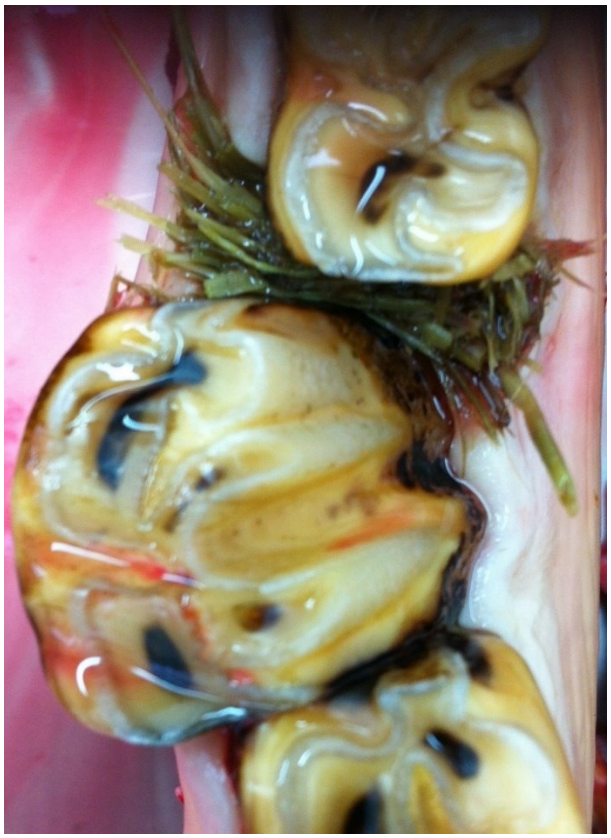


Figure 3.9 Buccally displaced 409 with associated diastema and secondary periodontal disease

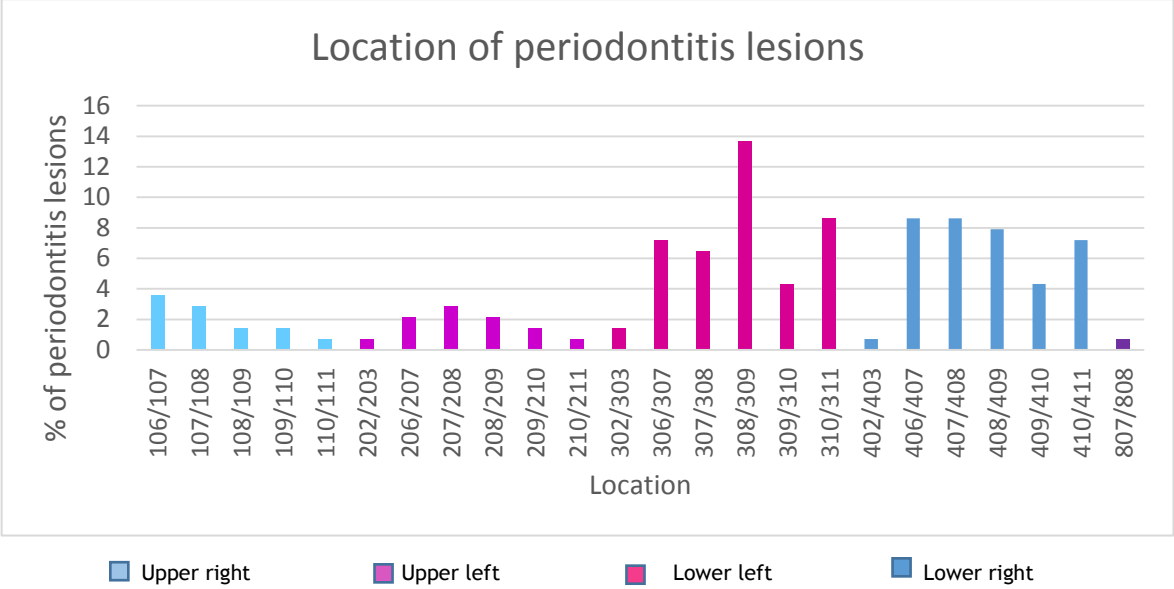


Figure 3.10 Location of periodontal lesions by Triadan positions within the equine oral cavity

3.3.3.2 Periodontal pocket depth and periodontal disease stage

The mean periodontal pocket depth was 12.7mm (SD 5.6) with a range of 6 to 44mm. The mean periodontal disease stage was 2 (moderate with pocket depth of 10-14mm). Using the staging criteria suggested by Cox *et al*. (2012), 56.8% of lesions were at Stage 2 (moderate disease), 21% of lesions were at Stage 1 (mild disease) and 23.1% were at Stage 3 (severe disease). Evidence of feed impaction was present in all periodontal pockets examined.

3.3.3.3 Tooth mobility index

The mean tooth mobility index score was 0.8 (Range 0-3) where zero is no tooth movement and 1 is the first distinguishable signs of movement as described in Table 3.2. Fifty-one percent of all lesions examined in this study had no associated tooth movement. In 11% of all lesions examined the tooth mobility index score was 1, in 31% the mobility index was 2 and 7% of lesions had associated Stage 3 tooth mobility.

3.3.3.4 Correlation analysis

Non-parametric correlation analysis revealed a significant positive correlation between age and number of periodontal lesions (Rho = 0.269; p = 0.038). Significant positive correlations were also seen between number of lesions and average periodontal pocket depth (Rho = 0.395; p=0.002) and number of lesions

and average tooth mobility score (Rho = 0.847; p<0.0001). Average pocket depth positively correlated with average tooth mobility score (Rho = 0.531; p< 0.00001).

When a partial correlation analysis was carried out, correcting for age of the animals, the number of lesions per animal positively correlated with average pocket depth (Rho = 0.287; p = 0.0027) and average tooth mobility score (Rho = 0.606; p < 0.00001). Average pocket depth also positively correlated with average tooth mobility score after correction for age (Rho =0.614; p < 0.00001).

3.4 Discussion

There is a well-known association between advancing age and presence of equine periodontal disease (Baker 1979). Despite this, there is little information regarding the breed and gender of horses suffering from periodontal disease and the link between these factors and presence of the disease has not previously been investigated.

The current study found no significant relationship between the presence of periodontal disease and either breed or gender of the horse. All periodontal pockets were associated with primary or secondary diastemata in the current study. Although the link between gender and periodontal disease has not previously been explored, Walker *et al*. (2012) investigated the prevalence and clinical characteristics of diastemata in 471 horses and found that there was no sex predisposition to the of presence of diastema. However, when considering only affected horses, females had a significantly higher mean number of diastema than males (Walker *et al*. 2012).

Little (1913) stated that periodontal disease was present in all breeds but suggested the condition may be more prevalent in heavy horses than lighter breeds. Walker *et al*. (2012) discovered that pony breeds had higher mean numbers of diastemata than Thoroughbred crosses and cobs. This is not surprising due to the facial conformation of some smaller native pony breeds such as Shetland and Welsh Mountain ponies that have small heads but relatively large teeth. Resultant dental overcrowding may lead to rotated and displaced

cheek teeth and subsequent periodontal disease (Dixon and du Toit 2011). No significant breed disposition was noted in the current study due to the wide variety of breeds represented and the relatively low numbers of animals per breed in the current study. Future large scale investigations into possible breed predisposition to equine periodontitis would be interesting.

Previous studies of equine periodontal disease revealed that its prevalence increases with advancing age in horses (Baker 1979; Wafa 1988) and also in donkeys (du Toit *et al*. 2009b; Rodriguez *et al*. 2013). Dental abnormalities are recognised to be an increasing problem as horses age. However, horse owners are often unaware of any problems in their animals (Ireland *et al*. 2012a) and consequently dental diseases, including periodontal disease, are left to progress untreated. This may partially explain the higher prevalence of periodontitis in older horses. As equine periodontal disease is most often a secondary consequence of other oral disorders (Dixon *et al*. 2008a), it is intuitive that if risk of concurrent dental disease increases with age, then risk of secondary periodontitis will also increase. Abnormalities in occlusion or masticatory movement as a result of pain or physical restrictions caused by other dental disorders such as overgrowths, cheek teeth fractures or conformation abnormalities may result in orthodontic movement of some teeth and creation of diastemata and secondary periodontal disease (Dixon *et al*. 2008a). Cheek teeth naturally narrow towards their apex and so with continued eruption senile diastemata may form with secondary periodontal disease. The number of periodontitis lesions also increased with age. As the likelihood of developing periodontitis increases with advancing age, it is understandable that an increasing number of lesions will also occur with advancing age. Walker *et al*. (2012) reported a significant increase in the prevalence and number of diastema with age, similar to the current findings. The pain associated with even one periodontitis lesion may result in abnormal mastication, placing abnormal forces on other teeth which can result in the formation of dental overgrowths over time with resultant food stasis. Consequently, further periodontitis lesions may occur in horses with untreated pre-existing periodontitis. Advancing age is also associated with periodontitis in other species such as man, (McDevitt *et al*. 2000; Borrell and Papapanou 2005; Eke *et al*. 2012) and dogs (Harvey *et al*. 1994; Kortegaard *et al*. 2008). Eke *et al*. (2012) reported that the increased

prevalence of human periodontitis with advancing age was influenced by the increasing prevalence of moderate periodontitis in older patients, a result of deterioration of previously mild cases. In addition, Schätzle *et al*. (2009) reported a decreased rate of regression of chronic periodontitis in older human patients. Age has also been associated with increased attachment loss (Grossi *et al*. 1994) and increased alveolar bone loss (Grossi *et al*. 1995). It has previously been suggested that the association between advancing age and prevalence of periodontitis in humans is representative of cumulative effect of prolonged exposure to other risk factors (Papapnou *et al*. 1991; Borrell and Papapnou 2005) including exposure to periodontal pathogens in the oral cavity. Age-related changes are also seen to affect periodontal tissue which may influence disease susceptibility with periodontal ligament stem cells harvested from older human patients having been reported to have reduced proliferative and migratory potential than those from younger patients (Zhang *et al*. 2012). The changes in immune and inflammatory responses to periodontal disease during aging are complex and increased activity of inflammatory mediators such as matrix metallopeptidase 9 (MMP-9) and prostaglandin E2 (PGE2) is associated with advancing age in even periodontally healthy human patients (Back 2007). It is important to remember, however, that periodontal disease in brachydont species such as man likely has a different aetiopathogenesis to that of periodontitis in the horse and so comparisons relating to the effect of age upon periodontitis prevalence are difficult to make between species.

The severity of periodontal disease of the horses in this study was assessed by using a grading system as developed by Cox *et al*. (2012) based on periodontal pocket depth assessment. This system was deemed the most suitable since a simple, accurate measurement could be obtained in both live horses and in horses examined *post-mortem*. A previous grading system developed by Wiggs and Loprise (1997) to assess brachydont teeth in veterinary species was then adapted by Klugh (2005) for use in hypsodont equine teeth and uses radiography to determine the percentage of periodontal ligament attachment loss. However, this system was impractical for the purposes of this study as radiographing every lesion in every horse for the sole purpose of disease staging would have been time- and resource-consuming and beyond the scope of this study. Mobility of the clinical crown is a simple measurement, easily performed in both live and

dead animals and also indicative of disease progression and attachment loss when adapted to equine teeth (Klugh 2005).

Tooth mobility was also increased with advancing age and these findings are again not surprising. In older teeth, the periodontal ligament is more firmly attached due to the presence of densely packed collagen fibres (Staszyk *et al*. 2015); however, as the horse ages, the tooth erupts and undergoes attrition of the occlusal surface during mastication, leaving a reduced length of reserve crown and a smaller area of (denser) periodontal ligament attachment. Consequently, destruction of the limited remaining periodontium is more significant in an older than a younger horse and so an increase in tooth mobility with periodontal disease in older horses is logical. Although the average pocket depth increased with advancing age in horses over nine years old, there was no significant association between pocket depth and age. This is somewhat surprising as the progressive and chronic nature of the disease would suggest that periodontal pockets would deepen over time and so deeper pockets would be expected in older horses with chronic disease. It is therefore possible that the lack of relationship between pocket depth and age is explained by the presence of periodontal pockets at a variety of stages, including in early lesions in aged horses, rather than a uniform progression to severe disease in aged horses. However due to the relatively small sample population, a larger scale study exploring these relationships would be useful.

A positive correlation between the number of periodontal lesions per horse and tooth mobility was noted, alongside a positive correlation between periodontal pocket depth and tooth mobility. This is to be expected as large periodontal pockets are indicative of significant periodontal ligament attachment loss leading to increased tooth mobility. In addition, an increased number of lesions would result in decreased overall support, especially if lesions affected adjacent teeth (i.e. lesions at 308/309 and 309/310 would result in significant attachment loss surrounding 309).

One possible limitation of this study is the lack of paired measurements from periodontitis lesions and periodontally healthy sites in diseased animals. Although healthy periodontal sites were assessed and measured in all horses in

the current study to ensure there was no presence of attachment loss or other signs of periodontal disease, this data was not carefully recorded and therefore could not be used in a paired statistical analysis. This may have been useful as although the accepted healthy equine periodontal pocket depth is less than 5mm, as proposed by Klugh (2005), it is likely that there is an overlap between physiological and pathological findings in equine periodontal anatomy (Cox *et al*. 2012; Staszyk *et al*. 2015). Further gross and histopathological studies are required to investigate this possible overlap in order to critically assess periodontitis lesions and the healthy periodontium in the horse (Staszyk *et al*. 2015). This would also require a larger number of animals than used in the current study to meet the statistical power required for such an analysis.

The majority of periodontitis lesions were located around the mandibular cheek teeth. There is limited information on the location of periodontitis lesions within the equine oral cavity, although all lesions in the current study were associated with diastemata. In a study of diastemata locations of which 43.7% had associated periodontal pockets, Walker *et al*. (2012) reported that 83.5% of diastemata were mandibular and 16.5% were maxillary. Dixon *et al*. (2008a, 2014) also reported a similar ratio, with 87% of diastemata being mandibular and 13% being maxillary. Du Toit *et al*. (2008) reported 63.6% of diastemata to be mandibular. In this study, 79.9% of periodontitis lesions associated with diastemata were mandibular with the remaining 20.1% maxillary. In contrast to this study and other previous studies, Simhofer *et al*. (2008) reported maxillary diastemata to be more prevalent. Walker *et al*. (2008) described Triadan position 07/08 being the most prevalent position for mandibular diastemata especially on the left side of the mandible (307/308) and Triadan 06/07 was the most prevalent site for maxillary diastemata. In the current study, maxillary periodontitis lesions were equally most prevalent at Triadan 06/07 and 07/08 with most mandibular periodontitis lesions located at Triadan 08/09. In agreement with Walker *et al*. (2012), most mandibular periodontitis lesions in the current study were located on the left (300) arcade, a finding which is inexplicable. Some authors have reported that the more caudal cheek teeth are more commonly affected by diastemata. Dixon *et al*. (2008) previously found 50% of diastemata to occur at either mandibular Triadan 09/10 or 10/11 which is in agreement with a *post-mortem* survey of donkeys carried out by du Toit *et al*.

(2008). A thorough examination with the assistance of a dental mirror or endoscope is essential in the live horse as caudal diastemata and associated periodontal disease can be easily overlooked due to their position within the oral cavity that is hidden by the tongue. Although a more thorough examination of the equine oral cavity is possible for *post-mortem* specimens, all examinations in live horses were carried out by experienced practitioners using the previously mentioned imaging techniques and so it is highly unlikely caudal lesions would have been missed in this study.

All periodontitis lesions in this study contained feed material. Walker *et al*. (2012) reported that 78% of maxillary and 94% of mandibular diastemata contained feed pocketing with 44% being associated with periodontal pockets. Du Toit *et al*. (2008) reported that 89% of diastemata with associated periodontal disease had food impaction. The diets of horses in the current study were highly variable and so were not examined in relation to the prevalence of periodontal disease or feed packing of lesions; however, this packing of various feed materials into periodontal pockets could be a worthwhile area of further investigation. Grass-fed horses affected with diastema have been reported to rarely lose weight in comparison to forage fed horses (Dixon *et al*. 2008a) and clinical signs associated with diastemata such as quidding have been reported to recur when pasture-based affected horses are reintroduced to forage in autumn/winter (Dixon et al. 2014). It is, however, unclear if the increase in reported clinical signs is due to dietary changes and increased trapping of feed alone or whether closer observation of stabled horses is also a factor (Dixon *et al*. 2014).

3.5 Conclusion

In conclusion, the positive relationships between advancing age and the presence of periodontal disease, tooth mobility and number of lesions per affected horse reported in this chapter were to be expected, meeting the hypothesis of the study. The lack of relationship between advancing age and periodontal pocket depth was unexpected. All lesions were associated with diastemata and were mostly adjacent to mandibular cheek teeth in location which is in agreement with previous studies (Walker *et al*. 2012). Further,

larger-scale studies would be useful to further examine these relationships, especially to investigate breed predisposition to equine periodontitis.

Chapter 4

Culture dependent identification of bacteria associated with equine oral health and periodontitis

4.1 Introduction

Bacteria have been shown to be important in the aetiopathogenesis of feline, canine and human periodontal disease and so it is highly likely they play a crucial role in equine periodontitis. The importance of investigating the role of bacteria in equine periodontitis has recently been highlighted by Cox *et al*. (2012) who discovered the presence of spirochaetes and Gram-negative cocci in equine periodontal pockets. In addition, Sykora *et al*. (2014) recently isolated *Tannerella* sp. and *Treponema* sp., which are frequently associated with severe human periodontitis lesions, from equine odontoclastic tooth resorption and hypercementosis (EOTRH) cases, a particular form of equine periodontitis affecting the incisor and canine teeth (Dixon *et al*. 2008b).

Knowledge of the bacteria inhabiting both the healthy equine oral cavity and equine periodontitis lesions is currently limited. Bacteriology of the equine oral cavity has mainly focused on culture of samples derived from apical abscesses. Baker (1979) performed initial culture studies with biochemical identification of isolates from both orally healthy and periodontitis samples as discussed in Section 1.4.2. A summary of bacteria previously isolated from the equine oral cavity of healthy and affected animals is provided in Table 1.3. More recently, modern molecular microbiology techniques have been used to identify bacteria from the equine oral cavity (Dorch *et al*. 2001; Sykora *et al*. 2014). By amplifying and sequencing the bacterial 16S rRNA gene it is possible to identify individual isolates to species level by comparing their sequence to the many others held in large online databases such as the EMBL database (Kulikova, *et al*. 2004) or GenBank (Benson *et al*. 2005). This approach allows a more accurate identification of isolates in comparison to traditional identification methods such as biochemical or phenotypic identification (Clarridge, 2004). In addition, using such methods allows identification of poorly described, rarely isolated, difficult

to grow or novel bacteria, providing clear advantages over traditional methods (Clarridge, 2004).

The aim of this study was to cultivate and identify the bacteria associated with a healthy oral cavity and periodontitis in horses using conventional aerobic and anaerobic microbiological culture and Sanger sequencing of the 16S rRNA gene.

4.2 Materials and Methods

4.2.1 Sample collection

As detailed in Section 2.2.1, after thorough oral examination a swab of the buccal aspect of the gingival margin of teeth 307 to 308 was taken from orally healthy horses using a Sterilin™ Amies transport swab (Fisher Scientific UK Ltd) and transported directely to the laboratory. Plaque samples from the periodontal pockets of horses affected by periodontitis were taken using an equine dental curette (Equine Blades Direct) and placed into 0.5 mL Fastidious Anaerobic Broth (FAB). In order to maximise survival of anaerobic organisms, when samples were taken at the University of Edinburgh, 100 µL of FAB was used to inoculate a Fastidious Anaerobic Agar plate which was then transported back to the laboratory using a GasPak™ EZ Anaerobe Container System (BD Medical Technology, Oxford, England). Samples from ten orally healthy horses and ten horses with periodontitis underwent bacterial culture and subsequent identification of isolates by Sanger sequencing.

4.2.2 Bacterial culture

Amies transport swabs were placed into 0.5 mL Fastidious Anaerobic Broth (FAB) and mixed for one minute to remove bacteria. From each sample, six ten-fold serial dilutions were prepared and 50 µL from each dilution was used to inoculate separate Columbia Blood Agar plates and Fastidious Anaerobic Agar (FAA) plates which both contained 7.5% defibrinated horse blood (E & O Laboratories, Bonnybridge, UK). Columbia blood agar plates were incubated at 37°C aerobically whereas FAA plates were incubated at 37°C in an anaerobic cabinet environment of 85% N_2, 10% CO_2 and 5% H_2. Plates were examined daily until formation of single colonies was apparent on at least one of the serial

dilution plates. After a maximum culture period of six days, the five most prevalent morphologically distinct colonies found on anaerobic and aerobic culture plates of each sample were identified and removed from the plate by using an inoculation loop and placed into 50 µL of sterile water (Thermofisher Scientific) giving a total of 10 isolates per horse. Colonies were then spun down by centrifuge for 2 minutes at 8000 rpm and the supernatant removed, leaving a bacterial cell pellet which was then stored at -20ºC. Where FAA plates had been transported from the University of Edinburgh, these were cultured for a maximum of four days at 37ºC in an anaerobic cabinet environment of 85% N2, 10% CO2 and 5% H2. The resulting growth was then scraped from the plate using a sterile inoculation loop and placed into 0.5mL of FAB from which six, ten fold serial dilutions were made and cultured anaerobically as previously described until individual colonies were apparent. Aerobic culture plates from the original FAB contining the plaque sample which had not undergone anaerobic transport were prepared as previously described.

4.2.2.1 Bacterial DNA extraction

Bacterial DNA was extracted and precipitated using a Masterpure™ Gram positive DNA purification kit (Epicentre, Cambridge, UK) and the corresponding manufacturer's instructions as follows. Each cell pellet was resuspended in 75 µl TE buffer and 0.5µl lysozyme (10mg/mL) and 2µl mutanolysin (1mg/mL) was then added. The isolates were subsequently incubated at 37ºC for one hour. Afterwards, 2.5µl of proteinase K (10mg/mL) was added, thoroughly mixed and incubated at 70ºC for 15 minutes, vortexing every 5 minutes. The isolates were then cooled to 37ºC before placing on ice for 5 minutes prior to DNA precipitation.

4.2.2.2 Bacterial DNA precipitation

Firstly, 90µl MCP precipitation reagent was added to each isolate before removing debris by centrifugation at 4ºC for 10 minutes at 8000rpm. The supernatant was then transferred to a clean 1.5mL micro-centrifuge tube and the pellet of debris discarded. 0.5µl RNase A (5µg/mL) was added and the isolate was incubated at 37ºC for 30 minutes. After incubation, 250µl isopropanol was added and the mixture inverted 30-40 times. Bacterial DNA was then pelleted by

centrifugation at 4ºC for 10 minutes at 10000 rpm. The supernatant was removed and a pipette tip was then used to remove the excess isopropanol mixture without dislodging the DNA pellet which was then rinsed in 70% ethanol. The pellet of bacterial DNA was dissolved in 35µl TE buffer. Three dilutions of bacterial DNA were prepared at dilutions of 1:10, 1:50 and 1:250 using sterile water.

4.2.2.3 Bacterial 16S rRNA gene amplification

Each dilution of bacterial DNA then underwent PCR amplification of the 16S rRNA gene. The 27f upstream primer (AGAGTTTGATCATGGCTCAG) and the 1387r downstream primer (GGGCGGWGTGTACAAGGC) were synthesized commercially by Invitrogen and used with Go Taq Flexi DNA Polymerase (Promega, UK) to amplify the 16S bacterial rRNA gene. Reactions were 50µl total volume and consisted of: 1µl dNTP PCR nucleotide mix, 5µl 1.5mM $MgCl_2$, 10µl 5x Go Taq Green Buffer (Promega, Southhampton, UK), 0.25µl Go Taq Polymerase, 0.25µl 27f upstream primer, 0.25µl 1387r downstream primer, 30.75µl Nuclease free water (Thermoscientific) and 2.5µl of bacterial DNA sample. The PCR reactions were run on a Primus 96 plus thermocycler (MWG biotech) and the sequence consisted of 2 minutes at 95ºC followed by 40 cycles of 95ºC 1 minute denaturation, 55ºC for 1 minute annealing, 72ºC for 1.5 minutes extension and finally an extension step of 10 minutes at 72ºC. In addition to bacterial DNA samples, two controls were also run, a positive control comprising 10ng known bacterial DNA (*Streptococcus mitis*) replacing the sample and a negative control containing with nuclease-free water replacing the sample.

4.2.2.4 Gel electrophoresis

The amplified DNA was detected by gel electrophoresis and visualisation under ultraviolet light. A 1% agarose gel was prepared by adding 0.5 g of agarose (Roche) to 50 mL 0.5x TBE Buffer (Sigma-Aldrich), mixing, then microwaving for 1 minute. When cooled sufficiently, 2.5 µL of ethidium bromide (Sigma-Aldrich) was added and the gel was poured and allowed to set. Once set, the gel was covered with an additional 100 mL TBE Buffer (Sigma-Aldrich) containing 1.75 µL Ethidium Bromide. Ten microliters of each sample were mixed with 2 µl 5x loading dye (Thermo fisher Scientific) and then loaded into separate wells. The

first well of each gel contained a 1000 base pair DNA ladder, prepared by adding 1 µL 1 kbp DNA Ladder (Promega), 9 µL nuclease free sterile water and 2 µl 5x loading dye. Both positive and negative controls were also run on each gel. The gels were run at 60V for thirty minutes. Gels were examined using Gel Doc ™ XR+ System machine with Image Lab ™ software (Bio-Rad Laboratories Inc., Hemel Hempstead, UK).

4.2.2.5 Purification of PCR products

PCR products were purified using a QiaQuick PCR Purification Kit (Qiagen, Crawley, UK). Five volumes of Buffer PB were added to one volume of PCR reaction and then mixed. This mixture was transferred to a QiaQuick column and centrifuged for 60 seconds at 10,000 rpm. The flow through was then discarded and the column placed back into the same 2mL collection tube. Next, 750µl of Buffer PE was added to the column which was then centrifuged again for 60 seconds at 10,000 rpm and the flow-through discarded. The column was centrifuged for another 60 seconds and then transferred to a clean 1.5mL micro-centrifuge tube. To elute bacterial DNA, 50µl of Buffer EB (10mM TrisCl, pH 8.5) was added to the centre of the QiaQuick membrane and the column was centrifuged again for 60 seconds. Purified PCR products were then stored at -20^{o}C until required for Sanger sequencing.

4.2.2.6 Sanger sequencing

PCR products then underwent Sanger sequencing of the bacterial 16S rRNA gene which was carried out commercially at DNA Sequencing and Services, College of Life Sciences, University of Dundee (Dundee, Scotland) using an ABI 3730 Capillary DNA Sequencer and the 357f sequencing primer (CCT ACG GGA GGC AGC AG).

4.2.2.7 Analysis of Sanger sequencing data

All sequence data were compared against the EMBL/GenBank sequence databases using the nucleotide MEGABLAST algorithm. The Basic Alignment Search Tool (BLAST) version 2.1 (Altschul et al. 1997) was used via the National Centre for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences with 98% or above identity to a sequence known to belong to a

particular species of bacteria were identified as that species with the highest score. Sequences with less than 98% identity to known species were designated as potentially novel species. Where sequences were identified as 'uncultured bacteria' this corresponded to sequences which matched those already entered into the database but had not been previously cultured, identified and named. Oxygen utilisation was assigned at genus level as previously described (Whitman *et al.* 2012). Where the sequence of the isolate corresponded to a previously uncultured bacterium the oxygen utilisation of that isolate was categorised as unknown.

4.3 Results

4.3.1 Sample population

The age, breed, sex and disease status of horses sampled for bacterial culture are shown in Table 4.1. The mean age of orally healthy horses was 7.2 years whereas the mean age of diseased horses was 15.5 years. Seventy percent of orally healthy horses were geldings and 30% were mares. In the periodontal disease group, 60% were mares and 40% were geldings. Of the orally healthy group, 60% were native ponies, 10% were cobs, 10% were Irish Sport Horses and 20% were grouped as miscellaneous. Of the diseased group, 10% were native ponies, 40% were cobs, 20% were of Thoroughbred type, 20% were Irish Sport Horses and 10% were grouped as miscellaneous.

HORSE	AGE	BREED	SEX	DISEASE STATUS	DISEASE GRADE
H1	11	Welsh section D	gelding	Orally healthy	0
H2	8	Cob	gelding	Orally healthy	0
H11	7	Dartmoor	mare	Orally healthy	0
H14	6.5	Welsh section A	gelding	Orally healthy	0
H15	4	Clydesdale	gelding	Orally healthy	0
H18	6	Dartmoor	mare	Orally healthy	0
H21	16	Irish Sports Horse	gelding	Orally healthy	0
H28	6	Exmoor	mare	Orally healthy	0
H50	5	Standardbred	gelding	Orally healthy	0
H66	2	Fell	gelding	Orally healthy	0
HM	21	Connemara	mare	Periodontitis	2
H3	14	Cob	gelding	Periodontitis	3

H6	5	Cob	mare	Periodontitis	2
H7	6.5	Arab	mare	Periodontitis	3
H9	14	Cob	gelding	Periodontitis	2
H10	16	Irish Sports Horse	gelding	Periodontitis	4
H19	12	Cob	mare	Periodontitis	3
H20	20	Thoroughbred	gelding	Periodontitis	2
H48	24	Irish Sports Horse	mare	Periodontitis	4
H49	22	Thoroughbred	mare	Periodontitis	2

Table 4.1 Age, breed, sex and disease status of horses sampled for bacterial culture.

4.3.2 Bacterial culture

All twenty samples demonstrated bacterial growth after three days (Table 4.2) using standard culture methods described in Section 4.2.2.1. Due to profuse growth of neat samples when plated and cultured both aerobically and anaerobically, serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were also cultured. Individual colonies could not be distinguished upon examination of any 10^{-1} dilution plates due to heavy growth and single colonies were most often identified when examining 10^{-3} and 10^{-4} dilution plates as shown in Table 4.2.

HORSE	DISEASE STATUS	INCUBATION TIME	SERIAL DILUTION OF PLATE USED TO OBTAIN ISOLATES	
			Blood Agar	Fastidious anaerobic agar
H1	Healthy	4	10^{-3}	10^{-3}
H2	Healthy	4	10^{-3}	10^{-3}
H11	Healthy	4	10^{-4}	10^{-3}
H14	Healthy	4	10^{-4}	10^{-4}
H15	Healthy	4	10^{-2}	10^{-3}
H18	Healthy	4	10^{-2}	10^{-2}
H21	Healthy	4	10^{-3}	10^{-4}
H28	Healthy	4	10^{-5}	10^{-4}
H50	Healthy	4	10^{-3}	10^{-3}
H66	Healthy	4	10^{-3}	10^{-2}
HM	Periodontitis	4	10^{-2}	10^{-2}
H3	Periodontitis	3	10^{-4}	10^{-4}
H6	Periodontitis	4	10^{-3}	10^{-4}
H7	Periodontitis	5	10^{-5}	10^{-5}
H9	Periodontitis	6	10^{-6}	10^{-5}
H10	Periodontitis	5	10^{-4}	10^{-4}
H19	Periodontitis	5	10^{-4}	10^{-5}
H20	Periodontitis	6	10^{-3}	10^{-3}
H48	Periodontitis	5	10^{-4}	10^{-3}
H49	Periodontitis	4	10^{-2}	10^{-2}

Table 4.2 Incubation time and serial dilution of plate used to obtain isolates

4.3.3 Amplification of 16S rRNA gene by PCR

In total, 200 isolates underwent amplification of the bacterial 16S rRNA gene, 100 from oral healthy horses and 100 from horses with periodontitis with 20 isolates from each horse. PCR amplification was successful for all isolates when visualised by gel electrophoresis.

4.3.4 BLAST analysis of sequencing data

In total, 73.5% of isolates seuqenced (n=147) were identified to species or strain level (Table 4.3). The sequences of 39 isolates tested (19.5%) corresponded to previously uncultured bacteria and eight of these isolates could be further classified as previously uncultured *Escherichia* (1 isolate), *Firmicutes* (1), *Klebsiella* (1), *Mannheimia* (1), *Neisseria* (1), *Pasteurella* (1), *Salmonella* (1) and *Vibrio* (1). The corresponding sequences of 14 isolates could not be matched to any sequence in the database.

Table 4.3 Identification of bacterial isolates in equine oral health and periodontitis

	Number of isolates	
Species	**Oral health**	**Periodontitis**
Acetobacterium woodii	1	
Acinetobacter lwoffii	1	1
Acinetobacter* sp. *4077		1
Actinobacillus genomosp.1		8
Actinobacillus hominis		1
Actinobacillus rossii	6	1
Aggregatibacter actinomycetemcomitans	1	3
Aggregatibacter aphrophilus	1	1
Amycolatopsis mediterranei	1	
Arthrobacter* sp. *LC143	1	
Bacterium 15as		1
Bisgaard Taxon 10 strain 24083-02		1
Bisgaard Taxon 10 strain 53665-03	7	
Bisgaard Taxon 10 strain 24322-03	5	1
Buttiauxella agrestis		1
Clostridium acetobutylicum		1
Dermacoccus nishinomiyaensis	1	
Endozoicomonas* sp. *SF102	1	
Enterobacter agglomerans	1	
Enterobacter amnigenus		1

Enterobacter cloacae strain FM 20		1
Enterobacter sp. *SMC27*		1
Escherichia fergusonii		1
Fusobacterium nucleatum	1	
Gordonia polyisoprenivorans	1	
Hafnia alvei		3
Lactococcus garvieae	1	
Lactococcus lactis	1	
Leuconostoc mesenteroides	7	
Micrococcus luteus		3
Micrococcus lylae	2	
Micrococcus sp. PASCW5		1
Micrococcus sp. PP5	1	
Moraxella canis	2	
Neisseria dentiae	2	
Neisseria sp. CCUG	1	
Neisseria weaveri	1	1
No matches	6	8
Pasteurella multocida		1
Pasteurella sp. Melo27	4	1
Pasteurella sp. Melo28		1
Peptostreptococcus anaerobius		4
Peptostreptococcus stomatis		1
Prevotella bivia		1
Prevotella dentalis		1
Prevotella oral taxon 229		1
Pseudomonas putida	1	
Rothia nasimurium	3	1
Salmonella enterica	1	
Salmonella typhimurium	1	
Serratia marcescens	1	3
Serratia sp. G5-10		1
Staphylococcus pasteuri	1	
Staphylococcus pseudintermedius		1
Streptococcus agalactiae	1	
Streptococcus criceti	5	1
Streptococcus devriesei		3
Streptococcus lutetiensis	1	4
Streptococcus mutans	2	
Streptococcus oriloxodontae	2	8
Streptococcus orisaini	1	
Streptococcus salivarius		1
Streptococcus sp. NK-2013	1	
Streptococcus sp. *NK-2014*	1	
uncultured Arthrobacter sp.		1
Uncultured bacterium	16	15
Uncultured Escherichia		1
Uncultured Firmicutes	1	
Uncultured Klebsiella		1

Uncultured Mannheimia		1
Uncultured Neisseria	1	
Uncultured Pastuerella	1	
Uncultured Salmonella		1
Uncultured Vibrio	1	
Veillonella denticariosi		1
Veillonella parvula		4
Veillonella rodentium	1	
Total	100	100

Table 4.3 Continued

4.3.5 Taxonomic classification of bacterial isolates

Isolates were grouped by phyla, genera, and species (Table 4.3). In total, 5 phyla, 35 genera and 66 species were identified. Orally healthy isolates belonged to 4 phyla, 25 genera and 39 species. Isolates cultured from periodontitis samples belonged to 4 phyla, 23 genera and 39 species. Twelve species were common to orally healthy and periodontitis samples while 27 species were unique to both oral health and periodontitis.

Three isolates cultured from periodontitis samples were *Bacteroidetes* in comparison to 0 in orally healthy horses. The number of isolates corresponding to *Firmicutes* was 28 isolates in oral health and 30 in periodontitis while *Actinobacteria* accounted for 11 isolates in oral health and 6 in periodontitis. *Proteobacteria* accounted for 38 isolates in oral health and 37 in periodontitis. Where isolates could not be classified, for example 'previously uncultured bacteria', phyla were designated as unknown, accounting for 22 isolates in oral health and 24 in periodontitis. In addition, in oral health, 1 isolate corresponded to *Fusobacteria* although no isolates cultured from periodontitis samples belonged to this phylum.

In oral health, the isolates predominantly comprised previously unidentified bacteria (16 isolates), *Streptococcus* (15 isolates), bacteria belonging to Bisgaard Taxon 10 (12 isolates), *Leuconostoc* (7 isolates), *Actinobacillus* (6 isolates) and *Pasturella* (5 isolates). In periodontitis, the isolates predominately comprised Streptococcus (18 isolates), previously unidentified bacteria (15 isolates), *Actinobacillus* (10 isolates) and *Veillonella* (5 isolates).

Veillonella sp. accounted for 1 isolate in oral health, and 5 isolates in periodontitis. Bacteria belonging to Bisgaard Taxon 10 accounted for 12 isolates in oral health but only 1 isolate in periodontitis. Seven orally healthy isolates were *Leuconostoc* sp. however this genus was not detected in periodontitis samples. In addition, 5 isolates derived from periodontitis samples were *Peptostreptococci,* but this genus was not isolated from any of the healthy horses.

4.3.6 Oxygen utilisation

The number of isolates corresponding to anaerobic organisms was 14 in oral health and 15 in periodontitis. Bacteria with an unknown oxygen utilisation accounted for 23 isolates in oral health and 26 isolates in peridontitits. Facultative anaerobes accounted for 44 isolates in oral health and 40 isolates in periodontitis. The number of aerobic organisms remained constant at 19 isolates in both oral health and periodontitis.

4.4 Discussion

Although bacteria are known to be crucial in the aetiopathogenesis of periodontal disease in other species, their importance has only very recently been acknowledged in equine periodontal disease (Cox *et al*. 2012; Sykora *et al*. 2014). Previous studies have relied upon biochemical or phenotypic identification of isolates cultured from equine oral clinical samples (Baker 1979) but more recent studies have employed modern molecular microbiology techniques such as 16S rRNA gene sequencing in order to more accurately identify isolates (Dorch *et al*. 2001; Sykora *et al*. 2014). The aim of this study was to identify the bacteria associated with a healthy oral cavity and periodontitis lesions in horses using conventional aerobic and anaerobic microbiological culture and Sanger sequencing of the 16S rRNA gene.

Twenty isolates per horse were randomly selected for further analysis and although this method provides useful information regarding the species present in the equine oral cavity in health and periodontitits, there are limitations of this qualitative data such that is is difficult to draw conclusion regarding the composition of the microbiome without quantitative data. It is therefore

difficult to make meaningful comparisons between the number of isolates of each bacterial species found in the healthy equine oral cavity and periodontitis lesions. Despite efforts to ensure selection of colonies was representative of the growth observed, it is also possible that those colonies picked were not representative due to similarities in morphological appearance.

Bacteroidetes accounted for 0 isolates in oral health but 3 isolates in periodontitis and this was due to the presence of *Prevotella* in periodontitis lesions. Several species of *Prevotella* have been shown to be involved in human periodontitis, such as *Prevotella intermedia* and *Prevotella melaninogenica* (Haffajee and αansky 1994) with *P. intermedia* able to stimulate cytokine production via activation of TLR 2. Growth of *Prevotella* is supported by the reduced oxygen tension found in periodontal pockets and proliferation of Gram negative anaerobes such as *Prevotella melaninogenica* (Loesche 1983) as well as spirochaetes in periodontal pockets is a well-known feature of human periodontitis (Listgarten and Hellden 1978). In the current study, only one more isolate was known to be anaerobic in the periodontitis samples in comparison with the number of anaerobic organsism in oral health. Although in the current study, efforts were made to support survival of anaerobic bacteria these organisms, particularly spirochaetes, are fastidious and difficult to culture and so standard culture may not truly reflect changes in bacterial populations in the oral cavity.

Bacteria associated with mild, moderate and severe periodontitis have previously been grouped into 'complexes', with 'red-complex' bacteria such as the anaerobes *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* being associated with the deep periodontal pockets seen in severe disease (Holt and Ebersole 2005). Although these 'red complex' bacteria are classically associated with severe periodontitis in man and have been detected in clinical samples from horses with EOTRH, a particular presentation of equine periodontitis, no sequences from isolates in the current study matched *P. gingivalis*, *T. denticola* or *T. forsythia*. Whether this is due to different requirements for the growth of these organisms from equine samples is moot. They may be absent or they may be present in lower numbers that makes detection difficult. However, despite a lack of identification of 'red complex'

bacteria, which are associated with periodontitis in man, several other species of bacteria that are known periodontal pathogens in man were identified in the horses involved in current study. *Aggregatibacter actinomycetemcomitans* has frequently been associated with aggressive periodontitis in humans (Zambon 1985) and was identified in both equine oral health (1 isolate) and equine periodontal disease (3 isolates). *A. actinomycetemcomitans* invades periodontal tissue, with cytotoxic effects on oral epithelial cells (Kato *et al*. 2000) and has been shown to increase activation of TLR 2 and TLR 4 (Kikkert *et al*. 2007). Kato *et al*. (2013) have also shown that the increased TLR 2 expression stimulated by A. *actinomycetemcomitans* invasion may lead to apoptosis in THP-1 cells via p38 activation and TNF-α production.

The sequences of 5 isolates cultured from periodontitis samples were matched to *Peptostreptococcus anaerobius,* although this species was not cultured from healthy samples. *P. anaerobius* is a Gram-positive anaerobic coccus and Mallonee *et al*. (1988) found increasing numbers of *P. anaerobius* to be associated with increasing severity of periodontal disease in the cat. *P. anaerobius* has also been associated with gingivitis (Moore et al. 1987), periodontitis (Wade *et al*. 1992) and endodontic infections (Sundqvist 1992) in humans. However, Riggio and Lennon (2002) developed a *P. anaerobius*-specific PCR assay and did not detect the organism in 60 subgingival plaque samples from patients with periodontitis or 43 pus samples from patients with apical infections, leaving the role of *P. anaerobius* in human periodontitis unclear. Downes and Wade (2006) supported this work, after isolating *P. anaerobius* from non-oral sites only. *Peptostreptococcus stomatis* was first described by Downes and Wade (2006) from a number of oral sites in man including apical abscesses and periodontal pockets and one isolate was detected in equine periodontitis samples in the current study.

Streptococci accounted for 15 of orally healthy isolates and 18 of periodontitis isolates. The relatively large number of *Streptococci* isolates is unsurprising as they represent a major component of the early oral biofilm and dominate initial colonisation of the tooth surface (Kreth *et al*. 2009). It is difficult, however, to comment on whether *Streptococci* represent a similarly large component of the biofilm in the horse due to the qualititatve methods used in this chapter. Several

species of interest were identified including *Streptococcus mutans,* which has long been associated with dental caries in humans *(*Loesche 1986*;* Forssten *et al*. 2010*)* although it has also been suggested that human caries is polymicrobial in nature (Fejerskov 2004). The closely related bacterium *Streptococcus devriesei* accounted for 3 isolates recovered from periodontitis samples, although this species was not cultured from orally healthy samples. Although not associated with equine periodontitis, *S. devriesei* was recently described for the first time by Collins *et al*. (2004) after 16S RNA gene sequencing of a Gram-positive *Streptococcus*-like organism recovered from equine teeth. *S. devriesei* has been discovered in 100% of samples taken from fifty horses with equine infundibular caries lesions and also in 4/40 control samples from orally healthy horses (Lundström *et al*. 2007). It was concluded by Lundström *et al*. (2007) that *S. devriesei* likely forms part of the normal, healthy, equine oral flora but under certain conditions may proliferate to colonise diseased equine infundibulae.

Veillonella species accounted for 1 of isolates in oral health and 5 isolates in periodontitis. *Veillonella* are also early colonisers in dental biofilm formation and may comprise up to 5% of the biomass of early plaque (Mager *et al*. 2003). Similar to *Prevotella,* proliferation of anaerobes such as *Veillonella* may also be supported by the reduced oxygen tension found in periodontal pockets (Loesche 1983). *Veillonella parvula* was not detected in orally healthy samples but accounted for 4 isolates from periodontitis samples. Several species of *Veillonella* have been isolated from both healthy gingival sulci and diseased periodontal pockets in man and *Veillonella parvula* has been significantly associated with chronic periodontitis (Mashima *et al*. 2015) and has been shown to stimulate cytokine production via activation of TLR 2 and TLR 4 (Kikkert *et al*. 2007). The 16S rRNA sequence of one isolate from the periodontitis samples was matched to *Veillonella denticariosi* which was initially described by Byun et al. (2007) following culture of carious human dentine.

Several species of bacteria isolated in the current study have been associated with caries, such as *S. mutans* (Loesche 1986) and *V. denticariosi* (Byun et al. 2007) in man and *S. devriesei* (Lunström et al. 2007) in the horse. Thorough dental examinations were carried out to ensure orally healthy horses sampled were free of any pathology and diseased horses were affected by periodontitis

only; however caries of peripheral cementum is commonly found adjacent to periodontitis lesions (Klugh, 2005) and so overlap in the bacteria associated with periodontitis and peripheral caries can be expected. In addition, it is likely, as suggested by Lundström et al. (2007), that these species are part of the normal oral flora and proliferate in environmental conditions created during development and progression of oral disease.

In the current study, 14% of all isolates sequenced could not be matched to any known sequence in neither the EMBL nor Genbank databases. Although 16S rRNA gene sequencing identifies around 90% of isolates to genus level in most cases (Drancourt et al. 2000; Janda and Abbott 2007) there are several possibilities as to why isolate sequences may not be matched to any of those existing in the databases. Inability to identify isolates to genus or species level may be due to novel or rare bacteria or lack of sequences deposited in databases (Janda and Abbott 2007). In addition, several authors have suggested that poor quality sequence deposition is a significant problem with Ashelford et al. (2005) identifying substantial anomalies in 1 of every 20 deposited sequences. In the current study, 40% percent of isolates sequenced were previously uncultured bacteria matching sequences previously deposited in the databases but not further characterised and of these 25% matched previously uncultured bacteria which had previously been identified at genus level. Since few investigations into the oral microbiome of the horse have been performed, it is to be expected that novel or previously uncharacterised bacteria would be present in the current study.

One limitation of this study is small sample size. Although 200 isolates were sequenced, only ten orally healthy and ten horses with periodontitis were sampled, with the ten most prevalent isolates per horse sequenced. It is likely that a larger sample population and number of isolates per horse would have given a more accurate overview of equine oral microbiology. As previously described, traditional culture of isolates from clinical samples can be inaccurate. Even when using modern molecular biology techniques to identity isolates, such as 16S rRNA sequencing, and thus avoiding errors present in traditional identification techniques, many species are fastidious and take prolonged periods of time to form colonies, require specialised conditions or are

simply uncultivable. Dewhirst et al. (2010) estimated that around 68% of oral bacteria cannot be cultured by conventional means and this is especially true for some well-known human periodontal pathogens, particularly spirochaetes and strict anaerobes. This means that both the current study and previous culture-dependent studies of bacteria involved in equine oral health and periodontitis do not reflect the entire equine oral microbiome. Indeed, species which may be important in the pathogenesis of equine periodontitis may not have been identified because these species are uncultivable, novel or previously uncharacterised. It is therefore highly important to use culture- independent methods to assist in identifying putative pathogens in equine periodontitis and to gain an insight into the healthy oral microbiome of the horse.

4.5 Conclusion

In conclusion, several putative pathogens have been identified in the current study and bacteria which have previously been associated with human periodontitis such as *A. actinomycetemcomitans*, *P. anaerobius* and *V. parvula* were detected in equine samples. Bacteria associated with human caries such as *S. mutans* and *V. denticariosi* and infundibular caries in the horse such as *S. devriesei* were also cultured from equine clinical samples. Although culture techniques have some potential advantages over molecular methods, that is it may provide several isolates of microorganisms that can be tested for their effects on the host response to infection in *in vitro, ex-vivo* and *in vivo* model systems and exploration of potential virulence mechanisms, there are distinct limitations. Culture dependent methods for identifying bacteria from clinical samples are laborious, lacks scope, can be inaccurate and may miss species which could be important in the aetiopathogenesis of disease. It is therefore important to use culture-independent methods to gain further insight into bacteriology of equine periodontitis and to characterise the equine oral microbiome in health and disease.

Chapter 5

Culture independent identification of bacteria associated with equine oral health and periodontitis

5.1 Introduction

The aetiopathogenesis of equine periodontal disease is currently unclear, although the possible involvement of bacteria has been recently acknowledged (Cox *et al*. 2012; Sykora *et al*. 2014) after initial investigations by Baker (1979). Bacteria have been shown to be the causative agents in feline, canine and human periodontal disease and so it is highly likely they play a crucial role in the pathogenesis of the condition in horses. An overview of bacteria previously detected in the equine oral cavity in health and periodontitis is given in Table 1.3.

Certain species of bacteria are known periodontal pathogens in humans and *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola, Veillonella dispar and Veillonella parvula* have all been implicated in the pathogenesis of human periodontal disease (Ledder *et al*. 2006; Sun *et al*. 2010*; You et al*. 2013 and Mashima *et al*. 2015). Sykora *et al*. (2014) recently used PCR to detect the presence of *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* in addition to unidentified *Tannerella* and *Treponema* species in gingival crevicular fluid collected from horses affected by EOTRH. However, the presence of known human periodontal pathogens in equine periodontal pockets has not been previously investigated.

Understanding of the equine oral microbiome continues to be limited and little is known about the role bacteria play in equine periodontitis (Cox *et al*. 2012). Studies in other species have estimated that around 70% of oral bacteria cannot be cultured by conventional approaches (Dewhirst *et al*. 2010) and thus the number and variety of bacterial species present in the oral microbiome has been greatly underestimated to date.

It is now possible to determine almost the entire community of bacteria, both commensal and pathogenic, that inhabit the equine oral cavity, in both health and periodontitis using culture independent methods. To date, the majority of approaches have used Sanger sequencing to determine bacterial 16S rRNA gene sequences. This approach allows detection not only of cultivable species but also of fastidious bacteria that may be uncultivable, and also of novel species that may be important in the pathogenesis of disease and has been used to determine the bacterial species present in canine (Riggio *et al*. 2011) and ovine (Riggio *et al*. 2013) periodontal disease lesions.

A further step in analysis of bacterial communities present in clinical samples is sequencing of the whole bacterial genome. Whole genome sequencing yields a vast amount of data allowing detailed species identification, taxonomic and functional classification of known and novel species (Didelot *et al*. 2012), as well as information regarding metabolic pathways and morphological components (Ren *et al*. 2003). It is also possible to determine the presence of bacterial virulence factors and anti-microbial resistance genes which is highly relevant in the clinical setting (Didelot *et al*. 2012). There is a low error rate associated with this technology and a high throughput is possible (Loman *et al*. 2012).

The aim of this study was to determine the microbial profiles associated with the healthy equine oral cavity and equine periodontitis using culture independent techniques, primarily high-throughput sequencing of the bacterial 16S rRNA gene. This approach provides far greater depth, coverage, accuracy and sensitivity than that offered by Sanger sequencing in assessing the composition of complex microbial communities, uncovering microbial diversities that are orders of magnitude higher and with considerably less bias (Song *et al*. 2013). This approach has recently been used by Gao *et al*. (2016) to initially investigate the healthy equine oral microbiome but the microbiome involved in equine periodontitis has not previously been investigated using this technique. Whole genome sequencing of a subset of samples was used to gain further information about the bacterial communities present in equine oral health and periodontitis. In addition to 16S rRNA and whole genome sequencing, a subset of samples was screened by qPCR to determine presence and abundance of DNA

belonging to known human periodontal pathogens at species level. By combining the culture independent methods described with conventional culture techniques a broad range of bacteria can be detected and identified, allowing an insight into the equine oral microbiome in both health and periodontal disease.

5.2 Materials and Methods

5.2.1 High-throughput 16S rRNA sequencing

5.2.1.1 Sample processing and DNA extraction

Supragingival and subgingival plaque samples were each vortex mixed for 30s and Amies transport swabs were immersed in 0.5 mL FAB and mixed to remove bacteria. A crude DNA extract was prepared from each sample by digestion with proteinase K (100 µg/mL) at 60ºC for 60 min, followed by boiling for 10 min. Further DNA purification was conducted using a bead beating technique where 150 µL of each sample was mixed with 200 µL phenol saturated with Tris-HCl (pH 8.0), 250 µL glass beads (0.1 mm) suspended in TE buffer and 200 µL lysis buffer. Samples were then placed in a BioSpec Mini-Beadbeater for 2 min at 2100 oscillations/min and DNA extracted with the AGOWA mag Mini DNA Isolation Kit (AGOWA, Berlin, Germany). In addition to samples from orally healthy horses and horses with periodontitis, five horses with periodontitis were also involved in a site comparison study where three samples were taken from each horse, one from a healthy site in the mouth, one from the diseased gingival margin and one from deep inside the periodontal pocket. Samples were stored at -20^{o}C prior to transportation to Academisch Centrum Tandheelkunde Amsterdam (ACTA) by courier on dry ice.

5.2.1.2 High-throughput sequencing

For each sample, the V3-V4 region of the bacterial 16S rRNA gene was generated by PCR with primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTWTCTAAT). Primers contained Illumina adapters and a unique 8-nt sample index sequence key (Kozich *et al*. 2013). Amplicon libraries were pooled in equimolar amounts and purified using the IllustraTM GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare, Eindhoven, The Netherlands).

Amplicon quality and size was analysed on an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

Paired-end sequencing of amplicons was conducted on the Illumina MiSeq platform using the v3 kit generating 2 x 301 nucleotide reads (Illumina, San Diego, USA) at ACTA.

5.2.1.3 Analysis of sequencing data

Sequencing reads were merged (Edgar and Flyvbjerg 2015), processed and clustered with USEARCH version 8.0.1623 (Edgar 2013). After merging (minimum and maximum merged length, 380 and 438, respectively), the sequences were quality filtered (max. expected error rate 0.002, no ambiguous bases allowed) and clustered into operational taxonomic units (OTUs) using the following settings: -uparse_maxdball 1500, only de novo chimera checking, usearch_global with -maxaccepts 8 -maxrejects 64 -maxhits 1. QIIME version 1.8.0 (Caporaso *et al*. 2010) was used to select the most abundant sequence of each OTU and assigned a taxonomy using the RDP classifier (Cole *et al*. 2009) with a minimum confidence of 0.8 and the 97% representative sequence set based on the SILVA rRNA database, release 119 for QIIME (Quast *et al*. 2013).

5.2.1.4 Statistical analysis

In order to normalise the sequencing depth, the dataset was randomly sub-sampled to 16000 reads per sample. Diversity analysis (Shannon Diversity Index, Chao-1 estimate of total species richness), data ordination by principal component analysis (PCA) and assessment of differences between microbial profiles of the two groups by one-way PERMANOVA were performed using PAleontological STatistics (PAST; v3.02) software (Hammer *et al*. 2001). PERMANOVA was used with Bray-Curtis similarity distance. For PCA, the OTU dataset was additionally normalized by log2-transformation. Diversity output was compared using Mann-Whitney test in SPSS (version 21.0). To determine which OTUs and taxa contribute to differences between the groups, Linear discriminant analysis Effect Size (LEfSe) (Segata *et al*. 2011) was used.

5.2.2 Whole genome sequencing

5.2.2.1 Sample Collection and Processing

As described in Chapter 2, Amies transport swabs from six orally healthy horses were washed in 0.5mL RNAlater as previously described then stored at -20°C until transport to ACTA. Subgingival plaque samples in 0.5mL RNAlater from eleven horses with periodontitis were also stored at -20°C until transported on dry ice by courier to ACTA for analysis.

5.2.2.2 Genomic DNA Extraction

The GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) with a partially adapted protocol was used for DNA isolation. The samples were thawed and centrifuged at 16000 rpm for 5 minutes. The supernatant was removed. The pellet was resuspended in 750 µL lysis solution and transferred to a 2.0-ml cryovial containing 0.1 mm glass beads (BioSpec Products, Inc., Bartlesville, OK, USA). Beadbeating was done in the MiniBeadBeater (BioSpec Products, Inc.) three times for 2 min. In between the beadbeating steps, the vials were incubated on ice for 5 min. Subsequently, proteinase K (10mg/mL) and RNase A were added to the vials and the isolation was continued according to the manufacturer's protocol (Gram-positive bacteria genomic DNA purification protocol).

5.2.2.3 Whole Genome Sequencing

The concentration of DNA was measured using picogreen (Quant It, InvitrogenTM, Carlsbad, CA, USA). DNA (200 ng) was fragmented by sonication (Covaris™ S2, Woburn, MA, USA) to 200 bp. The sheared DNA was processed with the Illumina TruSeq nano DNA library preparation kit (Illumina, San Diego, CA, USA). The Samples were sequenced using a 125 bp paired end modus (HT-v4-PE125, HiSeq 2500) at VUmc Cancer Research Center Amsterdam (the Netherlands).

5.2.3 QPCR

5.2.3.1 Sample collection

A subset of samples was screened for the presence of known periodontal pathogens. Samples from eighteen horses with periodontal disease and five orally healthy horses were included as shown in Table 2.4. Samples were collected as described in Chapter 2.

5.2.3.2 DNA extraction

Bacterial DNA was extracted from clinical samples and precipitated using a Masterpure™ Gram positive DNA purification kit (Epicentre, Cambridge, UK) and the corresponding manufacturer's instructions as follows. 0.5µl lysozyme (10mg/mL) and 2 µl mutanolysin (1mg/mL) were added to 75 µl of each sample. Samples were subsequently incubated at 37°C for one hour. Afterwards, 2.5 µl of proteinase K (10mg/mL) was added, thoroughly mixed and incubated at 70°C for 15 minutes, vortexing every 5 minutes. Samples were then cooled to 37°C before placing on ice for 5 minutes prior to DNA precipitation.

5.2.3.3 Bacterial DNA precipitation

90µL MCP precipitation reagent was added to each sample before removing debris by centrifugation at 4°C for 10 minutes at 8000rpm. Afterwards, the supernatant was transferred to a clean 1.5mL micro centrifuge tube and the pellet of debris discarded. 0.5µL RNase A (5µg/mL) was added and the isolate was incubated at 37°C for 30 minutes. After incubation, 250µL isopropanol was added and the mixture inverted 30-40 times. Extracted DNA was then pelleted by centrifugation at 4°C for 10 minutes at 10000 rpm. The supernatant was removed and a pipette tip was then used to remove the excess isopropanol mixture without dislodging the DNA pellet which was then rinsed in 70% ethanol. The pellet of bacterial DNA was dissolved in 35µL TE buffer. Concentration and purity of DNA was tested using NANODROP 1000 spectrophotometer (Thermoscientific, Renfrew, UK).

5.2.3.4 Putative pathogen selection and primer design

Seven species of bacteria were selected, based on data collected from bacterial culture of equine periodontitis samples presented in Chapter 4, preliminary high throughput sequencing data collected from 24 orally healthy horse and 24 horses with periodontitis (Kennedy et al. 2016), and previous studies showing these species to be periodontal pathogens in man. Previously validated primers were synthesized commercially by Invitrogen for *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola (*Nizam *et al*. 2016*)*, *Veillonella dispar (*Ammann *et al*. 2013*)* and *Veillonella parvula* (Igarashi *et al*. 2009). Primer sequences are shown in Table 5.1

Primer	Sequence (5' to 3')
A.actinomycetemcomitans **Forward**	5'-AACCTTACCTACTCTTGACATCCGAA-3'
A.actinomycetemcomitans **Reverse**	5'-TGCAGCACCTGTCTCAAAGC-3'
P. gingivalis **Forward**	5'-GCGCTCAACGTTCAGCC-3'
P. gingivalis **Reverse**	5'-CACGAATTCGCCTGC-3'
P. intermedia **Forward**	5'-CGGTCTGTTAAGCGTGTTGTG-3'
P. intermedia **Reverse**	5'-CACCATGAATTCCGCATACG-3'
T. forsythia **Forward**	5'-GGGTGAGTAACGCGTATGTAACCT-3'
T. forsythia **Reverse**	5'-ACCCATCCGCAACCAATAAA-3'
T. denticola **Forward**	5'-CCGAATGTGCTCATTTACATAAAGGT-3'
T. denticola **Reverse**	5'-GATACCCATCGTTGCCTTGGT-3'
V. dispar **Forward**	5'-CCCGGGCCTTGTACACACCG-3'
V. dispar **Reverse**	5'-CCCACCGGCTTTGGGCACTT-3'
V. parvula **Forward**	5'-GAAGCATTGGAAGCGAAAGTTTCG-3'
V. parvula **Reverse**	5'-GTGTAACAAGGGAGTACGGACC-3'

Table 5.1 Periodontal pathogen primer sequences.

5.2.3.5 Bacterial culture and standardisation

Aggregatibacter actinomycetemcomitans OSM 1123, was grown and maintained at 37°C on Colombia blood agar (Oxoid, Hampshire, UK) at 5% CO_2. *Veillonella dispar* ATCC 27335, *Veillonella parvula* DSM 2007, *Prevotella intermedia* ATCC 25611 and *Porphyromonas gingivalis* W83 which were maintained at 37°C on fastidious anaerobic agar (FAA [Lab M, Lancashire, UK]) in an anaerobic incubator (Don Whitley Scientific Limited, Shipley, UK) with an atmosphere of 85% N2, 10% CO2 and 5% H2. A colony forming unit count was performed and following extraction, the bacterial DNA content was determined using a NanoDrop ND-1000 (ThermoFisher Scientific, Wohlen, Switzerland). Laboratory stocks of *T. denticola* (ATCC 35405) and *T. forsythia* (ATCC 95137) were used to perform colony forming unit counts. Serial dilutions of bacterial DNA were prepared for each species and RT-QPCR was used to generate standard curves. RT-qPCR was performed using a MX300P™ real time PCR system (Agilent Technologies, Edinburgh, UK). SYBR® Select Mastermix (Life Technologies, Paisley, UK) was used with reactions performed in triplicate. Reactions consisted of 12 µl SYBR® Select Mastermix, 11.5 µl RNAse-free water (Qiagen), 0.5 µl bacterial DNA, 0.5 µl of forward primer and 0.5µl of reverse primer to give a total reaction volume of 25 µl. PCR was carried out using the following conditions: preliminary denaturation step of 95°C for 10 min followed by 50 cycles of denaturation at 95°C for 30 s, primer annealing at 59°C for 1 min and extension at 72°C for 1 min. The colony forming unit (CFU) equivalent for each species at a known concentration was calculated from the corresponding Ct value.

5.2.3.6 Real-time Quantitative PCR

RTQPCR was performed using a MX300P™ real time PCR system (Agilent Technologies, Edinburgh, UK). SYBR® Select Mastermix (Life Technologies, Paisley, UK) was used with reactions performed in triplicate. Reactions consisted of 12 µl SYBR® Select Mastermix, 11.5 µl RNAse-free water (Qiagen), 0.5 µl sample DNA, 0.5 µl of forward primer and 0.5µl of reverse primer to give a total reaction volume of 25 µl. PCR was carried out using the following conditions: preliminary denaturation step of 95°C for 10 min followed by 50 cycles of

denaturation at 95°C for 30 s, primer annealing at 59°C for 1 min and extension at 72°C for 1 min.

5.2.3.7 Data and Statistical Analysis

Microsoft Excel and GraphPad Prism 5 and SSPS (version 22) for Windows were used to analyse the data. Standard curves were obtained for each species of bacteria and used to calculate the colony forming unit equivalent (CFU eq.) in each sample from the Ct value. Equality of variance between the orally healthy group and the periodontitis group was assessed using the Levene's test to indicate the choice of independent sample test (t-test or the Welch's test) for determining whether differences between means were statistically significant. Differences were deemed significant if $p \leq 0.05$.

5.3 Results

5.3.1 QPCR detection of known human periodontal pathogens in equine orally healthy and periodontitis samples

5.3.1.1 Presence of pathogens in equine samples

The percentage of samples in both the orally healthy and peritonitis groups positive for the presence of the selected bacteria is shown in Table 5.2. All eight species were found in both groups, however not in every horse tested. Several species showed a larger abundance in samples taken from equine periodontitis cases. *P. gingivalis* was detected in 40% of orally healthy samples but 94.4% of equine periodontitis samples. Likewise, the presence of *T. forsythia* was detected in 20% of orally healthy samples and 66.7% of diseased samples. The percentage of samples positive for *P. intermedia, V. dispar* and *V.parvula* was also greater in periodontal disease than in oral health (Table 5.2). In contrast the percentage of samples positive for *A.actinomycetemcomitans* was slightly smaller in periodontitis (94.4%) in comparison to oral health (100%). Presence of *T.denticola* was also reduced in periodontitis (39%) in comparison to oral health (80%).

	% of samples positive for putative pathogen	
Bacteria	Orally Healthy	Periodontitis
A.actinomycetemcomitans	100	94.4
P. gingivalis	40	94.4
P.intermedia	80	100
T.denticola	80	39
T. forsythia	20	66.7
V.dispar	60	94.4
V.parvula	60	83.3

Table 5.2 Presence of putative periodontal pathogens in equine oral health and periodontitis

5.3.2 CFU equivalent counts in equine oral health and periodontitis lesions

Significantly larger CFU equivalent counts of *P. gingivalis* (p=0.04), *P. intermedia* (p=0.001), *T.forsythia* (p=0.006) and *V. dispar* (p=0.05) were noted in equine periodontal lesions in comparison to samples taken from orally healthy horses (Figure 5.1). CFU counts varied between horses and several outliers were noted in the data (Figures 5.1 and 5.2). Mean CFU equivalent counts for each species of bacteria increased in equine periodontal disease in comparison to oral health (Table 5.3).

	Orally Healthy		**Periodontitis**	
	Mean CFU eq. count	Standard deviation	Mean CFU eq. count	Standard deviation
A.actinomycetemcomitans	753.8	500.1	948.1	2433
P. gingivalis	56.3	115.2	156.8	291
P.intermedia	65.7	67.1	4310.7	8588
T.denticola	1194.7	581.1	1165.3	2885
T. forsythia	1	0	61.9	130.5
V.dispar	3253.4	5201.5	53405.3	124108.5

Table 5.3 Mean CFU equivalent counts and standard deviations (S.D). CFU equivalent counts were obtained by cross-referencing the Ct value obtained from the sample with the standard curve obtained for that bacterial species.

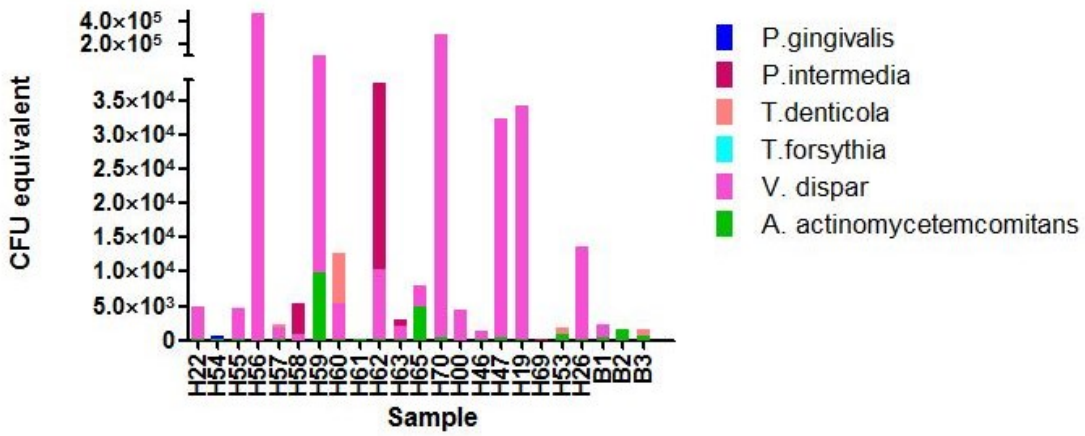


Figure 5.1 CFU equivalent counts per horse. CFU equivalent counts were obtained by cross-referencing the Ct value obtained from the sample with the standard curve obtained for that bacterial species.

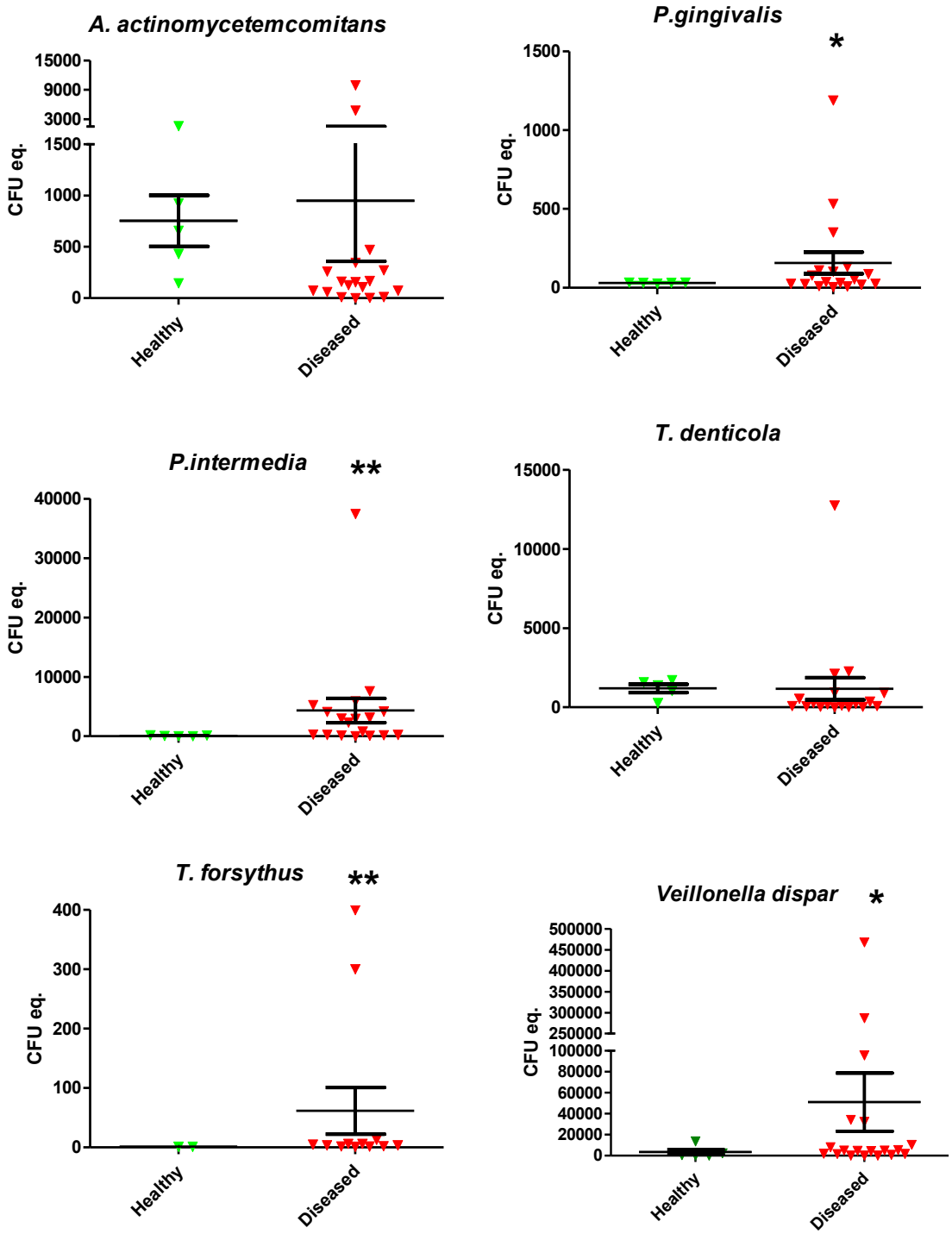


Figure 5.2 CFU equivalent counts in oral healthy (n=5) and periodontitis (n=18) samples CFU equivalent counts were obtained by cross-referencing the Ct value obtained from the sample with the standard curve obtained for that bacterial species.

Mean and standard error of the mean bars also shown * $p<0.05$ ** $p<0.01$

5.3.2.1 Mean CFUs equivalents per group

The mean number of CFU equivalent counts in the periodontitis group was over 11 times that of the orally healthy group. Mean numbers of CFUs for each species in oral health and disease are shown in Table 5.4 and Figure 5.3. Mean CFU equivalent counts were largest for *V. dispar* with a mean count in oral health of 3253 and 53405 in disease. The mean fold change was particularly large for *T. forsythia* (103-fold increase) in periodontitis. There was also a large mean fold change (65-fold) in detection of *P. intermedia* in equine periodontal disease in comparison to oral health. The only putative pathogen to show a mean fold decrease was *T. denticola* (mean fold change of 0.9). *A. actinomycetemcomitans* showed a very small mean fold increase of 1.25 between periodontitis and oral health.

	Orally Healthy	Periodontitis	Mean fold change
	Mean CFU eq.		
P.gingivalis	56.3	156.8	2.8
P.intermedia	65.7	4310.7	65.6
T.denticola	1194.6	1165.4	0.9
T.forsythia	0.4	41.3	103.3
A. actinomycetemcomitans	753.8	948.1	1.3
V.dispar	3253.4	53405.3	16.4

Table 5.4 Mean CFU eq. numbers in the oral health group and periodontitis groups. CFU equivalent counts were obtained by cross-referencing the Ct value obtained from the sample with the standard curve obtained for that bacterial species.

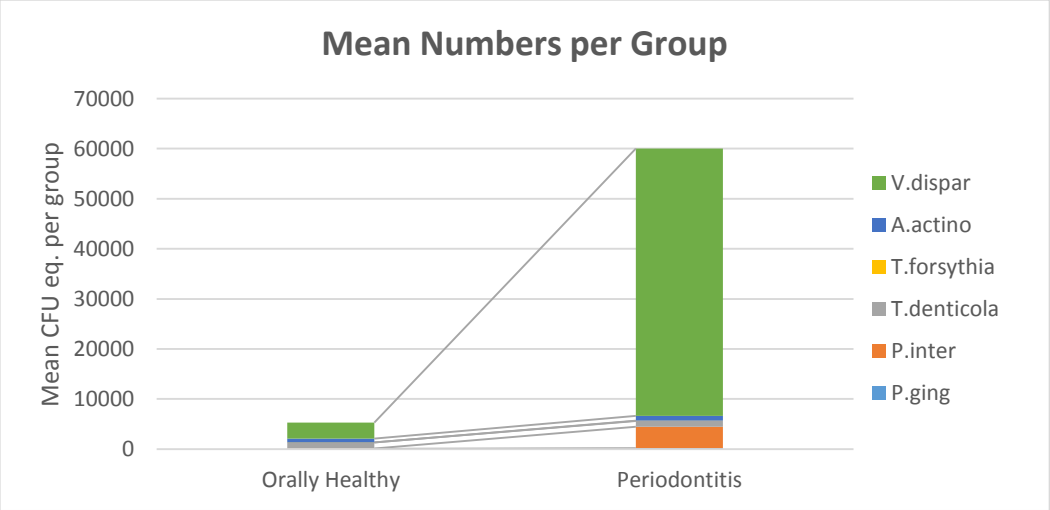


Figure 5.3 Mean CFU eq. per group

5.3.2.2 Proportions of CFU eq. per group and per horse

The proportion of each tested microbe comprising the samples differed between oral health and periodontitis Table 5.5 and Figure 5.4. In particular, the proportion of *P. intermedia* was larger in periodontitis (26.3%) in comparison to 2% in oral health. Larger proportions were also noted for *T. forsythia* (0.01% to 0.9%), *V. dispar* (35.8% to 49%) and *P. gingivalis* (1.8% to 5.2%). The proportion of *A. actinomycetemcomitans* was smaller in periodontitis (5.8%) in comparison with oral health (26.2%). The proportion of *T. denticola* was also smaller in periodontitis (34.2% to 12.5%). Proportions of microbes also differed between horses as well as between groups (Figure 5.5). V. dispar comprised the largest proportion of microbes tested in 12 horses (40% of orally healthy horses and 55% of diseased horses).

	Mean proportions per group	
	Orally Healthy (n=5)	Periodontitis (n=18)
A. actinomycetemcomitans	26.2	5.8
P.gingivalis	1.8	5.2
P.intermedia	2	26.3
T.denticola	34.2	12.5
T.forsythia	0.01	0.9
V. dispar	35.8	49.4

Table 5.5 Mean proportions of microbes per group

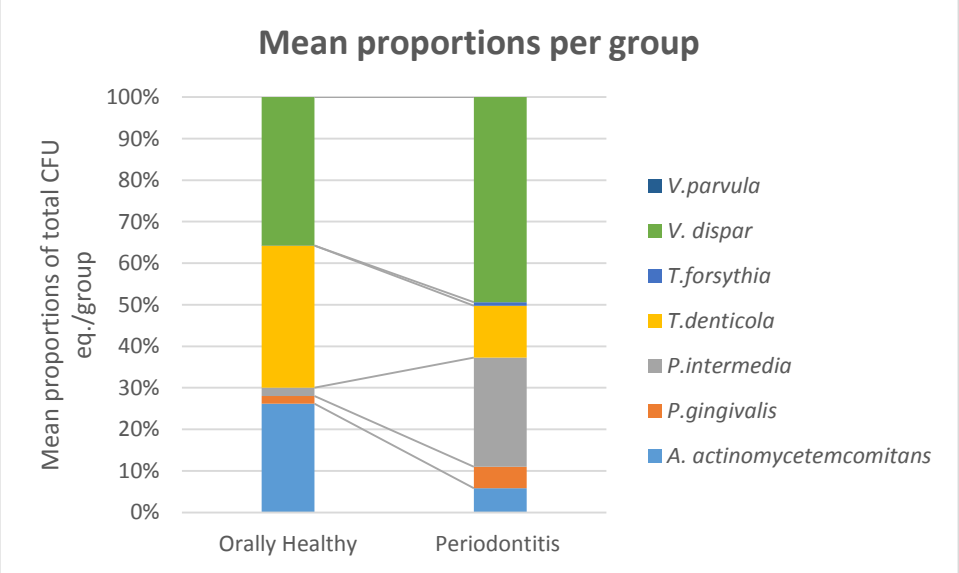


Figure 5.4 Mean proportions of microbes per group

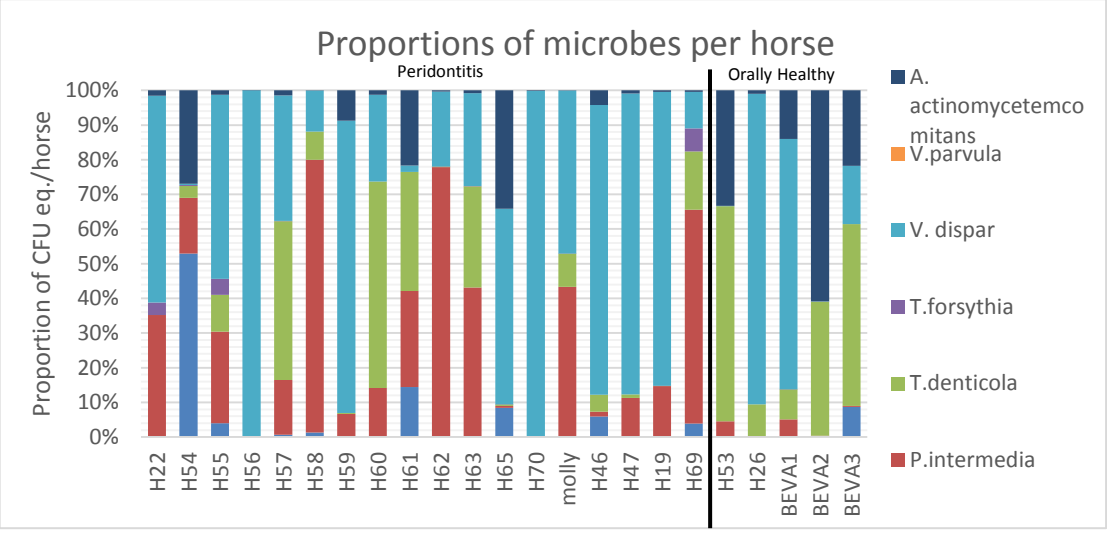


Figure 5.5 Proportions of microbes per horse

5.3.3 High throughput 16S rRNA sequencing

5.3.3.1 Sample demographics

Seventy horses were sampled in total with 30 of these horses being orally healthy 40 suffering from periodontal disease. Demographics of the sample population are shown in Table 5.6 and have previously been described in Chapter 3.

The grade of periodontal disease based on periodontal pocket depth as described by Cox *et al*. (2012) is also shown. Orally healthy horses with no sign of periodontitis were graded 0, clinically normal. Horses with periodontal pockets measuring 5-9mm were designated grade 1, those with 10-14mm pockets were recorded as grade 2 and those with severe disease and pockets of over 15mm were grade 3.

HORSE	AGE	SEX	BREED	PERIODONTITIS GRADE
H2	8	gelding	Cob	0
H10	11	gelding	Welsh Section D	0
H11	7	mare	Dartmoor	0
H12	6	mare	Welsh Section A	0
H13	22	gelding	Fell	0
H14	6.5	gelding	Welsh Section A	0
H15	4	gelding	Clydesdale X	0
H16	4	gelding	Shetland X	0
H17	4	gelding	Connemara	0

H18	6	mare	Dartmoor	0
H21	16	gelding	Irish Sports Horse	0
H26	22	gelding	Trakenher	0
H28	6	mare	Exmoor	0
H28	8	mare	Exmoor	0
H30	6	gelding	Welsh X	0
H31	14	gelding	Clydesdale X	0
H32	18	gelding	Haflinger X	0
H33	9	gelding	Cob	0
H34	11	gelding	Warmblood	0
H36	7	gelding	Shire	0
H37	22	mare	Welsh Section D	0
H38	16	mare	Welsh Section D	0
H39	22	mare	Welsh Section A	0
H40	16	gelding	Thoroughbred	0
H50	5	gelding	Standardbred	0
H51	1	stallion	Welsh Section A	0
H53	1	stallion	Welsh Section A	0
H66	2	gelding	Fell	0
H68	7	mare	Irish Draft	0
H79	1.5	gelding	Welsh Section A	0
H0	22	mare	Connemara	2
H3	15	gelding	Cob	3
H4	14	mare	Highland	2
H5	7	gelding	Cob	2
H6	5	mare	Cross	2
H7	6.5	mare	Arab	3
H8	8	mare	Arab	3
H9	14	gelding	Cob	2
H19	12	mare	Cob	3
H20	12	gelding	Thoroughbred X	2
H22	9	mare	Thoroughbred	2
H23	12	gelding	Welsh Section D	2
H24	15	mare	Thoroughbred	3
H25	11	gelding	Gerlander	3
H27	3	mare	Shetland X	2
H35	12	mare	Welsh Section C	1
H40	22	mare	Welsh Section A	3
H42	12	gelding	Irish Draft	2
H43	22	gelding	Icelandic	3
H44	22	mare	Icelandic	3

H45	16	gelding	Icelandic	2
H46	8	gelding	Warmblood	2
H47	14	mare	Arab	3
H48	24	mare	Irish Sports Horse	4
H49	22	mare	Thoroughbred	2
H54	8	mare	Irish Sports Horse	2
H55	9	mare	Icelandic	2
H56	16	gelding	Cob	3
H57	22	gelding	Icelandic	3
H59	15	mare	Thoroughbred	4
H60	7	gelding	Cob	2
H61	22	gelding	Cob	3
H62	6	mare	Cob	2
H63	22	gelding	Irish Sports Horse	3
H64	18	mare	Cob	2
H65	10	mare	Cob	2
H67	18	mare	Warmblood	3
H69	9	mare	Shetland	3
H70	7	mare	Shetland	4
H71	5	Mare	Shetland	4

Table 5.6 Signalment of horses involved in the study

5.3.3.2 Sequencing output

After quality processing the OTU table contained 1,620,552 reads that were clustered in 1946 OTUs with 783 distinct OTUs once duplicates were removed. These OTUs corresponded to 28 phyla, 52 classes, 174 families, 330 genera and 507 species. The number of reads per sample ranged from 6302 to 49,685 (median 15995.5, mean 15142.6, SD 4855.5). The mean number of reads per orally healthy sample was 14699 and the mean number of reads per periodontitis sample was 15,465.

5.3.3.3 Sample composition

The mean proportion of reads representing each phylum in the orally healthy and periodontitis groups is shown in Fig. 5.6

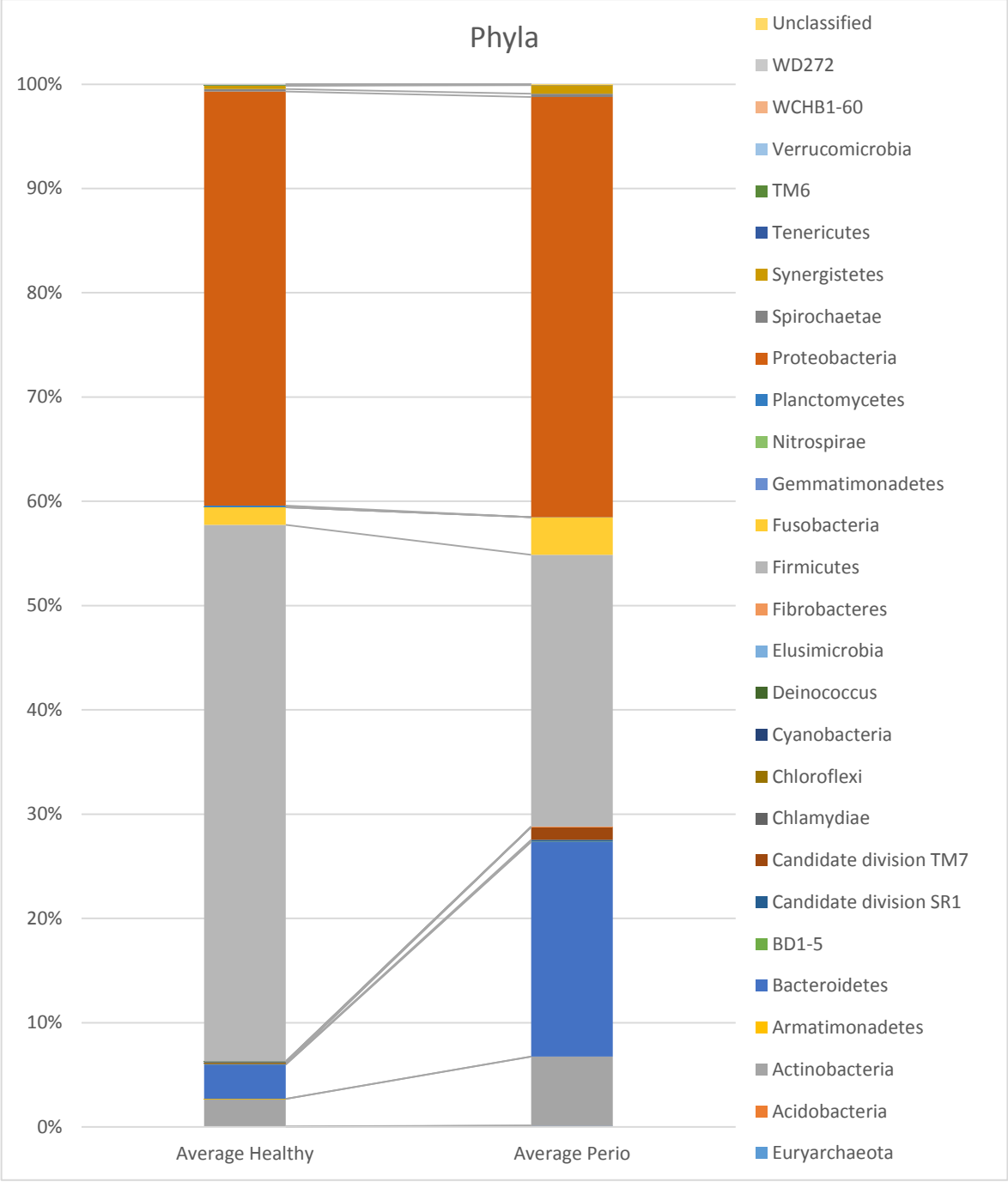


Figure 5.6 Composition of samples from orally healthy horses (n=30) and horses with periodontitis at phyla level (n=40) following high throughput 16s rRNA gene sequencing

When comparing the composition of orally healthy samples with those taken from horses with periodontitis, there were notably larger proportions of *Fusobacteria*, *Bacteroidetes*, *Candidate division TM7* and *Actinobacteria* in the periodontitis samples. There was a large decrease in the average percentage of reads representing *Firmicutes* from 51% in oral health to 26.1% in periodontitis whereas *Bacteroidetes* increased from 3.2% in oral health to 20.5% in periodontitis.

It was possible to identify 507 OTUs to genera level with 297 genera identified in oral health and 220 identified in periodontitis. In oral health, the most abundant genera identified were *Gemella* (36.45%), *Pseudomonas* (14%), *Acinetobacter* (8%), *Fructobacillus* (8%) and *Streptococcus* (6%) (Table 5.7). The most abundant genera present in the periodontitis samples were *Pseudomonas* (25%), *Prevotella* (14%), *Acinetobacter* (9%), *Streptococcus* (6%) and *Veillonella* (5%) (Table 5.8).

	Mean proportion in orally healthy samples (n=30) (%)
Gemella	36.46562
Pseudomonas	14.04524
Acinetobacter	8.061808
Fructobacillus	8.055669
Streptococcus	6.00893
Actinobacillus	5.01366
Citrobacter	3.71237
Enterobacteriaceae	2.956768
Psychrobacter	2.363856
Prevotella	1.74072
Leptotrichia	1.332222
Rothia	1.187949
Sphingobium	0.873902
Actinomyces	0.675556
uncultured bacterium	0.458556
Veillonella	0.395039
Porphyromonas	0.384413
Lautropia	0.340021
Moraxella	0.279337
Treponema	0.256433

Table 5.7 Mean proportion of genera in orally healthy samples (n=30)

	Mean proportion in periodontitis samples (n=40) (%)
Pseudomonas	25.12261
Prevotella	14.27231
Acinetobacter	9.456613
Streptococcus	6.89799
Veillonella	5.228511
Arthrobacter	3.945213
Flavobacterium	3.538444
Actinobacillus	2.032356
Gemella	1.928101
uncultured bacterium	1.844697
Succiniclasticum	1.630931
Actinomyces	1.584235
Enterobacteriaceae	1.568115
Fusobacterium	1.339543
Leptotrichia	1.175364
Fastidiosipila	0.842975
Dialister	0.814677
Shewanella	0.793213
Porphyromonas	0.747656
Pyramidobacter	0.725579

Table 5.8 Mean proportion of genera in periodontitis samples (n=40)

The abundance of *Prevotella* was larger in periodontits (14.3%) samples in comparison with oral health (1.7%). Bacteria belonging to the *Veillonella* genus increased from 0.3% in oral health to 5% in periodontitis. The abundance of *Treponema* and *Tannerella* remained relatively constant between oral health and periodontitis with *Treponema* accounting for 0.26% of identified bacteria in oral health and 0.25% in periodontitis and *Tannerella* accounting for 0.16% in oral health and 0.1% in periodontitis.

5.3.3.4 Microbial profile analyses

Principal component analysis (PCA) revealed a clear difference between the equine oral microbiomes in oral health and periodontitis (Fig. 5.7). Healthy samples clustered together and showed lower variability compared to periodontitis samples. The difference between microbial profiles of the two groups was statistically significant ($p<0.0001$, $F=10.55$, PERMANOVA) and Bray Curtis analysis revealed 89% dissimilarity between the two groups. Although the mean actual species richness (number of OTUs) (Fig. 5.8A*)* and mean estimated species richness (Chao-1) *(*Fig. 5.8B*)* was smaller in samples from periodontal

pockets in comparison to orally healthy samples this was not statistically significant (Species number p=0.07; Chao-1 p= 0.14). However, samples from periodontal pockets were shown to be significantly less diverse than orally healthy samples when analysed using Simpson (p=0.04) (Fig 5.8C) and Shannon Diversity indices (p=0.007) (Fig 5.8D).

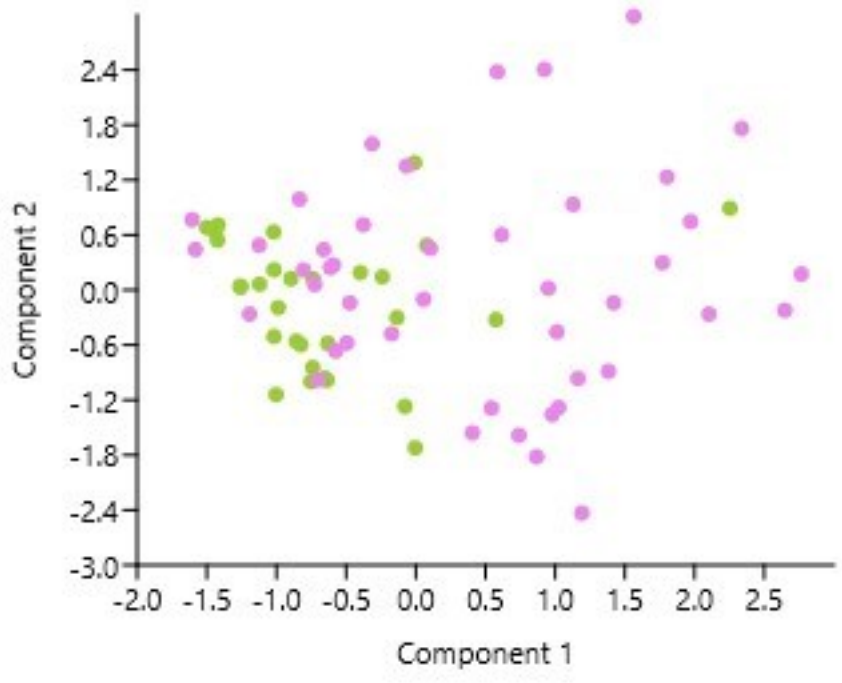


Figure 5.7 Two dimensional ordination of equine microbial profiles at oral health and periodontitis at OTU level by Principal Component Analysis. Green dots represent orally healthy samples and pick dots represent periodontitis samples.

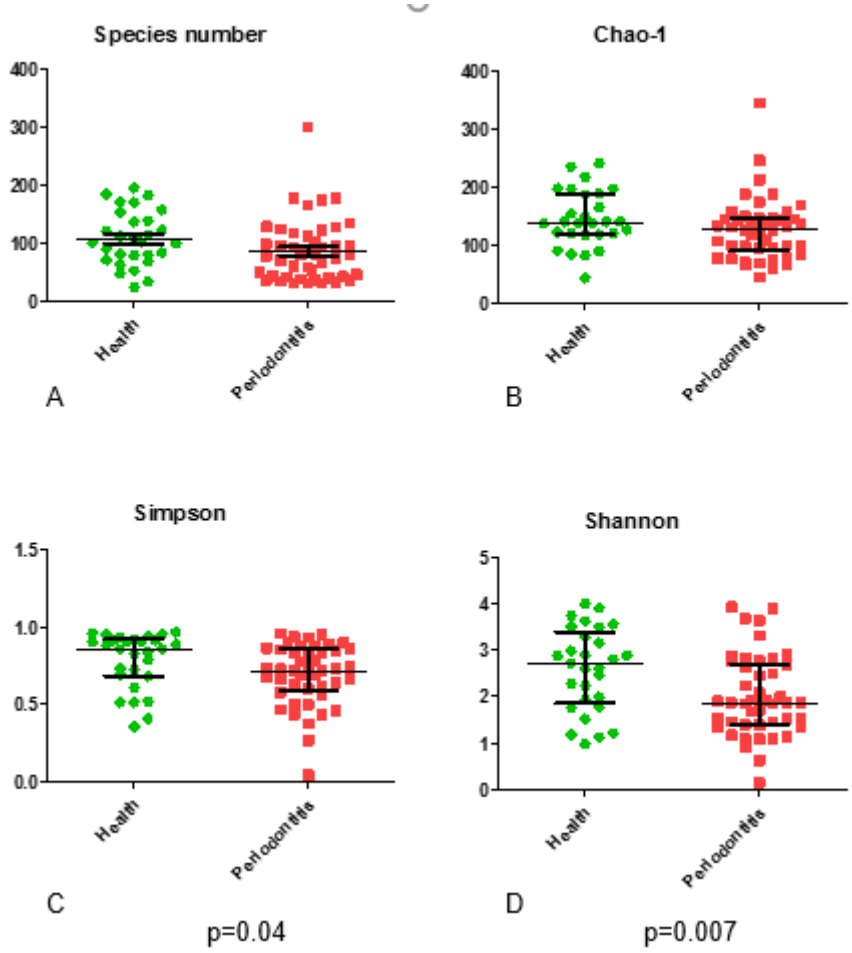


Figure 5.8 Diversity analysis on equine microbial profiles at health and periodontal disease. Error bars represent mean and standard error of the mean.

5.3.3.5 Compositional differences between the groups

Linear discriminant analysis (LDA) Effect Size (LEfSe) was used to assess the differences between the two groups of samples both, at the OTU level and at the genus or higher taxonomic level. In total 190 OTUs were statistically significantly different between the healthy and periodontitis groups ($p<0.05$, LDA>2). All 190 OTUs had an LDA score of over 4 and 166 were associated with periodontitis whilst 24 were associated with oral health (Figs 5.9 and 5.10). Seventy-six OTUs were significantly associated with disease at $p<0.01$ while 6 OTUs were significantly associated with oral health at this level. When OTUs significant at the $p<0.001$ level were considered, 12 OTUs were significantly different and all associated with periodontitis (Fig 5.11)

The most discriminative bacteria identified at genus level between health and disease were *Fusobacteria* and *Acinetobacter* at health and *Pseudomonas* and *Prevotella* in periodontitis. ($p<0.05$).

Interestingly, periodontitis samples had significantly higher relative abundance of *Bacteroidia* (Fig 5.12) *Peptostreptococcus* (Fig 5.13), *Prevotella* (Fig 5.14), *Spirochaetes* (Fig 5.15) and *Treponema* (Fig 5.16).

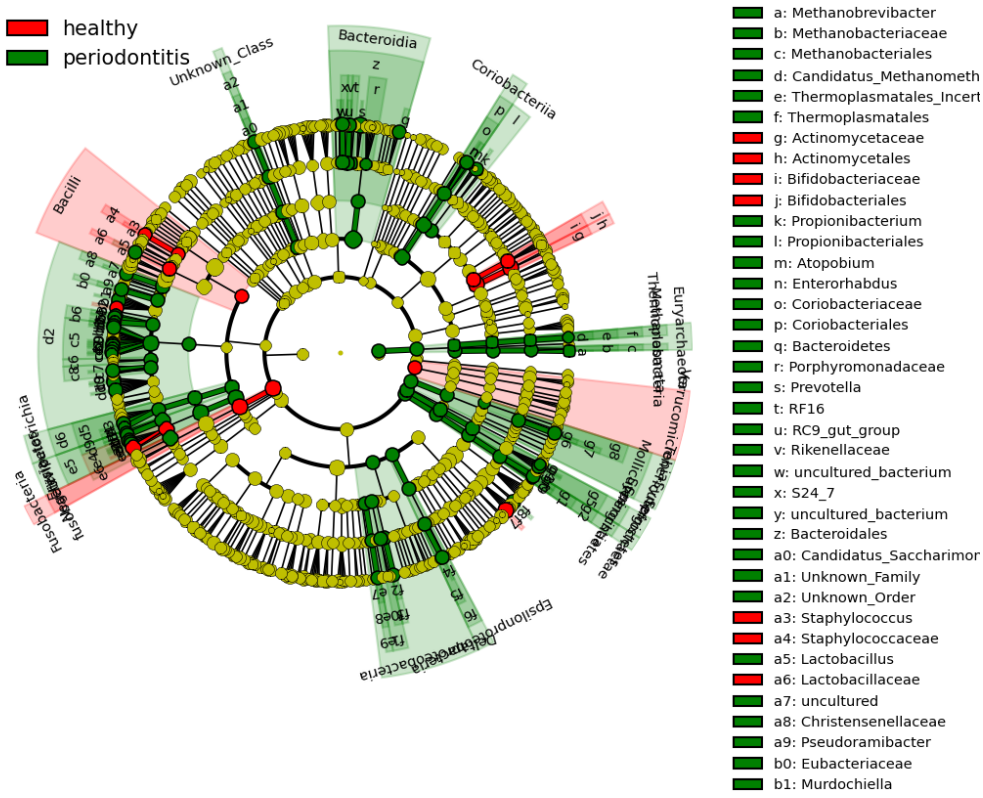


Figure 5.9 Taxonomic representation of statistically significant differences between healthy (red) and periodontitis (green) samples

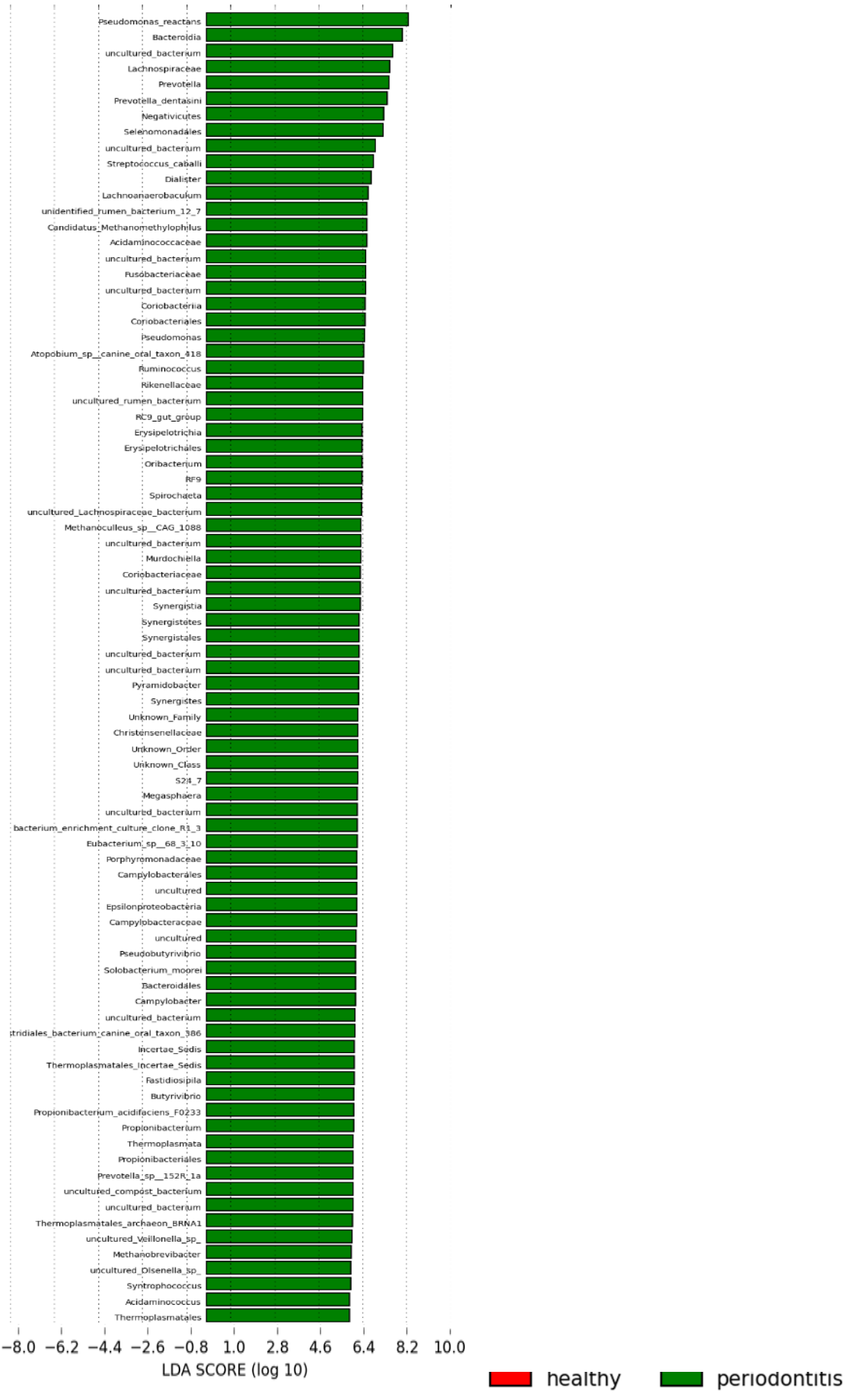


Figure 5.10 Visualisation of most significant taxa (genus or higher level) that differentiate between health and periodontal disease in equine microbiomes ($p<0.05$).

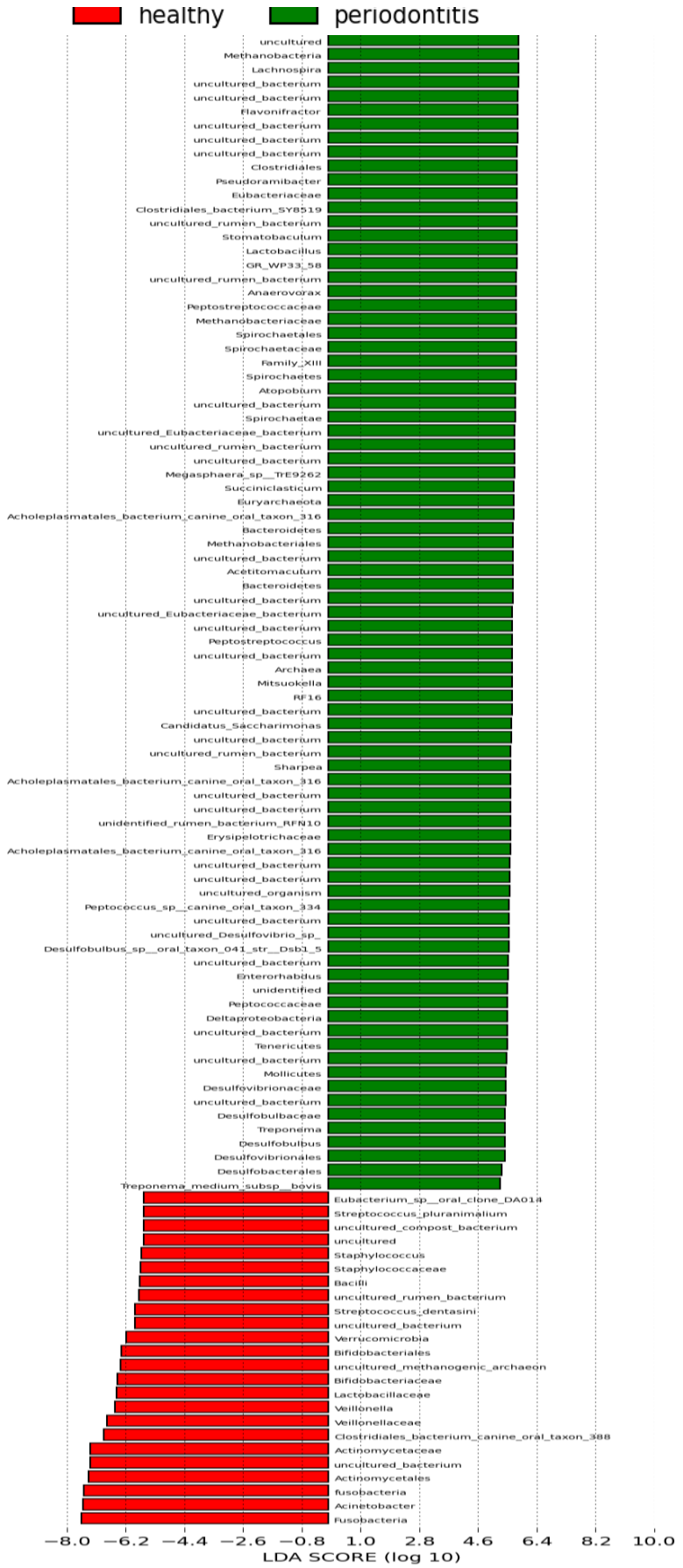


Figure 5.10 (Cont.) Visualisation of most significant taxa (genus or higher level) that differentiate between health and periodontal disease in equine microbiomes ($p<0.05$).

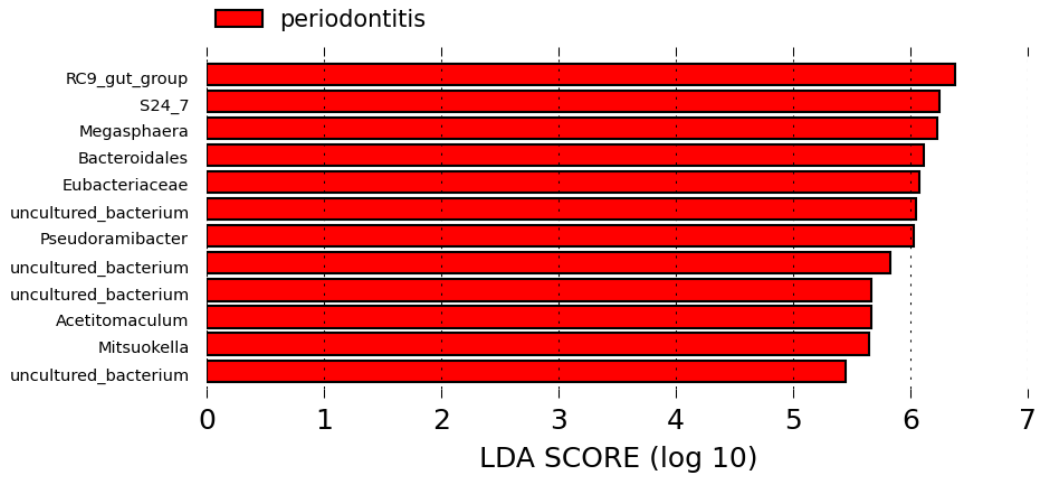


Figure 5.11 Visualisation of most significant taxa (genus or higher level) that differentiate between health and periodontal disease in equine microbiomes. (p<0.001).

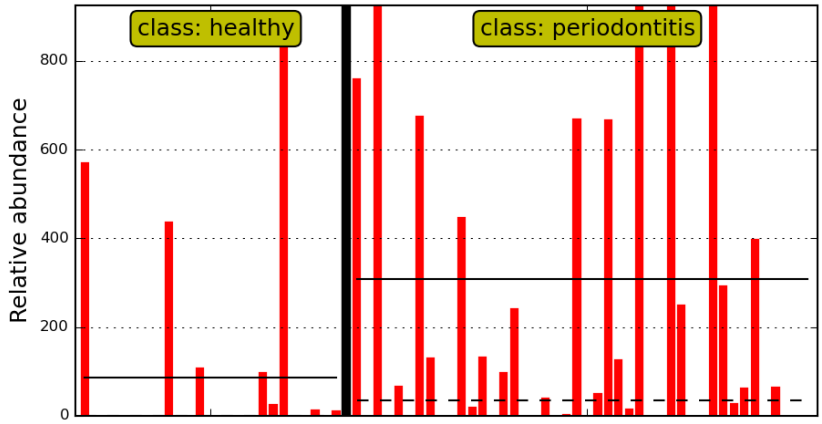


Figure 5.12 Relative abundance of *Bacteroidia* in oral health and periodontitis

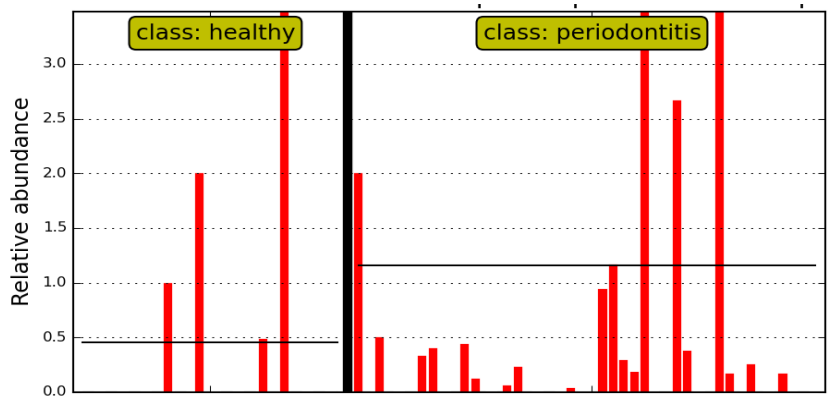


Figure 5.13 Relative abundance of *Peptostreptococcus* in oral health and periodontitis

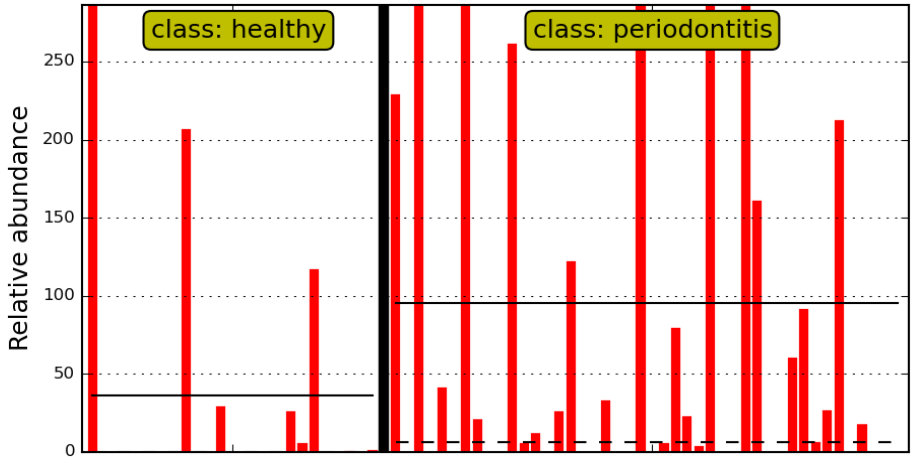


Figure 5.14 Relative abundance of *Prevotella* in oral health and periodontitis

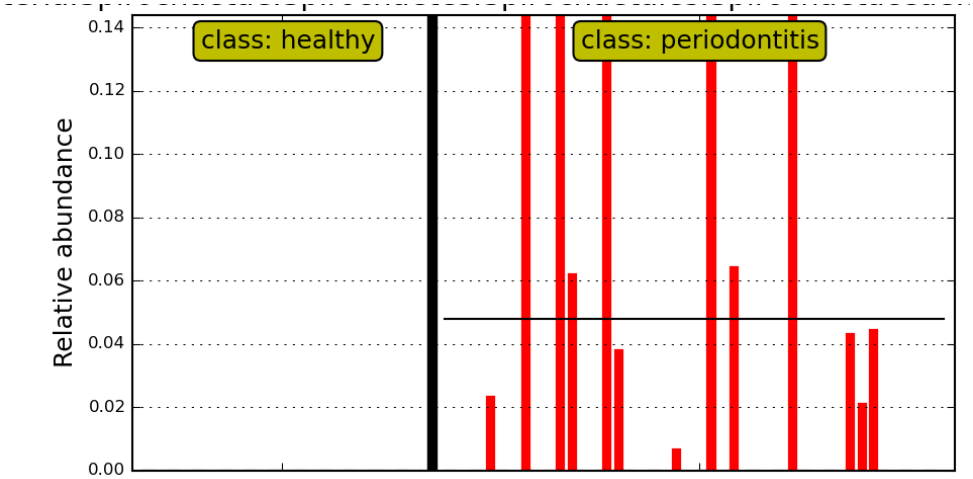


Figure 5.15 Relative abundance of *Spirochaetes* in oral health and periodontitis

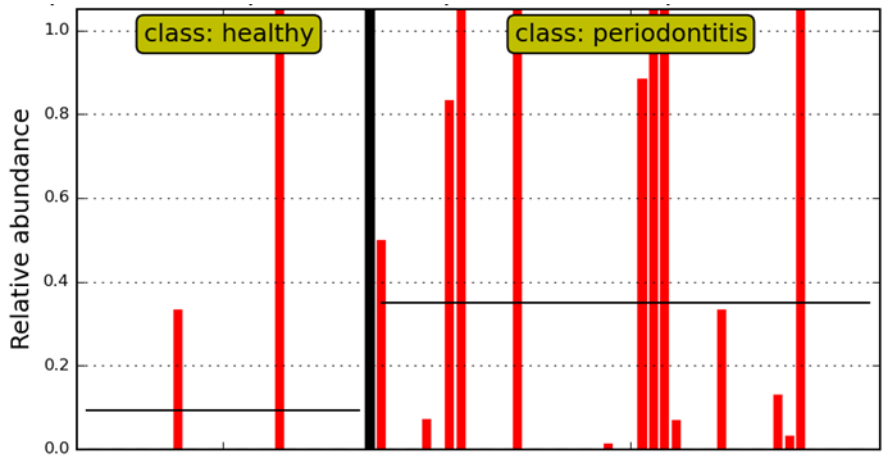


Figure 5.16 Relative abundance of *Treponemes* in oral health and periodontitis

5.3.4 Sample Site Comparison

5.3.4.1 Bacterial DNA yield

Unfortunately, a low level of DNA was obtained from one periodontal pocket site in one horse, yielding very few reads and this sample was excluded when calculating mean values for the bacteria present at each site between the five horses. All other samples yielded a satisfactory quantity and quality of bacterial DNA. In total, 15 phyla, 26 classes, 45 orders, 70 families, 118 genera and 102 species could be identified.

5.3.4.2 Comparison of Sample Composition

Comparison of a healthy site, the diseased gingival margin and deep within the periodontal pocket revealed subtle differences in the composition of the microbiota located at these sites.

When assessing composition by examining phyla of the microbiota present, a decrease in the proportion of *Proteobacteria* and *Fusobacteria* was noted from moving from healthy gingiva, to the diseased gingival margin and finally into the periodontal pocket (Fig 5.17).

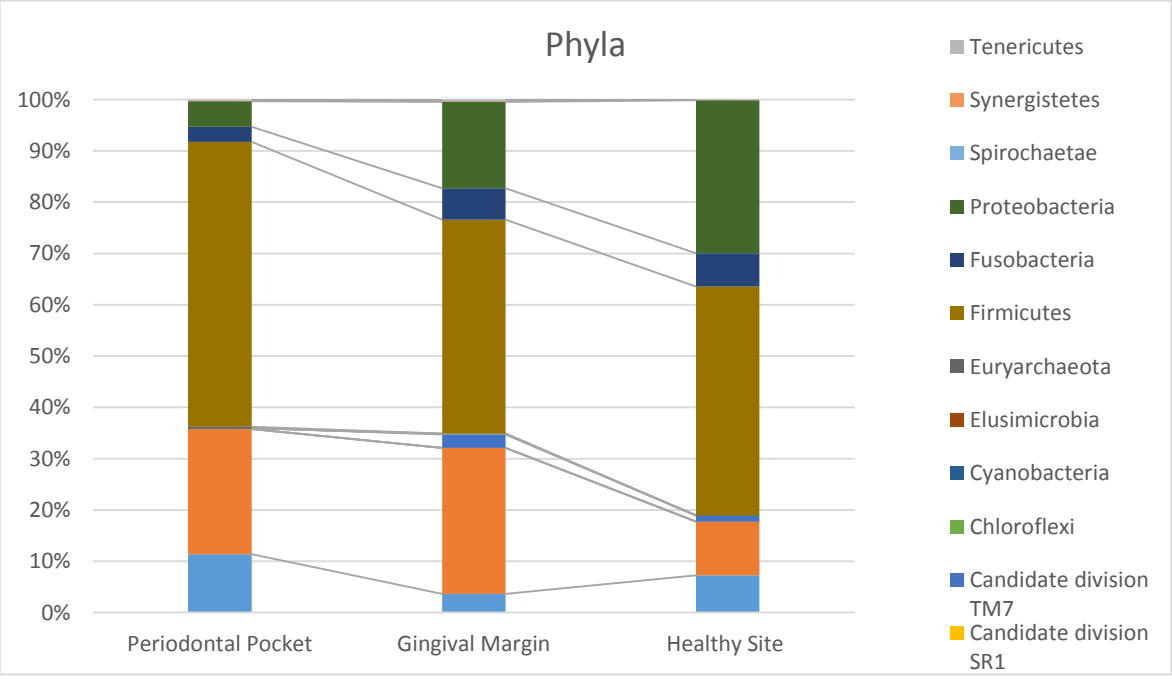


Figure 5.17 Composition of bacteria at different sites in the periodontally diseased equine mouth at phyla level following high throughput 16s rRNA gene sequencing

The mean composition of each sample site at order level can be seen in Fig 5.18 where decreases in the proportions of *Fusobacterales* and *Lactobacillale*s present were noted between healthy gingiva, diseased gingival margin and periodontal pocket. When moving to the healthy state from periodontal disease smaller proportions of *Selenomonadales* and *Sphingomonadales* were noted.

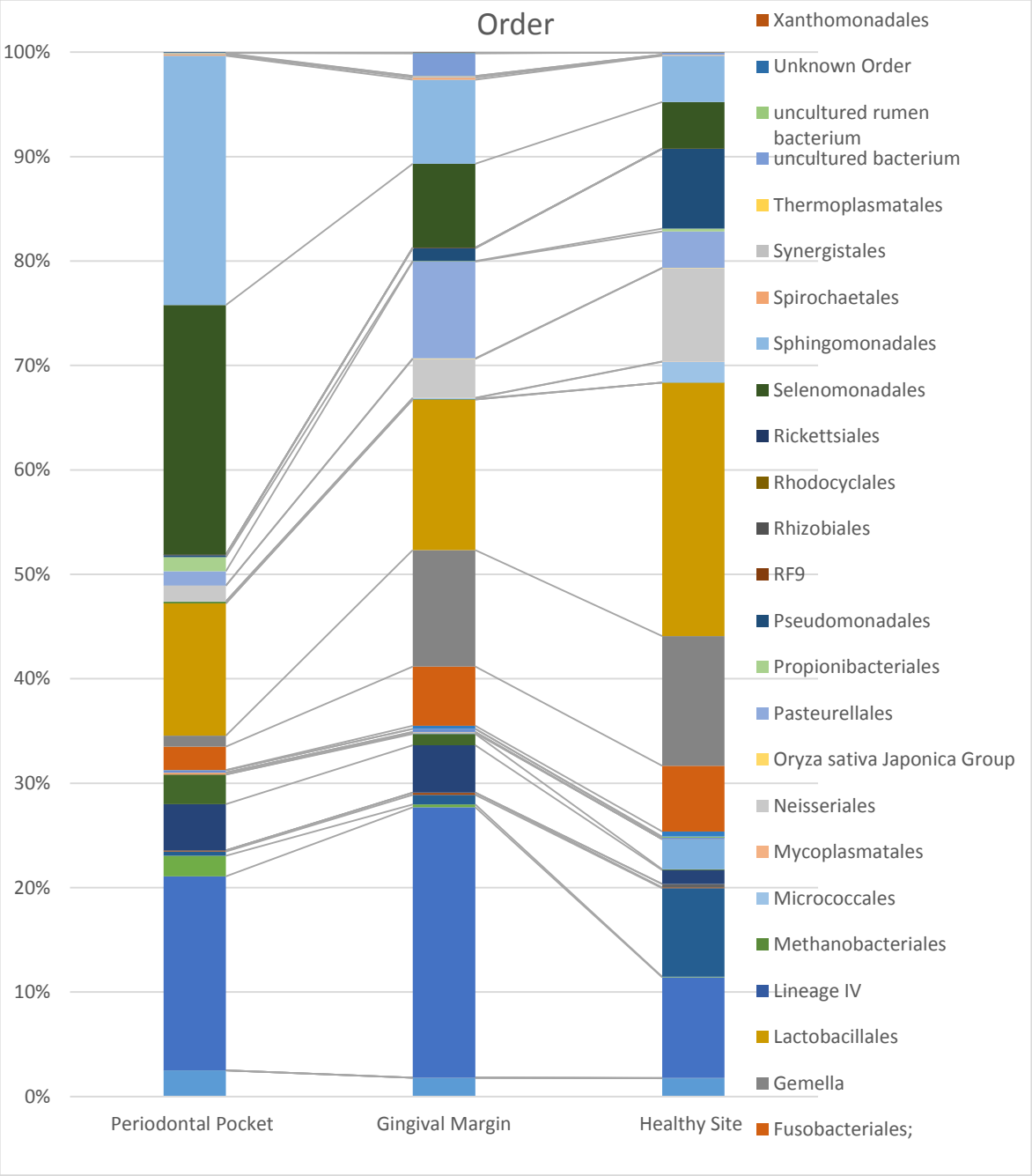


Figure 5.18 Composition of sites in the periodontally diseased equine oral cavity at order level following high throughput 16s rRNA gene sequencing

The genera comprising the highest proportion at the periodontal pocket site were *Veillonella* (31%), *Prevotella* (22%) and *Streptococcus* (17%). The most prevalent genera at the diseased gingival margin were *Prevotella* (25%),

Streptococcus (16%), *Gemella* (14%) and *Actinobaccillus* (10%) and at the healthy gingival site, the most prevalent genera were *Streptococcus* (29%), *Gemella* (15%) and *Lautropia* (10%). The proportion of *Veillonella* was 31% in periodontal pocket sites, 7% at the gingival margin and 5% at the healthy gingival sites. The proportion of *Gemella* was 1.4% in periodontal pocket sites, 14% in diseased gingival margin sites and 15% in healthy gingival sites.

With regards to the bacteria which could be identified at species level, previously uncultured bacteria accounted for the largest proportion at each site comprising 60% of the species identified at the periodontal pocket sites, 55% identified at gingival margin sites and 78% at healthy gingival sites. In particular, the presence of previously uncultured compost bacteria was noted and most prevalent in periodontal pockets (2.4%) in comparison to diseased gingival margin (1.2%) and healthy gingival tissue (0.2%). After previously uncultured bacteria, the predominant species identified in periodontal pockets were *Streptococcus criceti* (16%), *Parascardovia denticolens* (3.7%), *Propionibacterium acidifaciens* (2.6%) *and Prevotella dentasini* (2.4%). At the diseased gingival margin, after previously uncultured bacteria, the most prevalent identified species were previously uncultured methanogenic archaeons (14%), *Streptococcus criceti* (7.2%) and *Prevotella dentasini* (4.1%). At the healthy gingival sites, after previously uncultured bacteria the most prevalent species were previously uncultured methanogenic archaeons (4.3%), *Streptococcus* sp. *27284-01* (3.5%), *Streptococcus criceti* (3.5%) and *Streptococcus devriesei* (3%). Although present in small numbers, the proportions of *Prevotella intermedia* and *Treponema denticola* both increased in the diseased gingival margin 0.06% (*P. intermedia*) and 0.1% (*T. denticola*) and the periodontal pocket (both 0.9%) in comparison to the healthy gingival sites (*P. intermedia* 0.01%; *T. denticola* 0.03%)

5.3.4.3 Diversity indices and species richness

Despite the previously described differences in the microbial populations at each site, analysis using the Simpson Index, Shannon diversity, Chao-1 and estimated species richness revealed no significant difference in species richness or diversity between sample site groups (Fig 5.19).

Both dependent and independent t-tests revealed no significant differences following Bonferroni correction ($p > 0.05$).

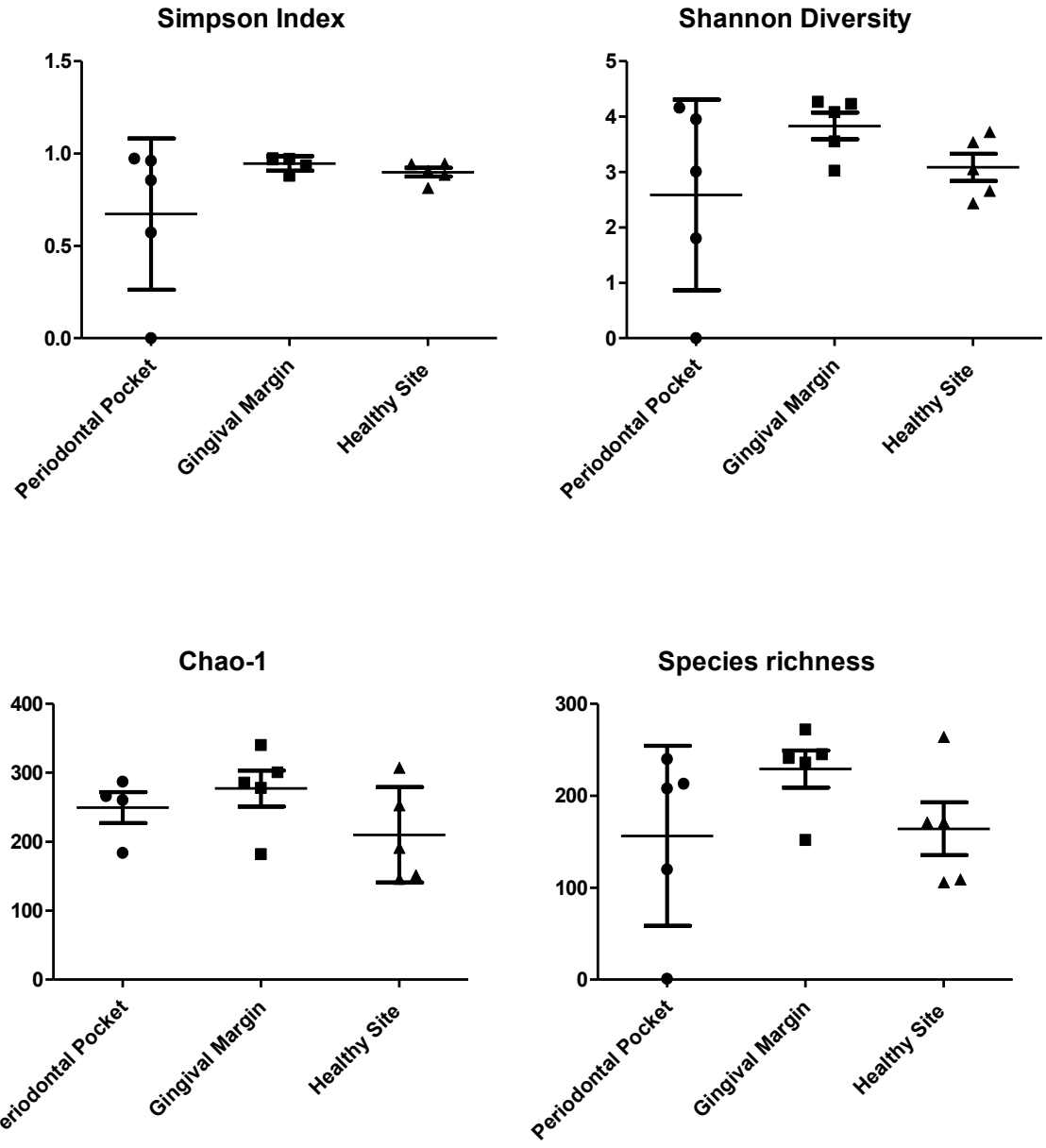


Figure 5.19 Diversity analysis on sites within the periodontally diseased equine orally cavity. Error bars represent mean and standard error of the mean.

5.3.5 Whole genome sequencing

5.3.5.1 Sequencing output

In total five samples from horses with periodontitis underwent whole genome sequencing in addition to three samples from orally healthy horses. Several additional orally healthy samples had been submitted for analysis but a satisfactory quality or quantity of DNA could not be obtained despite several attempts with a variety of healthy samples. A total of 6,971,311 reads were obtained from all samples of which 6,915,098 could be classified. The mean number of reads from orally healthy samples was 787342.2 (SD 35898.1) whereas the mean number of reads from the periodontitis samples was 1011530 (SD 178380.3) The bacteria detected in the eight samples could be classified into 63 phyla, 136 classes, 257 orders, 414 families 759 genera and 2001 species.

5.3.5.2 Sample Composition

The samples taken from horses with periodontitis had both a higher average number of reads per sample and more leaves on the taxonomy tree (i.e. were more diverse) than the samples taken from orally healthy horses (Fig 5.20). The relative abundance of each class of bacteria detected in the samples is shown in Fig 5.21. Bacteria belonging to the *Bacteroidia, Fusobacteria, Gammaproteobacteria, Actinobacteria, Bacilli* and *Clostridia* classes were commonly detected in all samples (Fig 5.21). A particularly high abundance of *Gammaproteobacteria* was noted in two healthy samples due to a high abundance of *Enterobacteriales*. This was not apparent in the other healthy sample and although stringent collection and processing procedures were implemented, there is a possibility that both samples could be contaminated as they were very similar in composition. However, the low number of healthy samples analysed means that it is difficult to build an accurate picture of the healthy equine oral microbiome using this study alone.

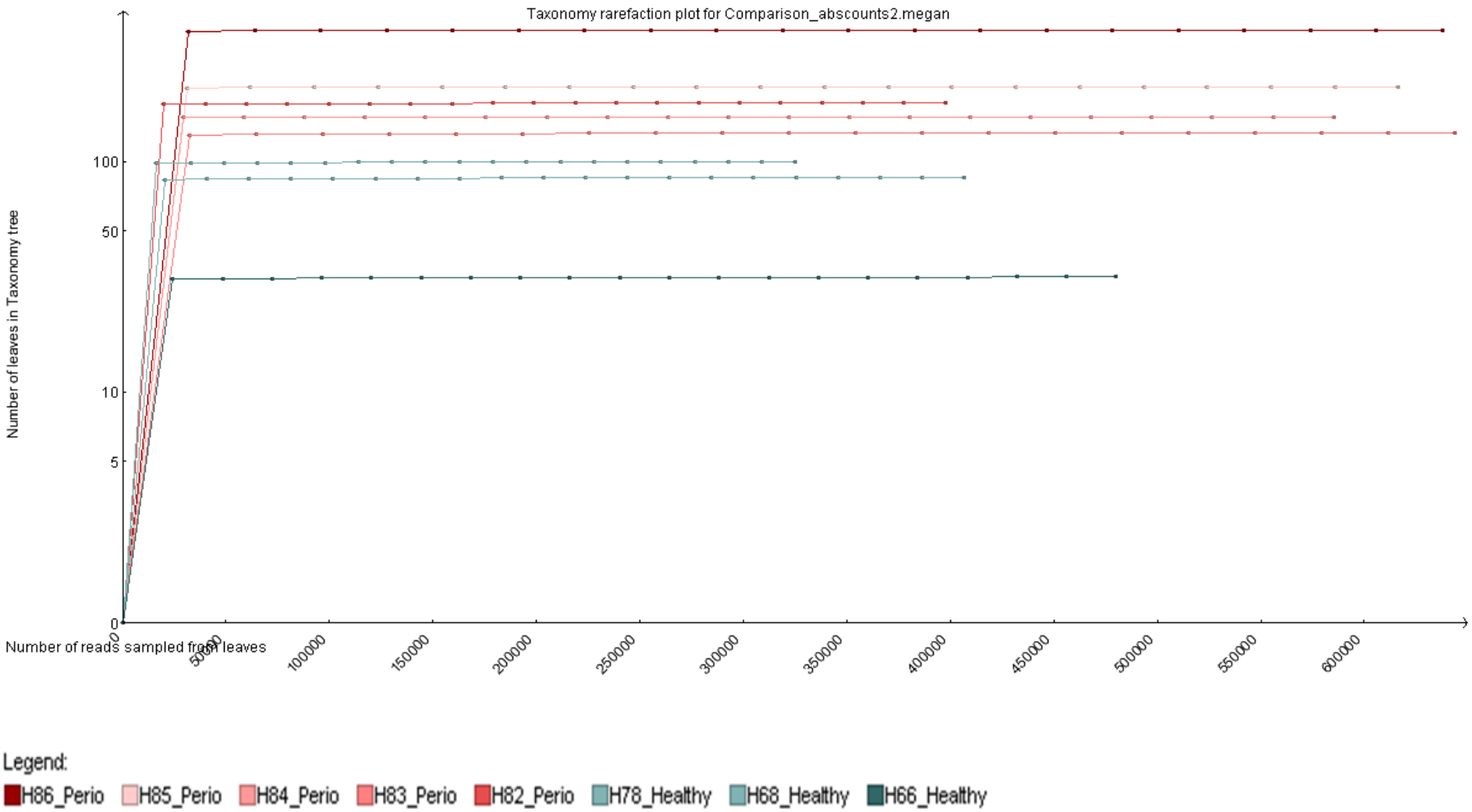


Figure 5.20 Number of reads and leaves in taxonomy tree following whole genome analysis of 5 periodontitis and 3 healthy samples

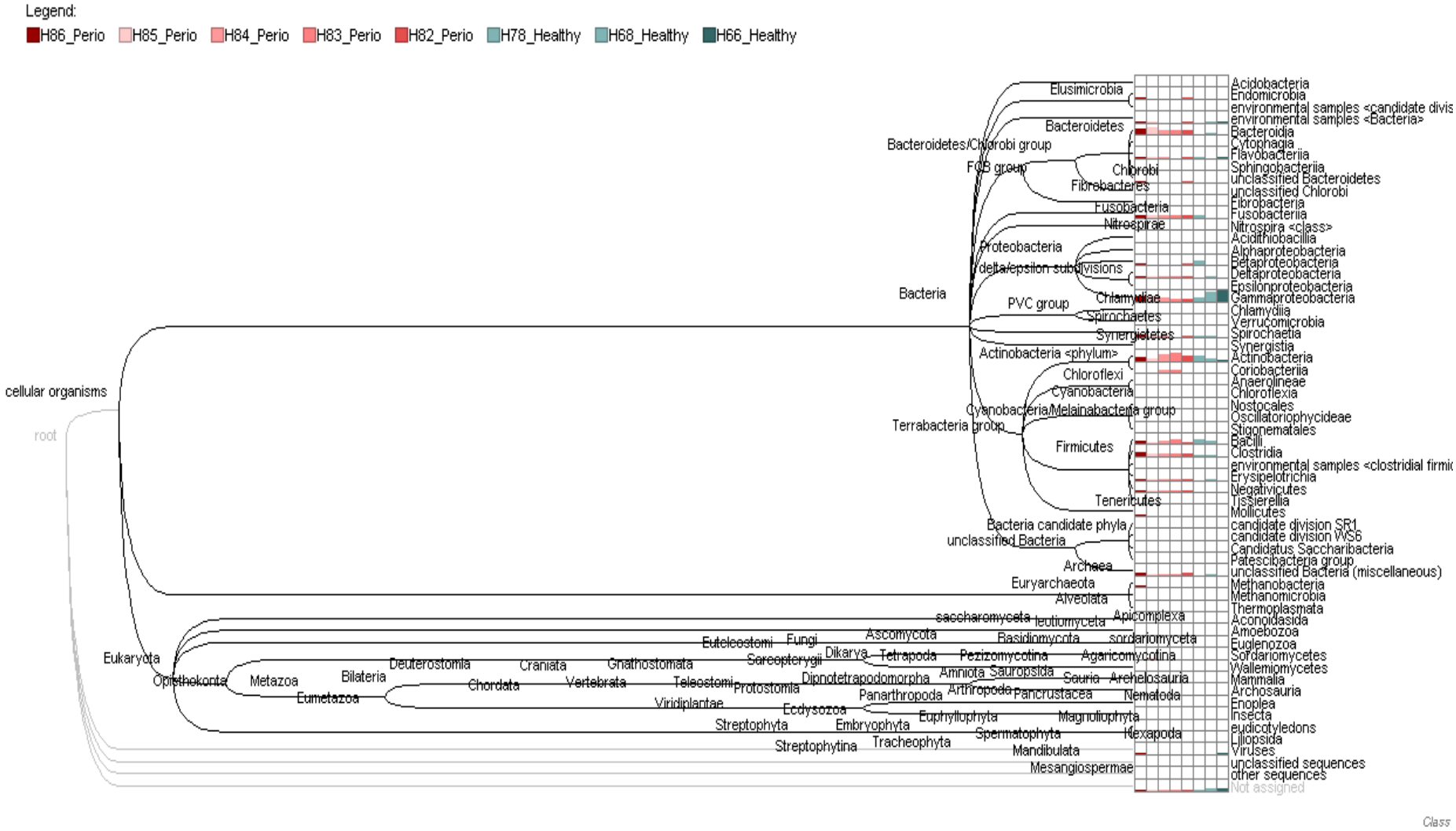


Figure 5.21 Taxonomy of bacteria present following whole genome analysis of 5 periodontitis and 3 healthy samples. Each box represents abundance of bacteria.

5.3.5.3 Presence of putative periodontal pathogens

Several known, human periodontopathogenic bacteria were detected during whole genome analysis of the samples (Table 5.9). The average number of reads was higher in the periodontitis groups for every putative periodontal pathogen except *Streptococcus mutans* (Table 5.9). In total, seventy-five species of *Prevotella* were identified. A significantly higher number of reads was detected in the periodontitis group for *Peptostreptococcus anaerobius* ($p=0.00007$), *Prevotella bivia* ($p=0.003$), *Prevotella dentalis* ($p=0.006$), *Prevotella denticola* ($p=0.007$), *Prevotella intermedia* ($p=0.003$), *Prevotella melaninogenica* ($p=0.002$), and *Prevotella nigrescens* ($p=0.003$). Several species including *Aggregatibacter actinomycetemcomitans*, *Fusobacterium periodontium*, *Peptostreptococcus anaerobius*, *Prevotella bivia*, *Prevotella dentalis*, *Prevotella denticola*, *Prevotella melaninogenica*, *Prevotella nigrescens*, *Treponema socranskii*, *Treponema vincentii* and *Veillonella dispar* were solely detected in the periodontitis samples with no reads detected in the orally healthy samples.

	Number of reads							
Putative periodontal pathogen	H86	H85	H84	H83	H82	H78	H68	H66
Aggregatibacter actinomycetemcomitans	82	0	0	193	155	0	0	0
Fusobacterium necrophorum	1831	5430	827	640	922	110	0	0
Fusobacterium periodontium	357	494	0	0	76	0	0	0
Peptostreptococcus anaerobius	312	402	307	286	254	0	0	0
Porphoromonas endodontalis	1194	658	94	167	435	104	0	0
Porphoromonas gingivalis	675	1021	154	133	512	0	355	0
Prevotella bivia	337	215	203	167	135	0	0	0
Prevotella dentalis	154	222	303	136	120	0	0	0
Prevotella dentasini	1739	23309	6391	6538	1006	88	212	0
Prevotella denticola	343	337	249	124	147	0	0	0
Prevotella intermedia	639	611	585	308	357	122	0	0
Prevotella melaninogenica	450	269	221	147	125	0	0	0
Prevotella nigrescens	544	303	188	159	170	0	0	0
Streptococcus devriesei	622	213	848	2526	483	1182	432	0
Streptococus mutans	386	166	704	716	286	1178	287	0
Tannerella forsythia	5966	3964	5160	1039	2006	368	1739	0
Treponema denticola	3200	992	628	0	1539	120	146	0
Treponema socranskii	1084	280	183	0	678	0	0	0
Treponema vincentii	356	161	0	0	133	0	0	0
Veillonella dispar	83	0	101	173	103	0	0	0
Veillonella parvula	315	92	353	167	374	118	0	0

Table 5.9 Number of reads of human periodontal pathogens in each whole genome sample

5.3.5.4 Presence of human and animal pathogens

Interestingly, a number of reads corresponding to well-known human and animal pathogens were detected in the samples as shown in Table 5.10. Only *Peptoclostridium difficile* showed any significant difference in abundance between health and disease, with a significantly higher abundance in periodontitis.

	Number of reads							
Pathogen	H86	H85	H84	H83	H82	H78	H68	H66
Mycobacterium tuberculosis	1220	1056	2750	4134	1991	2989	1569	1634
Streptococcus equi	239	0	0	190	120	147	0	0
Bordetella pertussis	960	994	1004	1079	1363	9258	2716	6448
Burkholderia mallei	133	0	204	0	0	0	0	0
Burkholderia pseudomallei	194	1047	451	911	107	955	364	834
Neisseria meningitidis	295	308	255	120	1028	2571	164	397
Campylobacter jejuni	254	225	0	0	587	0	0	0
Escherichia coli	4609	2215	193	199	1001	340	17241	10655
Salmonella enterica	5104	7186	3521	255	626	1686	23246	18611
Yersinia pestis	622	594	0	0	0	0	0	0
Mannheimia haemolytica	157	169	0	0	0	0	0	0
Peptoclostridium difficile	215	144	180	161	210	126	0	0

Table 5.10 Number of reads of human and animal pathogens in each whole genome sample

5.3.5.5 Cluster Analysis

Principal co-ordinate analysis (PCoA) (Fig 5.22) and construction of an unweighted pair group method with arithmetic mean (UPGMA) tree (Fig 5.23) showed clear grouping of the periodontitis samples which were dissimilar to the orally healthy samples. Within each group, some samples were also similar to each other such as H66 and H68 of the orally healthy group and H83 and H84 of the periodontitis group. The periodontitis samples did not group by severity of lesion, with the most severe (H86) and least severe (H85) appearing to be more similar in composition to each other than to the other diseased samples.

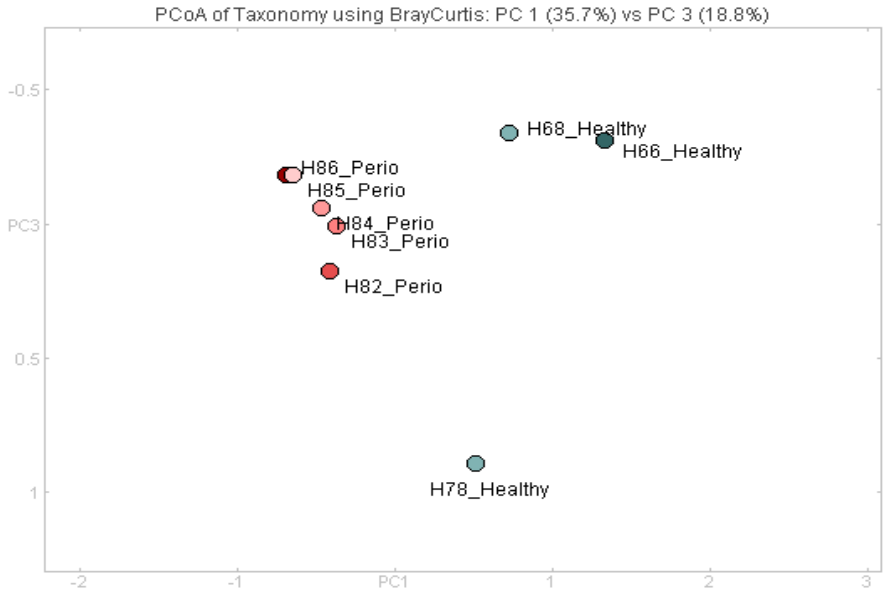


Figure 5.22 PCoA of 5 periodontitis and 3 orally healthy samples following whole genome sequencing

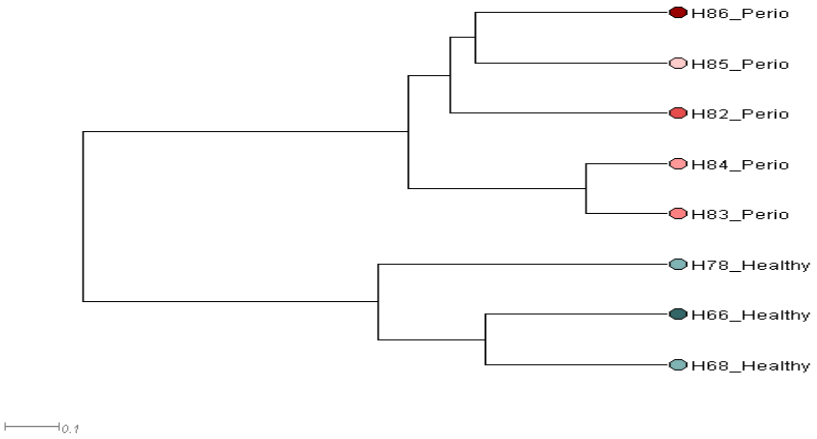


Figure 5.23 UPGMA tree of 5 periodontitis and 3 orally healthy samples following whole genome sequencing

5.3.5.6 Microbial Attributes

Attributes of the microbial populations detected in each sample such as disease association (Fig 5.24), Gram stain (Fig 5.25), known habitat (Fig 5.26) and oxygen requirement (Fig 5.27) were also used to compare the healthy and diseased groups. As shown in Figure 5.24 a high number of reads from the diseased samples were associated with dental diseases primarily associated with the periodontium such as periodontal disease, periodontal infections, gingivitis and presence of dental plaque and in addition dental caries and wound infections. In comparison, a relatively low number of reads from two healthy samples were associated with periodontitis only with no other reads corresponding to the diseases shown in Fig. 5.24

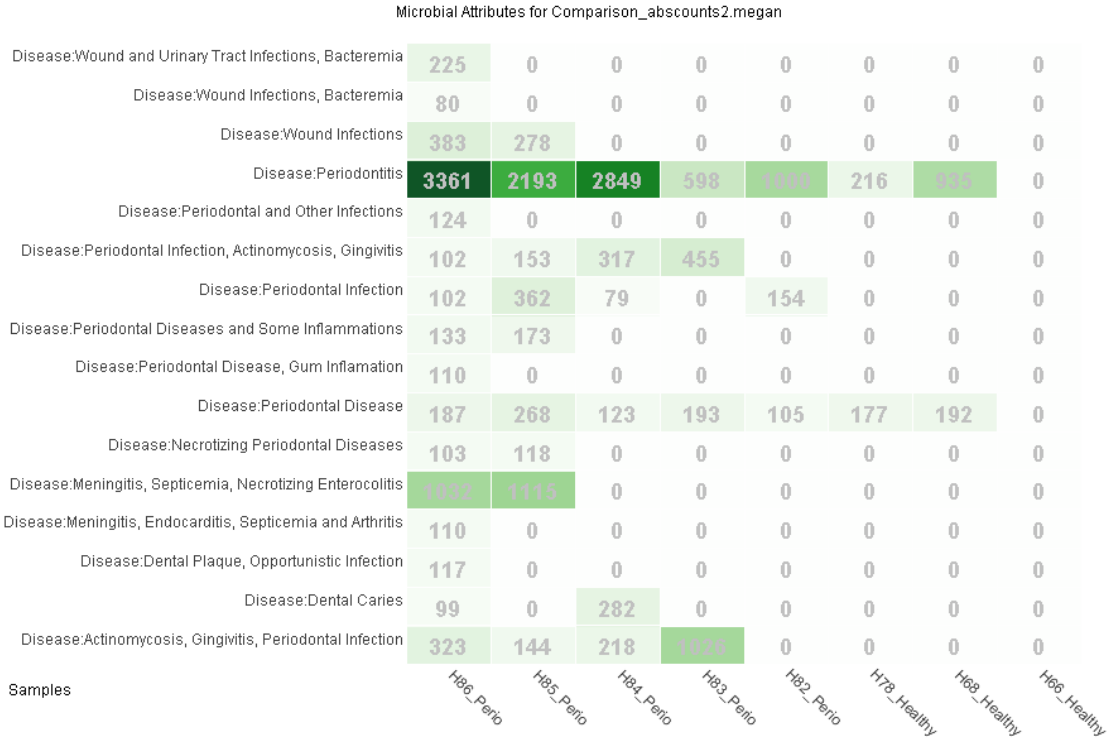


Figure 5.24 Disease association of bacteria and number of reads in equine oral health and periodontitis.

Although Gram +ve and bacteria of an unknown Gram stain status were present in high numbers in all samples, there was an increased number of reads corresponding to Gram -ve bacteria in the periodontitis samples in comparison to the orally healthy samples which increased with increasing severity of lesion (Fig 5.25). Strangely all reads from healthy sample H66 were of unknown Gram staining status.

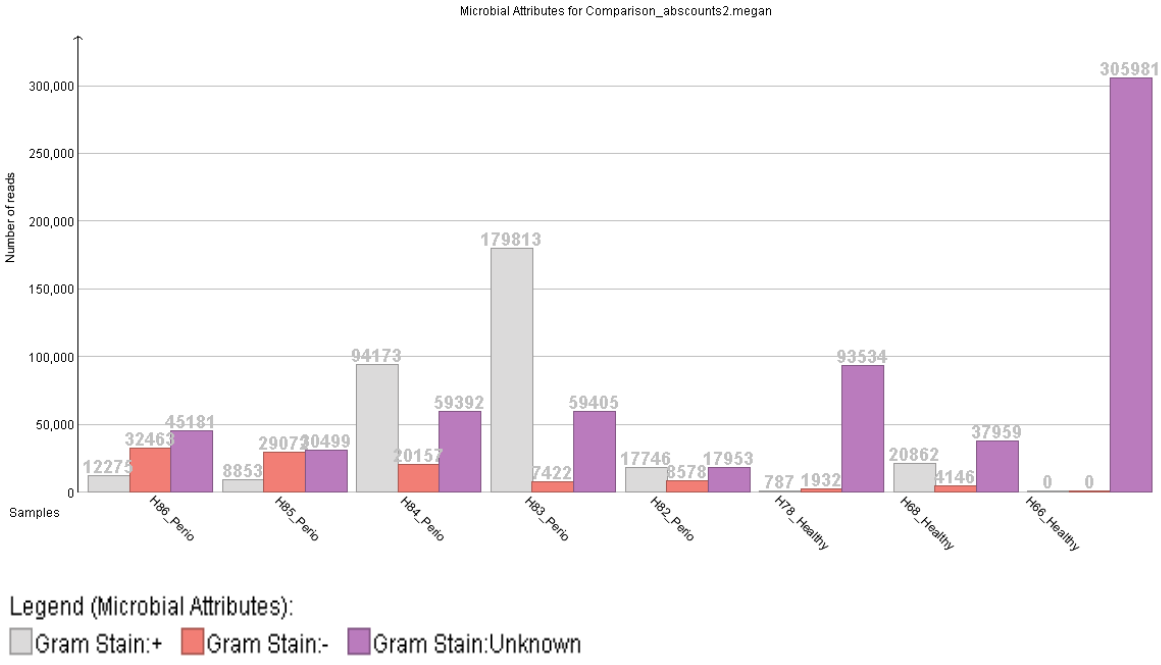


Figure 5.25 Gram staining of bacteria in equine oral health and periodontitis

The majority of reads from the periodontitis samples were host associated and their numbers decreased with decreasing severity of lesion (Fig 5.26). In diseased samples, terrestrial and aquatic species were also noted in addition to many reads corresponding to bacteria associated with multiple habitats. The healthy samples contained mainly host associated and aquatic bacteria although the reads from H66 could not be categorised by habitat.

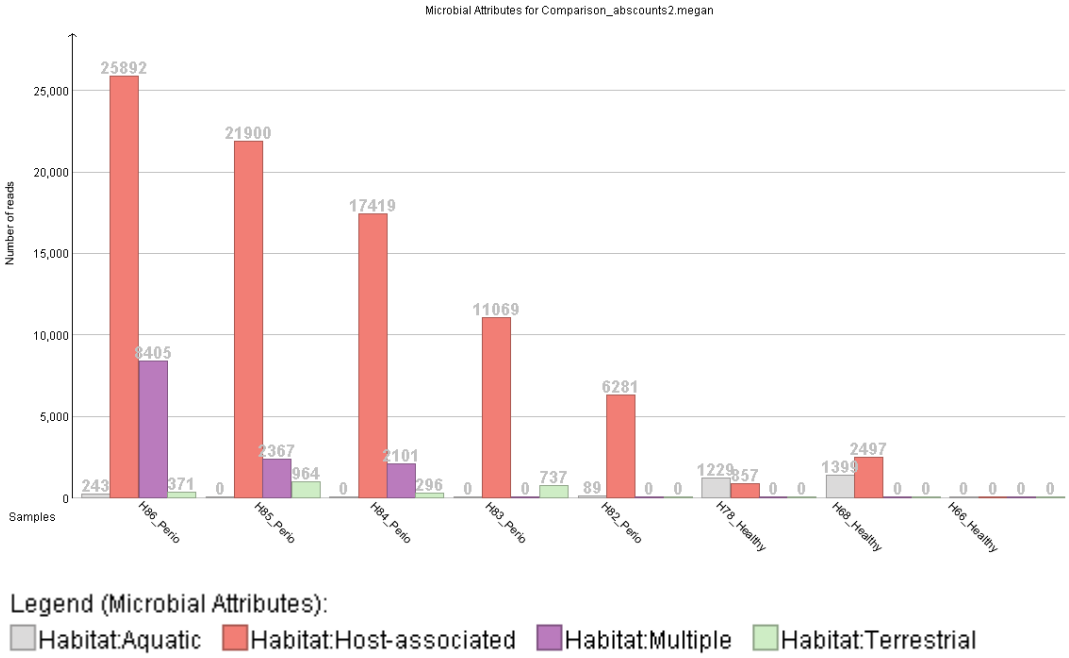


Figure 5.26 Habitat of bacteria in equine oral health and periodontitis

Aside from healthy sample H66 whose reads could not be categorised by oxygen utilisation, the majority of reads for all samples corresponded to anaerobic bacteria, with a higher percentage of reads corresponding to anaerobes in the diseased samples in comparison to the healthy samples (Fig 5.27). In addition, far fewer reads from the healthy samples corresponded to facultative anaerobes than in the periodontitis group.

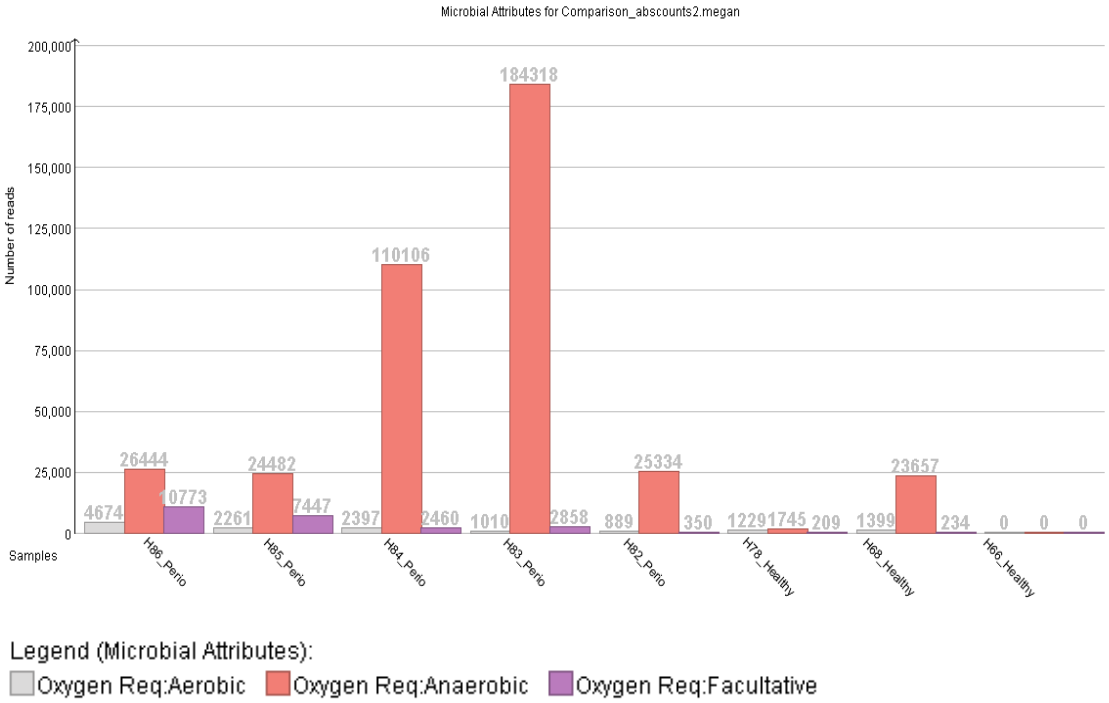


Figure 5.27 Oxygen requirement of bacteria in equine oral health and periodontitis

5.4 Discussion

Despite the difficulty in permanently resolving equine periodontitis and its high prevalence and substantial effect on welfare, few original research studies on its aetiopathogenesis have been published. In humans, the disease is known to be multifactorial and although bacteria play a major role in the aetiopathogenesis of periodontitis in other species, their role in equine periodontitis has only recently received investigation (Cox *et al*. 2012). Few studies have investigated the oral microbiome of the horse in oral health or disease. The current study was the first to use culture independent methods such as high-throughput 16S rRNA gene sequencing, whole genome sequencing and PCR in combination with culture dependent techniques to compare the bacterial populations present in equine oral health and periodontitis and thus represents a considerable advance on what has previously been documented for the oral microbial community in both healthy and diseased horses.

Previously, culture based studies have successfully isolated several bacteria from the healthy and periodontally diseased equine oral cavity including *Campylobacter* sp. (Baker 1979), *Peptostreptococcus* sp. and *Fusobacterium* sp. (Baily and Love, 1990), *Actinobaccillus equuli* (Bisgaard *et al*. 2009) and *Staphylococcus* sp. and *Streptococcus* sp. (dos Santos *et al*. 2014). In addition, Sykora *et al*. (2014) have recorded the presence of known and novel species of periodontal pathogens from horses with EOTRH (Sykora *et al*. 2014) using culture independent techniques. A novel species, *Streptococcus devriesei* was also discovered and identified on equine teeth by Collins (2004) following use of 16S rRNA sequencing. Although the previously described studies and several others, (as illustrated in Table 1.3), have identified many species present in the equine oral cavity, a survey of the microbiome present in oral healthy horses has only recently been performed, allowing assessment of the composition of the microflora present in the normal equine oral cavity. The microbiome of the equine gingival sulcus was investigated by pyrosequencing pooled samples from 200 sulcus sites in two orally healthy horses (Gao *et al*. 2016). Twelve phyla were identified, the most prevalent being Proteobacteria (37.7%), Firmicutes (27.6%) and Bacteroidetes (25.1%). The authors suggested that there are many similarities between equine subgingival microbiota and the subgingival

microbiota detected in human, feline and canine studies. The current study produced similar results to Gao *et al*. (2016) with the most prevalent phyla in oral health being *Proteobacteria* (39%), *Firmicutes* (51%) and *Bacteroidetes* (3.2%) (Fig 5.5). In periodontitis, these phyla were still predominant, however higher proportions of *Bacteroidetes* (20.5% of reads), *Firmicutes (*26.1%) and *Proteobacteria* (40%) were noted. (Fig 5.5). The higher abundance of *Bacteroidetes* is likely to be due to the higher abundance of *Prevotella,* accounting for only 1.7% of reads in oral health in comparison with 14% of reads in periodontitis.

In equine oral health, the most abundant genera were *Gemella* (accounting for 36.5% of reads), *Pseudomonas* (14%), *Acinetobacter* (8%), *Fructobacillus* (8%), *Streptococcus* (6%) and *Actinobaccillus* (5%) (Table 5.8) indicating that these genera comprise part of the normal oral flora of the horse. Bacteria belonging to the *Gemella* genus are known to be normal components of the human oral microflora and have been found to constitute high proportions of the microbiota of the dorsal surface of the human tongue (Mager *et al*. 2003).

Pseudomonas accounted for a high proportion of bacteria in both orally healthy samples (14%) and samples from periodontitis cases (25%) where it was the most common genus identified. Furthermore, *Pseudomonas* and *Prevotella* were the genera most highly associated with periodontitis. *Pseudomonas aeruginosa* is known to be a normal component of the oral biofilm in humans (Souto *et al*. 2014); however, it is also an important opportunistic respiratory pathogen (Sands *et al*. 2017) and is commonly isolated from equine wounds (Westgate *et al*. 2011). It is possible the increased abundance of *Pseudomonas* in equine periodontitis is due to opportunistic colonisation of already damaged tissue. However, *Pseudomonas* is also commonly isolated from the soil and the packing of feed into periodontal pockets could also increase the abundance of this genus in equine periodontal pockets. In human studies, the presence of *P. aeruginosa* in sub-gingival plaque was predictive of periodontitis in women (Persson *et al*. 2008) and strongly associated with chronic periodontitis in smokers (Souto *et al*. 2014). *Acinetobacter* has also been commonly isolated from orally healthy humans (Souto *et al*. 2014) and in addition to *Pseudomonas*, also presents a respiratory challenge to hospitalised human patients. In such patients, a strong

correlation between the presence of *Acinetobacter* sp. and *Pseudomonas* sp. was observed by Zuanazzi *et al.* (2010) and previous studies have indicated that these genera may communicate within formed biofilms sharing information through metabolic activity and genetic exchanges (Hansen *et al.* 2007).

Although *Acinetobacter* sp. have been associated with human nosocomial infections and periodontitis (Souto *et al.* 2006), the percentage of reads corresponding to *Acinetobacter* was similar in equine oral health and periodontitis and this genus was the second most highly associated with oral health in the horse. Both *Pseudomonas* and *Acinetobacter* were abundant in equine oral health and periodontitis and it is likely that communication within the equine oral biofilm is likely between these genera as has been found in other species. It is expected that *Pseudomonas* would be associated with periodontitis but it is surprising that the presence of *Acinetobacter* is highly associated with equine oral health, given the apparent close working relationship of these genera within the biofilm.

Alongside *Acinetobacter*, the other genus most highly associated with oral health was *Fusobacteria*. This genus is known to play a key 'bridging role' in the dental biofilm and is an important component of dental plaque in humans (Kolenbrander *et al.* 2010) and accounted for 1.7% of reads identified to genus level in oral health. Gao *et al.* (2016) reported that 5% of reads could be categorised as *Fusobacteria* and identified only *Fusobacterium necrophorum* at species level. In the current study, *F. necrophorum* was also identified in all whole genome periodontitis samples and one whole genome orally healthy sample and *Fusobacterium periodontium* was identified in all diseased but none of the healthy samples and so it is likely that the species of *Fusobacteria* associated with equine periodontal health are previously unknown or unidentified. Given that no previous studies have characterised the equine oral microbiome in such detail, it is highly likely that many novel or previously uncharacterised bacteria are present in both oral health and periodontitis.

Species belonging to the *Actinobaccillus* genus were previously found to account for 8.75% of bacteria identified in the healthy equine oral cavity (Gao *et al.* 2016) and *Actinobacillus equi* has been frequently isolated from the oral cavity

of healthy horses (Sternberg 1998; Bisgaard *et al*. 2009) and so it would appear that *Actinobaccillus* is also an important component of the healthy oral equine microbiome in contrast to the canine or feline oral microbiome where it is generally present at low levels or absent (Gao *et al*. 2016). *Actinobacillus* species are also frequently isolated from equine bite wounds with *Actinobacillus lignieresii, Actinobacillus suis,* and a *Actinobacillus equuli-like bacterium* all previously cultured (Abrahamian and Goldstein 2011).

Fructobacillus sp. are a group of lactic acid producing bacteria previously classed as *Leuconostoc* sp. commonly found in fructose rich environments (Enzo *et al*. 2015). It is likely that the presence of *Fructobacillus* in orally healthy horses may be due to the presence of fructose in the oral cavity following ongoing consumption of hay, haylage and commercial horse feed (Muller *et al*. 2016). A higher proportion may have been expected in the diseased group due to collection of feed in the periodontal pockets however the anaerobic environment of periodontal pockets would prove unsuitable for these bacteria which grow best under aerobic conditions.

Abundance of *Streptococcus* sp. was similar in oral health (6%) and periodontitis (6%). *Streptococcus* sp. have previously been identified in the healthy equine oral cavity (Baker 1979; dos Santos et al. 2014) and recently Gao *et al*. (2016) discovered *Streptococcus suis* and *Streptococcus minor* in orally healthy horses using high throughput 16S rRNA sequencing. Several *Streptococcus* species have been associated with dental caries, with a Streptococcus mutans-like bacterium, named *Streptococcus devriesei* by Collins (2004) being linked to infundibular caries (Lundstrom *et al*. 2007) in the horse. *S. mutans* and *S. devriesei* were both detected in all whole genome sequencing samples except one sample in which no reads from either species was present. There was no significant difference in the number of reads corresponding to either *S. mutans* or *S. devriesei* between the orally healthy and periodontitis samples which underwent whole genome sequencing. Ramzan and Palmer (2011) reported that the presence of peripheral cemental caries had no significant association with the presence of diastemata and the *Streptococcus* species associated with this condition were not associated with periodontitis in the current studies. Given the important role of feed stagnation and decomposition in relation to the

dental biofilm in periodontitis, and its potential role in peripheral caries it is perhaps unexpected that the two pathologies have no association.

The most abundant genera in equine periodontitis were *Pseudomonas* (25%), *Prevotella* (14%), *Acinetobacter (*9.4%), *Streptococcus* (7%) and *Veillonella* (5%). Bacteria belonging to the *Prevotella* genus are known to be associated with periodontitis in humans and the results of the current study indicate that this is also likely to be true in the horse.

Prevotella species accounted for 1.7% of bacteria identified at genus level and 14% in periodontitis and in addition, *Prevotella* was noted to be highly significantly associated with the presence of equine periodontitis following LefSe analysis with an LDA score of 6.8 (Fig. 5.10). When sampling two orally healthy horses, Gao *et al*. (2016) reported that 17.5% of reads corresponded to *Prevotella* species with unclassified species of *Prevotella* accounting for 33.5% of the total oral microbiota of one horse. The data generated from the current whole genome sequencing of both orally healthy horses and horses with periodontitis revealed the presence of 75 species of *Prevotella* with significant increases in abundance of *Prevotella bivia* (p=0.003), *Prevotella denticola* (p=0.007), *Prevotella dentalis* (p=0.006), *Prevotella intermedia* (p=0.003), *Prevotella melaninogenica* (p=0.002) and *Prevotella nigrescens* (p=0.003) in samples from diseased horses. In addition*, P. bivia, P. dentalis, P. denticola, P. melaninogenica* and *P. nigrescens* were only detected in horses with periodontitis, with no reads detected in the healthy samples. *P. intermedia* was also detected by PCR in the current study in 100% of diseased samples in comparison with 80% of healthy samples and CFU equivalent counts were found to be significantly increased in diseased samples (p=0.001) (Fig 5.2) with a 65 fold increase in disease. *Prevotella dentasini,* which Takada *et al*. (2013) discovered recently in the oral cavity of donkeys was highly significantly associated with periodontitis with an LDA score of 8. This organism was also detected in the whole genome samples except for one healthy sample and no significant association was noted, however, the whole genome study involved a much smaller number of samples. It is likely that there are many previously unidentified species of *Prevotella* in the equine oral cavity (such as *P. dentasini*) some of which could play a role in equine periodontitis as few studies of the

equine oral microbiome have been carried out although several species previously associated with periodontitis in humans were also significantly increased in the current study. Haffajee and Socransky (1994) showed several species of *Prevotella* to be involved in human periodontitis such as *Prevotella intermedia* and *Prevotella melaninogenica* and more recently 16S rRNA sequencing has revealed the association of *P. denticola* and other *Prevotella* oral taxons with chronic periodontitis in humans (Griffen *et al*. 2012; Abusleme *et al* 2013). *Prevotella* sp. are frequently detected in subgingival plaque in both humans (Dewhirst *et al*. 2010) and animals (Dewhirst *et al*. 2012; Kennedy *et al*. 2016) and there is evidence for the association between this genus and periodontitis in humans (Griffen *et al*. 2012; Pérez-Chaparro *et al*. 2014; Torrungruang *et al*. 2015), and horses (Kennedy *et al*. 2016). The results of the current study indicate that *Prevotella* sp. should be considered as potential periodontal pathogens in the horse; however it is currently unclear whether these Gram-negative anaerobic organisms play a direct role in the pathogenesis of the disease or simply flourish in the environment created by the deep periodontal pockets. In human studies, *Prevotella intermedia* and *Prevotella nigrescens* have been shown to stimulate cytokine production by activation of Toll-like receptor 2 (Kikkert *et al*. 2007). Sun *et al*. (2010) also reported increased expression of Toll-like receptors 2 and 4 in human periodontal ligament cells when challenged with *P. intermedia*. This is of potential importance as the production of a destructive inflammatory response in periodontal tissue by stimulation of the innate immune system by periodontopathogenic bacteria is thought to be central in disease pathogenesis in man (Graves and Cochran 2003). These studies link the microbiology and immunology of human periodontitis and indicate that these species of *Prevotella* are likely to be human periodontal pathogens. Immunological studies in the horse would provide useful information to elucidate the potential role of *Prevotella* in the equine disease. Interestingly, *Prevotella* sp. were the most commonly isolated genus from equine endodontic and apical infections by Biernert *et al*. (2003) and bacteria from this genus were also successfully cultured from equine dental extractions and in one horse, a corresponding blood culture taken during extraction was also positive for *Prevotella* sp. and so it is possible that there is also involvement in the pathogenesis of equine apical infections.

The abundance of *Veillonella* species was 0.3% in oral health and 5% in periodontitis although following LefSe analysis, the presence of *Veillonella* was significantly associated with oral health. RTQPCR revealed a significant increase in *Veillonella dispar* in diseased samples (p=0.05) and whole genome sequencing showed that *V. dispar* was only detected in diseased samples. *Veillonella parvula* was also detected but there was no significant difference in the number of reads corresponding to this species between orally healthy and diseased samples. Several species of *Veillonella* have been isolated from both healthy gingival sulci and diseased periodontal pockets in man and *V. parvula* has been significantly associated with chronic periodontitis (Mashima *et al*. 2015) and this species has also been shown to stimulate cytokine production by activation of both Toll-like receptor 2 and 4 (Kikkert *et al*. 2007). Further studies exploring the effect of *V. parvula* on equine gingival tissue would be required to elucidate whether *V. parvula* is pathogenic in equine periodontal disease or whether the environment of the periodontal pocket simply encourages growth. Gao *et al*. (2016) also detected *Veillonella* sp. in the healthy equine oral cavity using high throughput 16S rRNA sequencing with this genus accounting for 16.7% of reads in one horse and 2.9% of reads in a second horse and *Veillonella parvula* accounted for the majority of *Veillonella* reads. *Veillonella* sp. have also been cultured from the healthy equine oral cavity by Baker (1979) and Bailey and Love (1990).

Putative periodontal pathogens such as *Treponema, Tannerella* and *Porphyromonas* species were detected at low levels in this study. Tannerella species decreased slightly from 0.16% in oral healthy to 0.1% in periodontitis. *Tannerella forsythia* was significantly increased in diseased equine samples when detected by Q-PCR (p=0.006). Gao *et al*. (2016) also identified *Tannerella sp*. at very low levels (0.01% in one horse and 0.28% in the second horse) and both Gao *et al*. (2016) and Sykora *et al*. (2014) identified *T. forsythia* in both orally healthy samples (Sykora *et al*. 2014; Gao *et al*. 2016) and in samples from horses with EOTRH (Sykora *et al*. 2014). Although *Tannerella* sp. are classically associated with periodontitis, the *Tannerella* BO045 phylotype has previously been associated with periodontal health (Leys *et al*. 2002) and was noted to be present in the oral cavity of the horse (Gao *et al*. 2016).

In the high throughput 16S rRNA study, the percentage of reads corresponding to *Treponema* species was similar between oral health (0.26%) and periodontitis (0.25%) but the presence of *Treponema* sp. and other spirochaetes was significantly associated with equine periodontitis in the current study when data underwent LefSe anlysis. In addition, *Treponema socranskii* and *Treponema vincentii* were only detected in diseased samples undergoing whole genome analysis with no reads detected in healthy samples. Spirochaetes such as *Treponemes* have long been associated with human periodontitis (Listgarten and Hellden 1978) and more recently unidentified spirochaetes were detected within the epithelium of equine periodontal pockets (Cox *et al*. 2012). *Treponema denticola* is well recognised as a periodontal pathogen in man, acting as one of the three 'red complex' bacteria found in severe periodontitis lesions alongside *Porphyromonas gingivalis* and *Tannerella forsythia* (Holt and Ebsersole 2005). In a study performed by Sykora *et al*. (2014), DNA corresponding to *Treponema* species was detected in 78.2% of horses with clinically overt EOTRH compared to 38% of unaffected horses and *Tannerella* DNA was found in 38.4% of diseased horses compared to 19% of unaffected horses (Sykora *et al*. 2014). *Treponema medium, Treponema denticola, Treponema pectinovorum,* and *Treponema putidium* were identified by Sykora *et al*. (2014) in horses with EOTRH in addition to many previously unidentified *Treponema* and *Tannerella* species, which are likely to be equine specific. In addition, Gao et al. (2016) identified many *Treponema* human oral phylotypes previously unidentified in the horse in addition to equine oral taxons phylogenetically related to *T. denticola*; *T. putidum*; *T. pedis*; *T. medium*; *T. vincentii*; *T. lecithinolyticum*; *T. socranskii*; *T. parvum*; *T. succinifaciens* and *T. amylovorum*.

In the current whole genome study, there was no significant difference in abundance of *P. gingivalis* between equine oral health and periodontitis however a significant increase was detected in periodontitis samples (p=0.04) when detected by QPCR. *P. gingivalis* is a well-known periodontal pathogen in man, and has been well studied due to the organism's ability to subvert the host immune system and alter the local microflora (Hajishengallis *et al*. 2012). As two different sample groups were used, both with relatively low numbers it is difficult to assess the potential of *P. gingivalis* as a periodontal pathogen in the horse and a larger scale study would be required to achieve this.

The current study comparing the equine oral microbiome in health and periodontal disease has revealed that the genera found in equine periodontal pockets are similar to those found in human periodontitis even though many previously undetected and likely equine specific species are present. Furthermore, microbial attributes assigned during whole genome sequencing showed a high number of reads associated with human periodontal disease were present in diseased equine samples in comparison with healthy samples, further strengthening the link between the microbiota found in human and equine periodontitis. Additional attributes assigned during whole genome sequencing revealed an increase in reads corresponding to Gram negative, host associated bacteria and anaerobes in periodontitis which is to be expected given the increase in bacteria such as *Prevotella* in the diseased periodontal pocket. All reads in one sample were of unknown Gram stain status which is assumed to be an error.

Samples taken from horses with periodontitis were found to be significantly less diverse than orally healthy samples in the current high throughput 16S rRNA gene sequencing study. This contrasts with several human studies (Paster *et al*. 2006; Abusleme *et al*. 2013) which reported higher diversity in the periodontal pocket. In the current study, many reads corresponded to 'previously uncultured bacteria' which could not be further categorised and it is possible that this may result in an apparent reduced diversity. In addition, many previously uncultured bacteria were identified with some species not being closely related to other known bacteria. It is likely these may represent 'equine-specific' taxa. As few previous studies have been performed investigating the equine oral microbiome, it is highly likely that novel, previously undetected bacteria will be identified when using modern, culture-independent techniques. Further studies of the equine oral microbiome could identify such species and allow for more accurate representation of population diversity. In addition, the periodontal pocket found in diseased horses constitutes a new niche in an oral ecosystem that will select for a different, limited, microbiome and this may explain the significant decrease in microbiome diversity noted in the periodontitis cases in comparison with the orally healthy horses. Alterations in pH, nutrient availability and specifically oxygen tension may limit the species able to survive the periodontal

pocket, with deep pockets having a significantly lowered oxygen tension than even moderate pockets (Loesche *et al*. 1983). It is possible that during disease progression, the environmental changes occurring as a shallow gingival sulcus becomes a deep periodontal pocket allows a new group of bacteria to flourish whilst providing a less optimal environment for the growth of others and so it is difficult to assess whether a species is important in the aetiopathogenesis of the condition, or simply increased in abundance due to the formation of a more suitable environment. Environmental differences between the healthy equine gingival sulcus and diseased periodontal pockets may be particularly striking in the horse, as equine dental anatomy allows for the formation of particularly deep periodontal pockets which may measure over 15 mm in severe cases (Cox *et al*. 2012).

The current study demonstrated differing microbial populations existing in the healthy gingival margin, the diseased gingival sulcus and deep within the periodontal pockets of a small number of horses. Anaerobic organisms such as *Prevotella* sp. and *Veillonella* sp. predominated at the diseased gingival margin and periodontal pocket whereas aerobic *Streptococci* (29%) and *Gemella* (15%) predominated at the orally healthy sites. Although differing populations were found in each environment as expected, it is difficult in a study of this size and type to comment on whether an alteration in flora (dysbiosis) occurred first, predisposing to periodontitis or the condition developed due to an external cause such as the formation of diastema and subsequent decomposition of impacted feed material which is more likely in the horse. Longitudinal studies commencing with young healthy horses, and following-up on their periodontal status and microbiota of the oral cavity until the development of periodontal disease would be useful in future studies to monitor disease development and potential dysbiosis.

5.5 Conclusion

The two cohorts of horses examined harboured highly distinct microbial profiles, with samples from periodontally affected horses being less diverse than samples from the healthy horses. The microbiota present in equine periodontal pockets bore similarities to that present in human periodontitis, with similar genera and species present although many previously uncultured bacteria were also detected which are likely to be equine specific and potentially important in the aetiopathogenesis of the disease. Several species of bacteria belonging to the *Prevotella* genus were repeatedly seen to increase in diseased equine periodontal pockets using different microbiological methods and a great number of species were identified in equine periodontitis during whole genome sequencing. The results of this study indicate that species belonging to the *Prevotella* genus should be further investigated as potential periodontal pathogens in the horse. In order to identify potential equine periodontal pathogens, immunological studies should be performed to assess the local inflammatory response to these bacteria. Furthermore, additional, preferably longitudinal, studies are required to assess the changing population of bacteria present at the healthy site before and during development of disease.

Chapter 6

Expression of Toll-like Receptors and Cytokines in Equine Periodontal Disease

6.1 Introduction

The innate immune system plays an important role in the pathogenesis of periodontal disease in humans, with periodontopathogenic bacteria present in the dental biofilm capable of stimulating an inflammatory response (Kikkert *et al*. 2007). This response can also be destructive to host tissue, even resulting in marked periodontal ligament and tooth loss (Gemmell *et al*. 1997). Loss of support around the tooth results in deep periodontal pockets which support the growth of anaerobic bacteria, further stimulating an inflammatory response and exacerbating the disease.

Signature molecules of invading microorganisms are detected by host immune cells which recognise pathogen-associated molecular patterns (PAMPs) via specific pattern recognition receptors such as the Toll-like receptors (TLRs). Bacterial and viral classes produce distinct PAMPs such as lipopolysaccharides, lipotechioic acid and CpG dinucleotides which act as ligands, binding to and activating specific TLRs. This process allows the host to gain information on the nature of the invading pathogen, with subsequent induction or suppression of cytokines and other inflammatory mediators following TLR activation (Akira and Takeda 2004). By measuring TLR and cytokine expression in inflamed tissue, information can be gathered on the nature of putative pathogens involved in disease aetiopathogenesis and the immune response produced can be characterised (Akira and Takeda 2004).

Histopathological examination of diseased equine periodontium has revealed neutrophilic and eosinophilic inflammation, gingival hyperplasia and ulceration erosion and inflammation of the periodontal ligament (Cox *et al*. 2012). To date, there have been no studies of innate immunity in the equine oral cavity or of its possible involvement in the pathogenesis of equine periodontal disease.

In this chapter, TLR and cytokine expression in gingival tissue was assessed in orally healthy horses and horses with periodontitis.

6.2 Materials and Methods

6.2.1 Sample collection

Tissue biopsies (4 mm x 4 mm) were collected from the gingival margin between Triadan 306/7 teeth from thirteen orally healthy horses and from the periodontal pockets of 20 diseased horses and placed in 0.5 mL RNA Later (Sigma Poole UK) immediately after collection. Gingival biopsies were then stored at -20^{o}C until ready for RNA extraction and cDNA synthesis.

6.2.2 Tissue processing

Prior to RNA extraction, gingival biopsies were allowed to return to room temperature before tissue homogenisation. A new, sterile scalpel blade was used to dissect a 25mg portion each sample for homogenisation. After adding 300µL of Buffer RLT (Qiagen, Manchester, UK) a motorised pellet pestle (Sigma-Aldrich Poole UK) was used to macerate the tissue samples.

6.2.3 RNA Extraction

An RNeasy Fibrous Tissue Mini Kit (Qiagen) was used to extract RNA from the gingival tissue samples, according to the manufacturer's instructions. Following tissue homogenisation, 590µl of RNAse free water (Thermo-Fisher Scientific, Loughborough, UK) was added to the samples along with 10 µl proteinase K (100µg/mL). After mixing, the samples were then incubated in a water bath at 55^{o}C for 10 minutes. Next, the samples were placed in a centrifuge at 10,000 rpm for 3 minutes and the supernatant was then transferred to a new tube. A half-volume of 97% (v/v) ethanol was added to the supernatant, and mixed. Seven hundred microliters of this mixture were then added to an RNeasy Mini column which was placed into a 2mL collection tube. After 15 seconds of centrifugation at 10,000 rpm, the flow-through was discarded and this step was then repeated until all of the lysate had passed through. Next, 350µL of Buffer RW1 was added to the column and centrifuged for 15 seconds at 10,000 rpm and the flow-through discarded. Then, 10µl of DNase stock mixed with 70µL of Buffer

RDD was then added to the column membrane and the samples incubated for 15 minutes at 25°C. After incubation, 350µl of Buffer RW1 was added, the samples centrifuged once more and the flow-through discarded. Five hundred microliters of Buffer RPE were then added and the samples centrifuged at 10,000 rpm for 15 seconds. This step was then repeated and the samples were centrifuged for 2 minutes. The column was then placed into a fresh 1.5mL microcentrifuge tube and 50µl of RNase free water added carefully to the column membrane and the samples were centrifuged for a final time at 10,000 rpm for 1 minute.

6.2.4 cDNA synthesis

cDNA was synthesised from RNA samples using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Paisley UK). Firstly, the 2x RT Master Mix was prepared by allowing the following components to thaw on ice and then mixing: 10x RT Buffer (2 µl per reaction), 25x dNTP mix (0.8 µl per reaction), 10x RT Random Primers (1 µl per reaction), nuclease-free water (4.2 µl per reaction) to give a total of 10 µl 2x Master Mix per reaction. The master mix was then placed on ice and mixed gently. Ten microliters of sample RNA, along with 10 µl of master mix was added to a PCR tube, and mixed using a pipette tip before loading into a Primus 96 machine plus thermocycler (MWG biotech) and running a PCR cycling program of 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C and termination at 4°C.

6.2.5 Primer design, optimisation and efficiency

The National Centre for Biotechnology Information (NCBI) online tool at http://www.ncbi.nlm.nih.gov/tools/primer-blast/ was used to design primers for the following equine genes: TLR 2, TLR 4, TLR 9, IL-1ß, IL-4, IL-6, IL-10, IL-12 (subunits p35 and p40), IL-17, IFN-ɣ and TNF-α (Table 6.1). Primers were synthesised commercially (Invitrogen). Primer efficiency for effective cDNA amplification was tested with three serial dilutions of sample. Comparative Critical Threshold (Ct) measurements were used to calculate mRNA levels in gingival tissue and, in order to calculate primer efficiency, Ct values were plotted against log10 of the sample volume using the following formula: Efficiency = -1+10(-1/slope) (Fraga *et al*. 2014). Primer efficiencies of between 0.85 and 1.1 were considered acceptable. In addition, for each primer a melt

curve analysis was performed to ensure a single peak was produced during the reaction, indicating the formation of a single product. The NCBI BLAST tool available at http://www.ncbi.nlm.nih.gov/blast was also used to validate the primers.

Primer	Sequence (5' to 3')	Product Length (base pairs)
GAPDH Forward	ATTGCCCTCAACGACCACTT	195
GAPDH Reverse	GTTAGGGGGTCAAGTTGGGA	
18S Forward	CGGGGAATCAGGGTTCGATT	205
18S Reverse	CACCAGACTTGCCCTCCAAT	
TLR2 Forward	CCTGATCGACTGGCCAGAAA	156
TLR2 Reverse	CCAGTGAGCAGGATCGACAG	
TLR4 Forward	TTCTGCAGTGGGTCAAGGAC	145
TLR4 Reverse	GACCCGCCAACGATAGTCTT	
TLR9 Forward	ATGAAGGGACTGCGAGCAC	338
TLR 9 Reverse	AAGTCGGAATCGTGGAGGTG	
IL-1ß Forward	AGCTTCAATTCTCCCACCAACTT	100
IL-1ß Reverse	GAGCAGGGAACGGGTATCTT	
IL-4 Forward	TGGCCCGAAGAACACAGATG	108
IL-4 Reverse	AGTCCGCTCAGGCATTCTTT	
IL-6 Forward	TTCACAAGCACCGTCACTCC	128
IL-6 Reverse	GCAGTGGTGAGTAGTGGTCC	
IL-10 Forward	GAGCAGGTGAAGAGTGCCTT	141
IL-10 Reverse	TGCTTCGTTCCCTAGGATGC	
IL-12p35 Forward	CCACAAAAGCCCTCCCTTGA	103
IL-12p35 Reverse	TCCTGTTGATGGTCACTGCG	
IL-12p40 Forward	TTCAGGTCCAGGGCAAGAAC	153
IL-12p40 Reverse	ATACGGATGCCCATTCGCTC	
IL-17 Forward	ATTACCACAACCGCTCCACC	120
IL-17 Reverse	TCCCTTCGGCATTGACACAG	
IFN-ɣ Forward	CACCAGCAAGCTGGAAGACT	164
IFN-ɣ Reverse	TCCGGCCTCGAAATGGATTC	
TNF-α Forward	AGAGGGAAGAGCAGTTACCGA	189
TNF-α Reverse	CTGTCAGCTTCACGCCATTG	

Table 6.1 Equine TLR and cytokine primer sequences

6.2.6 Quantitative PCR

Quantitative PCR was performed using a MX300P™ real time PCR system (Agilent Technologies, Edinburgh, UK). SYBR® Select Mastermix (Life Technologies, Paisley, UK) was used with reactions performed in triplicate. Reactions consisted of 12 µl SYBR® Select Mastermix, 11.5 µl RNAse-free water (Qiagen), 0.5 µl cDNA, 0.5 µl of forward primer and 0.5µl of reverse primer to give a total reaction volume of 25 µl. PCR was carried out using the following conditions: preliminary denaturation step of 95°C for 10 min followed by 50 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C (TLR2, IL-1, IL-6, IL-12, 18S and GAPDH) or 60°C (TLR4, TLR9, IL-4, IL-10, IL-17 TNFα and IFNɣ) for 1 min and extension at 72°C for 1 min. GAPDH and 18S rRNA were used as housekeeping genes and amplified using the above conditions with the exception of primer annealing which was conducted at 55ºC for both genes.

6.2.7 Data and statistical analysis

Ct values obtained for equine TLR and cytokines genes were adjusted using the geometric mean of values obtained for housekeeping genes GAPDH and 18S rRNA and using the 2-Delta Ct method (Schmittigen and Livak 2008). To visualise the results, data were normalised to the control IL-12p35 mRNA levels. Fold change was also calculated. Cross tabulation was used to determine whether there was a statistically significant difference in the prevalence of periodontitis with the breed of horse and to determine whether there was a correlation between the presence of periodontitis with the age of the animals. Statistical analysis was carried out using SPSS Version 21 (IBM Chicago USA). The paired Students t-test was used for comparisons of healthy and diseased sites in the same animal. For independent group comparisons of parameters measured in healthy and diseased horses, the variance of the data was assessed by Levene's test to indicate the choice of independent sample test (t-test or the Welch's test) for determining whether differences between means were statistically significant. As the ages of the horses in both groups differed, age was recognised to be a potential confounding factor in the analysis. To investigate this possibility, the data were weighted by least squares regression using a General Linear Model. Appropriate parametric statistical tests were also utilised to analyse the weighted data.

Correlation between clinical severity of periodontitis and gene expression was measured using Bivariate Spearman correlation analysis. Data graphics were constructed using Microsoft Excel 2013, Minitab® Version 17 and GraphPad Prism 6. Differences and correlations were deemed significant when $p \leq 0.05$.

6.3 Results

6.3.1 Sample demographics

Twenty-four tissue samples originated from the School of Veterinary Medicine at the University of Glasgow and nine samples from the Royal (Dick) School of Veterinary Studies, University of Edinburgh. Twenty samples originated from horses with periodontitis and thirteen from orally healthy horses. All horses were humanely euthanised for reasons other than dental disease. Twenty horses were euthanised due to chronic disease: chronic musculoskeletal disease (n=10), poor condition (n=6), chronic ocular disease (n=2), chronic hepatic disease (n=1) and chronic gastrointestinal disease (n=1). The remaining 13 horses were euthanised due to congenital disease (n=5), dangerous behaviour (n=4), neoplasia (n=2), poor performance (n=1) and neurological disease (n=1). The age and disease status of horses sampled are shown in Fig 7.1. The mean age of horses with periodontitis was 16.8 years (range 1-27 years) and included 60% (n=12) mares, 35% (n=7) geldings and 5% (n=1) stallion. The mean age of orally healthy horses was 6 years (range 1-16 years) and included 58% (n=8) geldings, 25% (n=3) mares and 17% (n=2) stallions. The animals affected with disease were significantly older than healthy animals ($p=0.007$). The proportion of horses with periodontitis correlated with increasing age of the animals ($rho= 0.586$, $p<0.001$) (Fig 6.1).

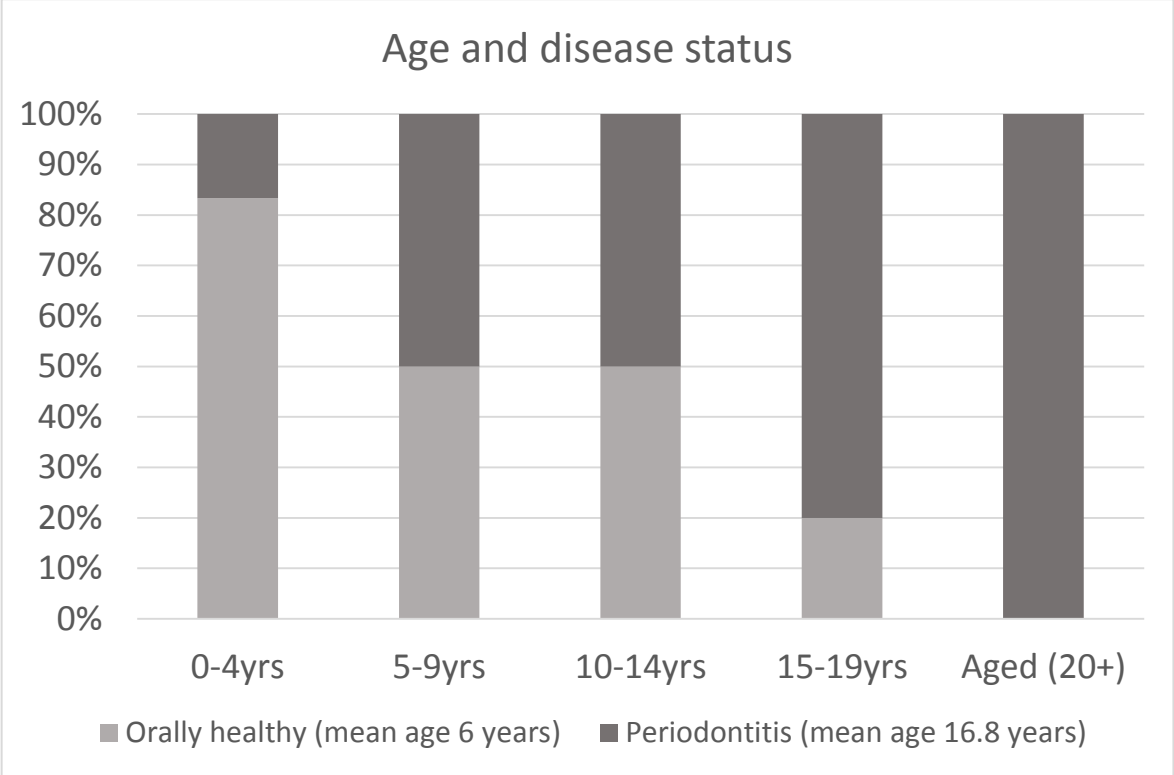


Figure 6.1 Age and disease status of 13 orally healthy horses and 20 horses with periodontitis.

Breed and disease status are shown in Fig 6.2. The breeds included 16 native ponies, 8 Thoroughbred (TB) or TB crosses, 3 Irish Sport Horses, 2 Icelandic horses, one Shire, one Selle Français, one Standardbred and one Irish Draft, the latter four of which were grouped together into a miscellaneous breed category. Due to the high number of breeds represented and relatively small sample population in this study, no meaningful data could be obtained regarding breed predispositions to equine periodontal disease.

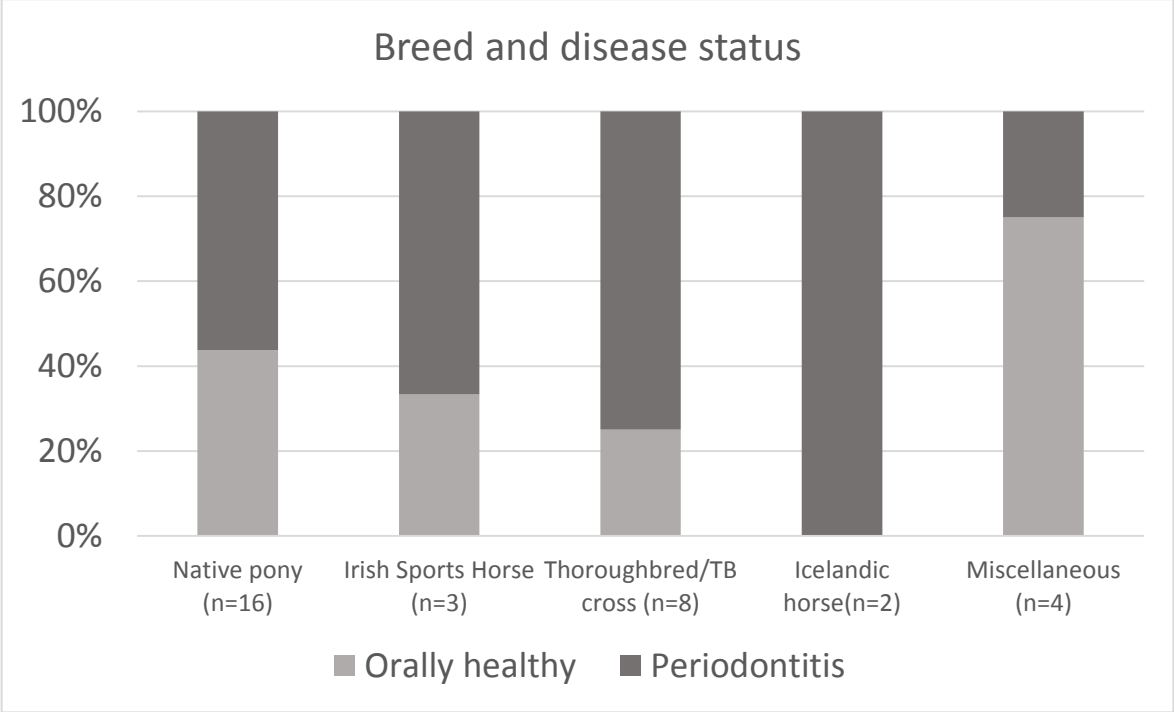


Figure 6.2 Breed and disease status of 13 orally healthy horses and 20 horses with periodontitis.

6.3.2 Grading of periodontal disease

The mean Periodontal Disease Grade as assessed by periodontal pocket depth (Cox *et al*. 2012) in horses with periodontitis was 2.2 (moderate). Nine horses had severe (Grade 3; i.e. periodontal pocket depth >15 mm) periodontal disease, six had moderate disease (Grade 2 i.e. periodontal pocket depth 10-14 mm) and four had mild disease (Grade 1 i.e. periodontal pocket depth 5-9 mm).

6.3.3 TLR mRNA expression

Expression of mRNA encoding TLR 2, TLR 4 and TLR 9 was higher in tissue samples from periodontitis lesions in comparison to orally healthy samples (Fig 6.3, 6.4). Statistically significant increases were seen in TLR 2 mRNA ($p=0.03$) and TLR 9 ($p=0.04$) mRNA before weighting for age. In the age-weighted analysis (Fig 6.3), TLR 4 mRNA was also significantly greater in disease ($p<0.001$) and p values for TLR 2 mRNA and TLR 9 mRNA decreased ($p<0.001$ and $p=0.013$, respectively) (Fig. 6.5). When fold change was calculated, levels of TLR2 mRNA showed a particularly large (389-fold) increase in periodontitis samples compared to orally healthy samples (Fig 6.4). In comparison, levels of TLR4

mRNA were only increased 2-fold and levels of TLR9 mRNA increased 3.8-fold in diseased samples.

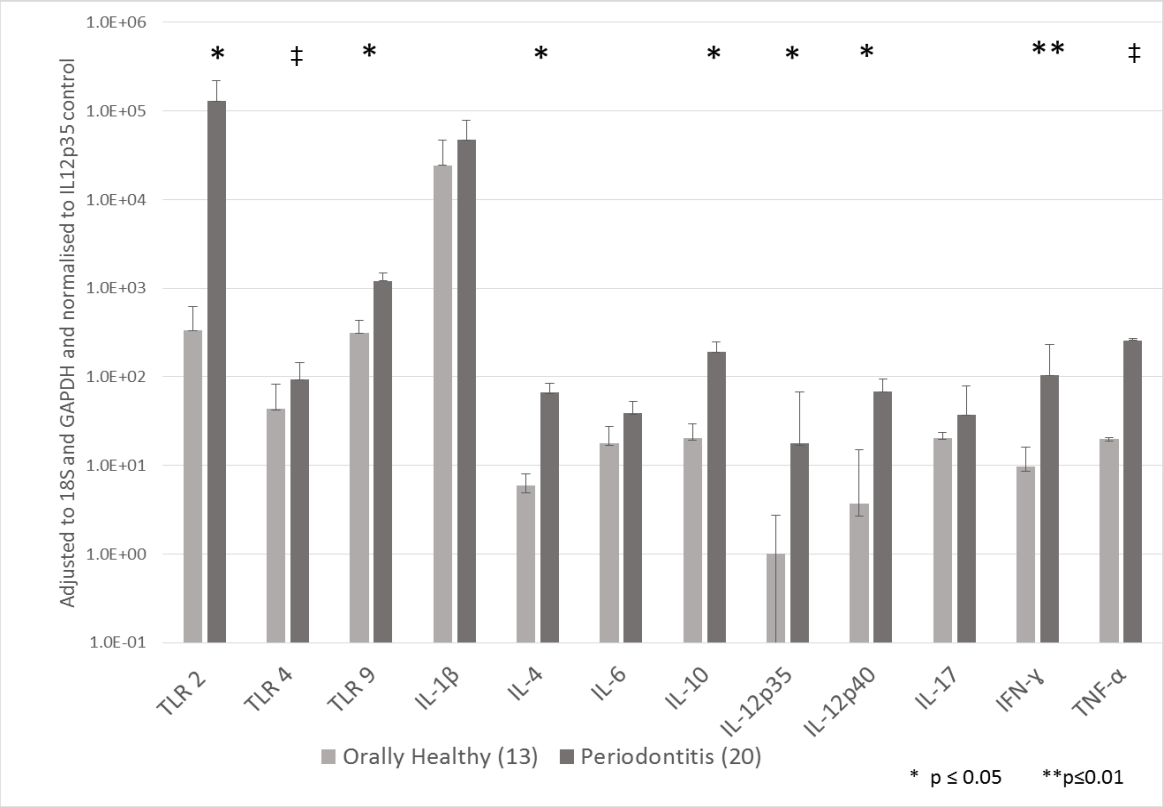


Figure 6.3 Gingival TLR and cytokine mRNA expression in 13 orally healthy horses and 20 horses with periodontitis. Levels of mRNAs were adjusted to the housekeeping genes (18S rRNA and GAPDH) and are shown relative to control IL12p35 mRNA levels. Error bars represent standard error.

*Statistically significant difference ($p<0.05$) between health and disease.

** Statistically significant difference ($p<0.01$) between health and disease.

‡ Statistically significant difference ($p<0.001$) observed only in the age-weighted analysis.

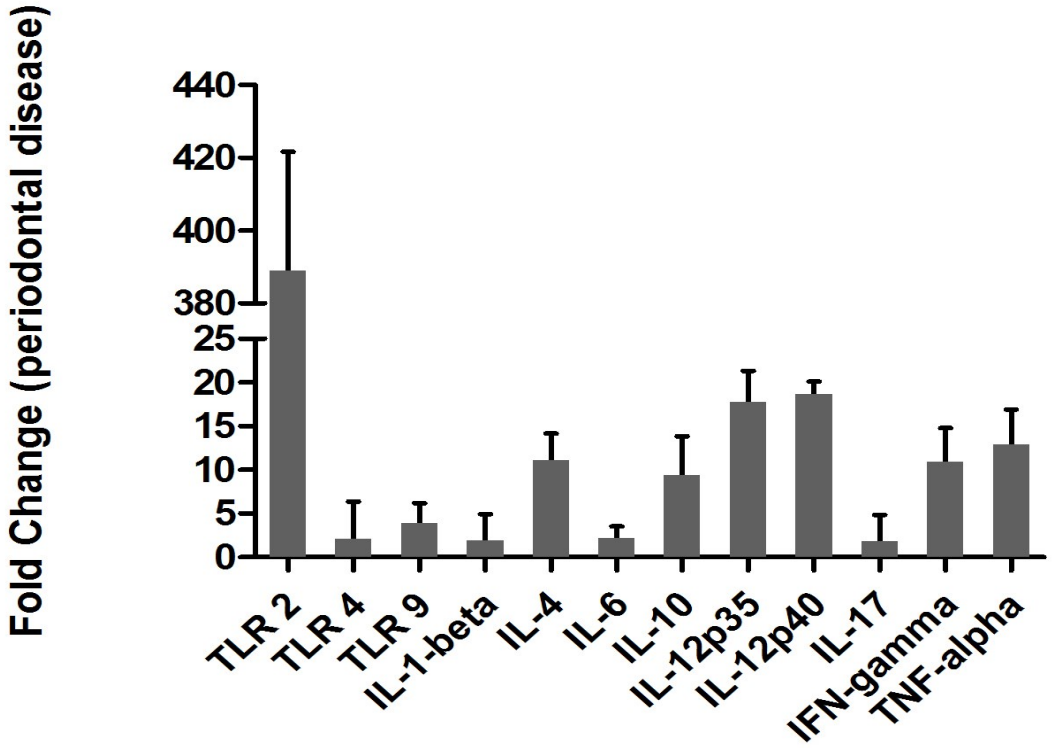


Figure 6.4 Fold change in TLR and cytokine mRNA expression in 13 orally healthy horses and 20 horses with periodontitis. Fold change in mRNA levels are shown for diseased sites compared to healthy sites with error bars representing standard error.

Control samples of healthy gingival tissue from horses with periodontitis lesions at other sites were used to provide site comparisons. When comparing healthy and diseased sites from the same animals, levels of TLR2 mRNA ($p=0.005$), TLR4 mRNA ($p=0.03$) and TLR9 mRNA ($p=0.02$) were significantly increased at diseased as compared to control sites (Figs 6.6 and 6.7). TLR2 mRNA levels showed a smaller fold increase between these sample groups, being increased 3.5-fold in diseased tissue. TLR4 mRNA was increased 17-fold and TLR9 mRNA was increased 16-fold in diseased tissue (Fig 6.8).

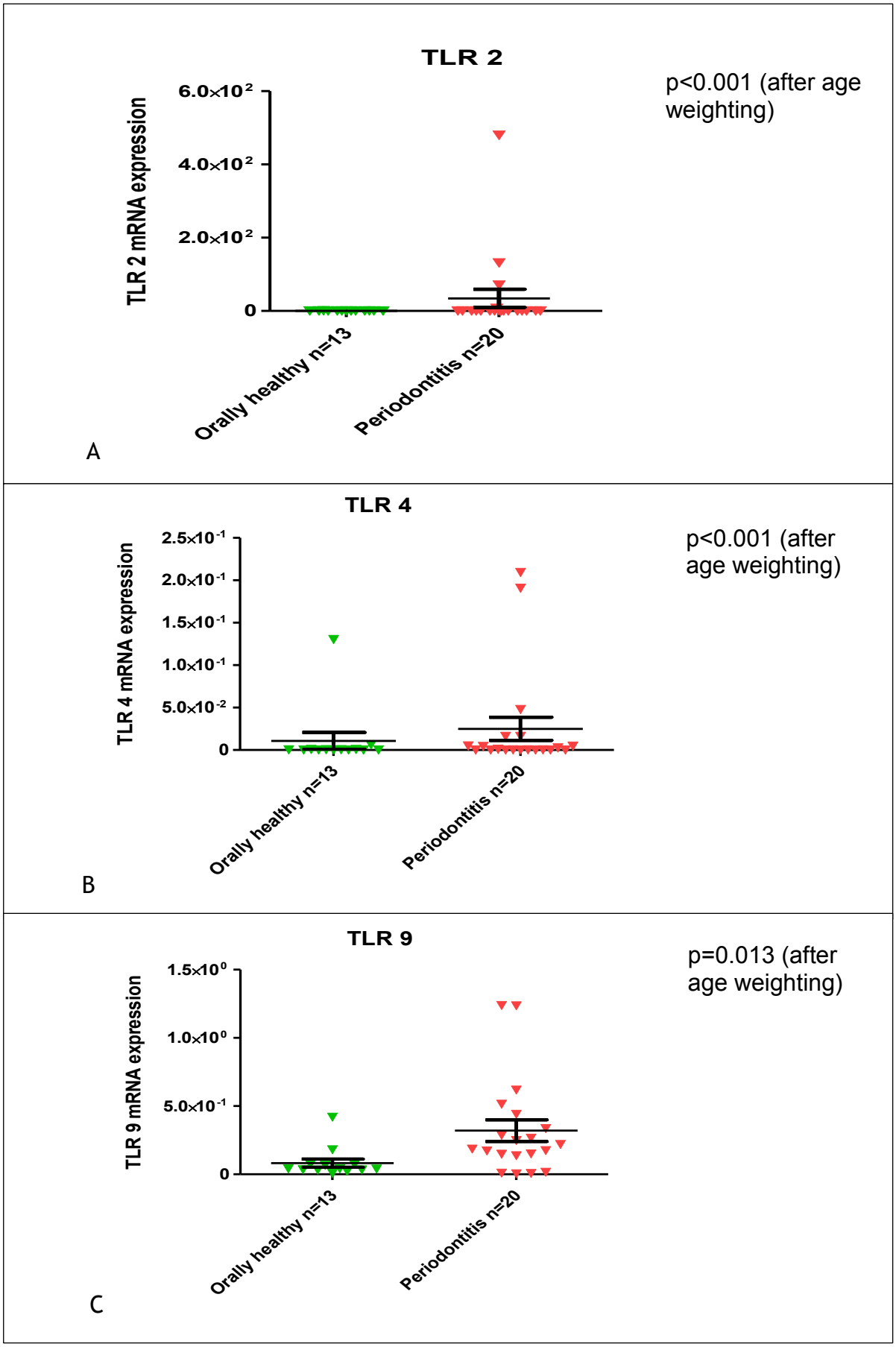


Figure 6.5 Gingival TLR mRNA expression (Ct value) in 13 orally healthy horses and 20 horses with periodontitis. Error bars show mean value and standard error.

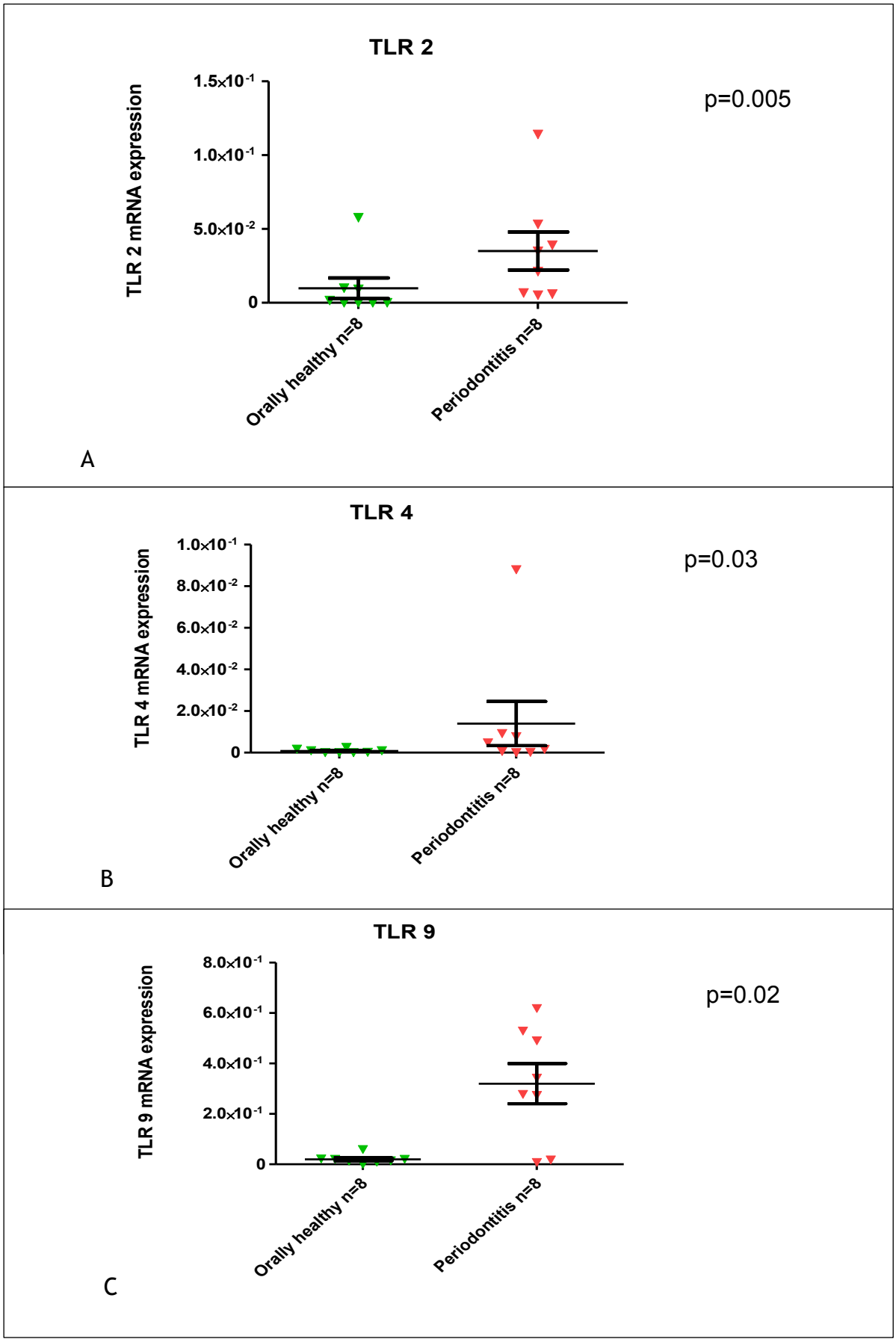


Figure 6.6 TLR mRNA expression (Ct value) in healthy gingival tissue and periodontitis lesions in the same animal. Error bars show mean value and standard error

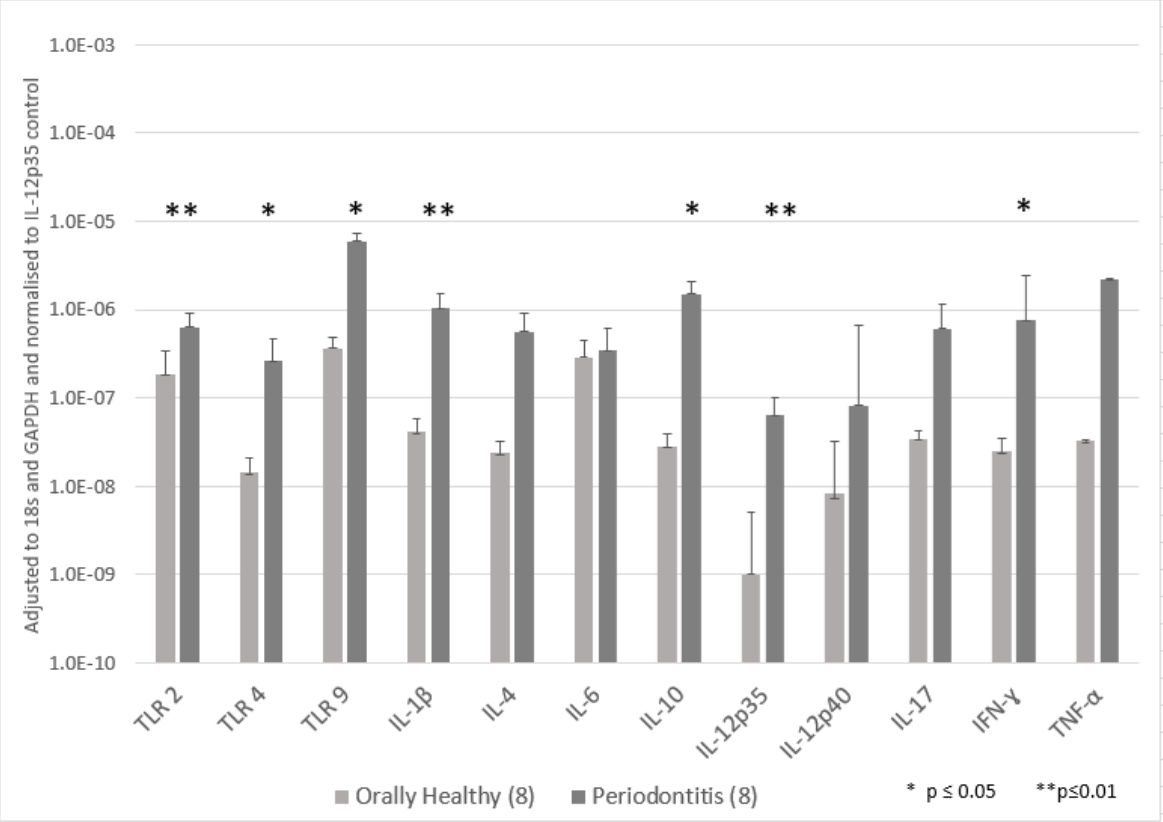


Figure 6.7 Comparison of TLR and cytokine mRNA expression in healthy gingival tissue and periodontitis lesions in the same animal. Levels of mRNAs were adjusted to the housekeeping genes (18S rRNA and GAPDH) and are shown relative to control IL12p35 mRNA levels. Error bars represent standard error

*Statistically significant difference ($p<0.05$) between health and disease.

** Statistically significant difference ($p<0.01$) between health and disease.

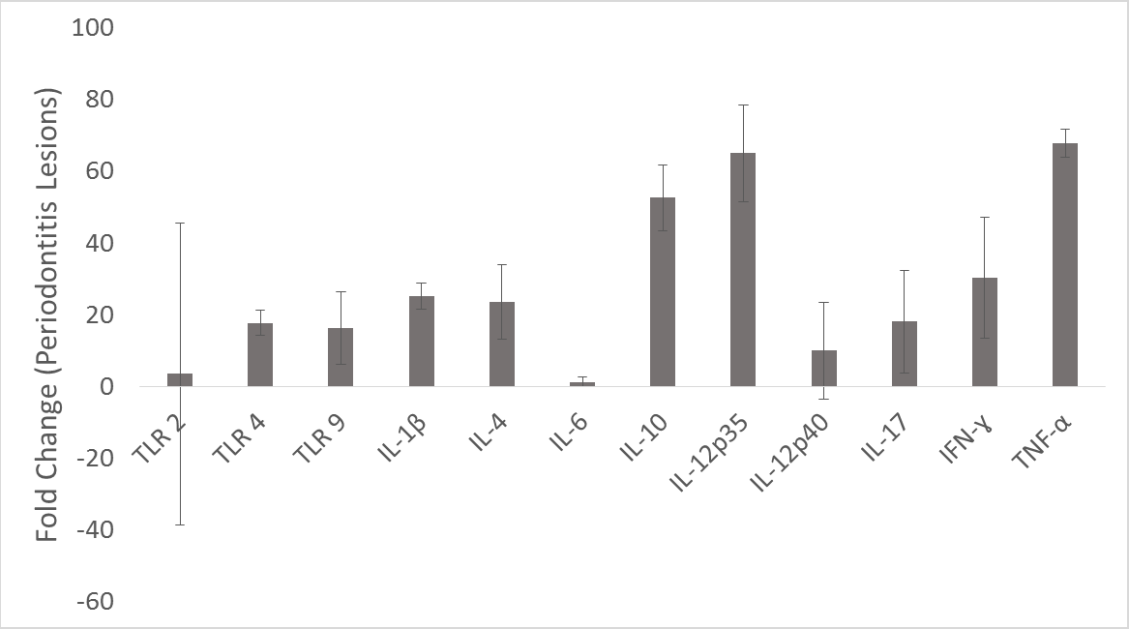


Figure 6.8 Fold change in TLR and cytokine expression between healthy and diseased gingival tissue from the same animal. Fold change in mRNA levels are shown for diseased sites compared to healthy sites with error bars representing standard error

6.3.4 Cytokine mRNA expression

Expression of mRNA encoding for all measured cytokines was higher in diseased as compared to healthy gingival tissue samples (Figs 6.3, 6.9, 6.10 and 6.11). A significantly larger number of mRNA transcripts of the following genes were observed in the age un-weighted analyses: IL-4 ($p=0.02$), IL-10 ($p=0.02$), IL-12p35 ($p=0.04$), IL-12p40 ($p=0.04$) and IFN-ɣ ($p=0.008$). In the age-weighted analysis, in addition to TNF-α mRNA ($p<0.001$), the levels of all the above gene transcripts were significantly greater in the periodontitis samples ($p<0.001$). When fold change was calculated, transcripts were increased as follows: IL-1ß mRNA (2-fold), IL-10 mRNA (9-fold), IFN-ɣ mRNA (11-fold), TNF-α mRNA (12-fold), IL-12p35 mRNA (18-fold), IL-12p40 (18-fold) (Fig 6.4). When comparing cytokine mRNA expression in healthy gingival tissue and periodontitis lesions from the same animal, mRNA expression of all cytokines measured was increased in diseased tissue, with statistically significant increases observed for the following genes: IL-1ß ($p=0.004$), IL-4 ($p=0.009$), IL-10 ($p=0.02$), IL-12p35 ($p=0.00007$) and IFN-ɣ ($p=0.02$) (Figs 6.7, 6.12, 6.13 and 6.14). When comparing diseased tissue with healthy tissue from the same animal, large fold increases were observed for transcripts of the following genes: IL-1ß (25-fold), IL-10 (52-fold), IL-12p35 (65-fold), IL-12p40 (22-fold), IL-4 (23-fold), TNF-α (65-fold) and IFN-ɣ (30-fold) (Fig 6.8).

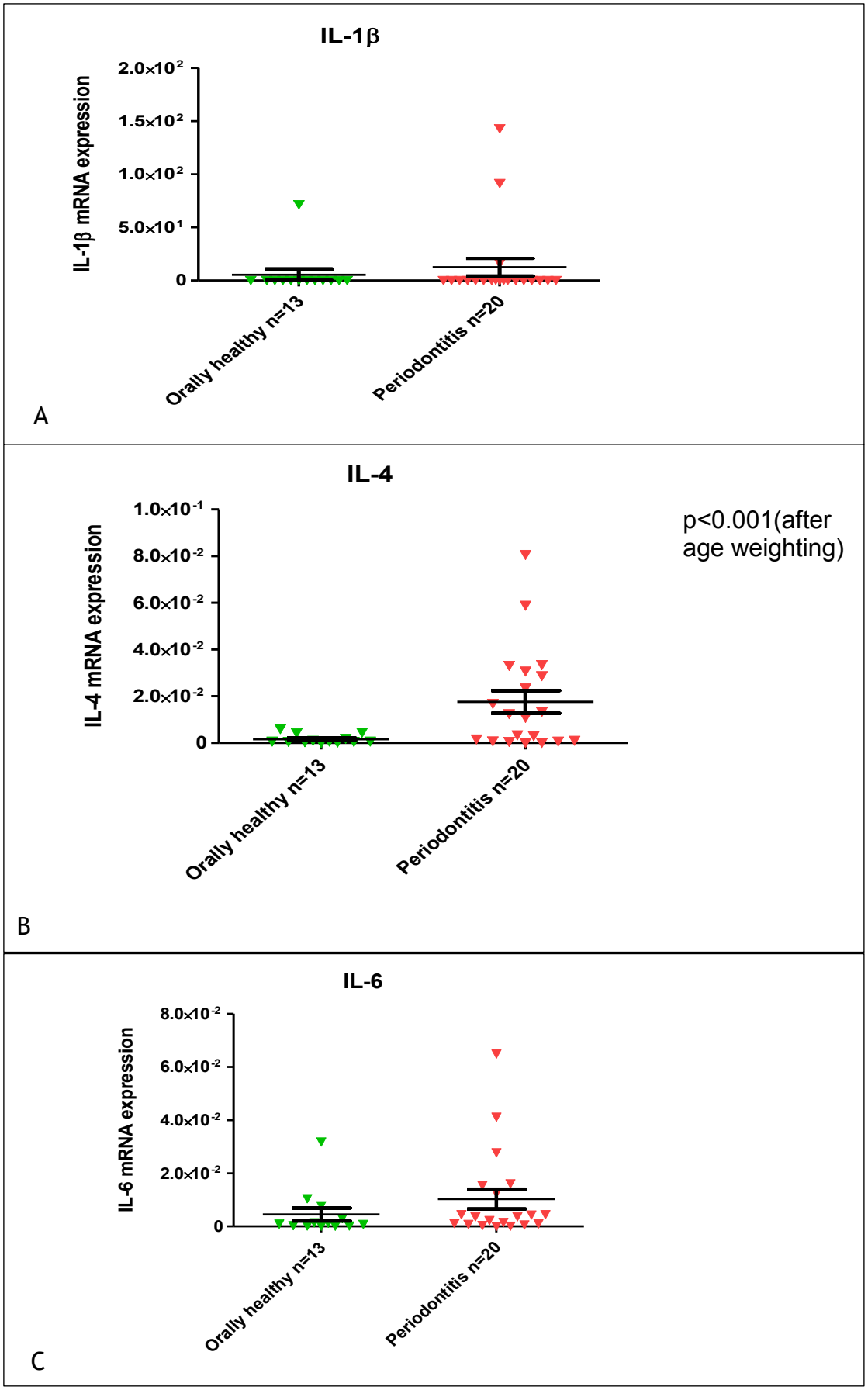


Figure 6.9 IL-1β (A), IL-4 (B) and IL-6 (C) expression (Ct value) in 13 orally healthy horses and 20 horses with periodontitis. Error bars show mean value and standard error of the mean.

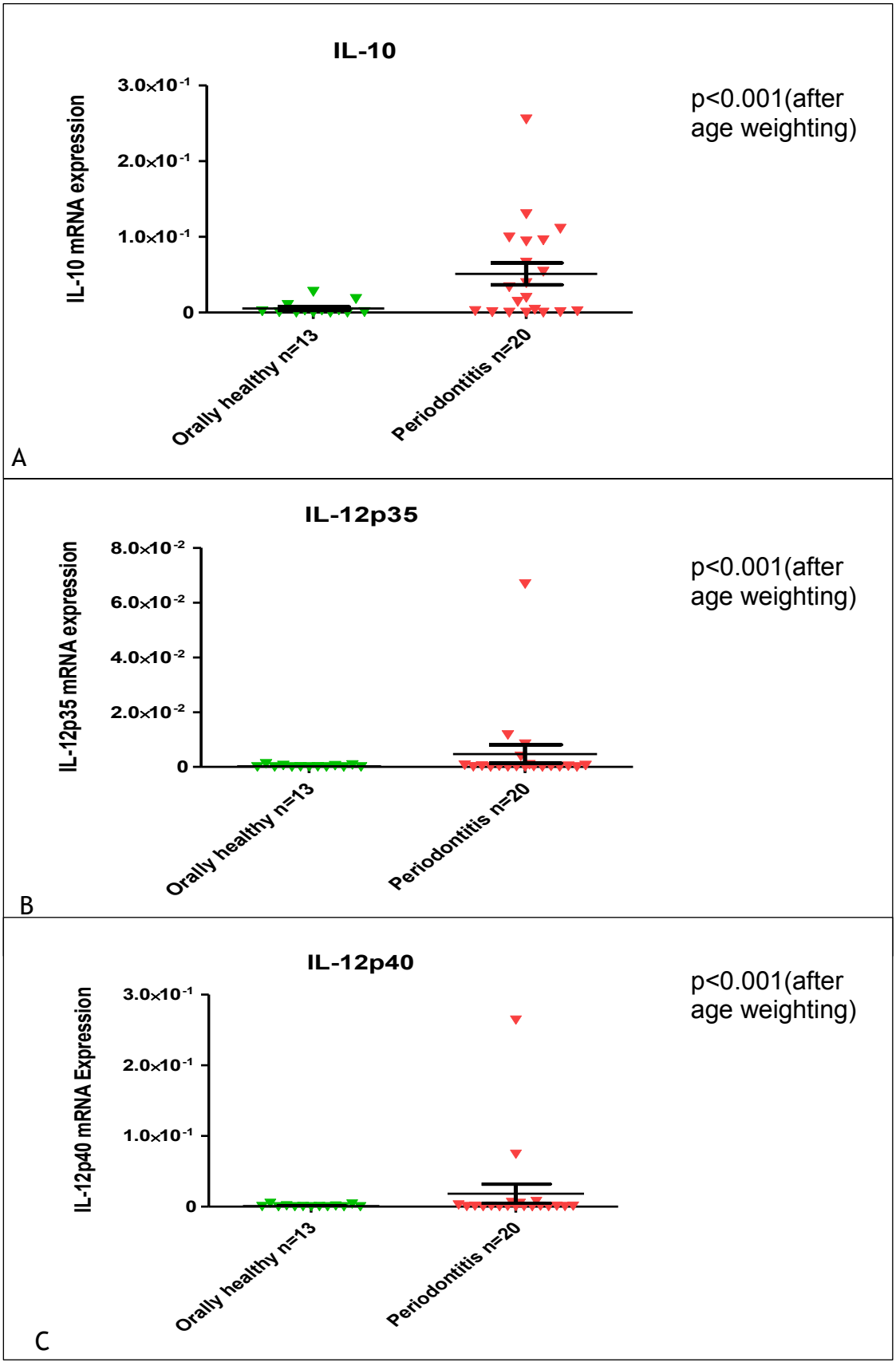


Figure 6.10 IL-10(A), IL-12p35 (B) and IL-12p40 (C) mRNA expression (Ct value) in 13 orally healthy horses and 20 horses with periodontitis. Error bars show mean value and standard error of the mean.

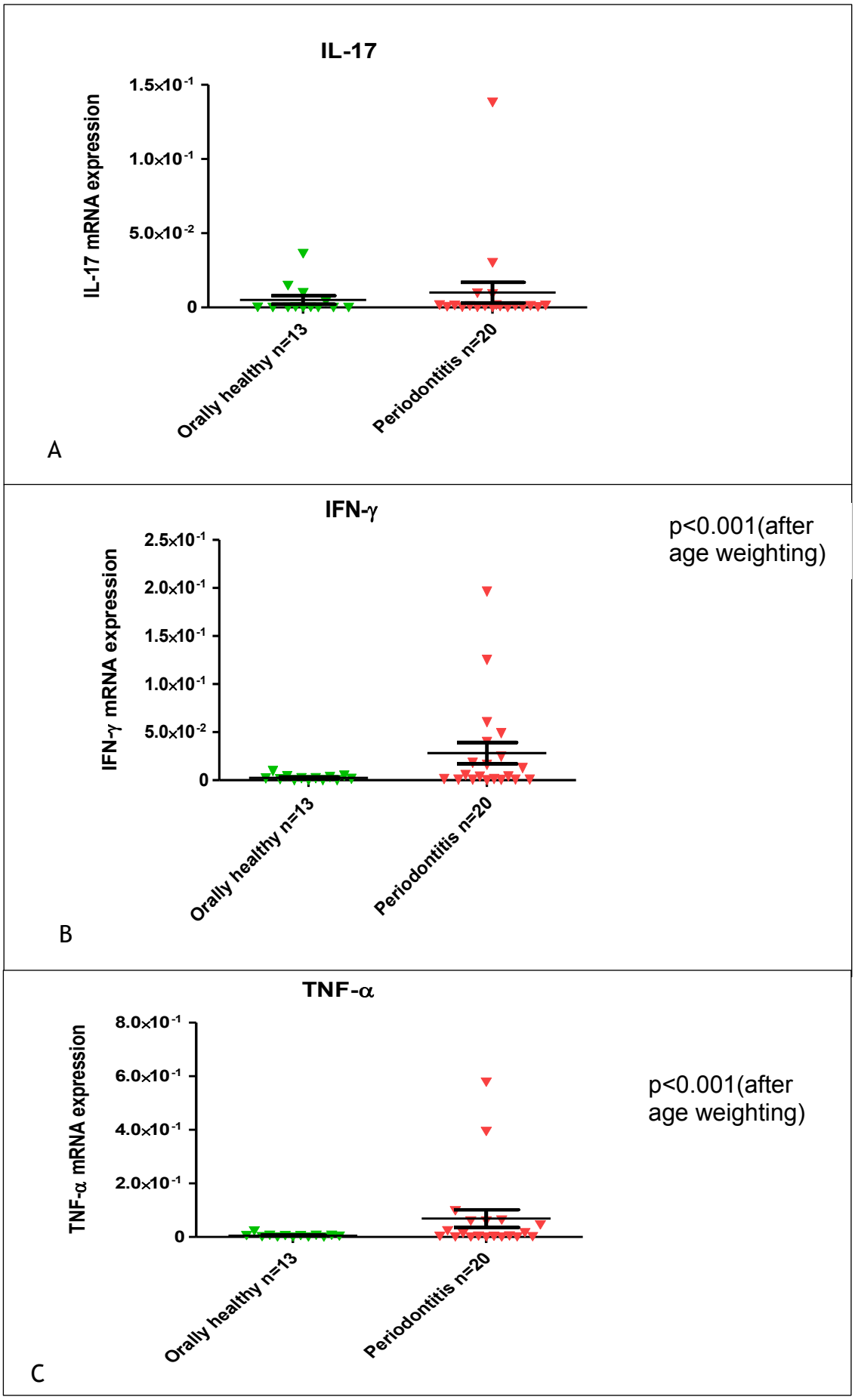


Figure 6.11 IL-17(A), IFN-γ (B) and TNF-α (C) expression (Ct value) in 13 orally healthy horses and 20 horses with periodontitis. Error bars show mean value and standard error of the mean.

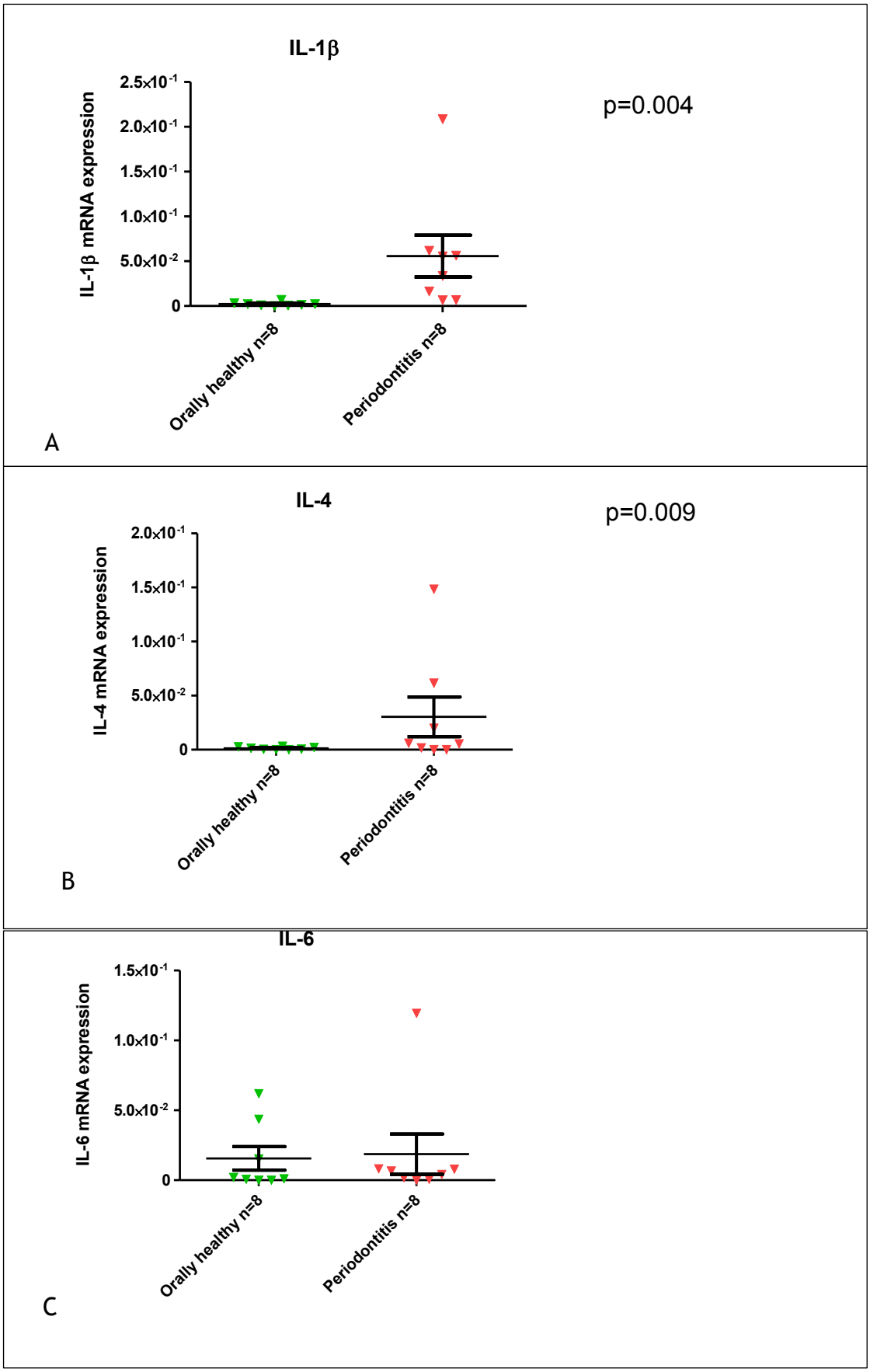


Figure 6.12 IL-1β (A), IL-4 (B) and IL-6 (C) mRNA expression (Ct value) in healthy gingival tissue and periodontitis lesions in the same horses. Error bars show mean value and standard error of the mean.

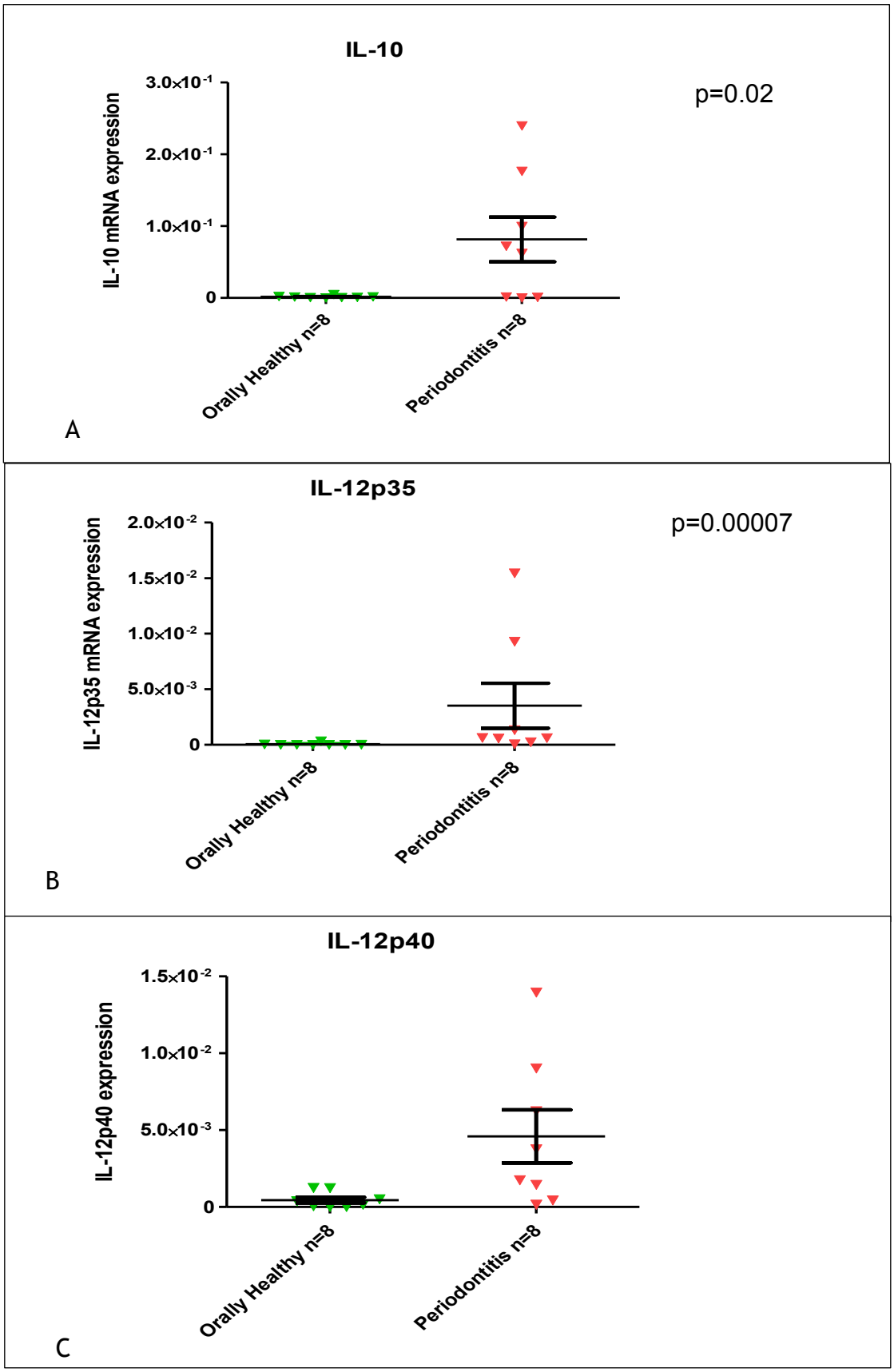


Figure 6.13 IL-10 (A), IL-12p35(B) and IL-12p40 (C) mRNA expression (Ct value) in healthy gingival tissue and periodontitis lesions in the same animal. Error bars show mean value and standard error of the mean.

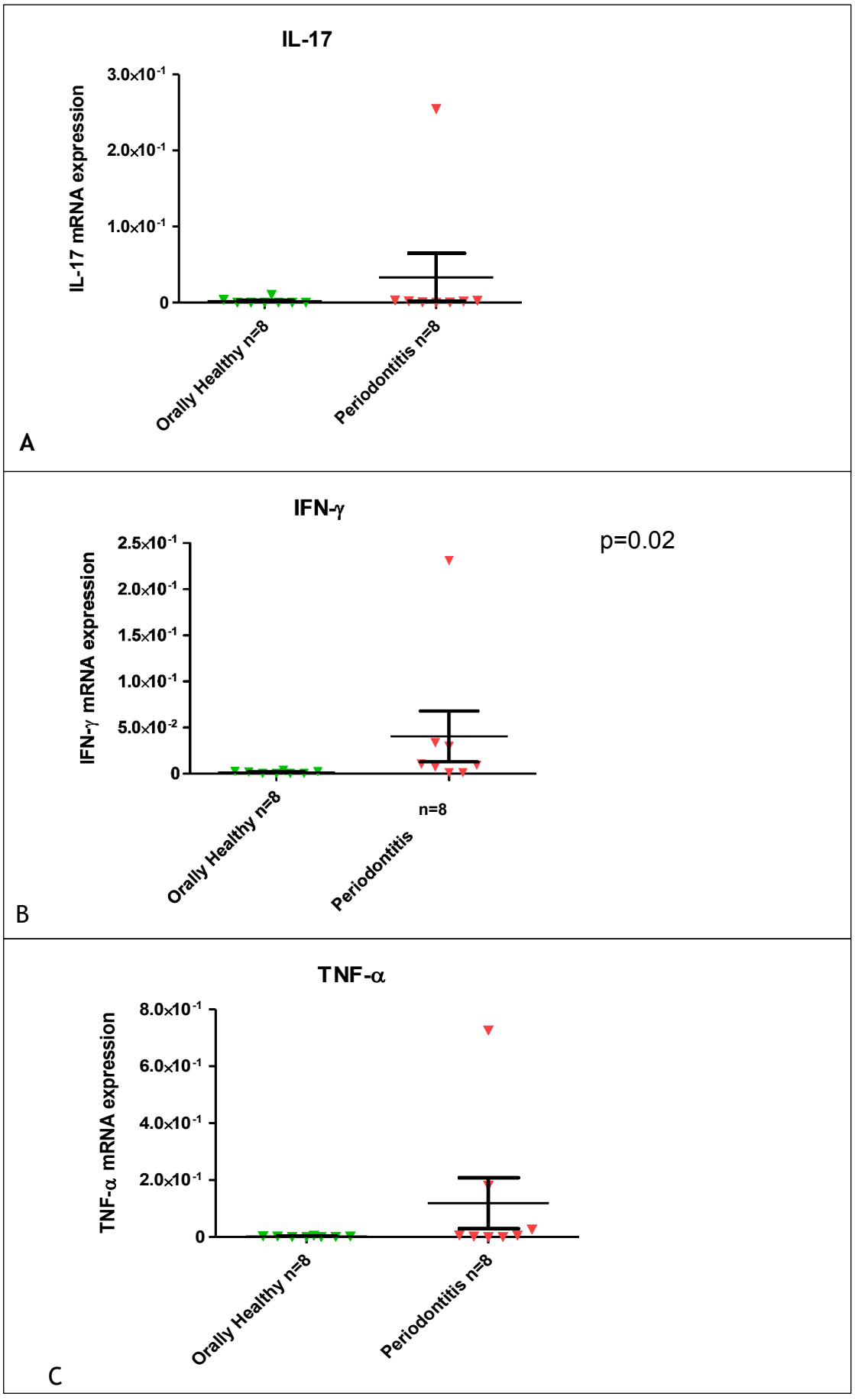


Figure 6.14 IL-17 (A), IFN- γ (B) and TNF-α (C) mRNA expression(Ct value) in healthy gingival tissue and periodontitis lesions in the same horses. Error bars show mean value and standard error of the mean.

6.3.5 Clinical severity and TLR and cytokine expression

Periodontitis cases were grouped according to disease grade (Cox *et al*. 2012): normal, mild, moderate and severe. TLR mRNA expression and clinical severity of periodontal disease showed no statistically significant correlations, except for TLR2 mRNA in an age-adjusted analysis ($p<0.001$) (Figs 6.15, 6.16, 6.17 and 6.18). Statistically significant positive correlations existed between disease severity and expression of IL-1ß mRNA and IL-4 mRNA, ($p=0.018$ and $p=0.026$) and in an age-adjusted analysis statistically significant positive correlations were recorded for IL-1 ß, IL-6, IL-10, IL-12p40, IL-17 and TNFα (all $p<0.01$).

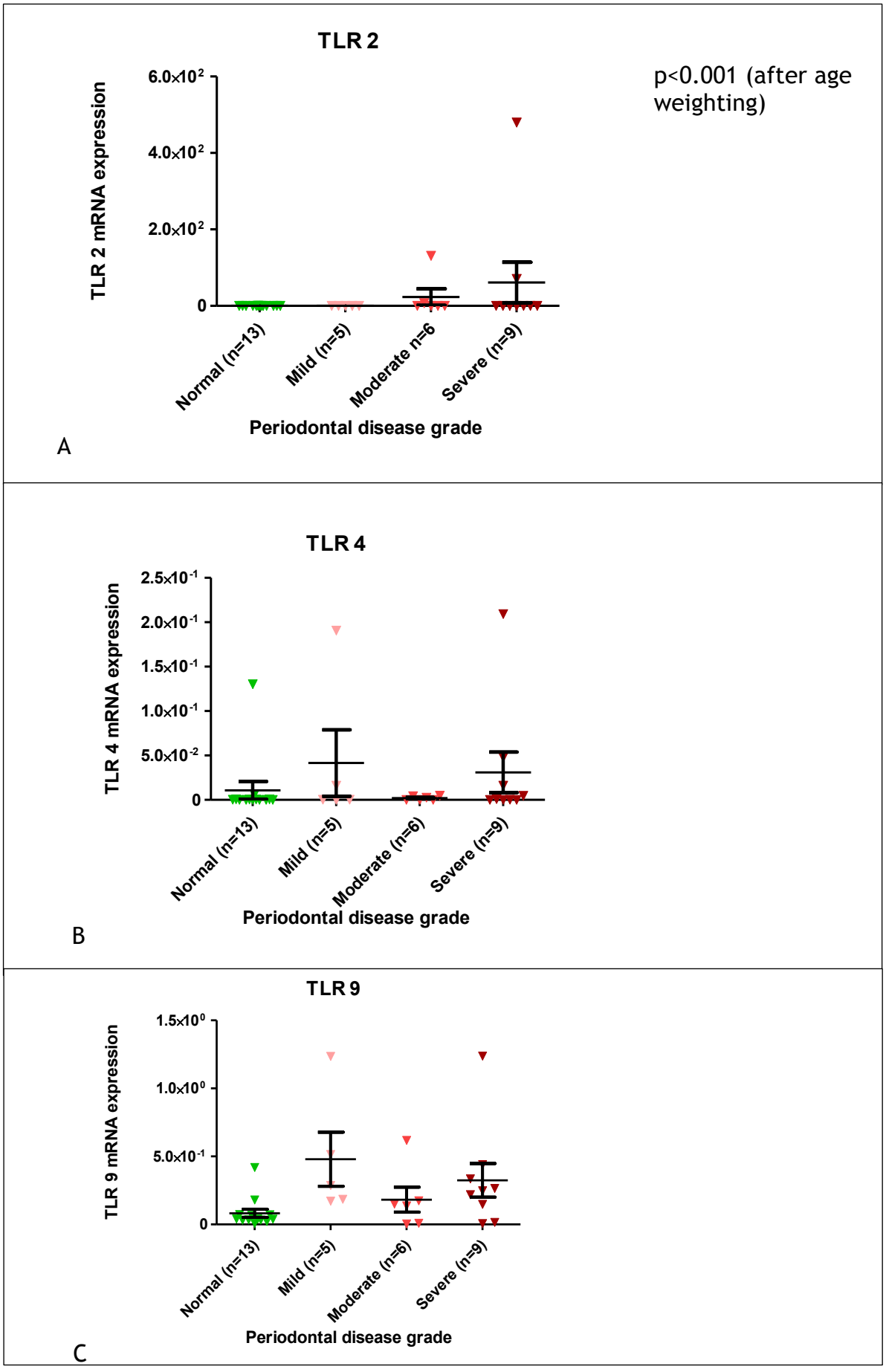


Figure 6.15 TLR 2 (A), TLR 4 (B) and TLR 9 (C) mRNA expression (Ct value) in orally healthy horses and horses with mild, moderate and severe periodontal disease. Error bars show mean value and standard error of the mean.

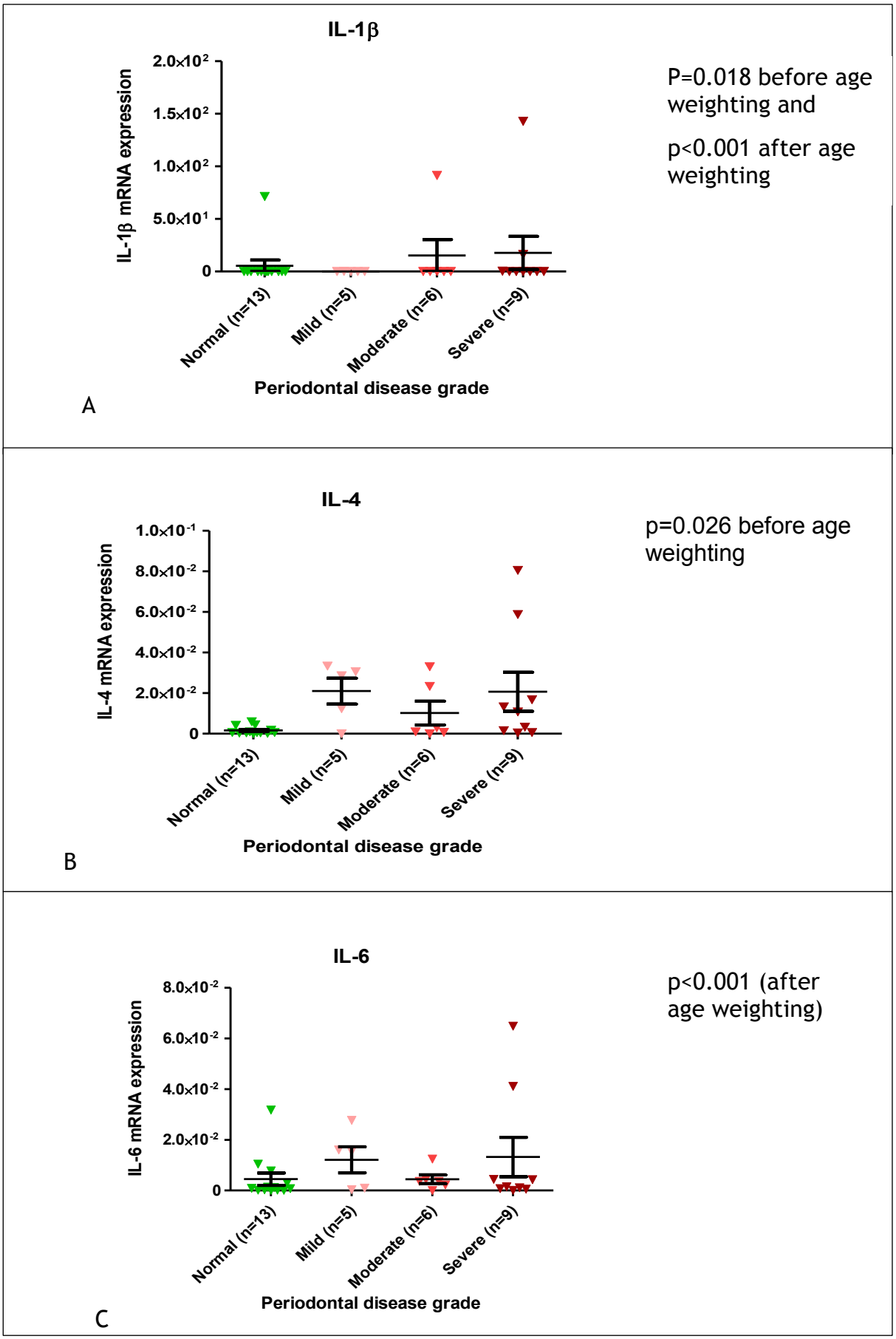
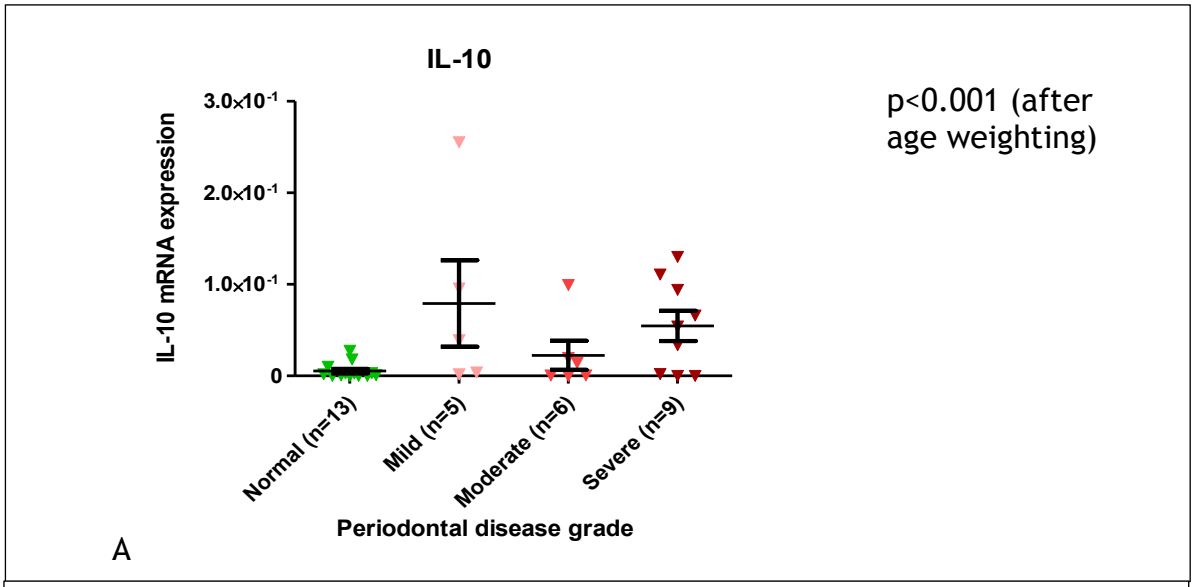
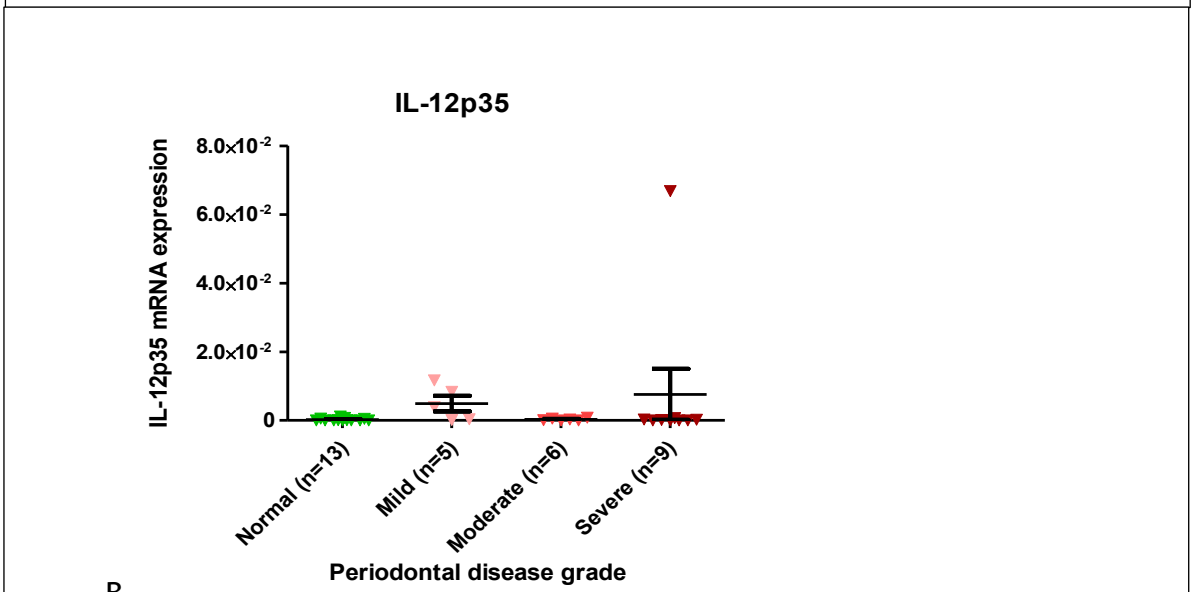


Figure 6.16 IL-1β (A), IL-4 (B) and IL-6 (C) mRNA expression (Ct value) in orally healthy horses and horses with mild, moderate and severe periodontal disease. Error bars show mean value and standard error of the mean.





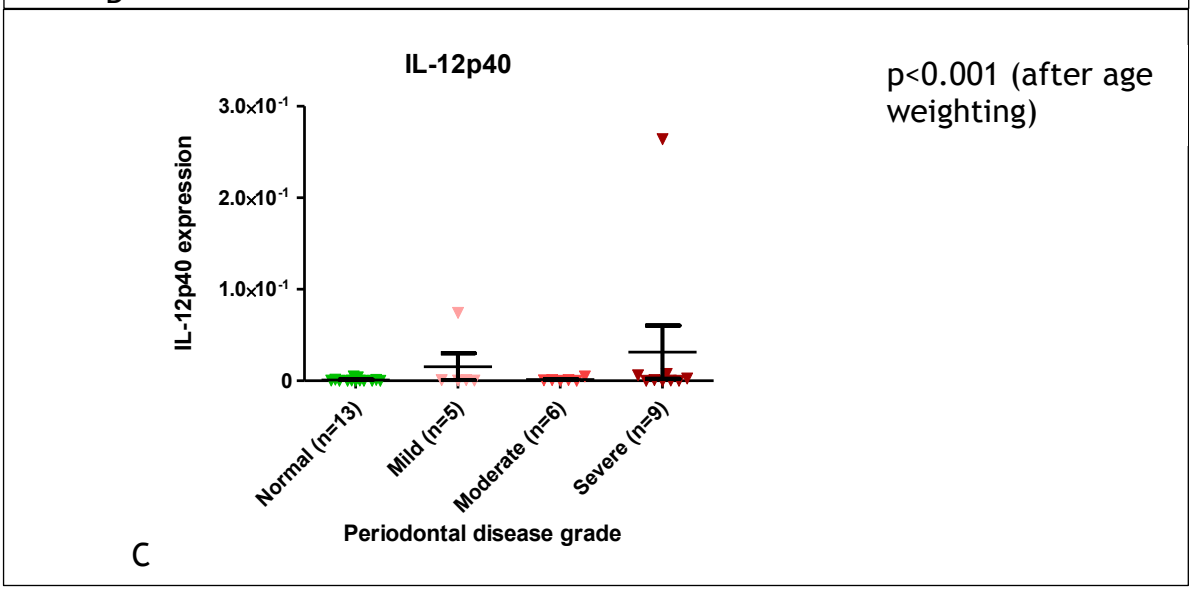


Figure 6.17 IL-10 (A), IL-12p35 (B) and IL-12p40 (C) TLR mRNA expression (Ct value) in orally healthy horses and horses with mild, moderate and severe periodontal disease. Error bars show mean value and standard error of the mean.

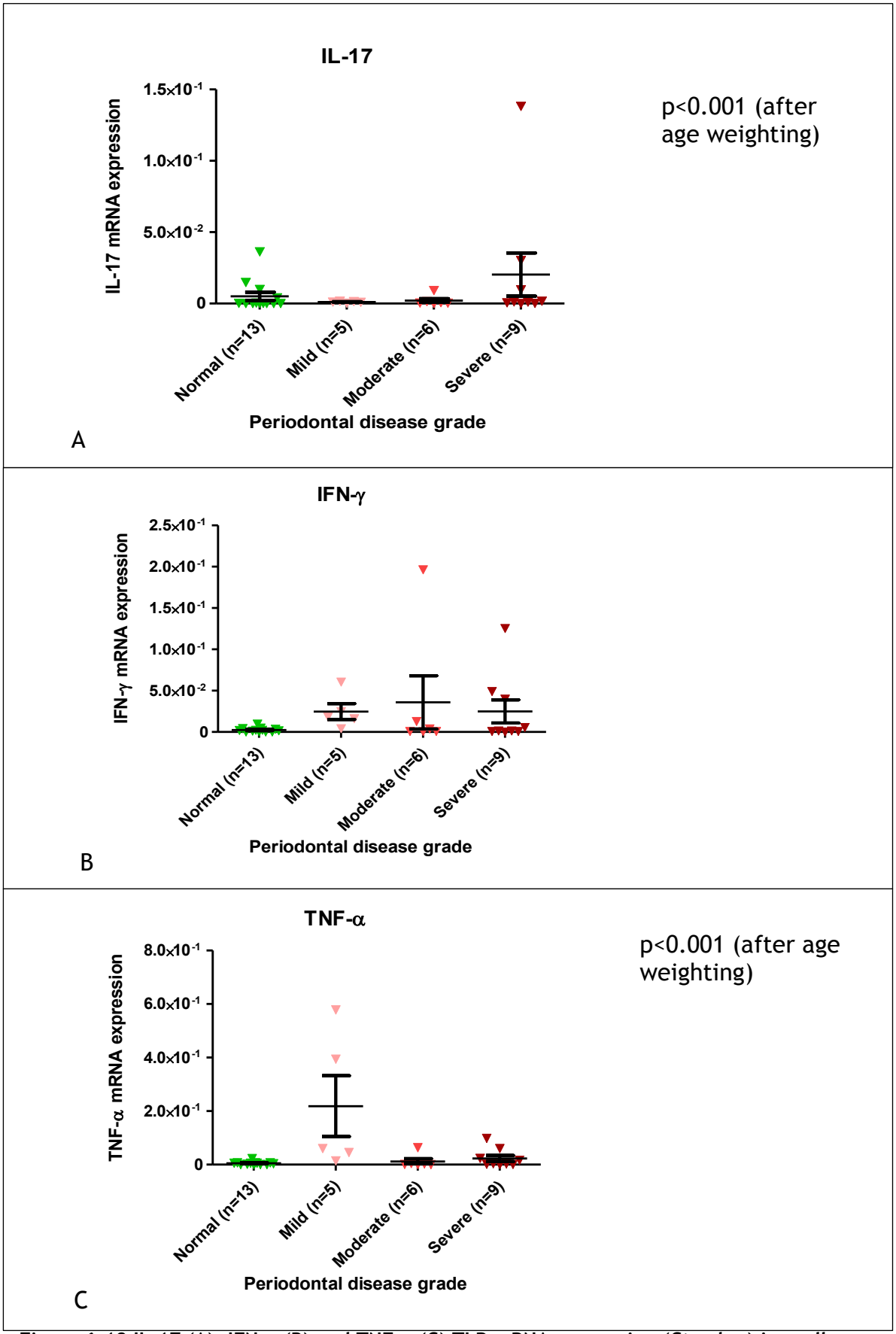


Figure 6.18 IL-17 (A), IFN-γ (B) and TNF-α (C) TLR mRNA expression (Ct value) in orally healthy horses and horses with mild, moderate and severe periodontal disease. Error bars show mean value and standard error of the mean.

6.4 Discussion

The aim of this chapter was to investigate and characterise the innate immune response in equine periodontal disease. Innate immunity plays an important role in the aetiopathogenesis of human periodontal disease as recently reviewed by Cekici *et al*. (2014). However, this aspect of equine periodontitis has received little attention to date.

Although expression of TLRs and cytokines has not previously been examined in equine periodontitis, a strong inflammatory reaction has been noted in histological examination of diseased periodontal tissue by Cox *et al*. (2012). This group discovered that erosion and ulceration of gingival tissue was significantly associated with periodontal disease grade and also that cellular degeneration was frequently present in the epithelial lining of the periodontal pocket. Transmigration of neutrophils through the periodontal pocket was also significantly associated with severity of disease. In addition to infiltration of inflammatory cells such as lymphocytes, plasma cells, neutrophils and occasional eosinophils Cox *et al*. (2012) also noted the presence of Gram negative and Gram positive bacteria, which were most numerous in the periodontal pockets, along with submucosal abscesses containing bacterial cocci. In addition, spirochaetes were detected within the gingival epithelium of the periodontal pocket of four horses with periodontal disease. This study by Cox *et al*. (2012) was the first to show the presence of bacteria, including spirochaetes, in conjunction with infiltration of inflammatory cells in the equine periodontal pocket and is supportive of the interaction between bacteria and the oral immune response playing an important role in the aetiopathogenesis of periodontal disease in the horse, as is also seen in man.

This chapter investigated gingival TLR and cytokine expression in horses with and without periodontal disease using real-time (RT) PCR. RT PCR is a reliable technique for measuring gene expression (Pusterla *et al*. 2006) and offers several advantages over other techniques, including the ability to produce rapid and accurate data, the requirement of only small amounts of sample and the ability to analyse more than one gene at a time (Fraga *et al*. 2014). Development and evaluation of a SYBR-Green RT-PCR assay for evaluation of equine cytokine

expression has been performed successfully by Sánchez-Matamoros *et al*. (2013). Several factors are important in order to achieve accuracy from such assays. One such factor is elimination of human technical error, such as pipetting inaccuracies. In order to minimise pipetting inaccuracies, in the current study, reactions were performed in triplicate (Fraga *et al*. 2014) using calibrated pipettes and means of Ct values were taken. In order to make valid comparisons between samples it is also important to consider primer efficiency. In the current study, an efficiency of 0.85 to 1.1 was considered acceptable, with 12 out of the 14 genes having optimal efficiencies of between 0.9 to 1.1 (Fraga *et al*. 2014). Consistent melt peak curves were obtained for all primers, indicating absence of any secondary products. Initially, primers for TLR 9, IL-1β, and TNF-α either produced no Ct value, or produced multiple or unexpected melt peaks during validation. These primers were redesigned, validated and accepted when a single peak was obtained.

To allow relative quantification of test TLR and cytokine genes and comparison between oral health and periodontitis, two housekeeping genes were used (Fraga *et al*. 2014). The criteria for selecting housekeeping genes are that their mRNA expression is stable and minimally regulated under experimental conditions, however no one housekeeping gene can be applied for normalisation of expression for every experimental condition (Zhang *et al*. 2009). Commonly used housekeeping genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S rRNA, beta-actin and hypoxanthine ribosyl transferase 1 (HPRT1). As little information was available regarding suitability of housekeeping genes in the horse, Zhang *et al*. (2009) evaluated a set of candidate genes as internal controls for gene expression studies in a variety of equine tissues using RT PCR. Zhang *et al*. (2009) demonstrated that 18S was the most stable housekeeping gene for data normalisation for equine gene expression studies, although equine gingival tissue was not used in that study. FigueiredoI *et al*. (2009) also used 18S rRNA as a reference gene when assessing gene expression in equine leukocytes. In addition, Beekman *et al*. (2011) evaluated several housekeeping genes as internal controls for gene expression studies in equine bronchoalveolar lavage cells from horses with inflammatory airway disease and found GAPDH to be the most stable gene. No data were available regarding selection of housekeeping genes in equine gingival tissue expression studies, although GAPDH has been

previously used as a reference gene by Bissell *et al*. (2004) to normalise data when investigating gene expression in human periodontitis. Expression of GAPDH and 18s rRNA did not differ between oral health and equine periodontal disease and therefore both genes were suitable for use as housekeeping genes in the current study. Single peaks were obtained for both reference genes, indicating a single product, and both were within the range of acceptable efficiencies.

As the risk of equine periodontitis increases with advancing age (Baker 1979; Wafa 1988; Ireland *et al*. 2012a) it was important to account for this when analysing the data from the current study. There was a significant ($p<0.001$) positive correlation between advancing age and presence of periodontitis in the horses sampled. A cross tabulation was used to exclude any effect of advancing age and to ensure any alterations in TLR and cytokine expression were due to the presence of periodontitis and not advancing age.

TLRs belong to the pattern recognition family of receptors (PRR) and each recognise specific pathogen-associated molecular patterns (PAMPs). Each TLR has specific ligands and after ligand binding a cell signalling cascade commences which ultimately induces or supresses the expression of genes involved in the inflammatory response, such as cytokines (Akira and Takeda 2004). In the current study, gingival expression of TLR 2, TLR 4 and TLR 9 in equine oral health and periodontitis was investigated. Before weighting for age, statistically significant increases were seen in mRNA expression of TLR 2 ($p=0.03$) and TLR 9 ($p=0.04$) in diseased periodontal tissue. In the age-weighted analysis, TLR 4 mRNA was also significantly greater in disease ($p<0.001$) and the p values decreased for TLR2 ($p<0.001$) and TLR9 ($p=0.013$) mRNA expression. A probable explanation for this was a reduction in the variance of the data in each group (i.e. the standard errors were reduced) with an increase in the observed effect size after correcting for the age of the animals. When comparing gingival tissue from an unaffected healthy site, all three TLRs were significantly increased in equine periodontal disease.

TLR 2 is found on the plasma membrane of monocytes, dendritic cells, mast cells, eosinophils and basophils and responds to a wide variety of molecules from

fungal, bacterial and mycobacterial pathogens. Ligands which activate TLR 2 include lipotechioic acid (LTA), lipoproteins and lipopeptides from Gram-positive bacteria (Takeuchi *et al*. 1999), lipopolysaccharide (LPS) from Gram-negative bacteria (Lappin *et al*. 2011), viral hemagglutinin protein, tGPI-mutin from parasites and glucuronoxylomannan and phospholipomannan from fungi (Akira *et al*. 2006). TLR 2 also associates with TLR 1 and TLR 6 to form functional heterodimers, TLR1/2 recognising triacyl lipoprotein (Kumar *et al*. 2009) and TLR 6/2 recognising diacyl lipoprotein (Kumar *et al*. 2009), fungal zymosan and LTA from Gram-positive bacteria (Akira *et al*. 2006). Expression of TLR 2 has also been shown to be upregulated after LPS stimulation of TLR 4 (Fan *et al*. 2003). In the current study expression of TLR 2 was significantly increased in periodontally diseased tissue in comparison to healthy gingival tissue from both unaffected animals and healthy (control) sites in diseased horses. In addition, a 389 fold-increase in TLR 2 expression was noted in horses with periodontitis compared to orally healthy horses. In the age-weighted analysis, TLR 2 expression showed a statistically significant positive correlation. Activation of TLR 2 by its ligands increases expression of TLR 2 mRNA (Weiss *et al*. 2004) and so it is likely that greater TLR 2 mRNA expression in the current study is a result of increased ligand binding and activation of the receptor due to the increased bacterial challenge present in diseased periodontal tissue. Higher levels of TLR2 expression have been noted in gingival tissue of human periodontitis patients (Sarah *et al*. 2006) and human gingival epithelial cells have been shown to recognise bacteria through activation of TLR 2 (Asai *et al*. 2001). The known human periodontal pathogens *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum* and *Veillonella parvula* have been shown to stimulate cytokine expression following activation of TLR 2 (Kikkert *et al*. 2007). Sun *et al*. (2010) have also shown significant increases in TLR 2 mRNA expression in human periodontal ligament cells following stimulation with the Gram-negative periodontal pathogens *P. gingivalis*, *P. intermedia*, *F. nucleatum* and *A. actinomycetemcomitans* (Sun *et al*. 2010). The increase in TLR2 mRNA expression in equine periodontitis could also be, in part, explained by activation of the receptor by ligands such as zymosan, glucuronoxylomannan and phospholipomannan present in fungi (Akira *et al*. 2006). As impaction of food into the periodontal pocket is common in the horse (Dixon *et al*. 2008a) it is

possible that in addition to acting as a bacterial nidus, impacted feed may also support growth of fungal organisms adjacent to the gingiva. In an age-weighted analysis, TLR 2 mRNA expression was significantly positively correlated with severity of periodontal disease in the horse and activation of TLR 2 by its ligands has previously been associated with alveolar bone loss as seen in advanced cases of periodontitis in other species. Myneni *et al*. (2011) studied the host response to the recognised periodontal pathogen *T. forsythia* and found that TLR 2 signalling and subsequent Th2 differentiation is responsible for *T. forsythia*-induced alveolar bone resorption. Signalling of TLR 1/2 and the TLR 2/6 heterodimers has been found to increase osteoclast formation and resorption of mandibular alveolar bone *in vitro* and stimulation with TLR 2 heterodimer ligands also induced periodontitis in an *in vivo* rat model (Matsumoto *et al*. 2012). Interestingly, TLR 2-deficient mice resisted alveolar bone loss when challenged with *P. gingivialis* whereas control mice showed significant bone loss following challenge (Burns *et al*. 2006).

TLR 4 is located in the plasma membrane of cells such as leukocytes and mainly detects the presence of Gram-negative bacteria by binding with LPS found on the bacterial cell surface (Takeuchi and Akira 2010). In addition to LPS from Gram-negative bacteria, other ligands which activate TLR include mannan (Kumar *et al*. 2009), glucuronoxylomannan from fungi (Akira *et al*. 2006), glycoinositolphospholipids from parasites (Kumar et al. 2009) and viral envelope proteins (Akira *et al*. 2006). TLR 4 is also activated by molecules originating from the host such as fibrinogen and heat-shock protein (Akira *et al*. 2006). In the current study, expression of TLR 4 mRNA was significantly greater in periodontal disease ($p<0.001$) than in unaffected animals after weighting for age. A much smaller fold increase (2-fold) of TLR 4 expression in disease was seen in comparison to the fold increase of TLR 2 expression (389-fold). When diseased sites were compared to healthy control sites from the same horse, TLR 4 expression was significantly increased ($p=0.03$) with expression increased 17-fold in disease. There was no significant correlation between expression of TLR 4 mRNA and severity of periodontal disease in the horse. Expression of TLR4 has been shown to be up-regulated in chronic periodontal disease in man (Sarah *et al*. 2006). In human studies, a higher number of cells expressing TLR 4 have been found in severely inflamed gingival connective tissue (Mori *et al*. 2003) and also

in the basal layer of diseased tissue compared to healthy controls (Beklen *et al*. 2008). Cluster of differentiation 14 (CD14) is a co-receptor associated with TLR 4 which assists in identification of LPS. When LPS is bound to CD14, TLR 4 is activated and this results in cytokine release. Becerik *et al*. (2011) found that the number of TLR 4 and membrane-bound CD14 positive cells were increased in the basal layer of diseased gingival tissue in comparison to healthy controls. However, previous studies have shown expression of CD14 to decrease with increasing severity of disease (Mori *et al*. 2003; Jin *et al*. 2004). As TLR 4 is primarily activated by LPS from Gram-negative bacteria (Takeuchi and Akira 2010) the elevated TLR 4 expression in diseased tissue in the current study is likely due to the increased presence and challenge of Gram-negative bacteria in the periodontal pocket. Gram-negative species such as *P. gingivalis, P. intermedia*, *A. actinomycetemcomitans*, and *F. nucleatum* are commonly implicated in the aetiopathogenesis of periodontal disease in man activating TLR 2 and 4 which results in the production of inflammatory cytokines (Kikkert *et al*. 2007; Sun *et al*. 2010). LPS produced by the keystone pathogen *P. gingivalis* has been shown to increase expression of TLR 4 in addition to TLR 2 (Sun *et al*. 2010) and anti-TLR 4 antibody inhibits *P. gingivalis* LPS-induced IL-1 production in human gingival fibroblasts (Wang *et al*. 2000). As with TLR 2, it is also possible that TLR 4 expression was higher in the current study due to activation by other ligands such as glucuronoxylomannan and phospholipomannan from fungi, whose growth could be supported in decaying and fermenting feed material usually present in equine periodontal pockets. Alongside increased bacterial challenge present in severe periodontitis cases, a positive correlation in TLR 4 expression with disease severity could be expected, however this was not found in the horse. One possible reason for this could be the relatively low number of samples in each group and therefore a larger scale study may be useful. It was not within the scope of this study to measure production of serum inflammatory markers in response to periodontitis in the horse, however this area of investigation could provide useful information on the systemic effects of the disease in the horse.

TLR 9 is an intracellular TLR which recognises and binds to unmethylated cytosine-phosphate-guanine bacterial, fungal and viral DNA (Hemmi *et al*. 2000). In an age-weighted analysis, TLR 9 expression was significantly higher ($p=0.0013$) in gingival tissue from horses with periodontal disease in comparison to unaffected animals. Expression in horses with periodontal disease was 3.8 times greater in orally heathy horses. In comparison to healthy control sites from diseased animals, TLR 9 expression showed a 17-fold increase ($p=0.02$) in diseased tissue. No significant correlation was noted between TLR 9 expression and severity of disease. Increased abundance of TLR 9-positive cells has been detected in periodontally diseased human gingival tissue (Beklen *et al*. 2008). TLR 9 and subsequent NF-κB activation has previously been detected in the epithelium of human periodontal pockets suggesting that the TLR is activated by the presence of bacterial, viral or fungal DNA within the periodontal pocket (Chen *et al*. 2012). In addition, bacterial DNA from A. *actinomycetemcomitans*, *P. gingivalis*, and *P. micros* was found to stimulate macrophages and gingival fibroblasts to produce TNF-α and IL-6. When CpG motifs on the DNA were methylated, this effect was not seen suggesting cytokine production following stimulation by the DNA of these periodontal pathogens was mediated through TLR 9 (Nonnenmacher *et al*. 2003) and it is possible that the increase in TLR 9 expression in equine periodontitis lesions is due to activation by bacteria within the periodontal pocket.

All three TLRs studied in this chapter (TLR 2, TLR 4, TLR 9) showed higher expression in equine periodontitis lesions in comparison to orally healthy tissues. While one explanation for greater expression is that the cell population at the site of inflammation differs, due to the increased presence of inflammatory and immune cells in the periodontal lesion, increased expression of TLRs could also result because of increased activation by their respective ligands and in such a case the results of this study might suggest that there is an increased abundance of TLR 2-, TLR 4- and TLR 9-activating ligands in the equine periodontal pocket, such as LTA, lipoproteins, LPS and unmethylated CpG motifs associated with bacteria. Several periodontal pathogens which have been shown to activate TLR 2 and TLR 4 (Kikkert *et al*. 2007) and TLR 9 (Nonnenmacher *et al*. 2003) and an increased abundance of *P. gingivalis*, *P. intermedia* and *T. forsythia* have been found in equine periodontal pockets as discussed in Chapter 5. It is possible that

the increased abundance of periodontal pathogens in addition to the significant change and increase in diversity of the microbiome of the diseased equine periodontal pocket is contributing a greater amount of TLR-stimulating ligands and accounting at least in part to the increased expression of TLR 2, TLR 4 and TLR 9 in equine periodontal pockets.

Binding of TLRs to their microbial ligands results in activation of second messenger systems which subsequently cause altered expression of a variety of immune and inflammatory genes, including cytokine genes (Akira and Takeda 2004). In order to further characterise the response of the innate immune system in equine periodontitis, expression of Th1, Th2 and Th17 cytokines were evaluated in the current study.

It has been suggested that the stimulation of Th1 cytokines in response to periodontopathogenic bacteria leads to the destruction of periodontal tissue (Graves and Cochran 2003). The levels of mRNA encoding the pro-inflammatory Th1 cytokines (IL-1ß, TNF-α, IFN-ɣ, IL-12p35, IL-12p40) were higher in diseased samples, although TNF-α was only significantly greater in the age-weighted analysis. In addition, IL-4 mRNA (a Th2 cytokine) and IL-10 mRNA (an anti-inflammatory cytokine) levels were also greater in periodontitis samples and a significant positive correlation between disease severity and IL-4 levels was observed. When comparing healthy control sites with periodontitis lesions in the same horses, significant increases were observed for mRNA levels of IL-1ß, IL-12p35 and IFN-ɣ in periodontitis. After age-adjusted analysis was performed, expression of IL-1ß, IL-6, IL-10, IL-12p40, IL-17 and TNFα showed significant positive correlation (all $p<0.001$) with disease severity.

In the current study, a positive correlation between disease severity and IL-1ß mRNA levels was observed and this correlation is in agreement with studies in human periodontitis by Sanchez *et al*. (2013) and Oh *et al*. (2015). In addition, expression of IL-1ß has been found to decrease after treatment of human periodontitis (Reis *et al*. 2014). IL-1ß plays a pivotal role in periodontal tissue destruction (Liu *et al*. 1996) by enhancing bone resorption and inducing synthesis of matrix metalloproteinases (MMPs) which may lead to further tissue destruction. However, collagen breakdown in the periodontal ligament induced

by MMP-1 may be central to the normal physiology of the healthy equine periodontium due to the long-term dental eruption and periodontal tissue remodelling occurring in hypsodont dentition (Warhonowicz *et al*. 2007). It is therefore possible that IL-1β plays important roles in both healthy and diseased equine periodontal tissue and this may explain its relatively high basal expression in healthy equine gingival tissue. It has been suggested that expression of TNF-α in gingival tissue, alongside IL-1β, plays an instrumental role in periodontal tissue destruction by stimulating osteoclast development and loss of alveolar bone (Graves and Cochran 2003), in addition to enhancing synthesis of collagenase by fibroblasts (Meikle *et al*. 1989; Okada and Murakami 1998). Further to these initial observations, TNF-α has also been found to increase in periodontitis GCF samples in man (Gokul *et al*. 2012) and rodents (Liao *et al*. 2014). In the current study, TNF-α mRNA levels were greater in periodontitis lesions, but only statistically significant in the age-weighted analysis. The mRNA levels of the IL-12 (NK stimulating factor) genes (IL-12p35 and IL-12p40) were found to be higher when comparing both unaffected healthy and periodontitis groups and healthy control sites and periodontitis lesions, with IL-12p35 mRNA levels increased 17-fold in periodontitis lesions. IL-12 acts as a pro-inflammatory cytokine, activating natural killer cells and inducing CD4 T-cell differentiation into Th1-like cells and inducing IFN-ɣ production (Honda *et al*. 2008). Elevated expression of IL-12p35 has been shown in human chronic periodontitis patients (Honda *et al*. 2008). The increased mRNA levels observed for both IL-12 genes in equine periodontitis may explain the significant increase in IFN-ɣ mRNA seen in periodontitis when compared to both orally healthy horses and healthy control sites. Produced by Th1 cells, IFN-ɣ acts to stimulate phagocytosis by macrophages and also acts as a positive regulator of the Th1 response, thus increasing IL-12 responsiveness (Mullen *et al*. 2001). Significantly higher levels of IFN-ɣ expression have been noted in active human periodontitis lesions (Dutzan *et al*. 2009; Zhang *et al*. 2010). A positive correlation with disease severity in man was seen by Ukai *et al*. (2001) and Papathanasiou *et al*. (2014). However, in the current study, expression of IFN- ɣ showed no significant correlation with equine periodontal disease severity. A larger scale study would be useful to examine this further.

Produced by Th-17 cells, the IL-17 cytokine family are thought to play important roles in host defence against microorganisms but they have also been implicated in the aetiopathogenesis of chronic inflammatory disorders (Gu *et al*. 2013). IL-17 is a pro-inflammatory cytokine which supports Th1 responses (Takahashi *et al*. 2005) and plays a key role in recruiting and activating neutrophils at the site of inflammatory responses (Aggarwal *et al*. 2003). In the current study, IL-17 mRNA levels were increased in equine periodontitis lesions compared to healthy control sites, but this was not statistically significant although following age adjusted analysis a positive correlation between IL-17 and disease severity was noted ($p<0.001$).

The Th2 response regulates the inflammatory response and counteracts the effects of the pro-inflammatory Th1 response, including regulation of cytokines such as IL-4, IL-6 and the anti-inflammatory cytokine IL-10. Although in the current study IL-6 mRNA levels were elevated in periodontitis tissue, this was not found to be statistically significant. This is somewhat surprising, as it has been suggested that IL-6 plays an important role in the pathogenesis of human periodontitis, inhibiting alveolar bone formation (Stefani *et al*. 2013). There was, however, a positive correlation between clinical disease severity and expression of IL-6 mRNA and so it is likely that IL-6 is important to the pathogenesis of equine periodontitis as it is in man. Further, larger scale studies may reveal significant increases in expression of IL-6 between oral health and periodontal disease in the horse. IL-4 mRNA levels were significantly higher in the periodontitis group when compared to the orally healthy group. IL-4 is thought to stimulate B-cell proliferation and subsequent immunoglobulin production (Dohnmann *et al*. 2000) whilst inhibiting phagocytosis and production of periodontally destructive Th1 cytokines such as IL-1β and TNF-α (Gemmel *et al*. 1997). IL-4 also stimulates production of cytokines with complementary, anti-inflammatory functions such as IL-10 (Pestka *et al*. 2004; Garlet 2010). In the current study, significantly higher levels of the anti-inflammatory cytokine IL-10 were recorded in diseased periodontal tissue when compared to both orally healthy horses and healthy control sites in horses with periodontitis. In addition, IL-10 levels were 9-fold higher in the periodontitis group compared to the orally healthy group. IL-10 also downregulates the pro-inflammatory response, having a protective role against disease progression in human periodontitis (Bozkurt *et al*.

2006) and IL-10-producing cells are widely distributed in periodontitis granulation tissue (Lappin *et al*. 2001). Sasaki *et al*. (2004) described an increased susceptibility to *P. gingivalis*-induced bone loss in IL-10 knockout mice. In addition to protecting against alveolar bone loss, IL-10 is also important in bone formation and repair (Garlet 2010) with Claudino *et al*. (2010) discovering reduced expression of markers associated with osteoblasts and osteocytes in periodontal tissues of IL-10 knockout mice.

In addition to microbial ligands, endogenous ligands are also capable of activating TLRs (Akira *et al*. 2006; Yu *et al*. 2010). Endogenous ligands are released from injured tissue and damaged cells during inflammatory conditions such as gingivitis and periodontitis. Several heat shock proteins are capable of activating TLR 2 (Yu *et al*. 2010) and TLR 4 (Akira *et al*. 2006). TLR 4 can also be activated by host fibrinogen and fibronectin and TLR 9 can be activated by host DNA (Yu *et al*. 2010) which is released from apoptotic host cells. Higher levels of serum fibrinogen have been found in humans (Sahingur *et al*. 2003) and rats (Keles *et al*. 2012) with periodontitis than in healthy controls. In addition, higher sero-positivity to heat shock protein 60 has been detected in human periodontitis patients than in healthy controls (Tabeta *et al*. 2000). As these proteins are inflammatory markers and TLR ligands it is likely that increased production in response to severely inflamed gingival tissue would result in increased activation of TLRs and subsequent alterations in cytokine expression in diseased gingival tissue. It has been suggested that these endogenous ligands act as an 'early warning system' to alert the host to tissue destruction and further stimulate the innate immune response to help combat the causative agent (Yu *et al*. 2010). Several additional TLRs and other PRRs such as scavenger receptors and Dectin-1 not examined in this study can also recognise some endogenous ligands and are important in host response to tissue damage. Dectin-1 has been suggested to be involved in apoptotic cell clearance, being able to recognise a ligand on apoptotic cells as well as fungal ligands (Weck *et al*. 2008).

Human periodontitis is often generalised, affecting a large proportion of teeth. However, in the horse, periodontal disease is focal and affects relatively small areas which usually contain substantial pre-existing diastemata lesions to which the periodontitis is secondary. This gives the opportunity to study both

periodontally diseased and unaffected, healthy areas in the same horse. When comparing TLR and cytokine expression between diseased sites and orally healthy animals with diseased sites and healthy sites from the same animal, TLR and cytokine expression followed similar profiles and in both sets of data significant increases in TLR 2, TLR 4 (after age-weighting), TLR 9, IL-10, IL-12 and IFN-γ were seen. Excluding TLR 2, the diseased samples showed much larger fold changes in comparison to their matched controls than those samples from orally healthy horses. As expression will vary between individuals, comparing diseased and healthy tissue from the same animal is useful and a larger scale study could more fully investigate the innate immune response to periodontitis in multiple sites within the oral cavity. In addition, a larger scale study with more horses within each disease severity group would provide useful information regarding correlation of TLR and cytokine expression with disease severity in the horse since in the current study group size was limited.

It is possible that individual variation may be important when investigating the equine innate immune response to periodontitis, as some genetic polymorphisms have shown to result in increased susceptibility to the disease in humans. Ozer *et al*. (2015) found an association with TNF-α (-308) allele 2 frequency and aggressive periodontitis and the CD14 CC genotype has been related to a susceptibility to chronic periodontitis (Sahingur *et al*. 2011). Polymorphisms of IL-1 (Yoshie *et al*. 2007), IL-4 (Anovazzi *et al*. 2010), IL-6 and IL-10 (Yoshie *et al*. 2007) have also been associated with periodontal disease susceptibility. Presence of cytokine and TLR genetic polymorphisms may result in inter-individual variation in host immune response to invading periodontopathogenic bacteria which could influence disease susceptibility and progression (Yoshie *et al*. 2007). A variety of horses from different breeds and backgrounds were sampled in this study and it is not known whether genetic polymorphisms in equine TLR and cytokine genes increase the susceptibility to periodontitis in the horse.

6.5 Conclusion

A mixed Th1/Th2/Th17 response was observed in gingival tissue samples from equine periodontitis cases, with statistically significantly higher levels of mRNA of both destructive pro-inflammatory and protective anti-inflammatory cytokines. It is likely that increased expression of these cytokine genes results from stimulation of TLR 2, TLR 4 and TLR 9 by their respective bacterial ligands. The results suggest that these receptors are also increased in equine periodontitis, although this was only demonstrated at the gene transcript level. In other species, recognition of periodontopathogenic bacteria by TLRs in gingival and periodontal tissue initiates cytokine production, often causing a destructive inflammatory response and thereby facilitating disease progression (Garlet 2010). The results of the current study suggest a similar bacterial aetiopathogenesis is likely in the horse although it is also possible that ligands such as molecules from fungi and host inflammatory markers will stimulate TLR activation and production of cytokines to a degree. The innate immune response of equine periodontitis is complex and further studies are required to further investigate the interaction of bacteria and innate immunity in the equine oral cavity.

Chapter 7

Toll-like receptor and cytokine activation in response to the presence of putative periodontal pathogens

7.1 Introduction

The interaction between periodontal pathogens and the innate immune system of the host plays a central role in the aetiopathogenesis of periodontitis (Kikkert *et al*. 2007; Sun *et al*. 2010; Gemmell *et al*. 1997). In the human oral cavity over 700 species of bacteria have been detected (Dewhirst *et al*. 2010) and the current study has reported the presence of over 2000 species in the equine oral cavity affected with periodontitis. Many of these species are likely to be commensal bacteria, existing in the healthy oral cavity of the host. It is important to distinguish which species are acting as periodontal pathogens, stimulating activation of Toll-like receptors (TLRs) and subsequent cytokine production which can lead to an excessive pro-inflammatory response and destruction of periodontal tissue, thus promoting the disease process.

It is possible to examine the interaction between putative periodontal pathogens and the host innate immune system by using cell-based assays. All the assays described in this chapter are commercially available and dependent on the activation of Nuclear Factor Kappa B (NF-κcanonical MYD88 adaptor protein, which is dependent on NFκB activation pathway. The various cells types described were transfected with one or more plasmids which confer specific antibiotic resistance to select and drive expression of: 1) specific TLRs, 2) Si RNA to knock down the adaptor protein for TLR-NF-κB activation MYD88, and in all cases 3) a reporter gene which responds to transcription factors NF-κB and AP-1 to promote the expression of an enzyme, inducible secreted embryonic alkaline phosphatase (SEAP), which is detected by substrate conversion and colour change using a microtitre plate assay.

THP-1X cells are derived from a human monocytic cell line and express the SEAP reporter gene. When stimulated by ligands for TLR 2, TLR 1/2, TLR 2/6, TLR 4,

TLR 5 and TLR 8, transcription factors are activated, resulting in the secretion of SEAP. This process can be detected by QUANTI-Blue™, medium which changes colour to purple in the presence of SEAP. By measuring absorbance of QUANTI-Blue™ using a spectrophotometer at 630 nm, the degree of TLR activation by different putative pathogens can be determined. MyD88Def THP-1 Blue cells are deficient in MyD88 and so are unable to respond to MyD88-dependent signalling, as used by TLR 2 and TLR 4. HEK Blue™ hTLR 2 cells and HEK Blue™ hTLR 4 cells express the human TLR 2 and TLR 4 genes, respectively, and so will respond in the presence of TLR 2 and TLR 4 ligands. These cell lines also express a SEAP reporter gene and so activation of TLR 2 and TLR 4 in response to the presence of putative periodontal pathogens can be monitored. Initial data from a pilot study using these cell lines to investigate activation of innate immunity by putative periodontal pathogens is presented in this chapter

Furthermore, by using matched data originating from the clinical, microbiological and immunological studies of equine periodontal disease presented in previous chapters of this thesis this chapter aims to establish if associations exist between the presence of bacterial species previously identified as putative pathogens, the clinical severity and the expression of TLR and cytokine mRNA.

It is hoped that use of cell-based assays to investigate activation of innate immunity by periodontal pathogens alongside cross-matched data derived from analysis of samples from clinical cases of equine periodontitis, will provide an equine specific-insight to the link between the innate immune response and equine periodontal bacteria.

7.2 Materials and Methods

7.2.1 Cell-line based assays

7.2.1.1 Microbial Culture

The following organisms were commercially sourced (DSMZ, Braunschweig, Germany) and cultured according to their specific growth requirements as advised by the source laboratory. *Aggregatibacter actinomycetemcomitans (* strain code NCTC9709) was cultured at 37°C on FAA supplemented with 7.5% defibrinated horse blood in 5% CO_2/air. *Escherichia coli K12*(ATCC 27325) , *Fusobacterium nucleatum* (NCTC10502), *Porphyromonas gingivalis* (ATCC11835), *Porphyromonas gingivalis* (W50), *Prevotella intermedia* (ATCC25611), *Prevotella bivia* (DSM No. 20514), *Prevotella oris* (ATCC 33573), *Tannerella forsythia* (ATCC95137), *Veillonella parvula* (DSM No. 2007) *and Wolinella recta* (DRWH) were grown anaerobically (85% N2, 10% CO2 and 5% H2) at 37°C upon FAA plates with 7.5% defibrinated horse blood. The agar for *T. forsythia* was supplemented with 6 mM N-acetylmuraminic acid (NAM). *Treponema denticola (*ATCC 35405) was grown at 37°C anaerobically as above on TYGVS (tryptone-yeast extract-gelatin-volatile fatty acids-serum) agarose (0.8% w/v) containing rifampicin 10 µg/ml. *Staphylococcus aureus* (NCTC 6571), *Streptococcus mitis* (ATCC 12261), *Streptococcus mutans* (NCTC10449), *Streptococcus salivarius* (NCTC8018), *Streptococcus sanguinus* (NCTC7163) were grown in 5% CO_2/air at 37°C upon Columbia blood agar plates. *Peptostreptococcus micros* (NCTC11808) was grown anaerobically (85% N2, 10% CO2 and 5% H2) at 37°C upon Columbia blood agar plates. *Lysobacter enzymogenes* (DSM1895), *Pseudomonas aeruginosa* (PAC611)), *Pasteurella multocida* (ATCC10544) were grown aerobically upon Columbia blood agar plates at 37°C. Candida albicans (SC5314) was cultured at 37°C upon tryptone soya agar. Culture plates were inspected to ensure that pure cultures of each species were achieved and no contamination had occurred. Individual colonies were removed from culture plates, placed into 5.0 mL PBS, mixed and then centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed, 1.0mL of PBS was added and mixed to disperse the cell pellet. The absorbance of the resulting suspension was then measured using a spectrophotometer and the number of colony forming units (CFUs) was calculated. The suspension was used immediately or stored at -20°C for future use.

7.2.1.2 Initial Cell Culture and Maintenance

THP1-X Blue ™ Cell line

Initial culture of THP1-X Blue ™ cells (InvivoGen, Toulouse, France) was performed according to the manufacturer's instructions. Firstly, the vial containing the cells was thawed at 37°C in a water bath. The cells were then transferred to a vial containing 15 mLs of pre-warmed growth medium comprised of RPMI 1640 (Thermofisher Scientific, Paisley, UK) with the addition of 10% foetal bovine serum (Sigma-Aldrich), 100 mg/mL Normocin™ (Invivogen) and Penicillin/Streptomycin (50 U/mL/50 µg/mL; Thermofisher Scientific). This vial was then centrifuged at 1500RPM for five minutes and the supernatant subsequently removed. The cell pellet was resuspended in 1 ml of pre-warmed growth medium as previously described and the vial contents transferred to a 25cm^2 tissue culture flask containing a further 5mL of pre-warmed growth medium. The culture was placed in an incubator at 37ºC in 5% CO_2.

Following primary culture, cells were maintained in growth medium supplemented with 200 µg/mL of Zeocin™ (Invivogen). The cells were passaged every three days by inoculating 7 x 10^5 cells/mL. All steps were performed using aseptic technique.

MyD88Def THP-1 Blue cell line

The MyD88Def THP-1X Blue ™ cell line underwent initial culture and maintenance using the same method as the THP-1X Blue cells described above, with the exception of the addition of 100 µg/mL of HygroGold™ (Invivogen) for cell maintenance as described in the manufacturer's instructions.

HEK Blue ™ hTLR-2 cell line

Initial culture of the HEK Blue ™ hTLR-2 cell line was performed using the same method as for the THP-1X Blue ™ cells, except that the culture medium used was DMEM (Sigma-Aldrich) with the addition of 10% foetal bovine serum, 50 U/ml penicillin, 50 µg/mL Streptomycin, 100 µg/mL Normomycin™ (Invivogen) and

2mM L-glutamine. HEK Blue ™ cells were maintained in growth medium supplemented with 1-X HEK-Blue™ selection medium and this was renewed twice weekly. Cells were passaged upon reaching 70-80% confluency.

HEK Blue™ hTLR 4/MD2 cell line

Initial culture and maintenance of the HEK Blue™ hTLR 4 cell line was performed using the same methods as for the HEK Blue™ hTLR 2 cell line.

7.2.1.3 Assays

THP-1X Blue™ assay

All assays were performed according to the manufacturer's instructions. Firstly, the cells were centrifuged at 1500 RPM for 5 minutes. The supernatant was removed and the cells were resuspended at 1 X 10^6 cells/mL in fresh, pre-warmed growth medium. The corresponding sample volume to give 100 multiplicity of infection (MOI) was calculated for each species, this was made up to 20µl using sterile, RNase-free water (Thermofisher Scientific) and added to each well of a 96 well plate. *V. parvula* was tested with THP-1X Blue™ cells only. Subsequently, 180 µl of cell suspension (200,000 cells) were added to each well and the plate was incubated at 37°C in a CO_2 incubator for 18-24 h. The following day, one sachet of QUANTI-Blue™ was prepared according to the manufacturer's instructions. Once prepared, 180 µl of QUANTI-Blue™ were added to each well of a fresh 96 well plate with 20 µl of THP1-XBlue™ cells supernatant. The plate was incubated at 37°C for four hours and the secreted embryonic alkaline phosphatase (SEAP) levels were measured using a spectrophotometer at 650 nm.

MyD88Def THP-1X Blue ™ assay

This assay was performed using the same method as the THP-1X Blue™ assay. In the THP-1X Blue™ and MyD88Def THP-1X Blue™ assays, sterile, RNase free water (Thermofischer Scientific) was used as a primary negative control. An additional negative control of cell culture medium was also included. TriDAP (3nM; Invivogen) and *E. coli* LPS (3nM; Invivogen) were included as positive controls. TLR 2 and heterodimers TLR 2/TLR 1 and TLR 2/TLR 6-specific ligand PAM C3

(3nM; Invivogen) were also included as TLR 2 positive controls. All procedures were carried out using aseptic technique and all assays were performed in triplicate.

HEK Blue ™ hTLR 2 assay

Firstly, 20 µl of each putative pathogen sample was added to each well of a 96-well plate. HEK-Blue™ hTLR2 cells were removed from the incubator, growth medium was discarded and the cells were rinsed gently with 10 mL of pre-warmed PBS. Then, 5 mL of fresh, pre-warmed PBS was added and the cells were placed into the incubator at 37˚C for 2 minutes before using a cell scraper to detach the cells. Any cell clumps were dispersed by gently pipetting up and down before counting the cells. A cell suspension containing approximately 280,000 cells per mL in HEK-Blue™ detection medium was prepared and 180 µL (approximately 50,000 cells) was added to each well. The plate was incubated at 37˚C in 5% CO_2 for 4 hours. SEAP was measured using a spectrophotometer at 620-655 nm.

HEK Blue™ hTLR 4/MD2 assay

The HEK Blue™ hTLR 4 assay was performed using the same method as the HEK Blue™ hTLR 2 assay. In the HEK Blue™ hTLR 4 and hTLR 2 assays, sterile, RNase-free water (Thermofischer Scientific) was used as a primary negative control. An additional negative control with cell culture media was also used. LPS (3 nM) was used as a positive control. All procedures were carried out using aseptic technique and all assays were performed in duplicate on at least three separate occasions.

7.2.2 Cross matched data collection

Data presented in previous chapters pertaining to clinical severity, gingival TLR and cytokine mRNA expression and RT-PCR targeting periodontal pathogens *P. gingivalis, P. intermedia, A. actinomycetemcomitans, V. dispar T. denticola* and *T. forsythia* were collated and matched to each horse.

Matched high throughput 16S rRNA gene sequencing data were available for the majority of the gingival biopsy samples. Over 500 species were detected including many oral taxons, oral clones and previously uncultured bacteria which meant cross matching data in other studies with the presence of a species, sub-species or clone proved difficult due to the number of individual strains present. Furthermore, this technique is more accurate when identifying at genus level and is often unable to discriminate between species. It was decided that the most useful information could come from correlating the clinical severity, periodontal pathogen PCR and immunology data.

7.2.3 Statistical analysis

7.2.3.1 Cell-based assays

A one-way analysis of variance (ANOVA) was performed in addition to Dunnett's Multiple Comparison Test. Any differences in the data between microbe/ligand-stimulated and control SEAP levels were deemed to be significant when a Bonferroni adjusted p value ≤ 0.05 was observed.

7.2.3.2 Crossed matched data analysis

Non-parametric correlation analysis was performed to assess potential correlations between presence of putative pathogens (detected by RT-PCR) and disease severity; presence of putative pathogens (detected by RT-PCR) and TLR and cytokine expression; TLR mRNA expression and cytokine mRNA. Data pertaining to correlations between clinical severity and TLR and cytokine expression and between TLR mRNA expression and cytokine mRNA expression is presented in Chapter 6. Age-adjusted analysis was performed to ensure any significant correlations were truly due to disease status and not age, as the diseased cohort of horses used in the immunological study were significantly older than the orally healthy group which is to be expected in equine periodontitis. Correlation was deemed to be significant at the $p \leq 0.05$ level.

7.3 Results

7.3.1 Cell based assays

7.3.1.1 THP-1X Blue™ assay

Significant increases in production of SEAP were noted when THP-1X Blue™ cells were cultured with *A. Actinomycetemcomitans, E. coli, F. nucleatum, L. enzymogenes, P. aeruginosa, P. gingivalis, P. intermedia, P. multocida, P. oris, S. aureus, S. sanguinus, T. denticola, T. forsythia* and *W. recta* when compared with the negative control ($p<0.0001$) indicating TLR activation (Figure 7.1). TLR 2 and heterodimers TLR 2/TLR 1 and TLR 2/TLR 6-specific ligand PAM C3 (3 nM) also resulted in a significant increase in SEAP when cultured with THP-1X Blue cells. Culture of LPS with these cells also produced a significant increase in production of SEAP (Figure 7.1).

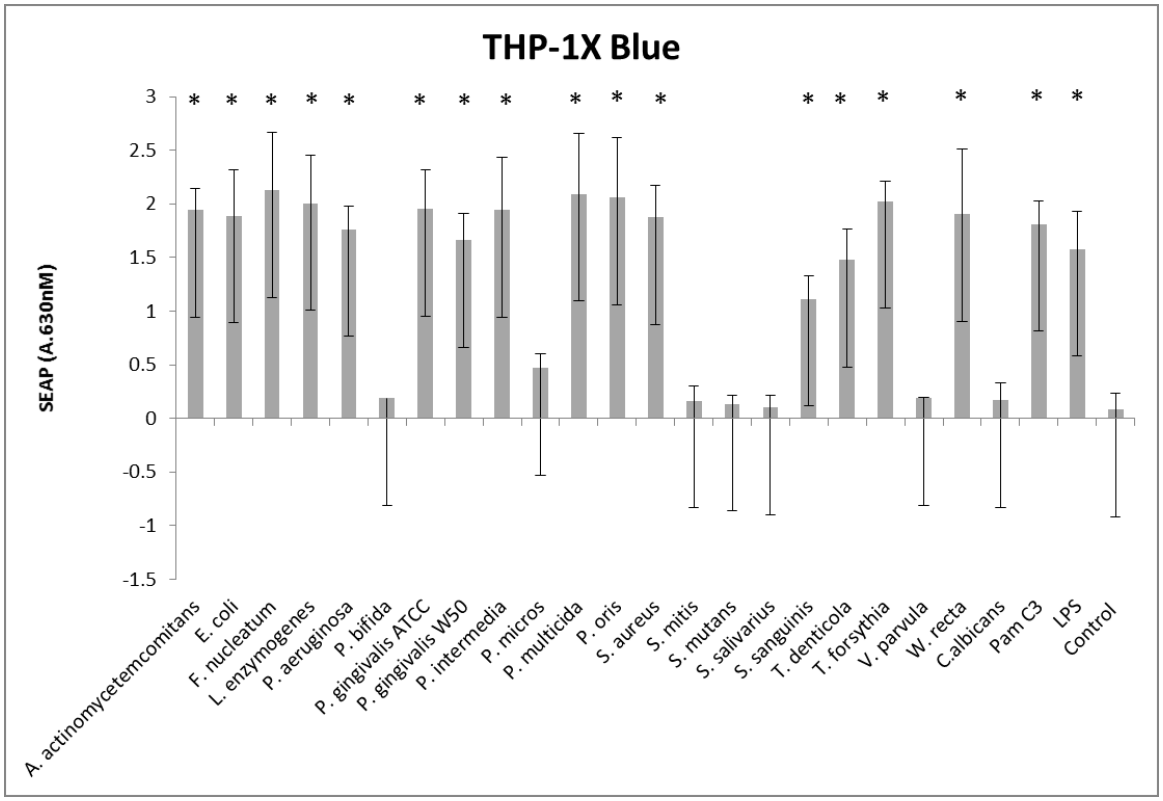


Figure 7.1 Measurement of SEAP following co-culture of THP-1X Blue™ cells and putative periodontal pathogens.Error bars represent standard deviation.

* $p<0.001$

7.3.1.2 MyD88Def THP-1X Blue™ assay

Much diminished responses to microbial and ligand stimulation were apparent, but significant increases in the production of SEAP when compared with the unstimulated control were noted when MyD88Def THP-1X Blue™ cells were co-cultured with *A. actinomycetemcomitans, E. coli, F. nucleatum, L. enzymogenes, P. gingivalis, P. intermedia, P. multocida* and *P. oris* ($p<0.001$) (Figure 8.2). TLR 2 and heterodimers TLR 2/TLR 1 and TLR 2/TLR 6-specific ligand PAM C3 (3 nM) did not produce any increase in SEAP. Culture of LPS with MyD88Def THP-1X Blue™ cells did not produce a significant increase in SEAP production. Positive control Tridap (3 nM) resulted in a significant increase in production of SEAP.

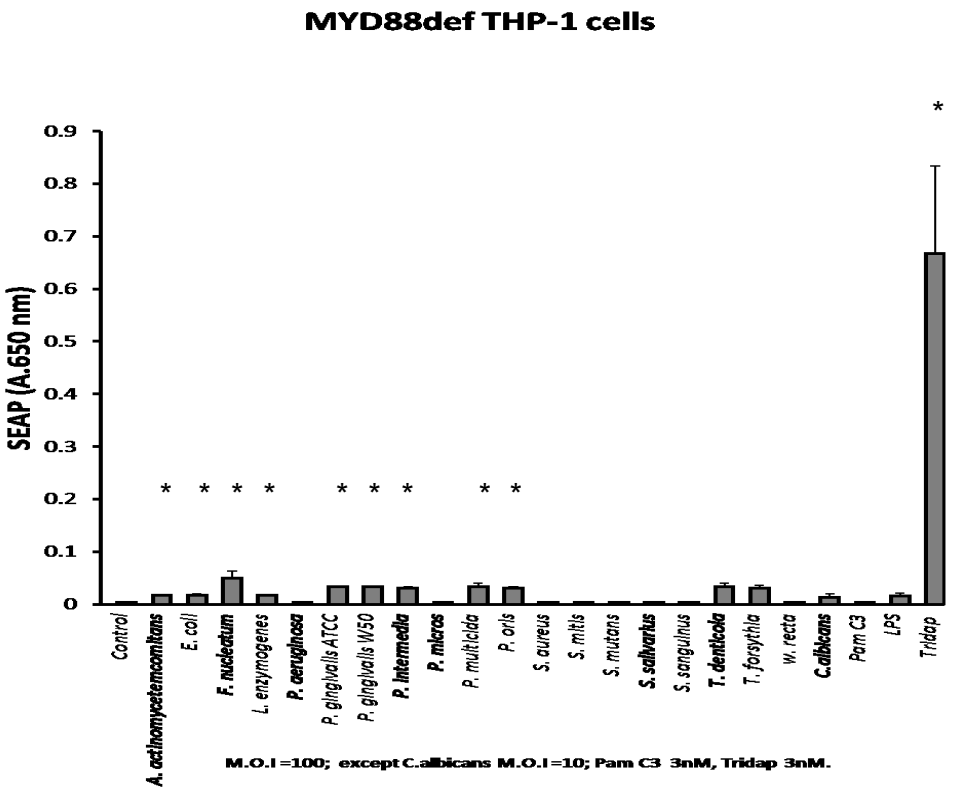


Figure 7.2 Measurement of SEAP following co-culture of MyD88 THP-1X Blue™ cells and putative periodontal pathogens. Error bars represent standard deviation.

* $p<0.001$

7.3.1.3 HEK Blue TM hTLR 2 assay

Significant increases in production of SEAP were noted when HEK Blue™ hTLR 2 cells were co-cultured *with A. actinomycetemcomitans, E. coli, F. nucleatum, L. enzymogenes, P. aeruginosa, P. gingivalis, P. intermedia, P. multocida, P. oris, S. aureus, S. sanguinus, T. denticola, T. forsythia and W. recta* (p<0.001) in comparison with the negative control (Figure 7.3). TLR 2 and heterodimers TLR 2/TLR 1 and TLR 2/TLR 6-specific ligand PAM C3 (3nM) produced a significant (P<0.001) increase in SEAP production, although *E. coli* LPS failed to produce a significant increase.

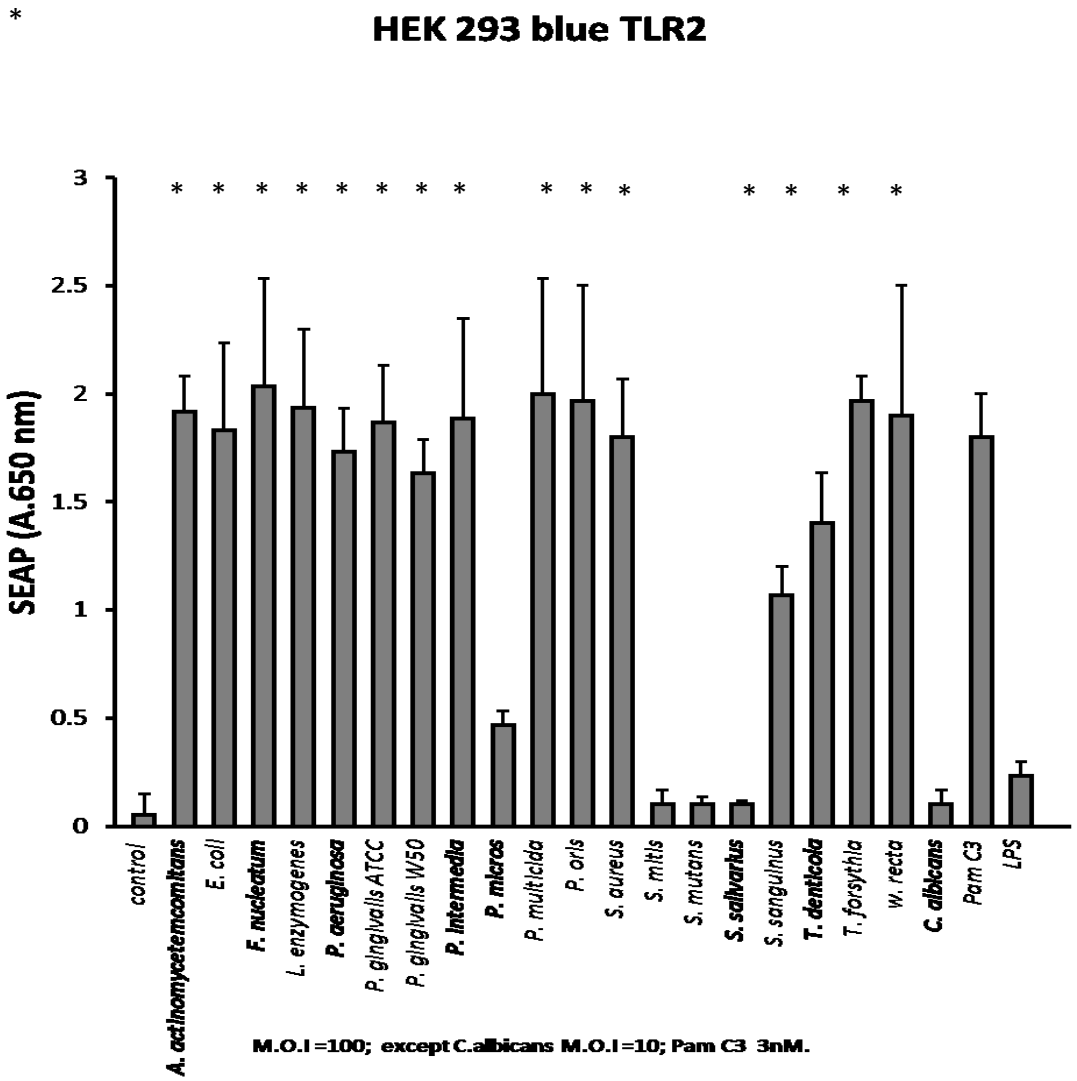


Figure 7.3 Measurement of SEAP following co-culture of HEK 293 Blue hTLR2 cells and putative periodontal pathogens. Error bars represent standard deviation.

p<0.001

7.3.1.4 HEK BlueTM hTLR 4/MD2 assay

Significant increases ($p<0.001$) in production of SEAP were noted when HEK Blue™ hTLR 4/MD2 cells were co-cultured with *E. coli*, *W. recta*, *F. nucleatum*, *P. multocida* and LPS (Figure 7.4).

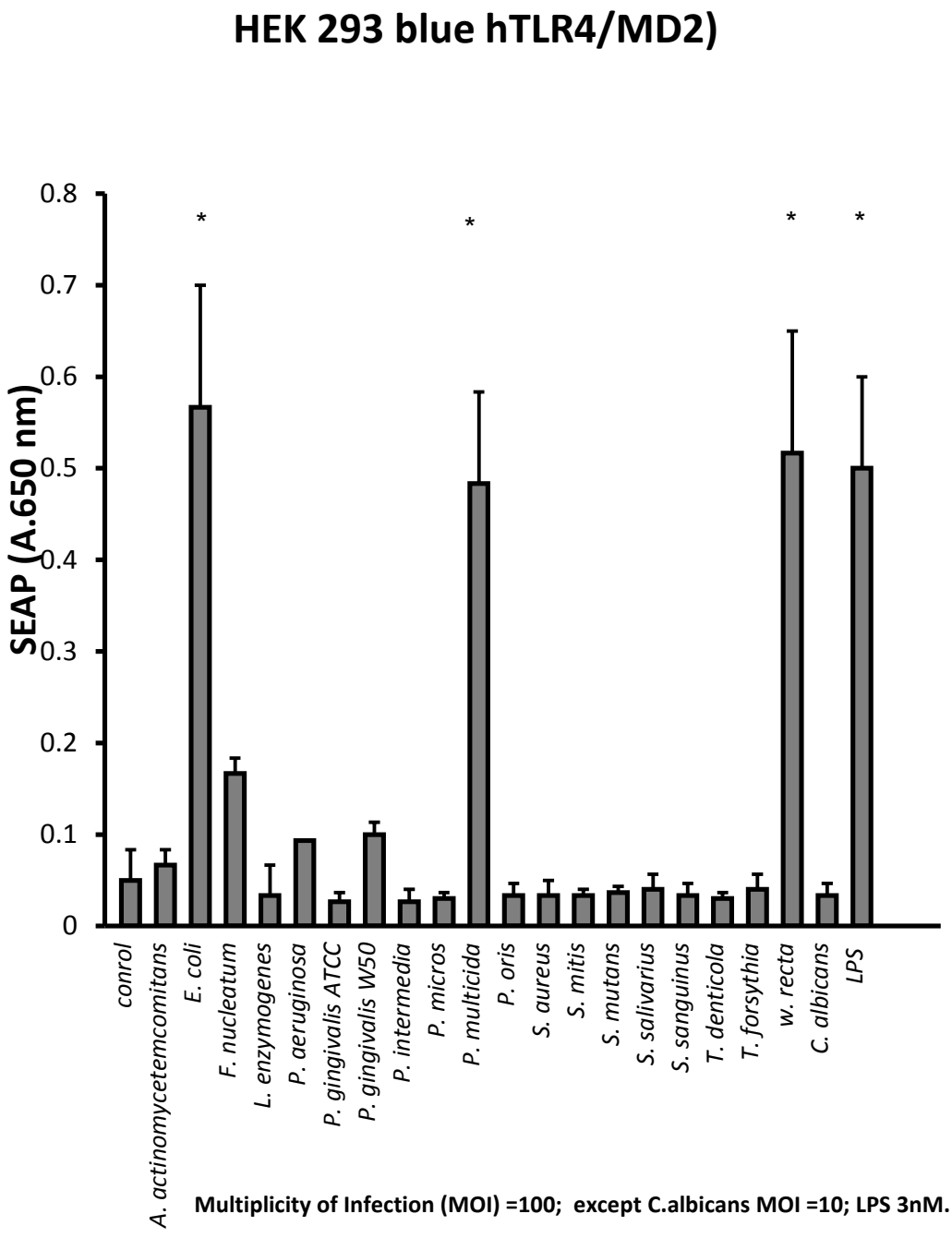


Figure 7.4 Measurement of SEAP following co-culture of HEK 293 hTLR 4/MD2 cells and putative periodontal pathogens. Error bars represent standard deviation.

* $p<0.001$

7.3.2 Correlation analysis

7.3.2.1 Disease severity, periodontal pathogen detection by RT-PCR and TLR mRNA expression

Associations between the presence of bacterial species (detected by real-time PCR), disease severity and TLR2 and TLR4 expression are shown in Table 7.1. Severity of equine periodontitis using the grading system proposed by Cox *et al*. (2012) was significantly positively associated with the presence of *A. actinomycetemcomitans* ($p \leq 0.05$) and presence of this species was positively associated with an increase in expression of TLR 4. The presence of *P. intermedia* was strongly positively associated with increased TLR 2 expression in diseased equine gingival tissue ($p \leq 0.01$). There were no other significant associations between putative periodontal pathogen, clinical severity, or expression of TLR 2 and TLR 4 in this study.

	Disease severity	TLR2	TLR4
P. gingivalis	0.019	-0.107	0.321
P. intermedia	0.368	0.955**	0.234
T. forsythus	0.225	-0.107	-0.143
A. actinomycetemcomitans	0.711*	0.393	0.679*
V. dispar	0.212	0.106	0.219

Table 7.1 Non-parametric correlations between putative periodontal pathogens (detected by real-time PCR) and TLR mRNA expression levels in healthy and diseased equine periodontal tissues.

* Correlation is significant at the 0.05 level.

** Correlation is significant at the 0.01 level (2-tailed).

7.3.2.2 Presence of putative pathogens and cytokine mRNA expression

Several significant correlations were observed between the presence of putative bacterial pathogens and cytokine expression levels (Table 7.2). There were significant negative correlations between abundance of *P. gingivalis* and the pro-inflammatory cytokine IL-1β and the anti-inflammatory cytokine IL-10. A negative correlation was also observed between *A. actinomycetemcomitans* and IL-1β and between *T. forsythia* and IL-10.

	IL-1β	**IL-10**
P. gingivalis	-0.786*	-0.786*
P. intermedia	-0.126	0.270
T. forsythus	-0.429	-0.500*
T. denticola	0.071	0.143
A. actinomyc	-0.536	-0.321
V. dispar	0.077	0.112

Table. 7.2 Non-parametric correlations between abundance of putative periodontal pathogens (detected by real-time PCR) and cytokine mRNA expression levels in the healthy and diseased equine periodontal tissues.

* Correlation is significant at the 0.05 level.

7.4 Discussion

The aims of this chapter were to assess the activation of Toll-like receptors (with a focus on TLR 2 and TLR 4) in response to putative periodontal pathogens and to identify any significant correlations between the presence of periodontal pathogens and clinical severity and TLR and cytokine expression. By discovering which species of bacteria provoke a significant inflammatory response in equine periodontal tissues and are related to severity of disease, one can identify which pathogens may be playing an important role in the aetiopathogenesis of the disease. The first stages of this work, described in this chapter, were to identify which species were capable of provoking TLR activation in established human cell lines and then to identify any existing correlations between microbiological, clinical and immunological data gained from equine clinical cases of periodontitis.

Cox *et al*. (2012) histologically examined both healthy and diseased equine periodontal tissue and described the presence of a strong inflammatory response in periodontitis, characterised by neutrophilic transmigration into gingival epithelium and monocytic and eosinophilic infiltration of the gingival lamina propria and submucosa. The presence of Gram-negative bacteria and spirochaetes in gingival samples were also associated with equine periodontal disease (Cox *et al*. 2012). As described in Chapter 6, there is significant activation of TLR 2, TLR 4 and TLR 9 in the diseased gingival tissue of horses with periodontitis, with a particularly large fold increase in TLR 2 mRNA observed. As described in Chapter 5, over 2000 species of bacteria have been found to inhabit the equine oral cavity. Many of these species are likely to be commensal organisms in the equine oral cavity, but some will be periodontal pathogens, stimulating the destructive inflammatory response that plays an important role in the aetiopathogenesis of periodontitis.

THP-1 cells have been widely used to investigate signalling pathways, monocyte/macrophage functions and immune modulation (Chanput *et al*. 2014). THP-1X Blue™ cells respond to TLR 2, TLR 1/2, TLR 2/6, TLR 4, TLR 5 and TLR 8 ligands and express an NF-kB- and AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Upon binding of these ligands and subsequent

respective TLR activation, SEAP is produced and can be measured. HEK 293 cells will also produce SEAP but can be modified to express only one TLR. In the current study, these cells were used to assess the activation of human TLR 2 and TLR 4 in response to putative periodontal pathogens.

Expression of both TLR 2 and TLR 4 has been reported to increase in diseased periodontal tissue (Sarah *et al*. 2006). In addition, Lappin *et al*. (2011) determined that soluble stimulants of TLR 2 and TLR 4 were significantly higher in the saliva of patients with periodontitis in comparison to healthy controls. In the current study, *A. actinomycetemcomitans, E. coli, L. enzymogenes, F. nucleatum, P. aeruginosa, P. intermedia, P. multocida, P. oris, P. gingivalis S. aureus, S. sanguinus, T. denticola, T. forsythia* and *W. recta* showed the ability to stimulate activation of Toll-like receptors in human cell lines. Previous work by Sun *et al*. (2010) showed that *P. gingivalis, P. intermedia, F. nucleatum, P. gingivalis* LPS and *A. actinomycetemcomitans* increased expression of TLR 2 and TLR 4 genes and cytokine production in human periodontal ligament cells and these effects were blocked by addition of anti-TLR 2 and anti-TLR 4 antibodies.

In the horse, the levels of *A. actinomycetemcomitans* present in the plaque was seen to correlate positively with expression of TLR4 mRNA and negatively with IL-1ß and was also associated with increased severity of clinical disease, thus indicating that this species may act as a periodontal pathogen in the horse as it does in aggressive human periodontal disease where it is known to stimulate TLR 2 and TLR 4 (Sun *et al*. 2010). It is likely that the high immunogenicity of this pathogen results in an increase in inflammatory cells within the lesion and therefore increased mRNA expression of TLR 4. A positive correlation with IL-1ß could have been expected but this may have not been apparent due to the relatively small sample size. Further correlation analysis revealed that *P. intermedia* was strongly associated with increased TLR 2 expression in equine periodontitis. This appears to underline the importance of the *Prevotella* genus in equine periodontal disease with the data presented in previous chapters strongly indicating that *Prevotella* sp. are involved in disease pathogenesis in the horse.

Co-culture of HEK CD14-TLR2 cells with *P. gingivalis, A. actinomycetemcomitans, T. forsythia, P. intermedia, F. nucleatum* and *V. parvula* by Kikkert *et al*. (2007) revealed TLR 2 activation and subsequent cytokine production by all tested Gram-negative bacteria therby, suggesting a role as periodontal pathogens. However, culture of these species with HEK CD14-TLR4 cells revealed TLR 4 activation by *A. actinomycetemcomitans* and *V. parvula* only (Kikkert *et al*. 2007). In contrast with this, co-culture of *V. parvula* with THP-1X Blue cells did not result in a significant increase in TLR activation in the current study. The reason for this remains unknown, but may be due to strain-specific differences or to subtle differences in the reporter assay system used and/or possibly the presence (or absence) of human serum in the cell culture fluid rather than foetal calf serum. It is impossible to exclude other mechanisms which may be responsible for increased cytokine/chemokine production in response to *V. parvula,* because the study by Kikkert *et al*. (2007) used an indirect method for assessing NFκB activation (namely cytokine/chemokine levels) unlike in the present investigation where NFκB activity was detected by expression of a reporter gene.

MyD88def THP-1X Blue™ cells are deficient in MyD88, a molecule which is essential in TLR 2-and TLR 4-mediated responses. In the current study, when these cells were cultured with putative periodontal pathogens, production of SEAP was greatly reduced for all species of bacteria as well as for LPS, indicating that TLR 2 and/or TLR 4 play important roles in the host response to these periodontal pathogens. Although *A. actinomycetemcomitans, E. coli, F. nucleatum, L. enzymogenes, P. gingivalis, P. intermedia, P. multocida* and *P. oris* produced a significant increase in SEAP levels, *P. aeruginosa, S. aureus, S. sanguinus, T. denticola, T. forsythia, W. recta* and LPS failed to stimulate SEAP production in MYD88 deficient cells when compared with unstimulated control cells. This suggests that *A. actinomycetemcomitans, E. coli, F. nucleatum, L. enzymogenes, P. gingivalis*, *P. intermedia, P. multicida* and *P. oris* are capable of activating NFκB by a TLR-independent pathway. However, the results demonstrate that the major part of the response to these species is probably mediated via TLR 2 and/or TLR 4. Although NFκB may also be activated by TLR 5 ligand receptor interactions involving some of these bacteria, it is also a MYD88-dependent process (Paredes-Juarez *et al*. 2014). Whilst, the mechanism by

which these bacterial species activated NFκB remains unknown, it is very probable that residual MYD88 expression by the MYD88-deficient cells would account for this activity since the reduced expression of MYD88 is due to hygromycin-dependent expression of a plasmid designed to silence the *myd88* gene, i.e., knock down levels of MYD88 mRNA (Paredes-Juarez *et al*. 2014).

Following co-culture of HEK 293 Blue™ hTLR 2 cells with putative human periodontal pathogens, significant increases in SEAP production were seen for *A. actinomycetemcomitans, E. coli, F. nucleatum, L. enzymogenes, P. aeruginosa, P. gingivalis, P. gingivalis W50, P. intermedia, P. multicida, P. oris, S. aureus, S. sanguinus, T. denticola, T. forsythia* and *W. recta*. However, only *E. coli, W. recta, F. nucleatum* and *P. multicida* stimulated a significant increase in SEAP production when cultured with HEK 293 Blue™ hTLR 4 cells and production of SEAP in response to these pathogens was much lower than in the HEK 293 Blue™ hTLR 2 cell assay, indicating a lower level of TLR activation. Since *P. aeruginosa, S. aureus, S. sanguinus, T. denticola* and *T. forsythia* significantly increased SEAP production in THP-1X Blue™ cells and HEK 293 Blue™ hTLR 2 cells, suggesting TLR activation, but did not produce significant responses in HEK 293 Blue™ hTLR 4 cells this suggests that the innate immune response to these pathogens is mediated mainly by TLR 2.

LPS was unable to provoke significant increases in SEAP production when cultured with HEK 293 hTLR 2 cells and MyD88def THP-1X Blue™ cells but did significantly increase SEAP production when cultured with THP-1X Blue™ cells and HEK 293 hTLR 4 cells, indicating that the inflammatory response to LPS is mediated by TLR 4. *E. coli* LPS is well recognised as a ligand of TLR 4 (Chow 1999; Akira *et al*. 2006; Takeuchi and Akira 2010) and TLR 4-deficient mice have been found to be hypo-responsive to the presence of LPS (Hoshino *et al*. 1999). There are, however, inter- and intra-species differences in the immune response to LPS and significant inter-individual differences exist between horses (Werners *et al*. 2006). Although Werners *et al*. (2006) suggested that genetic polymorphisms in TLR 4, MD2 and CD14 may be responsible for differences in the response to LPS between horses and also discovered mutations in the equine TLR 4 gene, a link between these mutations and an altered response could not be proved. TLR 4 activation by LPS in a variety of equine tissues has been observed,

with an increase in TLR 4 transcription in equine blood leukocytes noted following infusion of endotoxin (Fossum *et al*. 2012). Although there have been no studies investigating the effect of LPS on equine gingival tissue, LPS purified from periodontal pathogens such as *A. actinomycetemcomitans* (Gutiérrez-Venegas *et al*. 2006) and *P. gingivalis* (Wang *et al*. 2000) have been shown to provoke TLR 4 activation in human periodontal fibroblasts; however, these pathogens failed to produce a significant response in HEK 293 hTLR 4/MD2 cells in the current study. A possible reason for this difference is that in the present study the cells were stimulated by intact microorganisms with a mixture of TLR activators, including LPS and lipoproteins. In addition to TLR 4 activation, LPS has also been shown to activate TLR 2 (Kirschning *et al*. 1998) and more recent studies have shown many LPS- expressing Gram-negative periodontopathogenic bacteria can activate TLR 2 efficiently, in addition to TLR 4 (Kikkert *et al*. 2007; Sun *et al*. 2010; Lappin *et al*. 2011). Expression of TLR2 has also been shown to be upregulated after LPS stimulation of TLR4 (Fan *et al*. 2003).

In the oral cavity, bacteria exist in a biofilm which is defined as a 'biopolymer matrix-enclosed bacterial population, adherent to each other and surfaces' (Costerton *et al*. 1999). Multispecies biofilms colonise all areas of the oral cavity and their composition varies with location. Tooth associated biofilms can be further sub-divided into supragingival biofilms and subgingival biofilms (Kolenbrander *et al*. 2010). Although it has been demonstrated in this chapter that the putative periodontal pathogens studied here are most capable of provoking a response mediated by TLR 2, only a small selection of species were chosen for this work. Over 700 species exist in the human mouth (and the work in this thesis suggest that over 2000 species exist in the equine oral cavity), with many species co-existing in a biofilm colonising the diseased periodontal pocket and so the character of the innate immune response produced will be dependent on the potential response to all of these pathogens and their associated molecules. Complex microbial communities can result in a complex host response and some species such as *P. gingivalis* are capable of altering this host response. It has previously been reported that by altering functional levels of effector molecules and receptors *P. gingivalis* is capable of subverting the host response which may subsequently reduce the response to the periodontal biofilm, allowing growth and development and promoting a chronic inflammatory

state (Hajishengallis *et al*. 2012a; 2012b). In the current study, *P. gingivalis* showed significant negative correlations to IL-10 and IL-1ß mRNA expression and so it is possible that this organism is also able to subvert the equine oral immune system since it would be expected that increases in these cytokines would have occurred. Peyyala and Ebersole (2013) reported that *P.gingivalis* biofilms significantly inhibited the production of IL1, IL-6, TGF, Gro-1, IL-8, Fractalkine, and IP-10 whereas *F. nucleatum* biofilms stimulated significant increases in IL-1, IL-6, IL-8, and IL-10. The composition of the biofilm is well known to affect the host response (Eberhard *et al*. 2009). Eberhard *et al*. (2008) also demonstrated that levels of IL-8 gene transcripts varied according to stage of biofilm formation. It has also recently been shown by that bacterial cell viability is improved when bacteria exist in a multi-species biofilm, in comparison with single species biofilms (Ramage *et al*. 2016). Mixed species biofilms comprised of *S. mitis , P. gingivalis, F. nucleatum* and *A. actinomycetemcomitans* also provoked a larger response from human oral keratinocytes than single species biofilms with higher mRNA levels of interleukin IL-8, CXCL3, CXCL1, IL-1, IL-6, colony-stimulating factor 2, and TNF-α produced following co-culture.

The putative pathogens studied in cell based assays in this chapter primarily stimulated a TLR 2 response and data presented in Chapter 6 revealing a 389-fold increase of TLR 2 mRNA expression in horses with periodontitis. Significant elevations of TLR 4 and TLR 9 mRNA levels in equine periodontitis were also detected, indicating the presence of their respective ligands in diseased equine gingival tissue. The innate immune response to oral bacteria and their products in periodontal disease is highly complex and additional work is required to further understand the aetiopathogenesis of the disease, especially in the horse which has received little attention in the past.

The current study has shown activation of TLRs, especially TLR 2 and TLR 4, by putative periodontal pathogens in human cell lines and although this information provides a useful insight into the aetiopathogenesis of periodontitis, a crucial next step would be the development of an equine-specific model. In order to ascertain whether these pathogens also provoke an immune response in equine tissue, an equine gingival or periodontal cell line could be established and/or used, such as the equine periodontal cell line cultured by Stasyk and Gasse

(2007). The results of such further work would provide insight into the potential role of these pathogens in the aetiopathogenesis of equine periodontal disease. It is important to note, however, that a variety of different cell types will interact with the periodontal biofilm in equine periodontitis and so a model involving multiple cell types would be of greater use.

Cross matching clinical, microbiological and immunological data from Chapters 3, 5 and 6 provided valuable data relating to the potential role of putative human periodontal pathogens in the horse. Due to the relatively small number of horses involved in the study, a larger cross-matched sample group would be of great benefit in order to further investigate the effect of putative pathogens. As studies presented in this chapter (in addition to the 16S rRNA gene sequencing, whole genome and RT-PCR studies presented previously in this thesis), have strongly indicated that bacteria belonging to the *Prevotella* genus are important in the aetiopathogenesis of periodontitis in the horse. It would be useful to further focus on correlations between the presence of species from this genus on clinical severity and TLR and cytokine expression. Since *A. actinomycetemcomitans* was associated with more severe disease in the horse, further studies into the potential role of this periodontal pathogen in the horse are required.

7.5 Conclusion

The results of the current study have shown that *A. actinomycetemcomitans, E. coli, L. enzymogenes, F. nucleatum, P. aeruginosa, P. intermedia, P. multoicida, P. oris, P. gingivalis, S. aureus, S. sanguinus, T. denticola, T. forsythia* and *W. recta* can provoke an immune response in human cell lines by activation of TLR 2 and/or TLR 4. TLR 2 appears to be predominantly activated by periodontal pathogens. In this study, the response of culturing LPS with human cell lines was mediated by TLR 4.

Correlation analysis indicated that *A. actinomycetemcomitans* and *P. intermedia* can provoke a significant immune response in diseased equine gingival tissue. In addition, *A. actinomycetemcomitans* was linked with cases of more severe periodontitis in the horse, further suggesting a potential role in the aetiopathogenesis of the disease in the horse.

This pilot study has identified several potential periodontal pathogens and an important next step would be the development of equine-specific TLR assays to determine the potential of these species to stimulate an immune response in equine periodontal tissues.

Chapter 8 General Discussion

Periodontal disease is increasingly being recognised as a common and painful condition in the horse which negatively affects equine welfare. Early recordings by Aristotle (Carmalt 2007) initially identified the disease and the clinical features and high prevalence of the condition, especially within the urban working horse population, were first documented in the early 1900s by Colyer (1906), Little (1913) and Harvey (1920).

Recent studies have documented the disease in up to 75% of horses (Baker 1970; Ireland *et al*. 2011) with prevalence increasing with advancing age. Du Toit *et al*. (2008b) and Rodriguez *et al*. (2013) also reported an increased prevalence in older donkeys. Work presented in Chapter 3 of this thesis is in agreement with these studies, finding a positive association between advancing age and presence of periodontitis and number of lesions. It is possible that due to the frequent sub-clinical presentation of equine periodontal disease that lesions are allowed to progress untreated and so older horses may present with a higher number of more advanced lesions. Prevalence and number of diastemata were found to increase with advancing age by Walker *et al*. (2012) and all the periodontal lesions in the current study were secondary to diastemata. As risk of dental disease increases in older horses it is intuitive that risk of secondary periodontitis will also increase. The relationship between age and periodontitis prevalence is also reported in humans (Eke *et al*. 2012) with increased attachment loss (Grosssi *et al*. 1994) and alveolar bone loss (Grossi *et al*. 1995) and it has been proposed that this could be due to increased length of exposure to risk factors such as periodontal pathogens (Borrell and Papapnou 2005). It is difficult, however, to compare the effect of age on the prevalence of periodontitis in brachydont and hypsodont species due to the likely different aetiologies involved.

Advancing age was also found to be significantly associated with increased tooth mobility and the number of periodontitis lesions. In the older hypsodont equine tooth, the periodontal ligament is more firmly attached due to densely packed collagen fibres (Staszyk *et al*. 2015), but the surface area of attachment is reduced in comparison to a comparable tooth in a younger animal. Destruction

of this lesser area of attachment despite the denser collagen fibres would therefore be more significant with age and it would be expected that increased tooth mobility would occur in older horses with periodontitis. Tooth mobility was also associated with the number of lesions and the periodontal pocket depth, which is to be expected.

A lack of relationship between advancing age and periodontal pocket depth was noted and this was unexpected due to the increased number of lesions in older horses and the progressive nature of the disease. One possible explanation for this is due to the presence of periodontal lesions of variable severity, with new lesions forming in aged horses.

The current study detected no sex or breed disposition to periodontitis in the horse. In contrast, previous work by Walker *et al*. (2012) noted mares had a significantly increased number of diastemata than male horses when considering affected animals but no sex predisposition to the presence of diastemata was described when the whole study population was considered. Walker *et al*. (2012) also reported a higher prevalence of diastemata in pony breeds in comparison with Thoroughbred crosses and cobs although Little (1913) suggested the condition was more prevalent in heavy horses than lighter breeds. This disparity may be due to the differences in the populations investigated in the two studies as Little (1913) mainly examined urban working horses which would have included few ponies. The higher prevalence in ponies is not surprising due to the facial conformation of smaller native breeds with dental overcrowding leading to disorders of maleruption and subsequent periodontitis. The population size in the current study involved a wide variety of breeds but relatively few animals per breed; future larger scale studies would be required to fully investigate breed predispositions to equine periodontitis.

There are few reports on the location of periodontitis lesions within the oral cavity of the horse but all of the periodontitis lesions in the current study were associated with diastemata, the majority of which were located in the interproximal spaces between the mandibular cheek teeth. This agrees with both Walker *et al*. (2012) who reported 83.5% of diastemata were mandibular and 16.5% were maxillary and du Toit *et al*. (2008) who reported 63.6% of

diastemata to be mandibular. Dixon *et al*. (2008a) previously found 50% of diastemata to occur caudally in the mandible at either Triadan 09/10 or 10/11 and the current study found the 08/09 position was mostly likely to be affected. Thorough oral examination with either a dental mirror or endoscope is essential to identify caudal mandibular diastemata in horses.

Although this condition has been described as one of the most painful oral diseases of the horse (Dixon *et al*. 2008a), clinical signs such as quidding and weight loss can be subtle or even absent meaning that the disease may go undetected by owners (Dixon *et al*. 2008a; 2014). Unfortunately, periodontitis in the horse can be frustratingly difficult to treat (Dixon *et a*l. 2014; Jackson *et al*. 2016). Jackson *et al*. (2016) compared four methods of treating periodontitis associated with diastemata, including, clearing periodontal pockets by removal of impacted feed and flushing with chlorhexidine, clearing pockets and placing metronidazole, clearing pockets and placing dental impression material in the diastema and clearing pockets and widening diastemata. All treatments significantly reduced pocket depth but no method proved significantly better than any other. There is often a poor response to treatment with Dixon *et al*. (2014) reporting a partial response to diastema widening in 17% of cases and no response in 4% of cases. A high recurrence of periodontitis following this treatment was also noted upon follow-up of cases treated a mean of 20 months previously with 22% of cases showing recurrence of the disease.

There are some similarities in the aetiopathogenesis of periodontal disease in brachydonts and hypsodonts although the initiating factor is different. In brachydonts, accumulation of plaque in the gingival sulcus provokes an inflammatory response in the periodontal tissue (gingivitis). This response to periodontal pathogens present in the biofilm may become destructive, resulting in attachment loss, with gingival recession and formation of periodontal pockets which supports the proliferation of anaerobes thereby resulting in further inflammation. The disease commonly occurs in hypsodonts following food trapping and decomposition secondary to anatomical defects between adjacent teeth causing abrasion, mechanical damage and gingival inflammation. Dixon *et al*. (1999, 2000) noted periodontitis in the absence of concurrent dental disorders and without the presence of plaque in just 3 (0.9%) of 349 cases.

Subsequent feed decomposition and fermentation is supportive of bacterial growth providing further inflammatory stimuli and resulting in gingivitis, periodontal tissue breakdown and formation of periodontal pockets which allow proliferation of periodontal pathogens. 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 periodontitis is shown in Figure 8.1

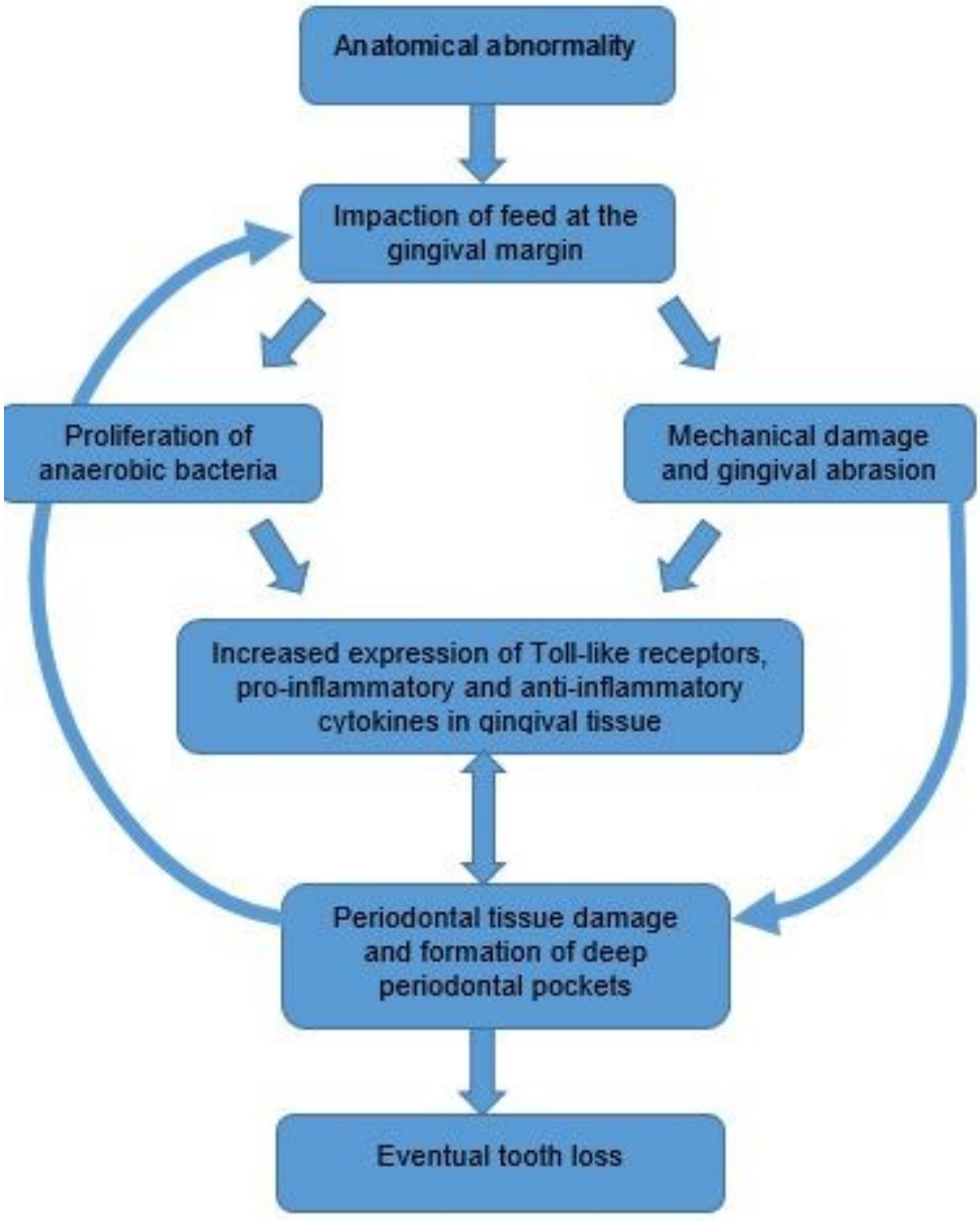


Figure 8.1 A proposed model for the aetiopathogenesis of equine periodontitis

Food packing was present in all periodontal pockets in the current study. Walker *et al.* (2012) reported feed packing in 94% of mandibular diastema. Approximately 90% of diastemata with associated periodontal disease were found to be packed with feed material by du Toit *et al.* (2008). Dixon *et al.* (2008a) reported recurrence of clinical signs associated with diastema and periodontitis when pasture fed horses were reintroduced to forage in the winter. However, it is unclear if this is due to increased feed trapping or closer owner observation. Future studies investigating the effect of diet on the presence or recurrence of periodontitis would be of much value in managing horses with periodontitis. Unfortunately, this was not possible in the current study due to lack of information for some horses and the large variety of feed materials in use.

In brachydonts, Gram negative bacteria and spirochaetes in particular (Holt and Ebsersole 2005) are well known to play an important role in the aetiopathogenesis of periodontitis, stimulating a destructive host inflammatory response which leads to degradation of periodontal tissues and loss of cementum and alveolar bone as observed in humans (Graves and Cohran 2003). The interaction between oral bacteria and the local innate immune system has been well studied in human periodontal disease but this had not previously been investigated in the horse and the equine oral microbiome had received relatively little attention in comparison with other species.

Previous work by Baker (1979) used culture-based methods to identify bacteria present in the healthy equine oral cavity and periodontitis lesions but more recently, several culture-independent methods have been used to investigate the equine oral microbiome, including high throughput bacterial 16S rRNA gene sequencing (Gao *et al.* 2016). A summary of bacterial genera and species previously detected in the equine oral cavity is shown in Chapter 1 (Table 1.3). The current study used a combination of culture dependent (Chapter 4) and culture independent (Chapter 5) methods to investigate the oral microbiome of the horse.

High throughput bacterial 16S rRNA gene sequencing revealed that the most prevalent genera present in the healthy oral cavity of the horse were *Proteobacteria* (39%), *Firmicutes* (51%) and *Bacteroidetes* (3.2%). Gao *et al*. (2016) also reported that these three phyla were the most prevalent in orally healthy horses with *Proteobacteria* accounting for 37.7% of reads, *Firmicutes* accounting for 27.6% of reads and *Bacteroidetes* accounting for 25.1% of reads. In the current study, the most abundant genera in equine oral health were *Gemella* (accounting for 36.5% of reads), *Pseudomonas* (14%), *Acinetobacter* (8%), *Fructobacillus* (8%), *Streptococcus* (6%) and *Actinobaccillus* (5%) with *Acinetobacter* and *Fusobacteria* being the genera most highly associated with equine oral health. The results of this study indicate that these genera comprise part of the normal oral flora of the horse.

The microbial profiles of equine oral health and equine periodontal disease were significantly different, with an 89% dissimilarity noted between the two sample groups. Whole genome sequencing was used in the current study to accurately identify bacteria at species level and gain additional information regarding microbial attributes and showed an increase in Gram negative, host-associated anaerobes in periodontitis samples which was to be expected. In total, the abundance of 190 OTUs was significantly different between oral health and periodontitis, with the majority (166) associated with periodontal disease and 24 OTUs associated with oral health. In periodontitis, the same three phyla as in oral health were still predominant but there was a notable increase in the proportion of *Bacteroidetes* (which increased to 20.5% of reads) while *Firmicutes* accounted for 26.1% of reads and *Proteobacteria* for 40% of reads. There was a large increase in *Prevotella* from 1.7% in oral health to 14% in periodontitis and the large increase in the abundance of *Bacteroidetes* was likely due to this. The most abundant genera in equine periodontitis were *Pseudomonas* (25%), *Prevotella* (14%), *Acinetobacter* (9.4%), *Streptococcus* (7%) and *Veillonella* (5%) with *Pseudomonas* and *Prevotella* being most significantly associated with equine periodontitis.

The results of several studies throughout this thesis have strongly suggested that some species of *Prevotella* can act as periodontal pathogens in the horse, with over 75 species of *Prevotella* detected in the equine oral cavity by whole

genome sequencing. In addition to being the second most discriminative genus for equine periodontitis and showing a large increase in abundance in periodontitis plaque samples, several *Prevotella* species which are known to be human periodontal pathogens were detected in equine periodontal pockets. Whole genome sequencing revealed that the number of reads corresponding to *Prevotella bivia, Prevotella dentalis, Prevotella denticola, Prevotella intermedia, Prevotella melaninogenica,* and *Prevotella nigrescens* were significantly increased in periodontitis. In addition, no reads corresponding to *P. bivia, P. dentalis, P. denticola, P. melaninogenica, P. nigrescens* were detected in oral health with these species only being detected in periodontitis samples. Significantly higher CFU equivalent counts of *P. intermedia* were also detected in periodontitis samples by QPCR and the abundance of this species increased from 0.01% at a healthy site, to 0.06% at the diseased gingival margin and to 0.9% deep within the periodontal pocket. *Prevotella* sp. were the most abundant genus at the diseased gingival margin and the second most abundant in the diseased periodontal pocket.

Gao *et al*. (2016) reported that 17.5% of reads corresponded to *Prevotella* species with unclassified species of *Prevotella* accounting for 33.5% of the total oral microbiota of one horse. 16S rRNA gene sequencing has revealed an association of *P. denticola* and other *Prevotella* oral taxons with chronic periodontitis in humans (Griffen *et al*. 2012; Abusleme *et al*. 2013) and species from this genus are frequently detected in subgingival plaque from humans (Dewhirst *et al*. 2010). Previous studies have reported involvement of *P. intermedia* and *P. melaninogenica* in human periodontitis (Haffajee and Socransky 1994; Griffen *et al*. 2012; Abusleme *et al*. 2013). Interestingly, *Prevotella* sp. were the most commonly isolated genus from equine endodontic and apical infections (Biernert *et al*. 2003) and bacteria from this genus were also successfully cultured from equine dental extractions. It is possible that there is also involvement of *Prevotella* sp. in the pathogenesis of equine apical infections (Kern *et al*. 2016).

In the correlation analysis performed in Chapter 7, the presence of *P. intermedia* was seen to significantly positively correlate with increased expression of TLR 2 mRNA in equine gingival tissue and when co-cultured with

THP-1X, MyD88 THP-1X Blue™ cells and HEK 293 Blue™ hTLR 2 human cell lines this species produced a significant innate immune response as seen by production of SEAP. Significant stimulation of TLR 2 was noted following co-culture of *P. intermedia* with HEK 293 Blue™ hTLR 2. *P. intermedia* was shown to be capable of TLR activation when cultured with THP-1X cells and also produced a significant but diminished response when cultured with MyD88 THP-1X BlueTM cells, indicating that while the innate immune response to *P. intermedia* is primarily mediated by TLR 2, activation of a TLR-independent pathway also occurs. *P. intermedia* and *P. nigrescens* have been shown to stimulate cytokine production by activation of TLR 2 (Kikkert *et al*. 2009). Sun *et al*. (2010) also reported increased expression of TLR 2 and TLR 4 in human periodontal ligament cells when challenged with *P. intermedia*. The presence of *P. intermedia* positively correlates with TLR 2 mRNA expression in the horse and it is therefore highly likely this species plays an important role in the aetiopathogenesis of equine periodontal disease.

Many previously uncultured species of *Prevotella* were also identified in the current study. This is not surprising considering the previous lack of research into the equine oral microbiome in health and periodontitis. *Prevotella dentasini,* was recently described by Takada *et al*. (2013) for the first time in the oral cavity of donkeys and was significantly associated with periodontitis in the current study. Many of these species are likely to be equine specific and could potentially play a role in the aetiopathogenesis of equine periodontal disease. The presence of *Prevotella* in the equine oral cavity and the potential periodontal pathogenicity of this genus in the horse strongly warrants further investigation.

In addition to *Prevotella* species, several known periodontal pathogens belonging to other genera were identified in equine periodontal disease. Spirochaetes have long been associated with periodontitis in man and the so called 'red-complex' bacteria *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* have been associated with severe periodontal lesions and deep periodontal pockets (Holt and Ebsersole 2005). Cox *et al*. (2012) recently identified spirochaetes in equine periodontal pockets. Equine dental anatomy results in the formation of deep periodontal pockets with the deepest mean

pocket depth recorded as being over 24mm in one horse (Chapter 3). It is therefore intuitive that 'red-complex' bacteria may be present in equine periodontitis and Sykora *et al.* (2014) reported that these bacteria were more commonly present in the gingival crevicular fluid of horses with clinically overt EOTRH than in orally health horses.

The abundance of *P. gingivalis* was noted to significantly increase in equine periodontal pockets when detected by QPCR although no significant change was noted in the samples analysed by whole genome sequencing. Although *P. gingivalis* was found to stimulate TLR activation in human cell lines, the presence of this pathogen was found to be negatively associated with the expression of IL-1β and IL-10 mRNA in the horse. The ability of *P. gingivalis* to subvert the host immune response and thereby subsequently reducing the response to the periodontal biofilm and promoting a chronic inflammatory state has been well studied in man (Hajishengallis *et al*. 2012a; 2012b). The lack of association between *P. gingivalis* and pro and anti-inflammatory cytokines in equine periodontal pockets could either indicate that this is also the case in the horse or that this species does not act as an equine periodontal pathogen. Further, larger scale studies would be required to investigate the potential role of this organism in equine periodontitis however it might be suggested that it is much more relevant in human periodontal disease than in equine periodontal disease.

Species from the genera *Tannerella* and *Treponema* were found to be present at similarly low abundances in both healthy and diseased samples. *Tannerella* species were also noted at low levels in the healthy equine oral cavity by Gao *et al*. (2016). *T. forsythia* has previously been detected in both orally healthy equine samples (Sykora *et al*. 2014; Gao *et al*. 2016) and in samples from horses with EOTRH (Sykora *et al*. 2014). The presence of *T. forsythia* was found to significantly increase in periodontitis in comparison to oral health when detected by QPCR in the current study. In addition, reads corresponding to *Treponema socranskii* and *Treponema vincentii* were only detected in periodontitis samples following whole genome sequencing.

T. denticola and T. forsythia were capable of activating TLRs in human cell lines (Chapter 7) and it was shown that this response was mediated through TLR 2. However, correlation analysis showed no relationship between TLR 2 mRNA expression in the horse and the presence of these pathogens. Expression of IL-10 mRNA did, however, correlate negatively with the presence of *T. forsythia*. IL-10 expression is thought to play a protective role in human periodontitis (Bozkurt *et al*. 2006), and a lack of expression results in increased alveolar bone loss in mice (Sasaki *et. al* 2004). However, a relationship between IL-10 expression or *T. forsthyia* and clinical severity was not demonstrated in the horse, and so one might be tempted to infer this species is not directly involved in the aetiopathogenesis of periodontal disease in the equid, despite its presence being noted in horses with EOTRH (Sykora *et al*. 2014). A *Tannerella* phylotype (BO045) previously associated with human periodontal health (Leys et al 2002) was noted to be present in the equine oral cavity (Gao *et al*. 2016). The results of the current study suggest that the 'red complex' pathogens classically associated with periodontitis in man may not play such an important role in the aetiopathogenesis of equine periodontal disease.

Veillonella species accounted for 0.3% of reads derived from HT 16S rRNA gene sequencing in oral health and this increased to 5% of reads in periodontitis. Reads corresponding to *Veillonella dispar* were only detected in periodontitis samples using whole genome sequencing and this species was seen to significantly increase in diseased samples when detected by Q-PCR, although there was no association between the presence of this pathogen and disease severity or expression of TLR 2, TLR 4, IL-1B or IL-10 mRNA, casting doubt that it is directly involved in equine periodontitis; however further studies would be required to explore this more fully. *V. parvula* has been significantly associated with chronic periodontitis in humans (Mashima *et al*. 2015) and this species has also been shown to stimulate cytokine production by activation of both TLR 2 and TLR 4 (Kikkert *et al*. 2007). This species was also detected in equine samples but no significant difference between orally healthy and diseased samples was observed.

Aggregatibacter actinomycetemcomitans is associated with aggressive periodontitis in humans where it has been shown to stimulate TLR 2 and TLR 4

expression (Sun *et al*. 2010) and this species was also detected in equine oral health and periodontitis. There is no previous description of the presence of this species in the equine oral cavity. There was no significant increase in abundance of this species between healthy and diseased animals detected by either Q-PCR or whole genome sequencing but correlation analysis revealed that the presence of the organism was significantly associated with increased expression of TLR 4 and increased severity of disease in the horse. *A. actinomycetemcomitans* was also shown to stimulate expression of TLR 2 in human cell lines but could also utilise a TLR independent pathway due to increased detection of SEAP when co-cultured with MyD88def THP-1 cells. Kato *et al*. (2013) also demonstrated the ability of *A. actinomycetemcomitans* to stimulate increases in TLR 2 mRNA expression and found that phagocytosis and apoptosis in *A. actinomycetemcomitans*-infected THP-1 cells were inhibited by the addition of an anti-TLR2 antibody, highlighting the importance of this receptor in the innate immune response to this pathogen.

It has been estimated that approximately 70% of the human oral microbiome cannot be cultured (Dewhirst et al. 2010) and one can safely assume a similar situation will exist in the horse. It is likely that an even larger proportion of bacteria present in periodontitis lesions will be non-cultivable due to the involvement of fastidious anaerobes and spirochaetes in the disease. Both culture-dependent and culture-independent methods were used in the current study. Culture of orally healthy and equine periodontitis samples showed several similarities to data derived from culture-independent studies including the presence of potential periodontal pathogens *A. actinomycetemcommitans, P. bivia* and *P. dentalis* in diseased samples. Several isolates represented were previously of uncultured bacteria or did not produce any matches with known species in the public access databases. This phenomenon is unsurprising due to the very few equine oral microbiology studies conducted previously. The limitations of the culture-dependent approach was clearly seen in this study since a much smaller number of genera were detected by this approach in comparison with high-throughput 16S rRNA gene sequencing and whole genome sequencing. Only three species of *Prevotella* were detected using culture based methods in comparison with 75 species detected by whole genome sequencing. High-throughput 16S rRNA sequencing allowed detailed investigation of the

equine oral microbiome in both health and periodontitis, building a more detailed picture of the oral microflora of the horse from phylum to genus level. It is acknowledged that this method is generally unable to discriminate well at species level (Woo *et al*. 2009) but the use of whole genome sequencing allowed investigation of the equine oral microbiome at this level and permitted the identification of putative periodontal pathogens. By using a combination of these identification methods, an assessment and comparison of the bacteria present in equine oral health and periodontal disease could be made for the first time.

The deep periodontal pockets seen in equidae constitute a new niche in an oral ecosystem that will select for a different and potentially limited, microbiome. Alterations in pH, nutrient availability and, more specifically, oxygen tension may limit the number of species able to survive the periodontal pocket, with deep pockets having significantly lower oxygen tension than even pockets of moderate depth (Loesche *et al*. 1983). Environmental differences present between the healthy gingival sulcus and diseased periodontal pockets may be particularly striking in the horse, as equine dental anatomy allows for the formation of particularly deep periodontal pockets which can measure over 15 mm in severe cases (Cox *et al*. 2012.) It is possible that during disease progression, the environmental changes occurring as a shallow gingival sulcus becomes a deep periodontal pocket allows a new group of bacteria to flourish whilst providing a less optimal environment for the growth of others.

The current study demonstrated that distinct microbial populations exist at the healthy gingival margin, the diseased gingival margin and deep within the periodontal pockets of a small number of horses. Anaerobic organisms such as *Prevotella* sp. and *Veillonella* sp. predominated at the diseased gingival margin and within periodontal pocket whereas aerobic *Streptococcus* (29%) and *Gemella* (15%) predominated at the orally healthy sites. This is to be expected given the growth requirements of these organisms. The abundance of *P. intermedia* increased at both the diseased gingival margin and again within the periodontal pocket. Although differing populations were found in each environment, it is difficult in a study of this size and type to comment on whether a disturbance in the microflora (dysbiosis) occurred first, predisposing to periodontitis or if the condition developed due to an external cause such as formation of diastema and

subsequent decomposition of impacted feed material, which is more likely in the horse. Longitudinal studies starting with young healthy horses, and following-up their periodontal status and microflora of the oral cavity until the development of periodontal disease, would be useful in future in order to monitor disease development and the presence of a dysbiosis.

Periodontopathogenic bacteria in the dental biofilm stimulate an inflammatory response in the periodontal tissues (Graves and Cochran 2003; Kikkert *et al*. 2007; Sun *et al*. 2010). This response is often severe and can be destructive to host tissues, resulting in loss of periodontal ligament attachment and alveolar bone. By examining the immune response to potential periodontal pathogens, it may be possible to ascertain which species may be important in the aetiopathogenesis of the condition and which simply increase in abundance due to the formation of a more suitable environment within the periodontal pocket. By correlating clinical, microbiological and immunological data it has been possible to identify *P. intermedia* and *A. actinomycetemcomitans* as potentially important periodontal pathogens in the horse due to their association with either increased TLR mRNA expression or clinical severity. Unfortunately, only a relatively small number of samples were available for use in the correlation studies and a larger scale study is suggested before a more firm conclusion could be drawn from this type of analysis. The co-culture of putative periodontal pathogens such as *A. actinomycetemcomitans, P. aeruginosa, P. intermedia, P. gingivalis, T. forsythia* and *V. parvula* with human cell lines showed the ability of these species to stimulate activation of TLRs, particularly TLR 2 which showed significant increases in mRNA expression in diseased equine gingival tissue. An important further step which was outwith the scope of this study would be to design equine specific TLR assays to assess the ability of potential pathogens identified in this study to stimulate an immune response in equine periodontal tissues.

Cekici *et al*. (2014) recently highlighted the importance of innate immunity in the aetiopathogenesis of human periodontal disease whilst Cox *et al*. (2012) reported marked infiltration of inflammatory cells into diseased periodontal tissue in the horse. There was a significant innate immune response produced in diseased equine gingival tissue with increased expression of TLR 2, TLR 4 and

TLR 9 mRNA observed in an age-weighted analysis in comparison to equine oral health. TLR 2 showed a 389-fold increase in diseased periodontal tissue in the horse and the innate immune response to periodontal disease appears to be mainly mediated by TLR 2 with increases in TLR 2 mRNA expression most highly significantly associated with disease. Previous studies have described increased expression of TLR 2 in human periodontitis patients (Sarah *et al*. 2006) with activation of this receptor being associated with alveolar bone loss (Myneni *et al*. 2011; Matsumoto *et* al. 2012). Comparison of diseased gingival tissue with healthy gingival tissue from the same animal also showed significant increases in TLR 2, TLR 4, TLR 9 mRNA expression. Increased expression of TLR mRNA is likely due to binding of the TLR to its ligands, which in periodontitis will mostly be derived from bacteria (Weiss *et al*. 2004)

In other species, recognition of periodontopathogenic species by TLRs in gingival and periodontal tissue initiates cytokine production, often causing an inflammatory response and thereby facilitating disease progression (Garlet 2010). The results of the current study suggest a similar aetiopathogenesis in the horse although it is possible that TLR stimulation may also result from the presence of fungi present entrapped within feed material in periodontal pockets. In the horse, TLR 2 seems to be particularly involved in the innate immune response to periodontal disease and this receptor will also respond to the binding of fungal molecules such as glucuronoxylomannan and phospholipomannan (Akira *et al*. 2006). Endogenous ligands such as fibrinogen can stimulate TLR 4 (Akira *et al* 2006) and this may also contribute to the innate immune response in damaged gingival tissue.

A mixed Th1/Th2 response was noted with increased expression of both pro-inflammatory cytokines which contribute to periodontal tissue destruction and anti-inflammatory cytokines which have a protective role in gingival tissue. Elevated expression of IL-4, IL-10, IL-12, IFN-γ and TNF-α mRNA was noted in diseased gingival tissue following an age-weighted analysis. Comparison of diseased gingival tissue with healthy gingival tissue from the same animal showed significant increases in IL-1ß, IL-10, IL-12 and IFN-γ mRNA expression in equine periodontitis. The increase in mRNA expression of these cytokines results from increased stimulation of TLR 2, TLR4 and TLR 9 following binding of their

respective ligands. Statistically significant positive correlations existed between disease severity and expression of IL-1ß, IL-6, IL-10, IL-12, IL-17 and TNF-α.

Stimulation of Th1 cytokines, such as IL-1ß and TNF-α, in response to periodontopathogenic bacteria leads to periodontal tissue destruction and loss of alveolar bone (Graves and Cochran 2003). A positive correlation between expression of IL-1ß and severity of periodontal disease was noted in the horse which is in agreement with observations in human studies (Sanchez *et al*. 2013; Oh *et al*. 2015). Liu *et al*. (1996) reported that IL-1ß plays a pivotal role in human periodontal disease, enhancing alveolar bone resorption and inducing synthesis of matrix metalloproteinases which may lead to further tissue destruction. In contrast, stimulation of Th2 cytokines such as IL-10 by periodontopathogenic bacteria produces an anti-inflammatory effect, protecting gingival tissue and reducing alveolar bone loss (Bozkurt *et al*. 2006). IL-10 mRNA expression also showed a positive correlation with disease severity. Lappin *et al*. (2001) reported that IL-10 producing cells are widely distributed in periodontitis granulation tissue and it is possible that a large area of longstanding granulation tissue is present in chronic equine periodontitis lesions. A truly mixed cytokine response was noted in the horse which may be a reflection of the chronicity of the equine condition.

The work presented in this thesis contributes important information to the current knowledge on the aetiopathogenesis of periodontal disease in the horse. The use of both culture dependent and culture independent methods to investigate the equine oral microbiome has provided significant breadth and depth of information on the microbiology of equine periodontal disease. The innate immune response produced in periodontally diseased equine gingival tissue has been characterised for the very first time in the horse. The link between the microbiological and immunological aspects of equine periodontal disease has identified potential equine periodontal pathogens and forms a basis for future studies on this important disease of the horse.

Future studies may include *in vitro* culture of equine gingival epithelial cells in order to investigate the initial innate immune response to the potential pathogens identified in this thesis either in isolation or in combination as a

biofilm which would provide further equine-specific insight into the aetiopathogenesis of the condition. In addition, there is potential for a 3D *in vitro* model of the equine gingival mucosa to be developed using stroma cells from *post-mortem* gingival biopsies. Consisting of fibroblasts within a collagen matrix and a superficial epithelial cell monolayer which could be induced to form stratified epithelium at the air/liquid interface, the models could be used in co-culture studies with putative pathogen biofilms and to study the effects of potential novel antimicrobial and anti-inflammatory therapeutics. Development of such a model could give a better understanding of the disease aetiopathogenesis and could aid in the development and testing of novel therapeutics which are needed in order to treat this frustrating condition.

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Appendix

Scientific papers published from this thesis

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VETERINARY RESEARCH

RESEARCH ARTICLE

Open Access

The microbiome associated with equine periodontitis and oral health

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Abstract

Equine periodontal disease is a common and painful condition and its severe form, periodontitis, can lead to tooth loss. Its aetiopathogenesis remains poorly understood despite recent increased awareness of this disorder amongst the veterinary profession. Bacteria have been found to be causative agents of the disease in other species, but current understanding of their role in equine periodontitis is extremely limited. The aim of this study was to use high-throughput sequencing to identify the microbiome associated with equine periodontitis and oral health. Subgingival plaque samples from 24 horses with periodontitis and gingival swabs from 24 orally healthy horses were collected. DNA was extracted from samples, the V3–V4 region of the bacterial 16S rRNA gene amplified by PCR and amplicons sequenced using Illumina MiSeq. Data processing was conducted using USEARCH and QIIME. Diversity analyses were performed with PAST v3.02. Linear discriminant analysis effect size (LEfSe) was used to determine differences between the groups. In total, 1308 OTUs were identified and classified into 356 genera or higher taxa. Microbial profiles at health differed significantly from periodontitis, both in their composition ($p < 0.0001$, $F = 12.24$; PERMANOVA) and in microbial diversity ($p < 0.001$; Mann–Whitney test). Samples from healthy horses were less diverse (1.78, SD 0.74; Shannon diversity index) and were dominated by the genera *Gemella* and *Actinobacillus*, while the periodontitis group samples showed higher diversity (3.16, SD 0.98) and were dominated by the genera *Prevotella* and *Veillonella*. It is concluded that the microbiomes associated with equine oral health and periodontitis are distinct, with the latter displaying greater microbial diversity.

Introduction

Periodontal disease has long been recognised as a common and painful equine oral disorder and its substantial welfare impact was acknowledged at the start of the twentieth century being described as "the scourge of the horse's mouth" [1, 2]. More recently, studies have shown the presence of periodontitis in up to 75% of horses [3, 4] with prevalence increasing with advancing age. A dental survey noted that classical (i.e. plaque-induced) periodontal disease was rare in horses, but periodontal disease induced by food impaction due to abnormal spacing between the cheek teeth was common [5]. The condition is often associated with the presence of cheek teeth diastemata [6] and can also be present secondary to other oral disorders such as supernumerary, displaced or rotated teeth [7]. Dropping of feed (quidding) and difficulty eating are the main clinical signs [8], although these can be subtle and easily overlooked. More recent clinical studies have reinforced the importance of equine periodontitis, currently recognised as a common and very painful equine dental disease [6, 8]. Two forms of periodontal disease exist, namely gingivitis and periodontitis. Gingivitis is completely reversible and is recognised by the classic signs of bleeding, inflammation, redness and swelling of the gums. Periodontitis attacks the deeper structures that support the teeth, damaging the surrounding bone and periodontal ligament, resulting in tooth loss. Despite the importance of this condition there have been few recent studies into its aetiopathogenesis.

Bacteria have been shown to be the causative agents in feline, canine and human periodontal disease and so it is highly likely they play a crucial role in the pathogenesis of

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the equine condition. Involvement of bacteria in equine periodontal disease was recently acknowledged [9, 10]. However, understanding of the equine oral microbiome is limited and merits further study and little is known about the role bacteria play in equine periodontitis [9]. Studies in other species have estimated that around 50% of oral bacteria cannot be cultured by conventional approaches due to nutritional and fastidious growth requirements [11] and thus the number and variety of bacterial species present in the oral microbiome has been greatly underestimated to date.

It is now possible to determine almost the entire community of bacteria, both commensal and pathogenic, that inhabit the equine oral cavity, in both health and periodontitis using culture-independent methods. To date, the majority of approaches have used Sanger sequencing to determine bacterial 16S rRNA gene sequences. This approach allows detection not only of cultivable species but also of fastidious bacteria that may be uncultivable, and also of novel species that may be important in the pathogenesis of disease. This method has already been used to determine the bacterial species present in canine [12] and ovine [13] periodontal disease lesions.

The aim of this study was to determine the microbial profiles associated with the healthy equine oral cavity and equine periodontitis using high-throughput sequencing of the bacterial 16S rRNA gene. This approach provides far greater depth, coverage, accuracy and sensitivity than that offered by Sanger sequencing in assessing the composition of complex microbial communities, uncovering microbial diversities that are orders of magnitude higher and with considerably less bias [14].

Materials and methods

Sample classification

Ethical approval was granted prior to the start of the study by the University of Glasgow School of Veterinary Medicine Ethics and Research Committee and by the University of Edinburgh Veterinary Ethical Review Committee. All horses involved in the study presented either to the Weipers Centre Equine Hospital, University of Glasgow or the Royal (Dick) School of Veterinary Studies, University of Edinburgh for routine dental examination, investigation of dental disease or had been humanely euthanatised for reasons unrelated to the oral cavity and sent for post-mortem examination. Following a thorough oral examination horses were categorised as either "orally healthy" or "periodontitis" and placed into two groups accordingly. The orally healthy group had no evidence of gingival inflammation, no periodontal pockets and no evidence of any other oral pathology. The "periodontitis" group had obvious gingival inflammation and periodontal pockets of over 5 mm in depth. No antimicrobial drugs had been given in the previous 8 weeks to any horse involved in the study.

Sample collection

Once food debris was removed, an equine dental curette was used to collect subgingival plaque samples from a single periodontal pocket (depth greater than 5 mm) of 24 horses with clinical periodontitis and placed into 0.5 mL fastidious anaerobe broth (FAB). A swab of the gingival margin with sufficient pressure to also collect material from the gingival crevice on the buccal aspect of cheek teeth 307–308 (Modified Triadan Numbering System) was taken from 24 orally healthy horses using an Amies Transport Swab (VWR International, Lutterworth, UK). One periodontitis affected sample was lost for further sample processing, resulting in 23 samples from periodontitis cases and 24 samples from healthy horses being available for analysis. Post-mortem samples were collected within 1 hour of euthanasia.

Sample processing and DNA extraction

Supragingival and subgingival plaque samples were each vortex mixed for 30 s and Amies transport swabs were immersed in 0.5 mL FAB and mixed to remove bacteria. A crude DNA extract was prepared from each sample by digestion with proteinase K (100 µg/mL) at 60 °C for 60 min, followed by boiling for 10 min. Further DNA purification was conducted using a bead beating technique where 150 µL of each sample was mixed with 200 µL phenol saturated with Tris–HCl (pH 8.0), 250 µL glass beads (0.1 mm) suspended in TE buffer and 200 µL lysis buffer. Samples were then placed in a BioSpec Mini-Beadbeater for 2 min at 2100 oscillations/min and DNA extracted with the AGOWA mag Mini DNA Isolation Kit (AGOWA, Berlin, Germany).

High-throughput sequencing

For each sample, the V3–V4 region (which gives optimal taxonomic coverage and taxonomic resolution) of the bacterial 16S rRNA gene was generated by PCR with primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTWTCTAAT). Primers contained Illumina adapters and a unique 8-nucleotide sample index sequence key [15]. Amplicon libraries were pooled in equimolar amounts and purified using the Illustra™ GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare, Eindhoven, The Netherlands). Amplicon quality and size was analysed on an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). Paired-end sequencing of amplicons was conducted on the Illumina MiSeq platform using the v3 kit generating 2 × 301 nucleotide reads (Illumina, San Diego, USA).

Analysis of sequencing data

Sequencing reads were merged [16], processed and clustered with USEARCH version 8.0.1623 [17]. After merging (minimum and maximum merged length, 380 and 438, respectively), the sequences were quality filtered (max. expected error rate 0.002, no ambiguous bases allowed) and clustered into operational taxonomic units (OTUs) using the following settings: -uparse_maxdball 1500, only de novo chimera checking, usearch_global with -maxaccepts 8 -maxrejects 64 -maxhits 1. QIIME version 1.8.0 [18] was used to select the most abundant sequence of each OTU and assigned a taxonomy using the RDP classifier [19] with a minimum confidence of 0.8 and the 97% representative sequence set based on the SILVA rRNA database, release 119 for QIIME [20]. Attributes such as oxygen utilisation, Gram stain and shape were assigned at genus level as previously described [21].

Statistical analysis

In order to normalise the sequencing depth, the dataset was randomly sub-sampled to 16 000 reads per sample. Diversity analysis (Shannon Diversity Index, Chao-1 estimate of total species richness), data ordination by principal component analysis (PCA) and assessment of differences between microbial profiles of the two groups by one-way PERMANOVA were performed using PAleontological STatistics (PAST; v3.02) software [22]. PERMANOVA was used with Bray–Curtis similarity distance. For PCA, the OTU dataset was additionally normalized by log2-transformation. The difference in diversity of the genera detected in both health and disease was compared and analysed statistically using the Mann–Whitney U test in SPSS (version 21.0). To determine which OTUs and taxa contribute to differences between the groups, linear discriminant analysis effect size (LEfSe) [23] was used.

Results

Sample demographics

The majority (16 of 24; 66.7%) of the periodontitis samples originated from the Royal (Dick) School of Veterinary Studies, University of Edinburgh, three (12.5%) originated from the Weipers Centre Equine Hospital, University of Glasgow and five (20.8%) were post-mortem samples. The mean age of sampled horses with periodontitis was 13.2 years (range 3–27 years); 13 (54%) of these horses were mares and 11 (46%) were geldings. Of the 24 orally healthy horses sampled, 20 (83.3%) were collected at the Weipers Centre Equine Hospital, University of Glasgow, two (8.3%) at the Royal (Dick) School of Veterinary Studies, University of Edinburgh and two (8.3%) were post-mortem samples. The average age of this group was 11.7 years (range 4–27 years); 16 (66.7%) of horses were geldings and eight (33.3%) were mares. Of all mares included in the study, 52% had periodontitis and 40% of all geldings had periodontitis. There was however no statistically significant difference between healthy and periodontitis affected horses by gender ($p = 0.383$; Chi square test) or by age ($p = 0.242$; Mann–Whitney test).

A diverse range of breeds were sampled, although 19 of 48 (39.6%) were native ponies: Welsh Cob ($n = 6$), Welsh Pony ($n = 4$), Dartmoor Pony ($n = 1$), Shetland Pony ($n = 2$), Connemara Pony ($n = 2$), Exmoor Pony ($n = 2$), Highland Pony ($n = 1$), Fell Pony ($n = 1$). Eleven of 48 horses (22.9%) were Cobs or Cob crossbreeds and four horses (8.3%) were Thoroughbred (TB) or TB crossbreeds. Icelandic horses accounted for three (6.3%) of the samples. The remaining 11 (22.9%) horses were of a variety of breeds: Arabian ($n = 3$), Irish Sports Horse ($n = 3$), Gelderlander ($n = 1$), Trakehner ($n = 1$), Warmblood ($n = 2$), Irish Draft ($n = 1$). No significant difference was observed between breed and the presence of periodontitis.

Sequencing output

Sequencing generated a total of 4 170 177 reads. After quality processing the OTU table contained 1 342 927 reads that were clustered in 1334 OTUs. The number of reads per sample ranged from 16 272 to 49 685 (median 27 855, mean 28 573, SD 7943). After subsampling at equal depth of 16 000 reads/sample, 1308 OTUs remained in the dataset that was used for the further analyses.

Microbial profile analyses

Principal component analysis revealed clear differences between the equine oral microbiomes in oral health and periodontitis (Figure 1). Healthy samples clustered together and showed lower variability compared to periodontitis samples. The difference between microbial profiles of the two groups was statistically significant ($p < 0.0001$, $F = 12.24$, PERMANOVA). Microbial profiles from healthy horses were statistically significantly less diverse ($p < 0.001$, Mann–Whitney test), both by actual species richness (number of OTUs) (Figure 2A) as well as by estimated species richness or Chao-1 (Figure 2B) and Shannon Diversity Index (Figure 2C). On average, samples from healthy horses harboured 161 OTUs (SD 116, range 64–568), while samples from periodontitis affected horses contained 252 OTUs (SD 81, range 85–380).

Compositional differences between the groups

Linear discriminant analysis (LDA) effect size (LEfSe) was used to assess the differences between the two

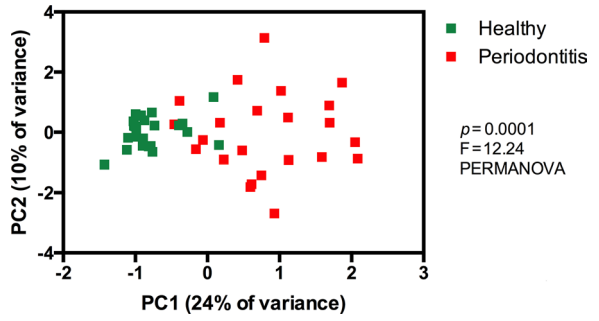

Figure 1 Two-dimensional ordination of equine microbial profiles at oral health and periodontal disease by principal component analysis (PCA). The OTU data was subsampled at 16 000 reads/sample and log2-transformed before the PCA.

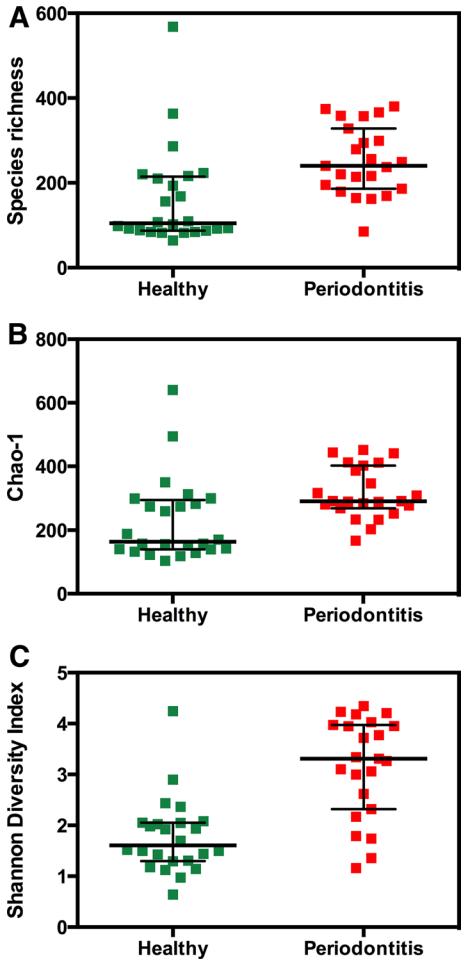

Figure 2 Diversity analysis on equine microbial profiles at health and periodontal disease. A Observed species richness or number of OTUs/sample; **B** estimated species richness or Chao-1 and **C** Shannon diversity index. Healthy microbiomes were statistically significantly less diverse than microbiomes with periodontal disease ($p < 0.001$, Mann–Whitney test).

groups of samples both at the OTU level and at the genus or higher taxonomic level. From all 1308 OTUs, 266 OTUs were statistically significantly different between the healthy and periodontitis groups ($p < 0.05$, LDA > 2). Of these, 64 OTUs had an absolute LDA score above 3 (Additional file 1), the majority of which (51 of 64 OTUs) were associated with disease.

At the genus level, from 356 genera or higher taxa, 107 taxa were statistically significantly different between the two groups ($p < 0.05$). Of these, 69 taxa had LDA scores above 3 and, again, the majority (52 of 69 taxa) were associated with disease (Figure 3). The most discriminative genera between health and disease were *Gemella* and *Actinobacillus* in health and *Prevotella* and *Veillonella* in periodontitis, respectively (Figure 4).

From 179 entries at the family level, 51 were significantly different between health and disease ($p < 0.05$) (Figure 5). The majority (N = 38) of these were associated with disease, while only 13 microbial families were positively associated with health (Additional file 2). Interestingly, periodontitis samples had significantly higher relative abundance of Methanobacteriaceae ($p = 0.0001$) and Thermoplasmatales ($p < 0.0001$) (both families belong to the domain Archaea).

With regard to inferred Gram stain and shape, strongly significant differences were observed between healthy and diseased samples (**$p < 0.0001$, *$p < 0.05$, Mann–Whitney test; data not shown).

Discussion

Despite the difficulty in permanently resolving equine periodontitis, its high prevalence and substantial effect on welfare, few original research studies on its aetiopathogenesis have been published. In humans, the disease is known to be multifactorial and although bacteria play a major role in the aetiopathogenesis of periodontitis in other species, their role in equine periodontitis has only recently received investigation [9]. Few studies have investigated the oral microbiome of the horse in oral health or disease. Recently, the microbiome of the equine gingival sulcus was investigated by pyrosequencing pooled samples from 200 sulcus sites in two orally healthy horses [24]. Twelve phyla were identified, the most prevalent being Gammaproteobacteria (28.8%), Firmicutes (27.6%) and Bacteroidetes (25.1%). The study suggested that there are many similarities between the equine subgingival microbiota and the subgingival microbiota detected in human, feline and canine studies. Putative periodontal pathogens such as *Treponema*, *Tannerella* and *Porphyromonas* species were detected at low levels in these samples. In addition, many bacteria identified were not closely related to other known bacteria and the authors suggested these may represent "equine-specific" taxa. As few previous

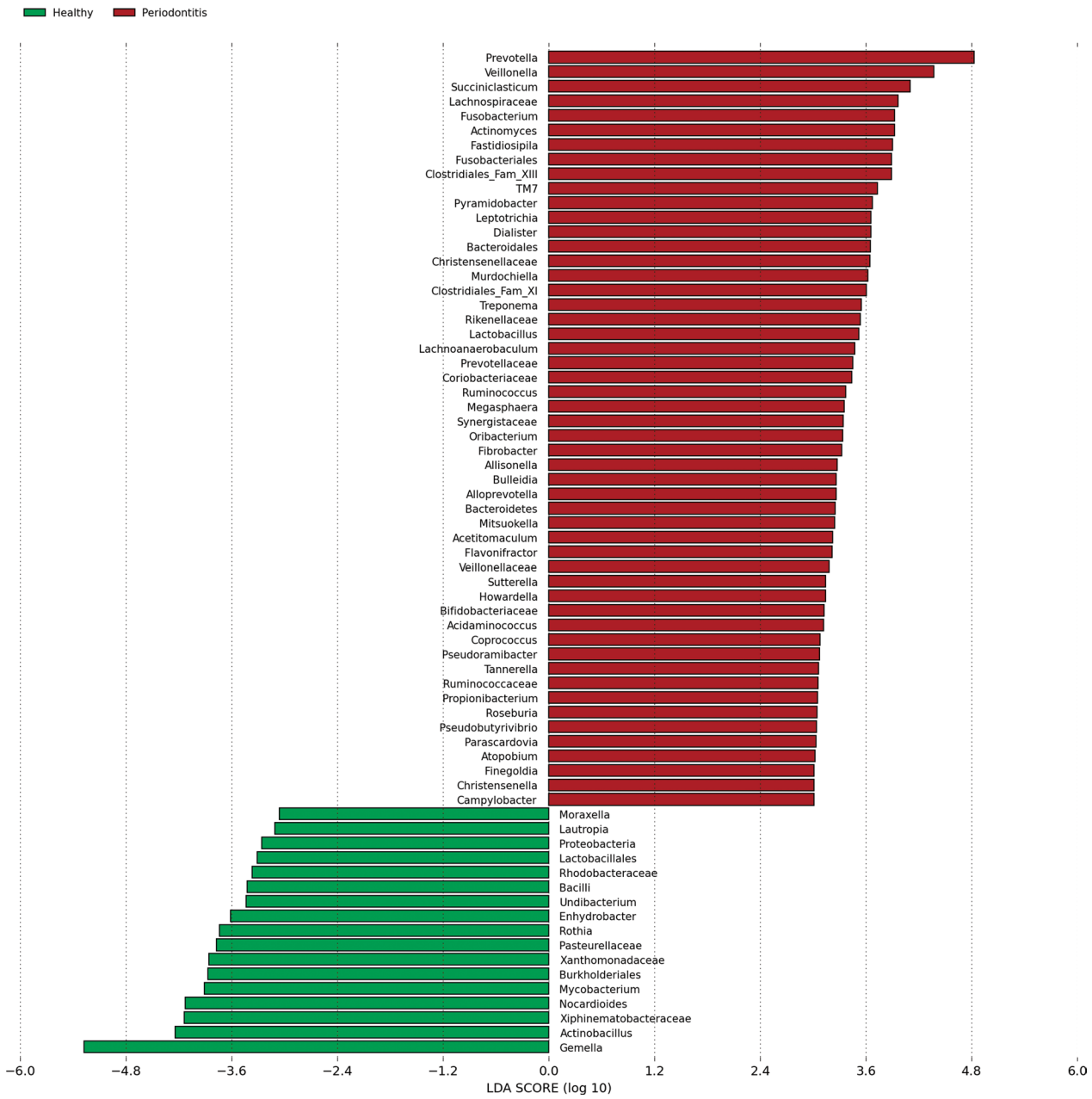

Figure 3 Visualisation of most significant taxa (genus or higher level) that differentiate between health and periodontal disease in equine microbiomes. In total 107 taxa were significantly different. Only 69 taxa that had LDA scores above 3 are shown. The taxa are ranked by the effect size in LEfSe.

studies have been performed investigating the equine oral microbiome, it is highly likely that novel, previously undetected bacteria will be identified when using modern, culture-independent techniques.

The current study was the first to use high-throughput 16S rRNA gene sequencing to compare the bacterial populations present in equine oral health and periodontitis and revealed a statistically significant dissimilarity between the bacterial populations found in equine oral health and in equine periodontitis lesions and represents a considerable advance on what has previously been documented for the oral microbial community in both healthy and diseased horses. In the current study, 60% of horses aged 10 years or above were affected by periodontitis and of all diseased horses, 70% were 10 years or older. Mares were found to be slightly more likely to

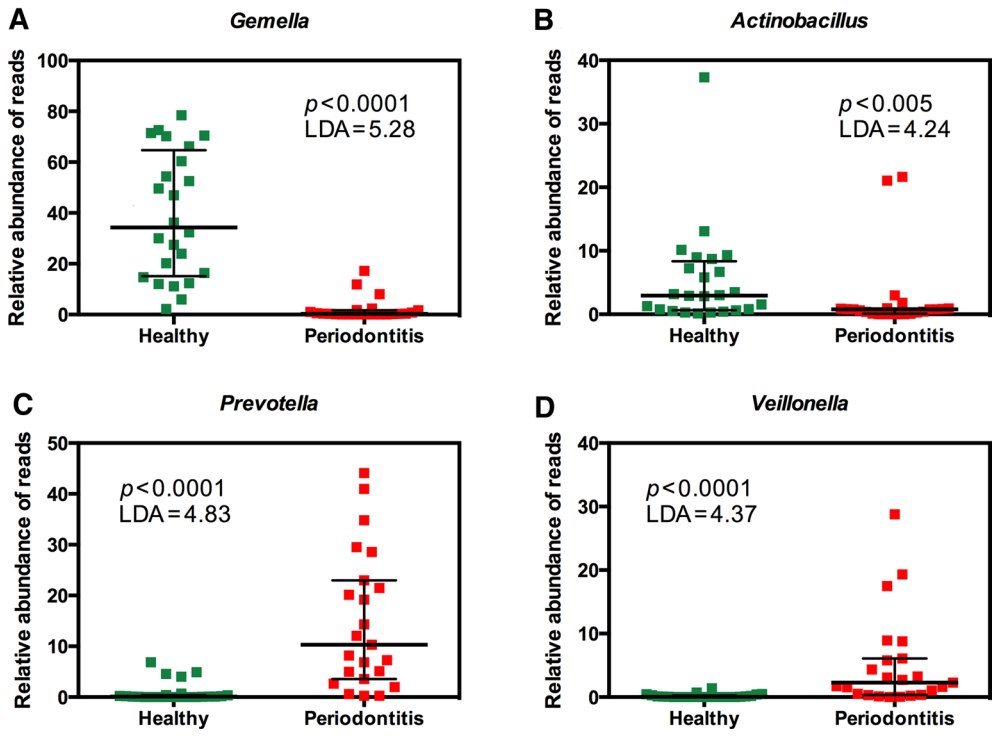

Figure 4 The relative abundance of most discriminative genera between health and disease. A *Gemella* and **B** *Actinobacillus* in health; **C** *Prevotella* and **D** *Veillonella* in periodontitis, based on LDA scores in LEfSe. Values are expressed as a percentage.

have periodontitis than geldings (52% of mares compared to 40% of geldings), although this difference was not statistically significant. Due to the large variety of breeds sampled and the relatively small sample numbers, no particular breed disposition to disease could be identified. Further larger scale studies may be useful to examine links between equine periodontitis and age, sex and breed.

In this cross-sectional study it is impossible to equate the results with disease aetiology and pathogenesis. A potential limitation of this study is that samples were collected from both live and dead horses and that this could add further variability to the results. However, all samples were collected within 1 hour of death (usually much quicker) and, since DNA from live and dead bacteria was detected rather than live cells per se, it is very unlikely that any changes in the microbiomes would be attributable to death of the horses. In any case, individual healthy oral samples (whether from live or dead horses) demonstrated noticeable variation in the composition of their microbiomes but were more similar to each other than to those from horses with periodontitis, and vice versa. Longitudinal studies starting with young healthy horses, and follow-up on their periodontal status and microbiota of the oral cavity until development of periodontal disease would be required. The periodontal pocket found in diseased horses constitutes a new niche in an oral ecosystem that will select for a different microbiome and this may explain the significant increase in microbiome diversity noted in the periodontitis cases in comparison with the orally healthy horses. Increased microbiome diversity has also been noted in samples taken from human periodontitis patients in comparison to orally healthy controls [25, 26].

Environmental differences present between the healthy equine gingival sulcus and diseased periodontal pockets may be particularly striking in the horse, as equine dental anatomy allows for formation of particularly deep periodontal pockets which may measure over 15 mm in severe cases [9]. It is possible that during disease progression, the environmental changes occurring as a shallow gingival sulcus becomes a deep periodontal pocket allows a new group of bacteria to flourish whilst providing a less optimal environment for the growth of others. In the current study, significant differences were seen in both the expected shape and Gram staining characteristics of bacteria detected in oral health and periodontal pockets, with Gram negative rods, spirochetes and mycoplasma more evident in periodontitis.

Spirochetes have long been associated with human periodontitis [27] and more recently spirochetes were detected within the epithelium of equine periodontal

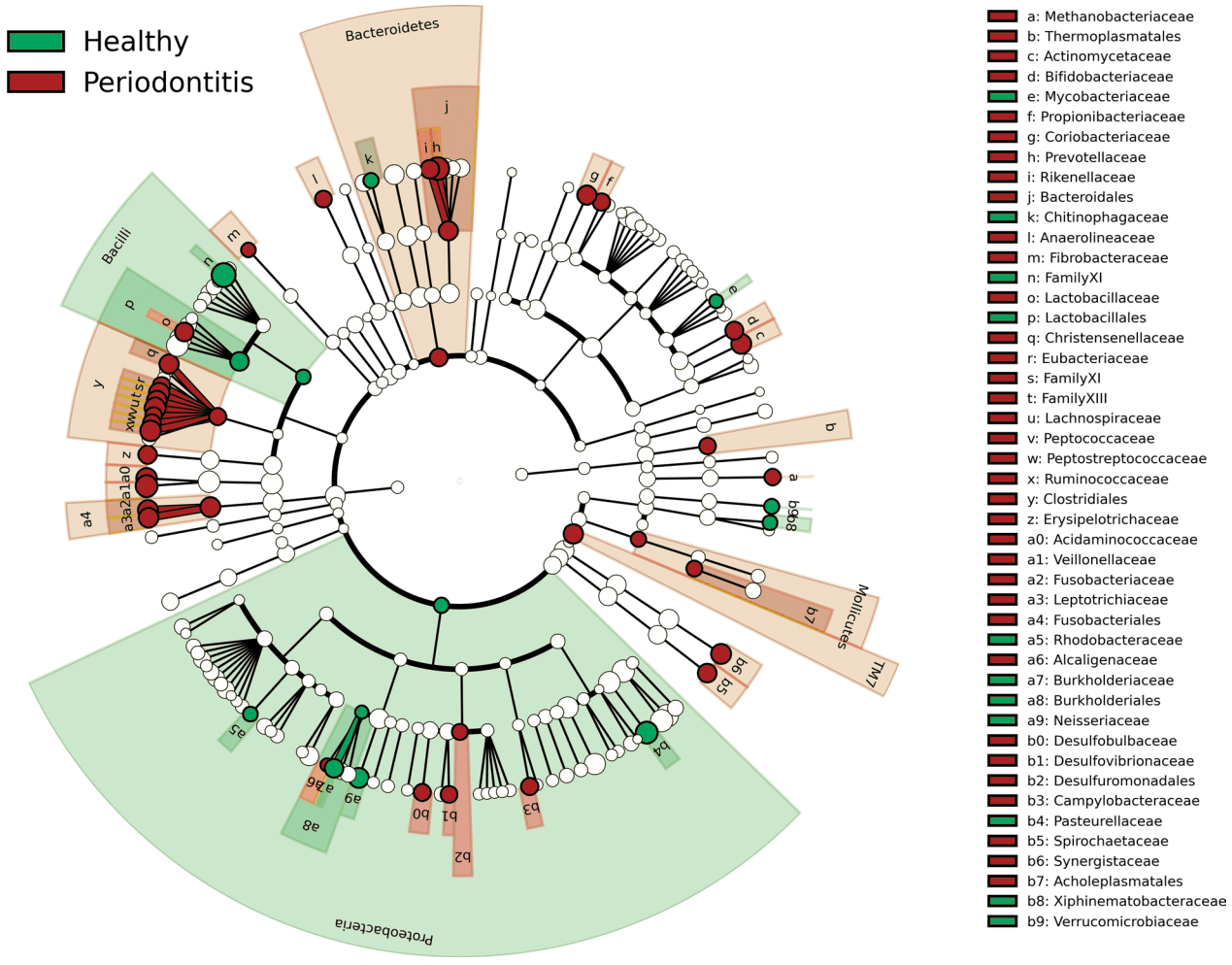

Figure 5 Taxonomic representation of statistically significant differences between the healthy and periodontitis samples at family or higher taxonomic level. All 51 significantly different taxa had LDA scores above 3. Differences are represented in colour (shades of red periodontal disease, green health).

pockets [9]. *Treponema denticola* is well recognised as a periodontal pathogen in man, acting as one of the three "red complex" bacteria found in severe periodontitis lesions alongside *Porphyromonas gingivalis* and *Tannerella forsythia* [28]. In another study, DNA corresponding to *Treponema* species was detected in 78.2% of horses with clinically overt equine odontoclastic tooth resorption hypercementosis (EOTRH) compared to 38% of unaffected horses and *Tannerella* DNA was found in 38.4% of diseased horses compared to 19% of unaffected horses [10]. In the current study, abundance of both the *Tannerella* and *Treponema* genera was significantly increased in periodontitis.

The most discriminative genera between health and disease were the genera *Gemella* and *Actinobacillus* in health and *Prevotella* and *Veillonella* in periodontitis, respectively. In equine periodontitis, the abundance of bacteria belonging to the *Prevotella* and *Veillonella* genera was significantly increased in comparison to oral health. Several species of *Prevotella* have been shown to be involved in human periodontitis, such as *Prevotella intermedia* and *Prevotella melaninogenica* [29]. Several species of *Veillonella* have been isolated from both healthy gingival sulci and diseased periodontal pockets in man. However, *Veillonella parvula* has been significantly associated with chronic periodontitis [30]. Interestingly, *Prevotella intermedia* and *Prevotella nigrescens* have been shown to stimulate cytokine production by activation of Toll-like receptor 2 and *Veillonella parvula* has been shown to stimulate cytokine production by activation of both Toll-like receptor 2 and Toll-like receptor 4 [31]. This is of potential importance as the production

of a destructive inflammatory response in periodontal tissue by stimulation of the innate immune system by periodontopathogenic bacteria is thought to be central in disease pathogenesis in man [32].

In equine oral health, significantly higher relative abundances of the genera *Gemella* ($p < 0.0001$) and *Actinobacillus* were noted in comparison to periodontitis, indicating that these genera comprise part of the normal oral flora of the horse. Bacteria belonging to the *Gemella* genus have been found to constitute high proportions of the microbiota of the dorsal surface of the human tongue [33]. In addition, *Actinobacillus equi* has been frequently isolated from the oral cavity of healthy horses [34, 35]. Given that no previous studies have characterised the equine oral microbiome in such detail, it is highly likely that many novel or previously uncharacterised bacteria are present in both oral health and periodontitis and additional studies would be required to further determine the composition of the equine oral microbiome.

In conclusion, the two cohorts of horses examined harboured highly distinct microbial profiles, with samples from periodontally affected horses being more diverse than samples from the healthy horses. Further, preferably longitudinal, studies are required to determine which bacteria are actively involved in the pathogenesis of disease.

Additional files

Additional file 1. OTUs that differed significantly between the groups. The output was created in LEfSe.

Additional file 2. Microbial families of higher taxa that differed significantly between the groups. The output was created in LEfSe. The same taxa are visualised in Figure 5.

Abbreviations

FAB: fastidious anaerobe broth; LDA: linear discriminant analysis; LEfSe: linear discriminant analysis effect size; OTU: operational taxonomic unit; PCA: principal component analysis.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RK conducted the experiments and was involved in preparation of the manuscript. DFL participated in study design, analysis of data and preparation of the manuscript. PMD collected and provided clinical specimens for the study and was involved in preparation of the manuscript. MJB conducted the high-throughput sequencing. EZ carried out bioinformatics analysis and was involved in preparing the manuscript. WC was involved in high-throughput sequencing and manuscript preparation. LEO assisted with interpretation of data. DB was involved in study design and preparation of the manuscript. BWB carried out bioinformatics analysis and was involved in preparing the manuscript. MPR conceived the study, participated in its design and was involved in manuscript preparation. All authors read and approved the final manuscript.

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