THE UNIVERSITY
of EDINBURGH

An Epidemiological, Pathological and Microbiological Study of Equine Dental Caries

Dewi Borkent

PhD - The University of Edinburgh - 2018

DECLARATION

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or processional qualification except as specified. Parts of this work have been published in Equine Veterinary Education, Equine Veterinary Journal, and in Veterinary Record.

Dewi Borkent

Edinburgh, 25th February 2018

ABSTRACT

Dental caries is caused by acidogenic oral micro-organisms which convert fermentable carbohydrates to acids that damage the tooth by causing a demineralisation and disintegration of the inorganic and organic substances of the tooth, respectively (Soames and Southam, 2005). In horses, two variants of dental caries occur: equine dental peripheral caries (PC) involving the periphery of teeth; and infundibular caries (IC) of the maxillary cheek teeth.

Twenty-five veterinarians and equine dental technicians experienced in equine dentistry were recruited to perform a UK-wide survey and examined 706 horses for the presence PC and IC, as well as for concurrent dental disorders. The recorded survey results for individual horses included data on potential risk factors including breed, sex, age, diet and the postcode of stables. The prevalence of PC and IC in this population, was 51.7% and 45.5%, respectively. The most commonly and severely PC affected teeth were the three caudal cheek teeth (Triadan 09-11) and for IC were the Triadan 09s. In a multivariable model without observer as a random effect, potential risk factors for the development of PC were: the presence of IC, the presence of diastema/periodontal disease (PD), the presence of multiple concurrent dental disorders other than IC, being fed 2.1-3.0 kg concentrates per day, and living in South East England and South West England. The risk factors which remained significantly associated with the presence of PC in a multivariable model with observer as a random effect, were: feeding 2.1-3 kg concentrates per day, multiple concurrent dental disorders other than IC, the presence of diastema/periodontal disease; additionally, dental fractures now became significantly associated with the presence of PC. In a multivariable model without observer as a random effect, potential risk factors for the development of IC were: the presence of PC, the presence of multiple dental disorders other than IC, and increasing age. Horses in North England and South West England were significantly less likely to have IC than horses in other regions of England and Scotland. In a multivariable model with observer as random effect, the only remaining risk factors were increasing age and geographical region, with a significantly lower likelihood for horses to have IC in South West England than in the other regions.

A molecular microbiological study on equine dental caries using a linear discriminant analysis effect size (LEfSe) at genus or higher level, showed *Gemella* and *Actinobacillus* to be the genera most associated with the PC study control group (no PC), and *Streptococcus, Olsenella* and *Scardovia* to be the genera most associated with PC. Additionally if LEfSe was performed at genus level only, then an additional genus shown to be associated with PC was *Mitsuokella*. The genus most associated with IC using LEfSe at genus or higher level was *Acidaminococcus*, while *Bacillus* was the genus most associated with the IC study control group (no IC).

A pathological study examined PC-affected cheek teeth grossly, histologically and, by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Dental plaque, containing bacteria was found to cover the PC lesions. In peripheral cementum, PC lesions were categorised by their shape on histological cross sections of affected teeth into: flake-type, flask-like, or ellipsoid-shaped lesions or combinations of these patterns. Bacteria from surface lesions penetrated between Sharpey's fibers of cementum in a direction perpendicular to the peripheral aspect of the tooth, destroying the intrinsic fibres and Sharpey's fibers. Bacteria also penetrated in a direction parallel to the peripheral aspect of the tooth, undermining the intrinsic fibres, including at the level of incremental growth lines. Bacteria were also found in ellipsoid-shaped lesions and in cemental lacunae of affected cementum.

In dentine affected by PC, bacteria were found within and between damaged dentinal tubules, sometimes causing flake-type lesions similar to those seen in cementum. Bacteria penetrated primary dentine and/or (regular/irregular) secondary dentine from the occlusal surface, or entered primary dentine through cementum and enamel from the peripheral aspect. Dental plaque containing bacteria were sometimes observed in dentinal fissure fractures.

PC of enamel was only visible using SEM, because this was the only method which did not require prior decalcification, that almost completely removed enamel.

In conclusion, PC and IC are prevalent dental disorders in the examined British equine population. The association between PC and concurrent dental disorders (multiple concurrent dental disorders, diastemata/periodontal disease and dental fractures), indicates that these should be addressed in affected horses. In horses

affected by IC, the focus should be on treatment of IC itself (like infundibular fillings), because associations of IC were found with risk factors which cannot (or not easily) be controlled such as age and region. Several bacteria could be identified which were associated with PC and other bacteria were more associated with IC or control groups. It was confirmed in the pathological study that bacteria penetrate the cheek teeth affected by PC and IC. So the focus of treatment of PC and IC can also be on reducing the bacteria associated with PC and IC respectively, or the focus could be on prevention by making the teeth more resistant against caries by fluoride treatment. A critical assessment of the use of chlorhexidine mouthwashes and supplementation of fluoride are needed to evaluate its potential effects on PC and IC.

LAY SUMMARY

Dental decay is caused by bacteria which produce acids from sugars which damage the teeth (Soames and Southam, 2005). In horses, two variants of dental decay occur: equine dental peripheral caries (PC) involving the periphery of teeth; and infundibular caries (IC) of the infundibulum (funnel shaped invagination of enamel) of the upper cheek teeth.

Twenty-five veterinarians and equine dental technicians, all experienced in equine dentistry were recruited to perform a UK-wide survey and they examined 706 horses for the presence PC and IC, as well as for other dental disorders. The survey results included data on potential risk factors including breed, sex, age, diet and the postcode of stables. The percentage of horses affected by PC and IC in this population, was 51.7% and 45.5%, respectively. The most commonly and severely PC affected teeth were the 3 (of the 6) cheek teeth in the back of the mouth and for IC was the 4th cheek teeth. Potential risk factors for the development of PC were: concentrates fed at a level of 2.1-3 kg per day, multiple other dental disorders other than IC, the presence of an abnormal space between the teeth/gum disease and the presence of broken teeth. Potential risk factors for the development of IC were: increasing age and geographical region, with a significantly lower likelihood for horses to have IC in South West England than in the other regions.

A bacteriological study on equine dental caries which did not require growing bacteria on plates, showed *Gemella* and *Actinobacillus* to be the bacteria most commonly associated with the peripheral control group (no PC), and *Streptococcus, Olsenella, Scardovia* and *Mitsuokella* to be the bacteria most associated with PC. *Acidaminococcus* was the bacterium most associated with IC, while *Bacillus* was the bacterium most associated with the infundibular control group (no IC).

A pathological study examined PC-affected cheek teeth grossly and microscopically. Dental plaque, containing bacteria was found to cover the PC lesions. In the outer layer of the tooth, PC lesions were categorised by their shape into: flake-type, flask-like, or ellipsoid-shaped lesions or combinations of these patterns. Bacteria were also found to have penetrated deeper into the tooth.

In conclusion, PC and IC are common dental disorders in the examined British equine population. The association between PC and multiple other dental disorders other than IC, the presence of an abnormal space between the teeth/gum disease and the presence of broken teeth indicates that these should be treated in affected horses. In horses affected by IC, the focus should be on treatment of IC itself (like infundibular fillings), because associations of IC were found with risk factors which cannot (or not easily) be controlled such as age and region. Several bacteria could be identified which were associated with PC and other bacteria were more associated with IC or control groups. It was confirmed in the pathological study that bacteria enter the cheek teeth affected by PC and IC. So the focus of treatment of PC and IC can also be on reducing the bacteria associated with PC and IC respectively, or the focus could be on prevention by making the teeth more resistant against caries by fluoride treatment. An evaluation of the use of certain mouthwashes and supplementation of fluoride are needed to evaluate its potential effects on PC and IC.

ACKNOWLEDGEMENTS

The research carried out for completion of this PhD thesis would not have been possible without the generous financial support of The Horse Trust. The research was carried out using the facilities at The University of Edinburgh, in the RDSVS, Roslin Institute and in Kings Buildings.

First of all I would like to thank my main supervisor Professor Padraic Dixon for being a major inspiration as an equine dental expert and as a person. Thanks for always being friendly, kind and patient, for showing me interesting equine dental cases and for improving the flow of sentences in articles and in this PhD thesis.

Thanks to Richard Reardon for all his help with statistics and for showing me interesting dental cases in the clinic.

I would like to thank Gerry McLachlan and Laura Glendinning for introducing me to the field of molecular bacteriology and bioinformatics, which was very hard for me in the beginning, but thanks to them, it became understandable.

Thanks to Darren Shaw for his very useful statistical course and for his help in writing more complicated codes using statistical programme R.

I would also like to thank Sionagh Smith especially for her help with the pathology chapter, and helping with interpreting histological slides. Thanks to Steve Mitchell who was always there to help a hand with creating good quality images using SEM and TEM.

Thanks to my Mum who always encouraged me to fight for my dreams and thanks to my Dad and sisters, grandparents, friends and boyfriend who always supported me. Thanks to Carola, Sally and Bonnie for the nice walks in the Scottish hills, which will be missed.

Thanks to the support of the Wellcome Trust Multi User Equipment Grant (WT104915MA) who made it possible to use the TEM.

The following reagent was obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project: Genomic DNA from Microbial Mock Community B (Staggered, Low Concentration), v5.2L, for 16S rRNA Gene Sequencing, HM-783D

CONTENTS

LIST OF FIGURE LEGENDS

LIST OF TABLE LEGENDS

LIST OF ABBREVIATIONS

AMOVA = Analysis of MOlecular VAriance

AP = acquired pellicle

DNA = DeoxyriboNucleic Acid

EDTA = ethylenediaminetetraacetic acid

HOMOVA = HOmogeneity of MOlecular Variance

IC = infundibular caries

LAG = line of arrested growth

LDA = linear discriminant analysis

LEfSe = linear discriminant analysis effect size

NGS = Next Generation Sequencing

PC = peripheral caries

PCR = Polymerase Chain Reaction

PCA = Principal Component Analysis

PDL = periodontal ligament

PERMANOVA = PERmutational Multivariate ANalysis Of VAriance

RTF = reduced transfer fluid

SEM = scanning electron microscopy

TAE = tris-acetate-ethylenediaminetetraacetic acid

TEM = transmission electron microscopy

1 CHAPTER 1: LITERATURE REVIEW

1.1 Evolution of the Horse

Equidae are part of the Order Perissodactyla (i.e. odd-toed ungulates) that also includes Tapiridae and Rhinocerotidae (MacFadden, 1992). Additional to the odd number of toes, common features of perissodactyls are: each foot terminates with an ungual padded or hooved phalanx (except for the extinct, clawed chalicotheres), concave, saddle-shaped navicular (central tarsal) facet on the astragalus (talus), axis of symmetry through the central metapodial (III), hind-gut fermentation and a specific cheek tooth (premolars 2-4, and molars) cusp morphology reflecting their herbivorous diet. In contrast to the current 7-10 equine species which all belong to the single genus Equus (that includes horses, zebras and asses), fossils of ancestors of the horse are classified into 32 extinct genera composed of more than 150 species. Prehistoric fossils reveal that the main evolution of Equidae occurred in North America from the early Eocene (55 million years ago) until the end of the last Ice Age (10,000 years ago) in the Pleistocene (MacFadden, 1992, 2010).

Hyracotherium (meaning "rabbit-like animal") which is the oldest known member of the family Equidae, was named and described by Richard Owen in 1839 (Bennet, 2008). The terms Eohippus or "Dawn horse" are also used for this earliest ancestor of the horse that appeared in the early Eocene. The teeth in *Hyracotherium's* small, short-snouted skull with a shallow mandible were brachydont (i.e. short crowned) and cusped. *Hyracotherium* browsed in open park woodlands and their diet consisted of leafs and ground cover (Gingerich, 1981). In the Middle Miocene (20-15 million years ago) the horse skull and dentition became more fully adapted to grazing. It resulted in a deeper skull with an expanded pre-orbital cheek region, more developed jaws and a relatively longer cheek tooth rows with hypsodont (i.e. high crowned) teeth.

The first appearance of cementum as a structural component of the clinical crown occurred in advanced *Parahippus* species, where it provided additional occlusal surface for mastication. With the development of hypsodont teeth, the role of cementum further developed (MacFadden, 2010). Hypsodonty has long been seen as

an evolutionary adaptation in browsers associated with a dietary change from softer leaves to grass because the silica-rich granules (phytoliths) present in grass causes increased dental wear (Stebbins, 1981; Jardine et al., 2012), and higher quantities of this coarse low-energy food has to be consumed for much longer periods. Recently it has been postulated that grit and soil attached to plants may be a more important cause of dental abrasion than grass, because they are more abrasive than phytoliths. Moreover, the great spread of grasslands across North America has been shown to occur 7 million years later than the first development of hypsodont teeth (Jardine et al., 2012).

Other simultaneous developments in the dental morphology of grazers that improved mastication and thus breaking down of long plant fibres included an increase in the number of cusps (lophs) with interconnections between them (styles). These resulted in a complex enamel pattern on the exposed occlusal surface. Self-sharpening enamel ridges are created on the tooth surface by a variation in hardness and thus wear of the three different calcified dental components (enamel, dentine and cementum). Other developments included an increased size of individual teeth and a uniform row of cheek teeth (Bennet, 2008). Grazing mammals need to utilise grass as their main food source but no mammals have the enzyme to break down the $\beta(1\rightarrow4)$-glycosidic bond in cellulose (the most common foodstuff on earth). However, the intestinal tracts of herbivores contain bacteria which produce cellulase that can break down cellulose (Stoker, 2016). In contrast to the very efficient gastro-intestinal tract that later developed in ruminants, horses have a simpler, smaller digestive system and developed a larger colon and caecum for cellulose digestion (Bennet, 2008). Sugars and starches not resistant to enzymatic hydrolysis are hydrolysed in the equine small intestine until an overload of the enzymatic capacity is reached. This excess of sugars and starches not resistant to enzymatic hydrolysis as well as fermentable carbohydrates (including fructans, starches resistant to enzymatic hydrolysis and cellulose) undergoes microbial fermentation in the colon and caecum and forms volatile fatty acids which are absorbed by the horse (Engelking, 2002; Hoffman, 2009).

During the early Eocene, primitive horses migrated via the then Bering bridge (that connected what is now Alaska to Russia) to Europe where different equid species developed, as well as a new genus *Paleotherium* (i.e. the first chalicothere-like equid genus). The various *Hyracotherium* descendants died out in the Old World by the early Oligocene. In the late Oligocene some chalicomorph browsers of the genus *Anchitherium* crossed westwards, also using the Beringian land route. In Eurasia *Anchiterium* gave rise to several different genera and species. In the early Miocene horses from the genus *Hipparion* migrated from North America to Eurasia via Beringian land route and lived in Africa until their extinction in the early Pleistocene. They were the last three-toed horses in the world. Horses from the genus *Equus* were the last migrating equids (Fig 1.1). They also used the Beringian land route to migrate to Eurasia and Africa but some back-migrations from Eurasia to North America also occurred and they even dispersed into South America via the Isthmus of Panama. In the Pleistocene (at the end of the last Ice Age, i.e. 10,000 years ago) Equidae became extinct except for Equus, the only surviving genus – which is the genus where the modern horse (Equus caballus), as well as donkeys and zebras, belongs to (Bennet, 2008).

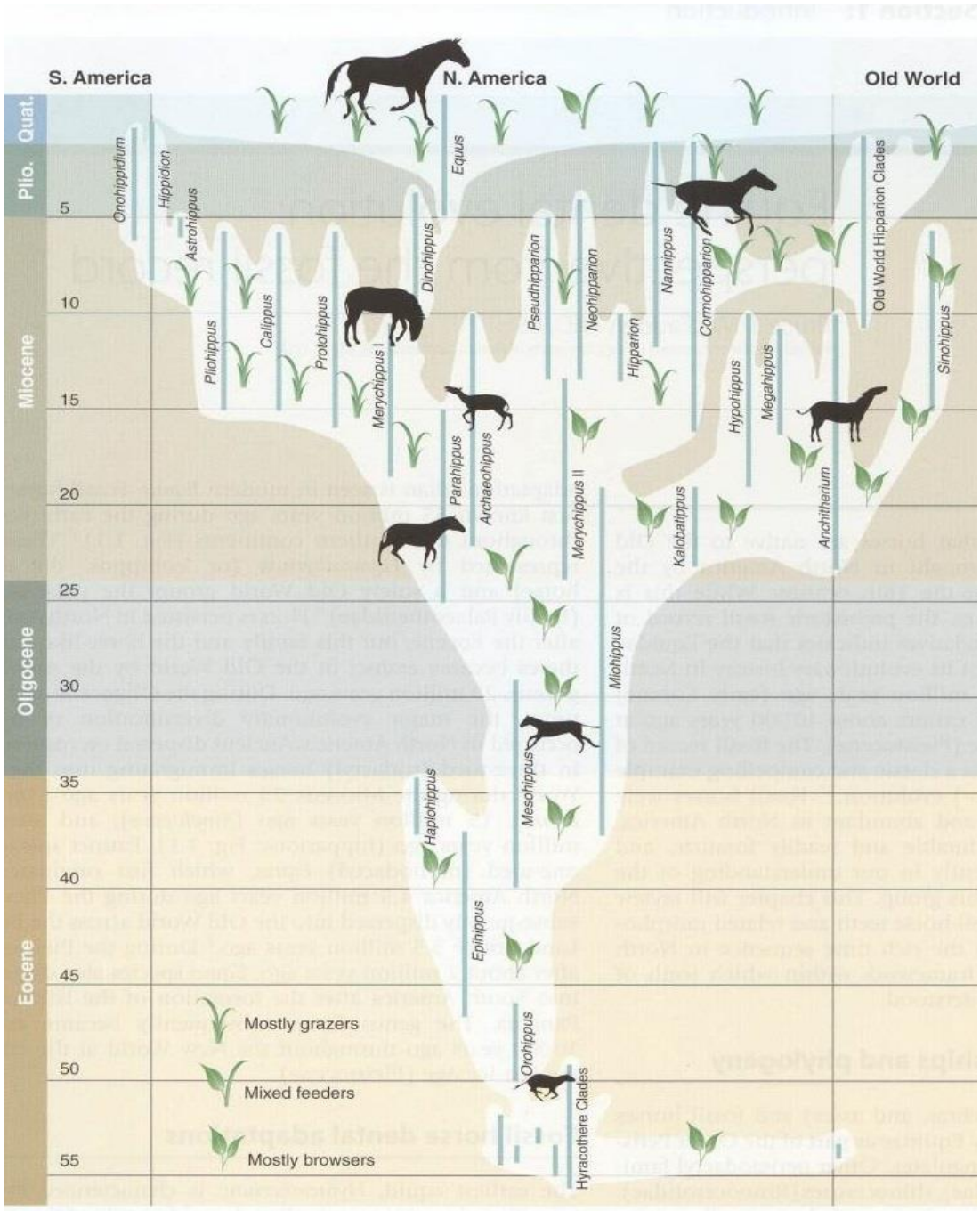


Fig 2.1. Phylogeny, geographic distribution, diet and body sizes of the Equidae over the past 55 million years (from Easley [1996]).

1.2 Embryology

During dental development, the primordial teeth of all mammals follow similar developmental phases consisting of an initiating, morphogenetic and cytodifferentiative phase. This is irrespective of the animal species or the tooth type involved although there are variations in timing and termination of these phases between species (Kollar and Lumsden, 1979).

1.2.1 Stages of Dental Development

Tooth formation starts with a primary epithelial band (i.e. epithelial thickening) invaginating into neural crest-derived mesenchymal tissue so that two ridges are formed: a rostral vestibular lamina and a caudal dental lamina, both of ectodermal origin (McGeady et al., 2006). The dental lamina gives rise to several epithelial swellings or tooth buds that project into the underlying mesenchyme. This is termed the bud stage of dental development. While the tooth buds extend into the underlying mesenchyme, the underlying mesenchymal cells proliferate and invaginate into the tooth buds so that the tooth buds become (inverted) cap shaped structures or enamel organs in what is termed the cap stage. These enamel organs will eventually give rise to all the deciduous teeth and the permanent molars (i.e. permanent caudal cheek teeth).

An enamel organ consists of a layer of internal enamel epithelium at its concave aspect and a layer of external enamel epithelium at its convex aspect, with a loose connective tissue called stellate reticulum interposed between them. The mesenchymal cells continue to proliferate within the concave area of the enamel organ, creating the dental papilla while other mesenchymal cells form the dental sac (follicle) after spreading peripherally around the enamel organ. This protective dental sac surrounds the enamel organ and dental papilla until dental eruption occurs (Berkovitz and Moxham, 1981; Bhaskar, 1991). The enamel organ, dental papilla and dental sac together form a tooth germ and each germ is the primordium of a single tooth.

As the enamel organ develops further, it acquires a bell shape in what is termed the bell stage of dental development and at first it remains connected with the oral

epithelium by a cord of cells from the dentinal lamina, but this cord gradually breaks down. Buds of permanent incisors, permanent canines and permanent premolars derived from outgrowths of the cord of cells from the dentinal lamina form separate enamel organs and stay in a dormant stage until the permanent tooth needs to develop. Cells of the internal enamel epithelium now become taller and more columnar in shape with large nuclei at their bases. Odontoblasts are formed out of the uppermost cells of the dental papilla, after molecular alterations in the dental papilla and the first layer of dentine occurs after the basal membrane disintegrates. Subsequently, the columnar cells of the internal enamel epithelium develop further into ameloblasts which in turn start to produce enamel (Ferguson, 1990). At first this is a structureless enamel and ameloblasts move away from the enamel-dentinal junction as they produce it, each creating a distal projection termed Tome's process. The proximal part of Tome's process secretes enamel that forms interprismatic enamel while the enamel secreted by the surface of Tome's process forms enamel prisms.

During dentine deposition, odontoblasts develop long fine extensions as they move away towards the pulp. These soft tissue extensions remain within the dentinal tubules and are called odontoblast processes. The mineralisation phase of dental development now starts, whereby crystals are formed, grow and fuse with each other to form a continuous mineralised layer. Since the mineralisation process lags behind dentine secretion, unmineralised predentine is found between odontoblasts and mineralised dentine (Nanci, 2008).

1.2.2 Differences in Dental Development between Hypsodont and Brachydont (e.g. Human) Teeth

Equine cheek teeth are square (maxillary) or rectangular (mandibular) on transverse section, but their incisors and canine teeth are circular or oval on transverse section similar to all classes of teeth in brachydont species (Latshaw, 1987). Other differences that distinguish hypsodont teeth from brachydont teeth are that hypsodont teeth (including the erupted [clinical] crown) become completely coated with cementum prior to eruption and that tooth eruption occurs before root formation is

complete (McGeady et al., 2006). The morphogenetic and cytodifferentiative phases have a delayed termination at the apical regions of hypsodont teeth compared to brachydont teeth in order to enable prolonged (but not continuous) growth and continuous eruption of equine teeth (Dixon and du Toit, 2010). The continuous growth and continuous eruption of teeth of rabbits and guinea pigs (elodont teeth) will be discussed in section 1.3.

1.2.3 Mineralisation of Equine (Hypsodont) Teeth

Mineralisation in multi-cusped teeth, such as equine cheek teeth starts separately at each cusp tip (Berkovitz and Moxham, 1981). These calcified aspects of the teeth later fuse together as the mineralisation process proceeds apically towards the dentinoenamel junction. Baker (1979) reported that mineralisation of deciduous teeth in the equine foetus is underway at 120 days and complete within 240 days of conception. While the formation and maturation of permanent equine tooth buds was first observed in the 9.5 months old foetus, the first permanent tooth buds (109 and 209) start to become calcified in the 10 months old foetus. Calcification of the mandibular opponents (309 and 409) starts up to 3 weeks later. It takes 360 days for an equine tooth to develop from a dental bud to a tooth ready for eruption (Baker, 1979).

1.2.4 Root Development in Brachydont and Equine Teeth

After crown formation is complete in brachydont teeth, root development occurs. Hertwig's epithelial root sheath in brachydont teeth is formed by a corono-apical extension of the internal and external epithelial cells over the dental papilla (Nanci, 2008). This double cell layer induces adjacent mesenchymal cells to differentiate into odontoblasts and these cells then produce predentine. Hertwig's epithelial rooth sheath now disintegrates and ectomesenchymal cells from the inner part of the dental follicle now come into contact with predentine and the mesenchymal cells of the dental sac now convert into cementoblasts and start to secrete cementum (Bhaskar, 1991). This differentiation into cementoblasts occurs after the brachydont tooth has erupted through the dental sac, causing the latter to collapse against the underlying root dentine.

Deposition of cementum on the equine tooth root is similar to that in brachydont teeth. However, the epithelial root sheath is an occluso-apical extension in hypsodont teeth and it can take a few years after eruption for roots to be well developed in equine teeth (Dixon and du Toit, 2010). Moreover, in horses the dental sac remains for a prolonged period compared to brachydont teeth (McGeady et al., 2006). The dental sac collapses before tooth eruption occurs, thus allowing the the entire tooth, including the occlusal surface to be covered with cementum (Dellmann and Brown, 1976). Once the epithelial root sheath disintegrates, no more enamel can be formed (Latshaw, 1987). After eruption of hypsodont teeth, the cementum at the occlusal surface is soon worn away. Thereafter, the occlusal enamel and dentine becomes exposed and comes in wear and the occlusal surface is now termed a secondary occlusal surface (Dellmann and Brown, 1976). In brachydont teeth, root formation ceases when the apices become very reduced in size and the tooth has reached its maximal length. However termination of root formation does not indicate the end of tooth eruption in hypsodont teeth (Muylle et al., 1999).

1.3 Differences in Anatomy between Brachydont and Hypsodont Teeth

Hypsodont teeth can be subclassified as aradicular and radicular teeth (Gorrel, 2013). Aradicular teeth can be found in rabbits and guinea pigs and these teeth will never develop true (i.e. anatomic) roots (i.e enamel free calcified structures) on their apices. The functional dichotomy between the root and crown is absent. Generally, a distinction between the crown and the root of a tooth can be made based upon the presence or absence of enamel on that part of the tooth, respectively. However, in rabbits and guinea pigs this distinction cannot be made since the full length of the tooth contains enamel. Tooth growth in these animals occurs continuously throughout life (i.e. elodont).

Equine teeth are described as radicular hypsodont teeth meaning that in addition to having a long crown, the teeth will eventually develop true roots. Equine teeth are anelodont meaning that they grow for a fixed period of time and for horses this usually is for approximately 7 years after eruption (Büdras et al., 2003), but then the

root apices close almost entirely and although still some blood supply remains, the teeth will not grow any further (Gorrel, 2013). Also, the dentition of a horse is heterodont and diphyodont, similar to rabbits and guinea pigs. Heterodont means that different classes of teeth have different forms (incisors vs cheek teeth) and function, while diphyodont is a term to describe the presence of primary (deciduous) and secondary (permanent) teeth (Verstraete, 1999). Equine teeth slowly erupt at a rate of 2-3 mm per year and attrition occurs approximately at the same rate when enough forage is provided (Baker, 1979; Dyce et al., 1987)

The dentition of humans, dogs and cats can be described as brachydont, heterodont, diphyodont and anelodont. These teeth have a distinct neck between the root and the crown. Dental tissues in brachydont teeth are arranged in layers with dentine surrounding the pulp, and cementum covering the dentine in the root and enamel covering the dentine in the crown (all crown [enamel containing part of the tooth] is supragingival in brachydont teeth), including the occlusal surface (Wiggs and Lobprise, 1997; Verstraete, 1999). This is in contrast to equine teeth with their longitudinally oriented, scroll-like arrangement of dental tissues. Enamel does not cover the entire occlusal surface in equine teeth but instead there are enamel infoldings from the periphery of the tooth, with dentine filling the internal areas of these folds adjacent to the pulp and overlying the pulp (subocclusal dentine). The periphery of the tooth including its infoldings is fully covered with cementum.

Equine incisors and upper cheek teeth also have additional cup-like infoldings of the peripheral enamel from the occlusal surface which are called infundibulae that are normally filled with cementum. The pulp in equine permanent teeth consists of one large common pulp chamber at eruption. One year after eruption several (5-8) separate pulp horns become formed in mandibular and maxillary cheek teeth and their sites (areas of secondary dentine) can be observed on their occlusal surfaces (Dacre et al., 2008a; Kopke et al., 2012). Incisors, wolf teeth and canine teeth only contain a single pulp.

While in brachydont teeth the anatomical crown is usually the same as the clinical crown (i.e. erupted crown), in horses most of the crown is unerupted and is referred to as reserve crown.

1.3.1 Dental Anatomy in the Horse

The standard mammalian dental formula consists of 3 incisors, 1 canine, 4 premolars and 3 molar teeth. The dental formulas of deciduous and permanent teeth in the horse are:

Deciduous teeth: 2(Di 3/3, Dc0/0, Dm 3/3) = 24 teeth
Permanent teeth: 2(I3/3, C 1/1 or 0/0, PM 3/3 or 4/4, M3/3) =36-44 teeth

In the Triadan System, three digits are used to identify each tooth (Floyd, 1991). The dental quadrants are numbered from 1 to 4 respectively for upper right, upper left, lower left and lower right arcade. The number of each quadrant forms the first digit of the code. However, in deciduous teeth the numbers 5-8 are used to refer to the tooth quadrants. The teeth in each quadrant are numbered in a rostro-caudal (mesio-distal) order thus from the central incisor (Triadan 01) to the last cheek tooth (Triadan 11) that make up the last two digits in the Triadan system.

1.3.2.1 Incisors

Incisors are embedded in the rostral aspects of the incisive (premaxillary) bone and the mandible (Büdras et al., 2003). Deciduous incisors are smaller, more triangular and wider than the permanent incisors and also have a distinct neck. They also appear whiter and their infundibulum is shallower than in permanent incisors (Baker, 1979). The incisor infundibulum is cone shaped from the occlusal surface to more apically (Klugh, 2010). It is made of enamel and is usually incompletely filled with cementum that appears dark due to food staining. With age, the infundibulum becomes worn by attrition, and thus becomes smaller, moves closer to the lingual border at the occlusal surface and eventually disappears. As the infundibulum wears away, a layer of secondary dentine that is termed the dental mark or dental star is laid

down to protect the underlying pulp from occlusal exposure. It first appears labial to each infundibulum at the occlusal surface of the incisor. It initially appears as two peripheral areas that later join to form a transverse, dark yellow (due to food staining) line of secondary dentine. The infundibulae in the upper incisors remain for longer, because they are deeper than those of the lower incisors (Baker, 1979). When the infundibular cavity is fully worn away a small, elevated ring of infundibular enamel remains present on the lingual aspect of the occlusal surface, which is called an enamel spot, enamel ring or enamel mark (Walmsley, 1993).

Galvayne's groove is a longitudinal groove that appears on the labial aspect of permanent upper 03s at about 10 years of age and was used for ageing horses, as was the development of a hook (focal overgrowth) on the caudolabial aspect of the occlusal aspect of the 03s. However, the timing of the development of these features are variable and therefore not very reliable for ageing horses (Dixon and du Toit, 2010). The shape of the occlusal surface of an incisor changes with age because of increasing wear and this change is more apparent in the lower 01s and 02s than in the lower 03s. The occlusal surface has an elliptical shape in a young horse, then becomes more round, triangular and eventually oval-shaped in an old horse (St Clair, 1975; Richardson et al., 1994).

1.3.2.2 First Premolars (Wolf Teeth)

The first premolars or wolf teeth (05s) are the only equine teeth which are brachydont. These rudimentary teeth erupt at 6-12 months of age, and have no deciduous precursor. Usually only the maxillary wolf teeth are present (one or both) while the mandibular wolf teeth are usually absent (Dixon and du Toit, 2010). Some wolf teeth never erupt, remaining subgingival and are termed *impacted* or *blind wolf teeth* (Linkous, 2006). There is much variation in location, shape and size of wolf teeth (Miles and Grigson, 1990) but usually their clinical crown is about 1-2 cm in length with a root that can be almost non-existent to more than 3 cm in length. Normally they are located just rostral to the 06s, but wolf teeth can be rostrally or rostrolaterally displaced. Moreover, they are not always orientated in a vertical

direction with some obliquely oriented in relation to the hard palate (Dixon and du Toit, 2010).

1.3.2.3 Canine Teeth

Deciduous canine teeth are vestigial, spicule-like structures and the lower deciduous canines that are situated caudal to the 03s (König and Liebich, 2007) do not erupt. Canine teeth are usually absent or vestigial in female horses, whilst male horses normally have 4 permanent canine teeth which erupt around 5 years of age. Although canine teeth are simple teeth without prolonged eruption as occurs with cheek teeth and without enamel infoldings, they cannot be classified as brachydont teeth since their clinical crown is covered by peripheral cementum and some slight degree of prolonged eruption can occur (Dixon and du Toit, 2010). Canine teeth have a pointed occlusal surface and while their buccal side is convex, their medial (lingual/palatal) side is slightly concave with a slight, caudal-facing curvature.

There is no occlusal contact between the lower and upper canine teeth since the lower canine teeth are located more rostrally (nearer to 03s, corner incisor) than the upper (that lie at the premaxilla - maxilla junction). This absence of contact is believed to make them more prone to calculus development (König and Liebich, 2007). Calculus is mainly found on the lower canine teeth and this may also be due to the location of ostia of the mandibular salivary glands that are located close to them, i.e. on the sublingual caruncle on the floor of the mouth, a few centimeters caudal to the incisors (Büdras et al., 2003).

1.3.2.4 Cheek Teeth

Deciduous premolars 2 to 4 (Triadan 06, 07, 08) are usually already erupted at, or erupt within a week of, birth (Dixon and du Toit, 2010). Unlike permanent cheek teeth, temporary cheek teeth have a distinct neck between their crown and roots (Huidekoper, 1981). The permanent cheek teeth (06, 07, 08) replace these deciduous cheek teeth at approximately 2.5, 3 and 4 years of age, respectively. The transverse areas of equine deciduous teeth are similar to the erupting permanent cheek teeth and thus it can be hard to distinguish retained remnants of deciduous cheek teeth (caps) from the underlying permanent cheek teeth.

The occlusal surfaces of all cheek teeth in a row act as a single functional unit as they are tightly compressed together occlusally, as a result of the different angles of their clinical crowns. In permanent dentition, the reserve crowns of the 06 cheek teeth are directed slightly rostrally (clinical crowns directed caudally); the long axes of the 07, 08 and 09 cheek teeth are relatively vertical and the reserve crowns of the two caudal cheek teeth are curved caudally to an increasing degree (clinical crowns directed rostrally). This is in contrast with the cheek teeth rows in omnivores and many carnivores where only the caudal molar (wisdom tooth) is directed rostrally (termed mesially). As these brachydont teeth form a true continuous arch, this caudal compression is sufficient to keep all the teeth compressed together occlusally. All ungulates have a physiological diastema as a result of their evolutionary increase in facial length and thus a need for the 06s to be directed in the opposite direction to the caudal cheek teeth in order to keep the occlusal aspects of the clinical crowns of the cheek teeth compressed together (Dixon and du Toit, 2010).

The occlusal surface of cheek teeth contain lophs and styles i.e. termed “transverse ridges” (2 on most cheek teeth but 1-3 on the 06s and 11s) and is slightly angled with the buccal sides of maxillary cheek teeth and the lingual sides of the mandibular cheek teeth being taller than, the palatal sides and the buccal sides of their clinical crowns, respectively. Maxillary cheek teeth are wider and squarer than mandibular cheek teeth which are more rectangular in shape (Baker, 1979). The mandibular arcades are straighter than their maxillary counterparts and mandibular arcades are on average 23% closer together than maxillary arcades, so that the lingual margins of mandibular cheek teeth lie medial to the palatal aspects of maxillary CT. This discrepancy in jaw width is called anisognathia (Dixon and du Toit, 2010).

The dorsal curvature of the occlusal surface of the 10s and 11s that increases in caudal direction to a variable degree is called the Curve of Spee. This is more pronounced in certain breeds, such as Arabian horses. Likewise, a rostro-dorsal curvature can be present on the occlusal surfaces of the rostral cheek teeth and this can also be considered as a normal variation (Dixon and du Toit, 2010).

The maxillary cheek teeth rows have a slightly convex buccal curvature. The buccal aspects of maxillary cheek teeth have outfoldings (vertical ridges) of cementum covered enamel called cingulae or styles that run in a vertical direction. Every maxillary tooth has two prominent ridges and a less prominent caudal ridge with two grooves between them. However the 06s can have three to four grooves and ridges.

Most maxillary cheek teeth have three roots, namely two small buccal (lateral) roots and one wide palatal (medial root). However, sometimes the medial root is divided into two separate roots and thus a maxillary cheek tooth can have four roots (Bradley, 1923). Roots elongate for years after the tooth has erupted. Most mandibular cheek teeth have two roots, a caudal and a rostral root which are equal in size, but the 311 and 411 can have three roots.

While the rostral cheek teeth 06s, 07s and often the rostral aspect (in older horses also the caudal aspect) of the 08s are embedded in the maxillary bone, the caudal maxillary cheek teeth (08), 09, 10 and 11 lie in the rostral and caudal maxillary sinuses. Usually the caudal part of the 08 alveolus and the complete alveolus of the 09 and the rostral aspect of the 10 are located in the rostral maxillary sinus. The caudal maxillary sinus normally contains the caudal aspect of the 10s and the 11 alveoli although much variation is possible in the position of alveoli and their cheek teeth in maxillary sinuses (Huidekoper, 1981; Perkins, 2001; Tremaine and Dixon, 2001). In a recent computed tomographic study Liuti et al. (2017) found that the alveoli of the 11s were consistently present within the caudal maxillary sinus, the 10s were variably present in the rostral or caudal maxillary sinus, and the alveoli of the 09s were in most cases completely present in the rostral maxillary sinus, all 08s were fully or partly within the rostral maxillary sinus.

Cheek teeth taper in towards their apices and thus the crown decreases in size with age, and so as cheek teeth erupt, they drift more rostrally in the sinuses (mesial drift) (Huidekoper, 1981; Perkins, 2001; Tremaine and Dixon, 2001).

The fully developed 07s, 08s 09s and10s cheek teeth all contain 5 pulp horns, while the 06s and mandibular 11s usually contain 6 pulp horns and the maxillary 11s have 7 pulp horns (Dacre, 2005b; Dacre et al., 2008a). A modified pulp horn classification system described by du Toit et al. (2008a), is commonly used to refer to the different pulp horns (Fig 1.2).

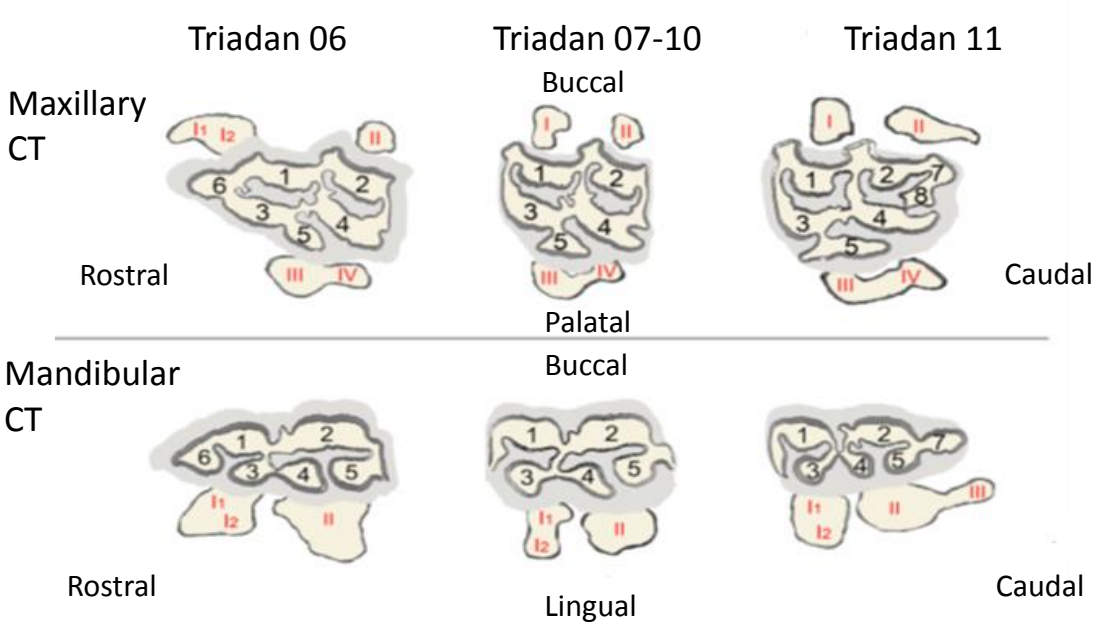


Fig 1.2. Numbering system of root canals and pulp horns for maxillary cheek teeth (top) and mandibular cheek teeth (bottom), adapted from Du Toit et al. (2008a). The occlusal surface is illustrated by dentine (cream), cementum (light grey) and enamel (dark grey). Root contours are schematically displayed adjacent to the occlusal surfaces. Positions of pulp horns are indicated with Arabic numerals (1 to 8). Positions of root canals are indicated with Roman numerals I to IV (from Kopke et al. [2012]).

1.3.2 Dentine

1.3.2.1 Composition of Dentine in Brachydont Teeth

Dentine is a calcified dental tissue, yellowish in colour that is somewhat harder than bone but softer than enamel. It is composed of 70% inorganic (mainly hydroxyapatite crystals) and 30% organic material (mainly collagen fibrils and mucopolysaccharides) and water. The collagenous component is mainly made up of type I collagen fibrils, with small amounts of type III and IV collagen and also some

inclusions of non-collagenous matrix proteins and lipids. Some of the matrix proteins are similar in bone and dentine. Dentinal collagen type 1 is a scaffold for 56% of its mineral content, holding it within pores of its fibrils, while the non-collagenous matrix proteins can upregulate, downregulate or stabilise mineral deposition. Dentine has some elastic properties which, along with cementum, prevents the overlying hard but brittle enamel from fracturing. Dentine and enamel are tightly connected at the scalloped dentino-enamel junction. The roots of brachydont teeth are not covered by enamel so that cementum is directly attached to root dentine (Nanci, 2008).

1.3.2.2 Histology: Structures of Dentine in Brachydont Teeth

As noted, odontoblast processes are attached to odontoblasts that lie at the pulp periphery and then run through canaliculi (dentinal tubules) transversing the dentine across the full thickness of the dentine to the dentinoenamel junction in the crown and to the cementodentinal junction in the root. Dentinal tubules create a profuse anastomosing system for diffusion of nutrients through dentine. During dentinogenesis the odontoblasts and thus also the dentinal tubules follow a S-shaped path (Fig 1.3) towards the centre of the pulp in coronal dentine (Nanci, 2008). Near the root tip and along incisal edges and cusps the odontoblasts follow a straight course, creating straight dentinal tubules (Bhaskar, 1991). At the tip of the tooth this means that they run perpendicular to the occlusal surface. Dentinal tubules are absent in predentine but their outline is formed by a network of collagen fibrils surrounding each odontoblast process (Nanci, 2008).

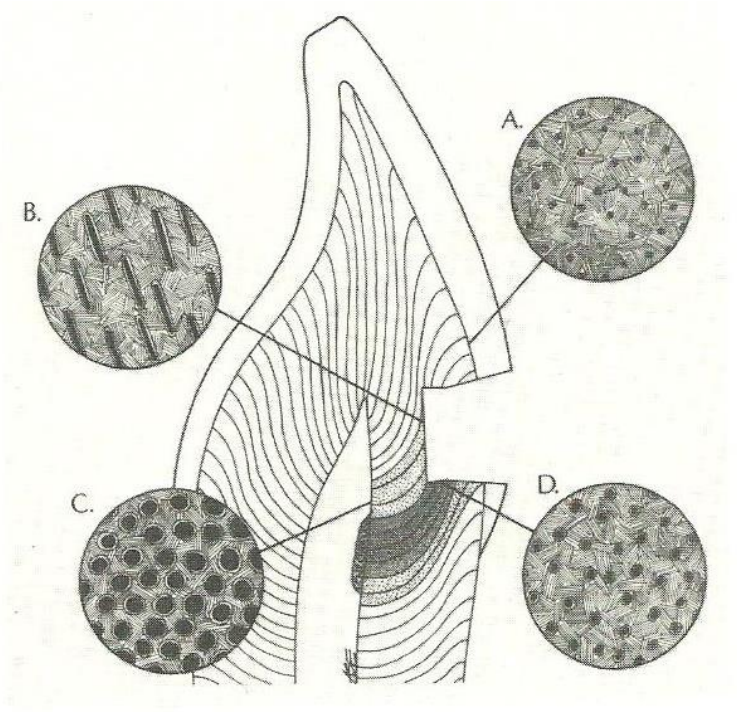


Fig 1.3. Diagram illustrating the curvature, size and distance between dentinal tubules in human outer, mid and inner dentine (from Bhaskar [1991]).

Intratubular dentine is a hyper-mineralised tissue surrounding the dentinal tubules. In some more peripheral areas of dentine, intratubular dentine completely occludes the lumina of the dentinal tubules, commonly halfway between the dentinoenamel junction and the pulp, especially in the apical third of the root. Dentine with occluded tubules is referred to as sclerotic dentine (Avery, 1992; Nanci, 2008).

Intertubular dentine is the primary product secreted by the odontoblasts and is deposited between the dentinal tubules. It forms the main mass of dentine and although it also contains collagen I fibrils and hydroxyapatite crystals, it is less mineralised than intratubular dentine. The collagen fibrils in intertubular dentine run perpendicular to intratubular dentine (Avery, 1992).

The granular layer of Tomes represents an area of dentine with high numbers of granules, that in brachydont teeth is situated between the cementoenamel junction and the apex of tooth, just below the dentinal surface where cementum covers the root. This distinct area can be observed in undecalcified ground dentinal sections under transmitted light and is believed to be caused by coalescing looped terminal

portions of dentinal tubules or a special arrangement of collagen and non-collagenous matrix proteins (Nanci, 2008).

1.3.2.3 Histology: Types of Dentine in Brachydont Teeth

Primary dentine constitutes most of the dentine and forms the main part of the tooth. It can appear translucent like enamel because it contains large amounts of highly mineralised intratubular dentine. Mantle dentine is the term for the outer layer of brachydont primary dentine that is first deposited beneath enamel (in the crown) and beneath cementum underlying the granular layer in the roots. Mantle dentine is about 20µm thick and its collagen fibrils are oriented perpendicular to the dentino-enamel junction. The remaining primary dentine is called circumpulpal dentine since it surrounds the pulp chamber**.** It contains crowded collagen fibrils that are smaller in diameter and slightly more mineralised compared to those of mantle dentine and also has more defects than mantle dentine (Bhaskar, 1991; Nanci, 2008).

Globular dentine contains globular calciforites that failed to coalesce. This results in areas of hypomineralisation between the globules which are called interglobular spaces (Avery, 1992). Despite these defects of mineralisation, dentinal tubules are uninterrupted in this zone, indicating that normal matrix formation has occurred. Globular dentine is located in the circumpulpal dentine adjacent to the mantle dentine in the crown and may even extend into the root (Bhaskar, 1991; Avery, 1992).

Secondary dentine is formed after root formation has completed and contains fewer dentinal tubules than primary dentine, which are for the most part continuous with the tubules of primary dentine but not as regularly oriented. At the interface of primary and secondary dentine the dentinal tubules can bend sharply (Bhaskar, 1991). However, the ratio of mineral to organic substance is similar to primary dentine. Deposition of secondary dentine occurs slowly and continuously, but is not uniform. Greater amounts of secondary dentine can be found on the roof and floor of the coronal pulp chamber. This results in pulp space recession that increases with age

and this additional subocclusal secondary dentine helps prevent pulp exposure in older teeth.

Because the pulp and dentine act as a single functional unit, the term pulpodentinal complex is commonly used to describe both tissues and reflects their close relationship (Nanci, 2008). Another pulp protective mechanism is the above-noted development of sclerosis of the more peripheral dentinal tubules, which reduces the permeability of dentine. This feature appears to occur more readily in secondary rather than in primary dentine (Nanci, 2008).

Tertiary dentine is formed by pulp in reaction to pulpar insults. Such noxious stimuli could be dental disease (such as caries) or trauma (iatrogenically caused or by extensive erosion, attrition or abrasion) if the odontoblast processes in the insulted dentine are exposed or damaged (Bhaskar, 1991). Some affected odontoblasts will die but the surviving odontoblasts can produce tertiary dentine, also known as reactionary dentine. If the tertiary dentine is formed by undifferentiated mesenchymal pulpar cells that migrate to the zone of injury and transform into odontblasts, it is termed reparative dentine. Tertiary dentine can have varying histological appearances, including irregular dentinal tubules, less numerous or even absence of dentinal tubules, osteodentine (odontoblasts that produced tertiary dentine have become included in dentine), or a combination of these features (Bhaskar, 1991; Nanci, 2008). The quality and quantity of the tertiary dentine that is formed reflects the intensity and duration of the stimulus that initiated its formation (Nanci, 2008).

1.3.2.4 Dentine in Equine Teeth

The composition of dentine in equine teeth is similar to that of brachydont teeth. Brown staining can occur in secondary dentine from food dyes and, as noted, is seen in incisors as a “dental star” and on the occlusal surface of cheek teeth that are in wear as 5-8 brown, linear zones (Dixon and du Toit, 2010). Although it is not clear in other species how far odontoblast processes extend into primary dentine, in equine teeth odontoblast processes were found to run through the full thickness of dentinal tubules as far as the dentino-enamel junction (Kilic et al., 1997a). The odontoblasts

reside in predentine at the periphery of the pulp cavity and retain the capacity to produce dentine even when they seem to be in a morphological resting phase.

Although intratubular dentine is present in the dentition of several mammalian Orders, including Primates and Carnivora, it is found in highest amounts in the dentition Artiodactyla and Perissodactyla species (Bradford, 1967) and this feature is highly developed in equine teeth (Takuma et al., 1966; Boyde, 1997). The term peritubular dentine was formerly used because the dentine forms a collar around the dentinal tubules (Avery, 1992). However, intratubular dentine is a more accurate term since the dentine is formed within the dentinal tubules. Intratubular dentine is deposited around odontoblast processes in the dentinal tubules after the collagen in intertubular predentine is formed. Intratubular dentine is more wear-resistant compared to intertubular dentin, due to its higher degree of mineralisation. The absence of collagen in intratubular dentine is an important factor in achieving this higher mineralisation, since collagen can accommodate but also obstruct dental mineralisation (Boyde, 1997).

In a scanning electron microscopy (SEM) study of equine incisors, higher levels of intratubular dentine were found more peripherally than centrally (Muylle et al., 2001). The amount of intratubular dentine deposited within the tubules is asymmetrical. The dentinal tubule walls (composed of intratubular dentine) that face the dentino-enamel junction were thicker than the tubule walls closest to the centre of the tooth, and this asymmetry increased towards the dentino-enamel junction. The maximum thickness of intratubular dentine was found in the peripheral aspects of the tubules, approximately 300-500 μm from the dentino-enamel junction. This is in contrast to the SEM findings of Kilic et al. (1997a) who showed that the asymmetry in intratubular dentine distribution was greatest closest to the pulp. Moreover, it was observed that deposition of intratubular dentine increased gradually from the dentino-enamel junction to the primary-secondary dentine interface (i.e. more centrally) and that the maximum thickness of cheek teeth intratubular dentine was found halfway between the dentino-enamel junction and the pulp.

Muylle et al. (2001) also studied the course of the dentinal tubules in equine incisors that was found to be almost straight, from the occlusal tip of the pulpal chamber towards the occlusal surface, whereas at the labial and lingual sides of the tooth the tubules were s-shaped from the pulp towards the dentino-enamel junction. Crowding of the tubules occurred near the pulp.

Similar to the above described brachydont dentine, equine dentine can be divided into primary dentine, secondary dentine (regular or irregular) and tertiary dentine (reactionary or reparative) (Fig 1.4). Odontoblasts in equine teeth normally deposit regular secondary dentine and later irregular secondary dentine on the periphery of the pulp horn. Secondary dentine gradually reduces the size of the pulp and eventually the pulp horn becomes completely filled. Irregular secondary dentine is deposited last subocclusally in the centre of the pulp cavity. Just like regular secondary dentine, it is a physiologic type of dentine (although it grossly appears like tertiary dentine) that prevents pulp exposure with normal wear and it has been shown to be present in every normal equine and donkey cheek tooth histologically examined (Dacre et al., 2008a; du Toit et al., 2008b).

The median depth of subocclusal secondary dentine in equine mandibular and maxillary cheek teeth was 10.8 and 9.0 mm respectively and did not increase with age (White and Dixon, 2012). This is in contrast with donkey teeth where an increase in subocclusal secondary dentine appears to occur with age and where the mean depth of occlusal secondary dentine was similar: 14.6 mm in mandibular and 13.4 mm in maxillary cheek teeth (du Toit et al., 2008a). A thinner median depth of occlusal secondary dentine was found over matched pulp horns in 49% of overgrown equine teeth compared to control teeth. Moreover, the height of dental overgrowths was greater than the depth of occlusal secondary dentine in at least one pulp horn in 14/24 overgrown teeth (Marshall et al., 2012).

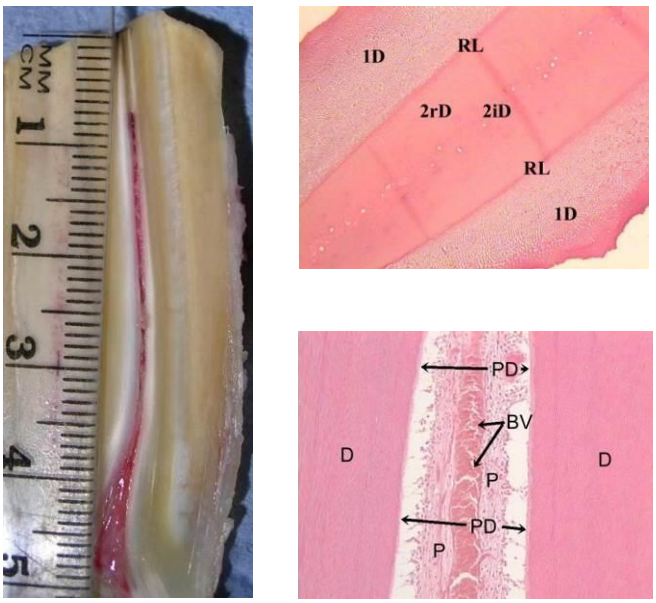


Fig 1.4. Decalcified transverse histological section through pulp horn at two levels of a mandibular cheek tooth, more occlusally (top right), the dental pulphorn is fully sealed by secondary regular (2rD) and irregular (2iD) dentine. 1D= primary dentine; RL= rest line; more apically (bottom right) the pulp horn remains open D=dentine; BV= blood vessel; P=pulp; PD=predentine (Courtesy of P.M. Dixon).

While infection of the pulp is uncommon in horses, apparently intact odontoblast process-like structures protrude from the dentinal tubules of primary and regular secondary dentine on the occlusal surface and are exposed to the oral environment with all of its microorganisms and biochemical and pH effects (Kilic et al., 1997a). It is still not entirely clear what prevents oral microorganisms from accessing these dentinal tubules and entering the pulp. It has been proposed that the odontoblast processes at the occlusal surface are calcified and thus their tubules are impervious. Other studies suggest that these structures (also termed “laminae limitantes”) are not odontoblast processes but actually consist of collagen fibrils that represent the inner un-mineralised layer of the intratubular dentine (Muylle, 2000). If microorganisms could access the intratubular dentine, other mechanisms could possibly stop them from reaching the pulp cavity. These protective mechanisms may comprise a smear layer of ground dental tissue sealing off dentinal tubules and/or a retrograde flow of fluid in these tubules from the pulp to the occlusal surface (Holland, 1994).

1.3.3 Enamel

1.3.3.1 Composition of Enamel

Enamel is the hardest, most densely calcified tissue in the body due to its high (circa 96%) mineral content (i.e. impure hydroxyapatite) and crystalline arrangement with 4% organic and water components (Bhaskar, 1991). However, enamel is brittle and is also permeable to certain dyes (Tarbet and Forsick, 1971), other organic components (Borggreven et al., 1977), inorganic ions (Byers and Yoon Lin, 2003), radioactive tracers (Braden et al., 1971; Joyston-Bechal et al., 1971), light (Bhaskar, 1991) and aqueous fluids (Poole et al., 1963; Lindén, 1968; Shellis, 2000). It can act as a semipermeable membrane that permits complete or partial passage of certain molecules (Bhaskar, 1991) and this permeability appears to decrease with age (Bertacci et al., 2007). Enamel is shiny and because it is translucent, it gains its colour from the underlying dentine. Immediately after enamel substrate is secreted, mineralisation starts. Enamel has no cellular inclusions making it an inert or "dead" tissue. While dentine and cementum contain collagenous proteins (i.e. connective tissue) reflecting their mesodermal origin, the small organic component of enamel consists of keratin proteins revealing its ectodermal origin. The organic matrix of enamel consists of several enzymes and enamel proteins. Amelogenin makes up 90% of these enamel proteins, the remaining 10% of enamel proteins are enamelin and ameloblastin (Nanci, 2008).

1.3.3.2 Histology: Structure of Enamel

Enamel hydroxyapatite crystallites are much longer than those present in dentine, cementum and bone. Individual enamel crystallites, that are approximately 0.05-1 µm long and 30 nm wide, are organised in somewhat cylinder-shaped bundles, known as rods (or prisms). These rods are approximately 4-12 µm wide (9 µm long) although the apparent width of each rod depends on the plane of section. From the dentino-enamel junction to the enamel surface the diameter of the rods increases twofold (Bhaskar, 1991).

In brachydont teeth, enamel rods are orientated at right angles to the dentinal surface, but seldom run in an entirely straight line. Instead they follow a wavy pattern, with alternating clockwise and counter-clockwise orientation of rods. These changes in

orientation can be seen as alternating dark and light strips or "Hunter-Schreger bands" under oblique reflected light (Bhaskar, 1991).

The crystallites run mainly with their long axes in the general direction of the longitudinal axis of the rods, although there is a variation in orientation of crystallites within and between rods. The inter-rod enamel surrounds each rod and contains crystallites which are oriented in a different direction from those in the rods. The differences in direction of crystallites between rod and interrod enamel are especially significant in this area where rod and interrod enamel are not connected, but are separated by a narrow space containing the organic substance, known as rod sheath.

The deposition of enamel during crown formation results in brown-stained bands visible on ground sections which are known as incremental lines of Retzius or Striae of Retzius. These lines are concentrically arranged on transverse ground sections and resemble the growth rings of a tree. Incremental lines reflect differences in structure and degree of mineralisation during enamel growth. Although they are generally considered as a normal feature due to a weekly rhythm in enamel production, metabolic disturbances can cause the incremental lines to broaden and be more prominent, indicating abnormal enamel deposition (Bhaskar, 1991).

The enamel surface may be smooth or may have fine brownish grooves (Avery, 1992). Those grooves are orientated horizontally in a wave-like pattern and result from the Striae of Retzius terminating on the enamel surface, but at the enamel surface they are called perikymata. They are continuous around the tooth and are directed parallel to each other and the cementoenamel junction.Their regular course and the distance between perikymata decreases near the cervical margin in brachydont teeth (Bhaskar, 1991).

Between the Striae of Retzius, less distinct lines can be observed in ground sections when rods are cut longitudinally, that are known as cross-striations (Bhaskar, 1991). Since enamel matrix is formed in a rhythmical manner, cross-striations appear between rod segments as dark transverse striations, especially in regions that are insufficiently calcified or after the action of mild acids on enamel (Chandra et al.,

2004). The distance between adjacent cross-striations reflects the daily enamel deposition by ameloblasts (FitzGerald, 1998).

Fissure-like structures or "cracks" which actually represent the outer surfaces of enamel lamellae can be seen on almost all tooth surfaces (Bhaskar, 1991). Enamel lamellae are thin, leaf-like structures that originate from the dentino-enamel junction and extend at right angles through the enamel to the enamel surface. It is possible to differentiate between artefactual cracks caused by sample processing and lamellae in an enamel section by decalcification, after which iatrogenic cracks will disappear but the lamellae can still be observed (Kumar, 2014). The best way to visualize lamellae is in a horizontal (transverse) dental section, since these lamellae extend in a longitudinal and radial direction from the tip of the crown to the cervical region. Type A lamellae are only present in enamel and contain poorly calcified rod segments. Type B lamellae may reach into dentine and consist of degenerated cells. Type C lamellae may also reach into dentine and contain organic matter that presumably originates from saliva, which fills up the fissures in erupted teeth. If connective tissue from the dental sac remains in an enamel fissure, cementum may be formed, creating lamellae partly or entirely made up of cementum (Bhaskar, 1991).

1.3.3.3 Histology: Types of Enamel

Enamel in general can be divided into different patterns according to the morphology of its rods, and by the arrangement and proportions of the rod and inter-rod enamel. In pattern 1 enamel the rods have distinctive cylindrical boundaries, which is in contrast to pattern 2 and 3 enamel where the rods have incomplete cylindrical boundaries so that they appear horseshoe shaped or resemble fish scales when observed from the enamel surface. In pattern 2 enamel, the rods are arranged in vertical rows whereas in pattern 3 enamel the rods show a horizontal arrangement. In pattern 1 enamel the interrod enamel envelops tunnels of rods, in pattern 2 the vertical rows are divided by sheets of interrod enamel and each rod is connected by narrow bridges to the interrod enamel on either side. Interrod enamel in pattern 3 is attached as a tail below each rod head (Boyde, 1997). Hydroxyapatite crystals are

orientated parallel to the long axis of the heads of rods, where the tails of rods show a deviation of 65 degrees (Bhaskar, 1991). The tails of each row of rods fit between the heads of the rods in the row below and all are connected by narrow enamel bridges (Boyde, 1989). These bridges reflect a small area of the circumference of the rod where is no space or rod sheath between the rod and inter-rod enamel, and in this region the crystals of rod and interrod enamel are confluent (Nanci, 2008).

All these enamel patterns can be found in most (brachydont and hypsodont) teeth, although different groups of mammals generally show one predominant pattern and different gradations may exist between and within teeth. In Primates, Carnivora, Pinnipedia and Proboscidea pattern 3 predominates, while pattern 2 is the most common form in Perissodactyla (includes horses), Artiodactyla, marsupials, lagomorphs and rodents. Pattern 1 enamel can often be found just under the coronal surface in most mammals, but in Sirenia, Tapiridae, Chiroptera and Insectivora this pattern seems to be more abundant throughout the teeth (Boyde, 1989; Hillson, 2005a).

1.3.3.4 Enamel in Equine Teeth

In equine teeth peripheral cementum that is dull and chalk-like, overlies the shiny enamel except on the occlusal surface, the labial aspect of the incisors and more occlusally on the sides of cheek teeth, where it is usually worn away. Deciduous incisors have a whiter appearance than the permanent incisors because they have less (peripheral) cementum covering the enamel (Dixon and du Toit, 2010).

The enamel patterns in equine teeth are described as Equine Type-1, -2 and -3 enamel (Kilic et al., 1997b) (Fig 1.5). Equine teeth mainly consist of Equine Type-1 and Type-2 enamel with smaller amounts of Equine Type-3 enamel. Equine Type-1 enamel is located at the dentinoenamel junction on the inner aspect of enamel folds. On transverse dental sections, it can be recognised by its rounded or oval rods arranged in parallel rows between laminae (sheets) of inter-rod enamel. The enamel rods run parallel to each other with no decussation (i.e. interweaving). Its rods are usually angled at 45 degrees to the dentinoenamel junction and the occlusal surface. Equine Type-1 enamel is found in high amounts in cheek teeth.

Equine Type-2 enamel can be found at the cementoenamel junction on the periphery of the enamel layer with horseshoe to keyhole shaped rods that are oriented at several oblique angles. Decussation of enamel is present in this type, meaning that the direction of bundles of enamel rods changes and extends in all three planes. This feature makes it more resistant to cracks than Equine Type-1 enamel where cracks can occur along rod and inter-rod lines. Type-2 enamel contains minimal or no interrod enamel. Equine Type-3 enamel is inconsistently present as a thin layer at the dentinoenamel and cementoenamel junctions and is composed of rods surrounded by large amounts of interrod enamel in a honeycomb-like structure.

Variable amounts of Equine Type-1 and Type-2 enamel are present throughout the cheek teeth. Almost all cheek teeth enamel folds contain both Equine Type-1 and Type-2 enamel although the thickness of Equine Type-2 enamel increases in peripheral enamel folds (ridges) and decreases in invaginations of folds towards the centre of the tooth. Maxillary cheek teeth contain higher amounts of Equine Type-1 enamel, while Equine Type-2 enamel is the predominant type in mandibular cheek teeth; Equine Type-2 enamel is the main enamel type present in incisors, with a much higher Equine Type-2/Equine Type-1 ratio compared to maxillary and mandibular cheek teeth (Kilic et al., 1997a).

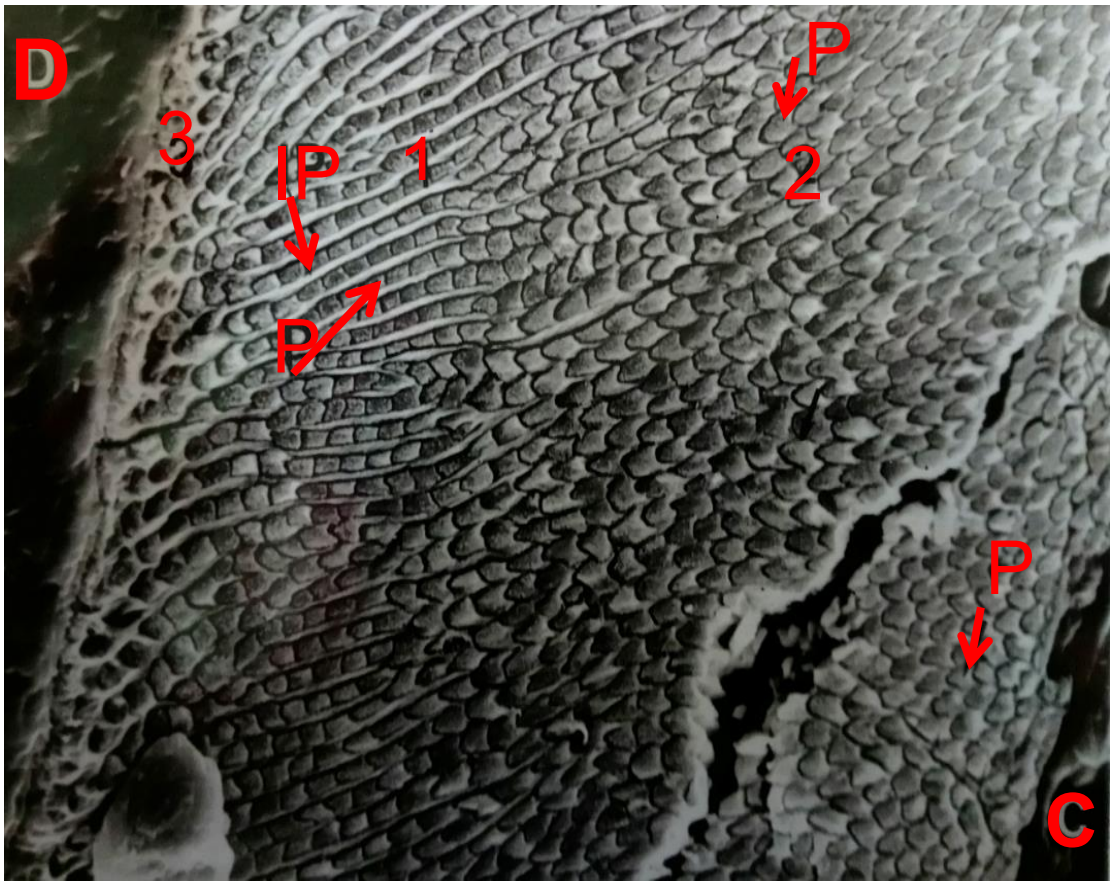


Fig 1.5. SEM image of an acid etched section of a maxillary cheek tooth (08) showing all three different types of enamel: Equine Type-1 (1), Equine Type-2 (2) and Equine Type-3 (3) enamel. D: Dentine, E: Enamel; C: Cementum; IP: Interprismatic enamel plates; P: Prisms (adapted from Kilic et al., 1997a).

1.3.4 Cementum

1.3.4.1. Composition of Cementum in Brachydont Teeth

Cementum is a cream-coloured, calcified dental tissue of mesodermal origin (like dentine) that is softer than enamel or dentine, and contains 45-50% inorganic material and 50-55% organic substance and water. The inorganic component is mainly hydroxyapatite crystals while the organic part is mainly type I collagen and proteoglycans. Cementum has the highest fluoride content of all mineralised tissues (Bhaskar, 1991). It resembles bone both grossly and histologically in that it is also a hard dense tissue containing cells residing within lacunae and their cell processes in canaliculi. However, cementum is more resistant to resorption than bone (Avery, 1992). Brachydont cementum does not contain nerves or blood vessels (although

equine cementum can [Mitchell et al., 2003]) and although there are many blood vessels near its surface in the periodontal ligament, they do not penetrate the cementum. The periodontal ligament and cementum form a single functional unit (Stanley et al., 1966).

Cementum only covers the root in human teeth, but in about 60% of teeth, cementum also overlaps enamel for a short distance in the cervical area of the tooth. Unlike other mineralised tissues, cementum continues to grow throughout life and increases in thickness with age (Zander and Hurzeler, 1958; Azaz et al., 1974; Schroeder, 1986; Chandra et al., 2004). Cementum is usually thicker around the apex of all teeth especially in the furcation of multi-rooted teeth in both unerupted and newly erupted teeth (Bhaskar, 1991). Cementum can also be quickly deposited in response to different insults, including (chronic apical) infection, trauma or extensive occlusal stress, thus making cementum the most adaptable calcified dental tissue (Jones, 1981; Bhaskar, 1991; Dacre et al., 2008b; Dacre et al., 2008c).

1.3.4.2. Histology of Cementum in Brachydont Teeth

Intermediate cementum sometimes also called “cementoid layer” or “hyaline layer of Hope-Well Smith” is produced by the inner layer of epithelial cells of the root sheath. Thereafter the epithelial cell layer disintegrates and the epithelial cells migrate into the periodontal tissues. Intermediate cementum is therefore the first layer of calcified dental tissue deposited, sealing off the underlying dentinal tubules. It is an amorphous, acellular, highly calcified layer with a harder consistency than the adjacent cellular cementum and dentine. The predominant protein in intermediate cementum is an enamelin protein, unlike in secondary cementum where collagen is the main protein (Avery, 1992).

Acellular extrinsic fibre cementum (also called primary cementum) is found from the cervical margin to the apical third of the tooth and is formed after Hertwig’s epithelial root sheath disintegrates. Cementoblasts (fibroblast-like cells) appear along the predentine and their cell processes contact and penetrate between the fibres of unmineralised dentine. They produce and deposit collagen fibrils and fibres between

these structures, so that cementum and dentine intermingle. Thereafter the mineralisation process that started in the predentine reaches the primary cementum and mineralisation of the intermingled dentine and cementum fibres occurs. This brachydont deposition pattern is in contrast to rodent cementum, where no intermingling of fibres occurs as the dentine is already mineralised before any cementum is formed (Nanci, 2008). The organic component of primary cementum is mainly made up of Sharpey's fibres. These are bundles of large extrinsic fibres produced by fibroblasts of the periodontal membrane which cross the periodontal space and become anchored in the alveolar bone (Butler, 1991). Cellular intrinsic fibre cementum or secondary cementum covers the middle to the apical third and furcations of the brachydont tooth. Cementoblasts become entrapped in the matrix they produce, their secretory activity then decreases and these mature cells are then called cementocytes. They are located in irregular shaped lacuna and their cell processes lie within canaliculi that extend into the matrix and are nourished by diffusion (Nanci, 2008). Secondary cementum is only formed in brachydont teeth after the tooth comes into occlusion. Sharpey's fibres can also be found in secondary cementum but only in small amounts. Intrinsic fibres predominate in secondary cementum which are smaller than extrinsic fibres (Butler, 1991).

Mixed cementum (alternating layers of acellular and cellular cementum) is located at the apical portion and furcations of the tooth. Acellular afibrillair cementum forms spurs and patches over enamel and dentine in proximity to cementoenamel junction (Nanci, 2008).

Incremental growth lines in cementum are caused by incremental (appositional) growth, meaning that the growth is accomplished by the addition of new layers to previously formed layers. The formation of these incremental growth lines have an annual rhythm and are in synchrony with the annual incremental growth cycle of periosteal bone and sometimes of dentine (Burke and Castanet, 1995). Under polarised light the subsequent layers appear as alternating light and dark bands, in part due to changes in orientation of the sites of insertion of Sharpey's fibres. Other reasons for this layered pattern are differences in:

- Density of cementocyte lacunae
- Density of collagen fibres versus ground substance
- Density of extrinsic fibres versus intrinsic fibres
- Degree of mineralisation of fibres

Growth zones appear as thick, opaque, chromophile, isotropic bands (uniformity in all directions; dark bands under polarised light). More cementocytes are incorporated in cementum when cementum is growing rapidly than when growing slowly (Jones, 1981; Hillson, 2005b). Rapidly growing cementum contains more Sharpey's fibres with poorer mineralisation and more intrinsic fibres relative to ground substance compared to areas with slower growing cementum (Hillson, 2005b).

1.3.4.3. Cementum in Equine Teeth

Under polarised light in transverse undecalcified ground sections, two regions can be distinguished in equine cementum, especially near the occlusal surface in older teeth. Adjacent to the peripheral cementoenamel junction, there is an irregular orientation of hydroxyapatite crystals similar to that present in maxillary cheek teeth infundibular cementum. More peripherally, the hydroxyapatite crystals have a more regular pattern and are arranged in similar concentric orientations forming peripheral lines (Dixon and du Toit, 2010).

Cementum deposition in equine teeth occurs throughout life, both on the reserve crown before it erupts and on the roots, respectively termed coronal cementum and root cementum (Dixon and du Toit, 2010). This means that new Sharpey's fibres are formed on the reserve crown, thus allowing the prolonged eruption of equine teeth, while the newly formed cementum also contributes to the structure and mechanical strength of the clinical crown, protecting the underlying peripheral enamel from cracking and helping to form the enamel ridges on the occlusal surface (Dixon and Copeland, 1993). Once the tooth exits the alveolus with its tight spatial restriction, additional cementum is deposited on the clinical crown, creating a thicker peripheral

cemental layer on the clinical than the reserve crown (Mitchell et al., 2003). In this area many lines of arrested growth can be observed. These lines are continuous from apical to occlusal and run parallel to the peripheral aspect of the tooth (Fig 1.6).

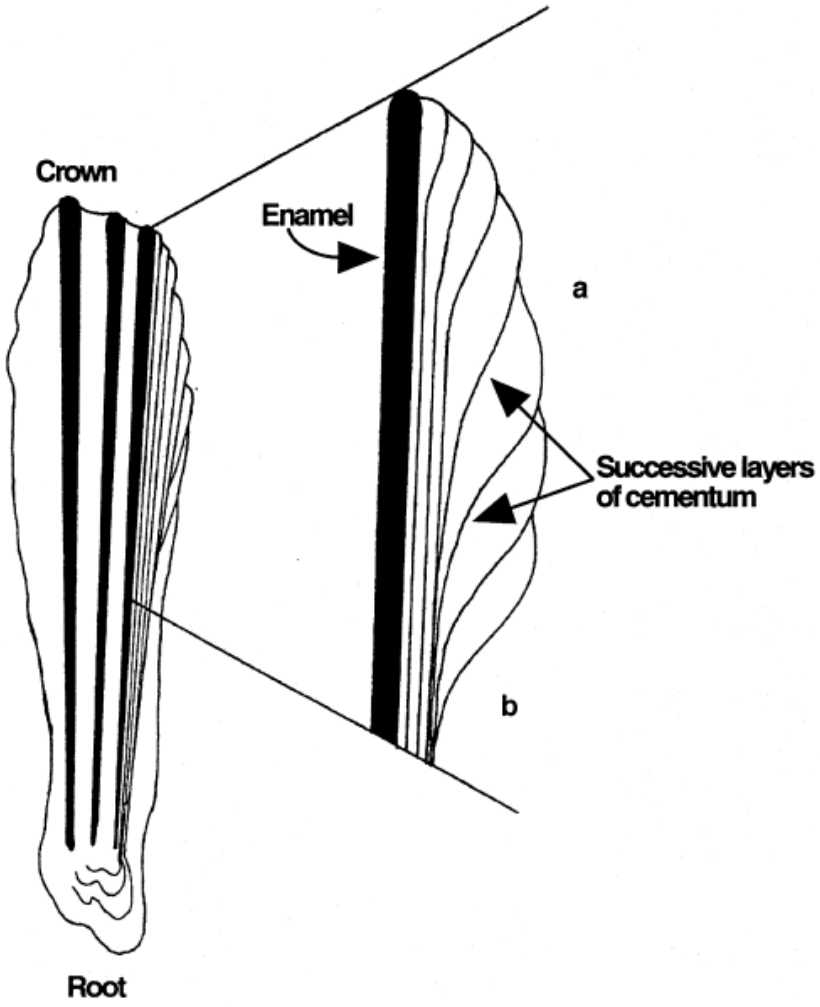


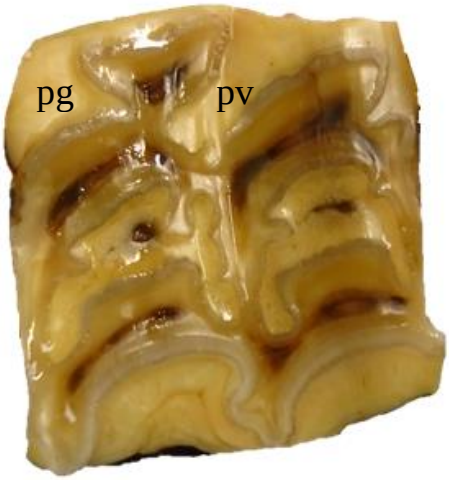
Fig 1.6. Equine dental cementum deposition in successive layers shown in a longitudinal cross section of a mandibular cheek tooth. a) Successive layers of cementum deposited above the gingiva are not parallel in section; b) Successive layers of cementum deposited below the gingiva are parallel in section. From Burke and Castanet (1995).

Because cementum deposition only can occur if there is an effective blood supply, cementum cannot be deposited more than a few millimeters away from the gingival vasculature on the clinical crown (du Toit et al., 2008a) nor on the more occlusal or apical aspect of the infundibulum, unless some gingival (Suske et al., 2016b) or apical vasculature remain, respectively (Fitzgibbon et al., 2010). The peripheral

cementum on the clinical crown can therefore be regarded as an inert tissue although active vasculature has been shown to extend from the gingival margin beneath the cemental surface on a few mm of the clinical crown (Mitchell et al., 2003; du Toit et al., 2008b) and Suske et al. (2016b) showed that infundibular cementum of newly erupting cheek teeth still had blood supply through a lateral channel so that infundibular cementum deposition still can occur for some years in the erupted clinical crown, after loss of the occlusal dental sac vasculature. In older horses with advanced dental wear, the roots (containing dentine and cementum) with heavy cemental deposits can become exposed on the occlusal surface which quickly wear away since it lacks (hard) enamel, thus creating a smooth occlusal surface also known as a smooth mouth.

Mandibular cheek teeth contain more peripheral cementum than maxillary cheek teeth (du Toit et al., 2008a), but maxillary cheek teeth and mandibular cheek teeth proportionately contain much more peripheral cementum than incisors and canine teeth. However, the thickness of cementum in cheek teeth varies greatly and depends mainly on the degree of peripheral enamel infoldings (Fig 1.7, Fig 1.8). If these enamel infoldings are deep, then a thicker cementum layer will be present in them. This is most obvious in the two deep lingual (medial) infoldings in each mandibular cheek tooth (entoflexid, metaflexid) where especially towards the tooth apex the peripheral cementum can become fully enclosed by the enamel folds, resembling infundibulae of the maxillary cheek teeth (Dixon et al., 2010). In the maxillary cheek teeth, the preprotoconal groove and the postprotoconal valley are the two major enamel infoldings filled by periperhal cementum. Most infundibulae are incompletely filled with cementum at eruption (Fitzgibbon et al., 2010).

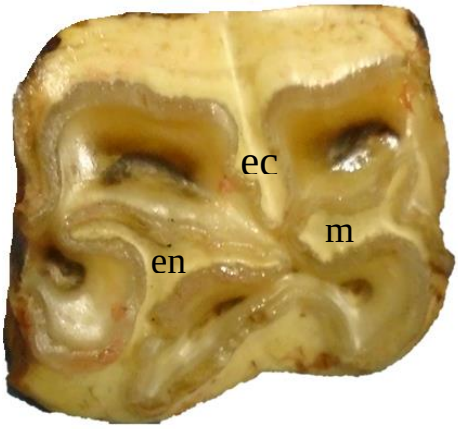
Palatal aspect



Buccal aspect

Fig 1.7. Occlusal surface of a maxillary cheek tooth showing the preprotoconal groove (pg) and the postprotoconal valley (pv).

Buccal aspect



Lingual aspect

Fig 1.8. Occlusal surface of a mandibular cheek tooth showing the entoflexid (en), metaflexid (m) and ectoflexid (ec).

1.3.4.4. Infundibular Cementum in Equine Teeth and Cemental Hypoplasia

Each infundibulum has a central occlusal blood supply originating from the dental sac in the unerupted tooth. However, the occlusal blood supply is lost after eruption by loss of the overlying dental sac. As noted, Suske et al. (2016b) have recently described an additional infundibular blood supply coming from the rostral (mesial) aspect of the tooth for the rostral (mesial) infundibulum and from the caudal (distal) aspect of the tooth for the caudal (distal) infundibulum. This lateral opening of the distal infundibulum, is wider and more apical than the lateral opening of the rostral infundibulum and thus remains functional for a longer period of time. This allows more complete cementogenesis in the distal than in the rostral infundibulum. When the blood supply is eventually lost at the lateral aspects of infundibulae, the infundibular cementum now becomes an inert tissue.

Cementum is deposited within the infundibulae in an occluso-apical direction (Suske et al., 2016b) and Joest (1915, 1926) and Joest et al. (1922) state that there is an incomplete filling of maxillary cheek teeth infundibulae at the time of eruption (i.e. cemental hypoplasia). Baker (1974) found incomplete cementum filling in the depths of the infundibulae in 43% of erupted teeth, even in teeth with a normal occlusal surface. Connective tissue remnants were present in the infundibular cementum but there was no cementoblast activity within these infundibular defects that often contained food material. Moreover, later studies showed an even higher prevalence of 85% (Fitzgibbon et al., 2010) and 90% of apparently normal maxillary equine cheek teeth to have infundibular cemental defects (Windley et al., 2009). Because of its high prevalence in healthy cheek teeth, cemental hypoplasia (Fig 1.9) could even be considered as a normal anatomical variation (Kilic et al., 1997c).

Infundibular cemental hypoplasia is most often seen in maxillary 09's and any cementum that is present adjacent to these cemental defects is usually porous. Premature removal/loss of the overlying deciduous tooth (only for the premolars- i.e. first three cheek teeth) or premature eruption of a cheek tooth that damages the dental sac causing a premature loss of infundibular blood supply could lead to

underdeveloped infundibular cementum in the underlying permanent tooth (Kilic et al., 1997c; Dixon, 2002).

In contrast to this most common type of cemental hypoplasia termed central infundibular hypoplasia, where hypoplasia is found at the site of the previous central infundibular vasculature (Kilic et al., 1997c), a less common type of cemental hypoplasia termed junctional cemental hypoplasia can occur at the cementoenamel junction. Junctional cemental hypoplasia appears to be caused by a failure of disintegration of the reduced enamel epithelium or failure of resorption of the adjacent enamel surface (Kilic et al., 1997c). This type of hypoplasia is mainly found in incisor infundibula and its clinical importance is doubtful because equine incisors rarely show evidence of infundibular caries (IC).

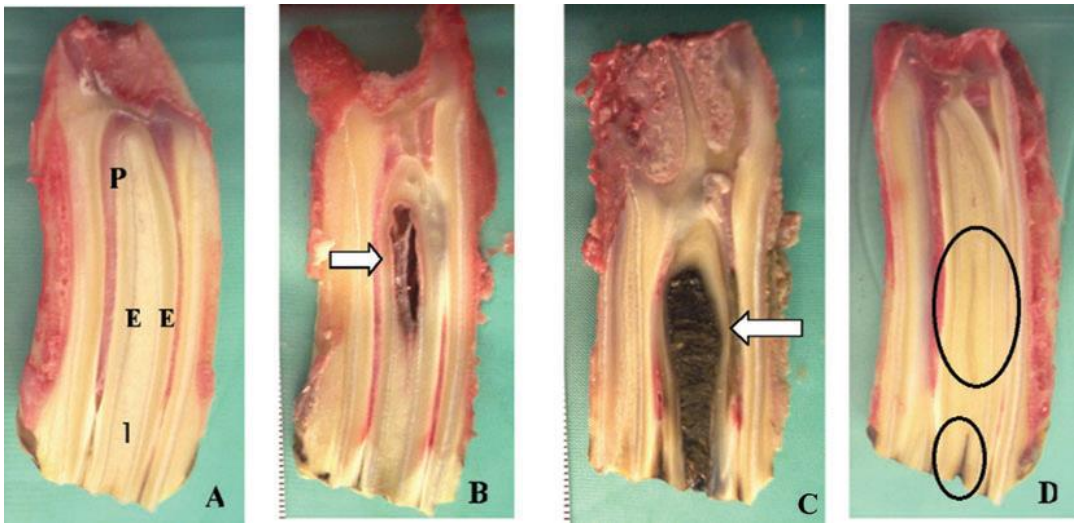


Fig 1.9. Transverse sections of 4 equine maxillary cheek teeth with different infundibular cementum features. A: Infundibulum (I) totally filled with grossly normal cementum, P = pulp horn, E = infundibular enamel. B: Infundibulum with extensive hypoplasia of infundibular cementum at its apical aspect (arrow), that contains folds of soft tissue. C: Infundibulum (with apical dilation) that has occlusal exposure and is completely filled with food debris and carious cementum with extension of caries into enamel in some areas (arrows). D:Infundibulum with a small, discoloured cemental defect on the occlusal surface (small oval) and a linear, central area of pale brown, porous appearing cementum running through much of the length of infundibulum (large oval). From Fitzgibbon et al. (2010).

1.3.4.5. Deposition of Peripheral Coronal Cementum Following (Partial) Enamel Resorption in Equine Teeth

According to Kilic et al. (1997c), cementum can be deposited both directly and indirectly on the enamel surface. When indirectly deposited, a thin, smooth, mineralised layer was found between the cementum and the enamel, which caused a weak connection between these tissues. This layer of cementum could easily be separated from the enamel, especially in dried specimens.

However, a later SEM study (Sahara, 2014) reported that only a direct attachment of equine cementum to enamel was present, suggesting that the observation of an indirect attachment was due to the small size of the cementoenamel junction in the sectioned or crushed specimens used by Kilic et al. (1997c). Direct deposition of cementum could occur on a resorbed or an unresorbed enamel layer.

The surface of resorbed enamel contains depressions of different shapes and sizes, whereas unresorbed enamel has a honeycomb-like, pitted appearance surrounding these areas of resorbed enamel (Kilic et al., 1997c; Sahara, 2014). X-ray emission micro-analysis showed that once cementum is laid down the underlying enamel cannot mineralise any further (Jones and Boyde, 1974).

The cementoenamel junction possesses great mechanical strength because of the enlarged interface area created by the interlocking of the enamel depressions and the overlying cementum (Jones and Boyde, 1974). The 7-fold difference in strength between equine and bovine teeth in the region of the cementoenamel junction could also be explained by the strong mechanical interlocking provided by the hemispherical-shaped cementum protrusions present in equine teeth (Wang et al., 2006).

The resorption of enamel may be an immunologically-mediated process, where the disintegrating enamel epithelium exposes the enamel and so attracts odontoclasts because of its inert nature (Kilic et al., 1997c). Sahara (2014) also observed that the detachment of strands of enamel epithelial cells was followed by resorption of enamel. TRAP (i.e. tartrate-resistant acid phosphatase)-positive mononuclear cells infiltrated and became attached to the remaining ameloblasts of the enamel surface. Thereafter these cells became osteoclasts and began the enamel resorption, creating

excavations (Howship's lacunae) in the enamel surface (Jones and Boyde, 1974, 1994).

Enamel resorption begins at the occlusal aspect, where the odontoclasts moved laterally, leaving a thin TRAP-positive and toluidine blue-positive layer and proceeds in an apical direction (Sahara, 2014). The resorption process occurred in waves within a short period of time, creating a pattern of bands and rings of resorption bays. The thin organic (TRAP-positive and toluidine-positive) layers are also called cementum lines/reversal lines (Sahara, 2014) and in brachydont teeth these are known to be filled with osteopontin and sialoprotin which are major matrix proteins containing the cell adhesion motif arginine-glycine-aspartic acid (Nanci, 2008). They appear to promote the adhesion of certain cell types, including osteoclasts.

1.3.4.6. Histological Classification of Peripheral Coronal Cementum According to its Order of Deposition in Equine Teeth

Coronal cementum can be classified histologically by order of its deposition at the level of the reserve crown. Primary cementum is formed on the irregular, rough cementoenamel junction surface of the reserve crown. This relatively thin layer consists of intrinsic cellular cementum, without Sharpey's fibres and can be found over the apical, middle and subgingival aspects of the reserve crown. The middle aspects of the reserve crown is then covered by a layer of secondary cementum with a large amount of (extracellular) Sharpey's fibres that is oriented perpendicular to the tooth surface. Incremental lines are present on the border area from the mid to the subgingival regions. Tertiary cementum is deposited in thick layers subgingivally with asymmetrical lenticular incremental growth lines. Sahara (2014) reported that in horses the PDL fibres were mainly inserted into secondary and tertiary cementum.

Blood vessels are present in equine primary, secondary and tertiary cementum, especially in secondary cementum (Mitchell et al., 2003). Areas which were particularly well-vascularised included cementum of the reserve crown as well as the infoldings of cementum (i.e. entoflexid and metaflexid of mandibular cheek teeth

and postprotoconal valley and preprotoconal groove of the maxillary cheek teeth) (Mitchell et al., 2003).

1.4 Oral Environment

1.4.1 Saliva

Besides having a role in digestion and taste, saliva is also important for protecting the teeth and other oral structures, which potentially is an entrance port for micro-organisms. Saliva contains lysozyme, an enzyme that hydrolyses bacterial cell walls, and lactoferrin that has the capacity to bind iron that many bacteria need to survive. Additionally, a physical barrier is provided by the salivary mucins (Nanci, 2008). In contrast to some other mammals, little or no secretory IgA was found in equine parotid saliva (Genco et al., 1969).

Its lubricant properties helps saliva mechanically flush the mouth. Saliva also has a buffering effect because it contains $HCO3^-$, ammonia, urea, calcium, phosphate and other ions. The pH of human saliva is approximately 6.0-7.5 (usually measured ex vivo) and in horses is between 7.3-7.9 (Baker, 1979; Bardow et al., 2004). However, the pH and buffering capacity of saliva also depends on its secretion rate. An increased saliva secretion rate is associated with higher pH values and an increased buffering capacity due to higher levels of sodium and bicarbonate (Kidd, 2005). The calcium, phosphate and fluoride components of saliva also play an important role in maintaining and restoring tooth integrity.

1.4.2 Pellicle, Plaque and Bacteria

A biofilm adhering to the tooth surface is termed pellicle or plaque, depending on the thickness and constitution of the layer. The initial phase of pellicle formation starts within seconds of a tooth being exposed to saliva. Acquired pellicle (AP) is a thin (0.5-1µm) proteinaceous layer, composed of proteins, carbohydrates and lipids that forms on the surface of teeth. The sources of these compounds are salivary secretions, gingival crevicular fluid, oral epithelial cell products, and products of micro-organisms (Hannig and Joiner, 2006; Siquera et al., 2012). Bacteria are observed to adhere to acquired pellicle within three minutes of exposure of teeth to

saliva (Hannig et al., 2007) and the proteins in the pellicle have specific receptors for bacterial adhesins that facilitate this process (Douglas, 1994; Hannig et al., 2007). Then pellicle plays an important role in oral lubrication, regulation of mineral homeostasis and host antimicrobial defense (Siquera et al., 2012).

Plaque is a biofilm that mainly consists of an organic matrix of salivary mucins (mucopolysaccharides, the major glycoprotein components of mucus) and extracellular polysaccharide polymers with attached micro-organisms (Soames and Southam, 2005). As the biofilm matures, the microbial community becomes more complex. The rate of growth of dental plaque depends on the availability of nutrients, competition with other micro-organisms and environmental conditions in the biofilm (Chávez de Paz et al., 2008). In humans the microbial community of supragingival plaque differs from that of subgingival plaque (Costalonga and Herzberg, 2014). Predilection sites for plaque to accumulate are often at mechanically protected areas (Buchalla, 2013). In horses these sites mainly appear to be the inderdental (interproximal) spaces.

Supragingival plaque can have a structured architecture with polymere-containing channels or pores/voids (black holes) connecting the dental surface with the oral cavity (Auschill et al., 2001; Marsh, 2005). The micro-organisms in this biofilm can have an uneven spatial arrangement (Auschill et al., 2001). The most viable bacteria were observed in the central part of the plaque and lining the voids, where effective diffusion of nutrients could take place. Dead bacteria surrounded the viable bacteria, meaning that these dead bacteria were found both closest to the tooth surface and the oral cavity. The function of this dead biological material might be to protect the underlying living micro-organisms (Auschill et al., 2001).

1.5 Dental Abnormalities

1.5.1 Calculus

Calculus is composed of mineralised dental plaque (in horses predominantly calcium carbonate) and since the mineral component is mainly derived from saliva, calculus

is most abundant on teeth that are close to the orifices of salivary ducts (Jubb et al., 1985).

1.5.2 Hypercementosis

Hypercementosis is an abnormal thickening of cementum that may be diffuse or circumscribed in a certain areas of a tooth but it can also occur in multiple areas within a single tooth, in several teeth or all teeth can be affected (Bhaskar, 1991). The term cemental hypertrophy is used if the cemental overgrowth improves the functional qualities of cementum, while cemental hyperplasia means that the overgrowth is not correlated with increased function or occurs in nonfunctional teeth. In cemental hyperplasia, decreased amounts of Sharpey's fibres are embedded in the cementum. If cemental hypertrophy is localised, then a spur can develop that provides a larger surface area for fibre attachment which improves anchorage of the tooth to the surrounding alveolar bone, especially in areas exposed to great stress.

Localised hypercementosis may involve embedded calcified bodies developing around degenerated epithelial cell rests which are termed excementoses (Bhaskar, 1991).

Extensive cemental hyperplasia is a circumscribed form of hypercementosis surrounding the root like a cuff and can sometimes be associated with chronic periapical inflammation (Bhaskar, 1991). Irregular overgrowth of cementum with spike-like extensions, calcification of Sharpey's fibres and the presence of cementicles may occur in response to injuries to the cementum (Bhaskar, 1991).

1.5.3 Dental Caries

Dental caries is defined as a demineralisation of the calcified dental tissues (i.e. inorganic part/hydroxyapatite crystals) and destruction of its organic components, and in humans it is mainly caused by acid-producing bacteria, that often causes brown staining of affected dental tissues (Soames and Southam, 2005).

The four prerequisites for a caries lesion to develop are: tooth, substrate, plaque (Keyes, 1960) and bacteria. Although different factors such as location and position, composition and morphology of the tooth may play a role in the development of

caries, caries will not develop without the presence of acidogenic bacteria and substrate (monosaccharides, disaccharides and other fermentable carbohydrates). Miller (1889) first proposed the acidogenic theory of dental caries and postulated that dietary carbohydrates were fermented by oral microorganisms and converted to acids, primarily lactic acid, but also acetic and propionic acid. These acids cause a pH drop of the pellicle/plaque and when it crosses the critical level of pH of 5.5, mineral ions are released from the hydroxyapatite crystals of enamel. The same occurs in cementum at the much higher pH level of 6.7 (Tanzer, 1992), whereas the critical pH in dentine is about 6.0 (Vanuspong et al., 2002). However the critical pH levels are not strict, since demineralisation also depends on the levels of phosphate and calcium ions in the fluid (such as plaque fluid and saliva) surrounding the tooth (Dawes, 2003). The higher these calcium and phosphate levels are, the lower the critical pH will be. Plaque fluid contains more calcium and phosphate than saliva, so the critical pH in a tooth covered by plaque will be lower than salivary pH. The concentrations of these ions in saliva and plaque fluid also vary among individuals.

The opposite effect is also possible: remineralisation of teeth occurs when the pH increases above the critical value (Soames and Southam, 2005), although this process can only take place in a subsurface lesion that creates a suitable matrix for crystal growth (such a white-spot lesion in human enamel). However, if acid has eroded the tooth surface and pellicle (or plaque) later covers this lesion, this organic layer will prevent the tooth from remineralising, even in a supersaturated mineral environment (Dawes, 2003).

Dental erosion is a different process from dental caries. Dental erosion is caused by the direct action of acids, without the mediation of bacteria dissolving exposed calcified dental surfaces (cementum, enamel and/or dentine). It occurs over a larger area compared to caries, affecting all/most of the caudal teeth with gastric reflux or all/most of the anterior teeth with dietary acids. An acidic substance is a greater challenge for the exposed tooth surface than a cariogenic substrate (i.e. fermentable carbohydrates) and the rate of acid demineralisation caused is highest in areas not covered by dental plaque. Dental plaque can form a buffer since it is partly saturated

with respect to tooth minerals so that the pH rarely falls as low as 4.0, in contrast to areas not covered by plaque where the pH can fall to 2.4 after exposure to an acid. Moreover, the rate of demineralisation caused by acids will be high since the calcium and phosphate which become dissolved will be detached from the dental surface very quickly and thus become lost immediately, especially if it is a liquid acid, whereas in caries the dissolved minerals are transported away from the tooth more slowly partly because of the overlying plaque (Buchalla, 2013).

1.5.3.1 Substrate

The pH changes on exposed dental surfaces in response to dietary fermentable carbohydrates are similar in teeth with and without caries. However, the starting pH in the plaque overlying teeth suffering from caries will be lower and therefore the pH will be under the critical level for a longer period of time (Soames and Southam, 2005).

In horses, it was proposed that the frequent feeding of high levels of concentrates (mainly containing simple carbohydrates such as grain starches) can cause caries (Dixon et al., 2010), which is supported by the acidogenic theory. Dental damage caused by low pH haylage (silage) due to the addition of excessive amounts of preservative acid should be termed dental erosion, as noted above. Gere and Dixon (2010) showed in a post-mortem study that the prevalence of equine peripheral caries (PC) in Swedish horses was highest in trotting horses, which were fed high levels of concentrates and silage as compared to less affected horses not on such diets. Diets consisting mainly of simple carbohydrates caused severe generalised severe peripheral caries in equids (horses and donkeys) working in rubbish dumps in Mexico city (Dixon et al., 2010). The computed tomography (CT), histopathological and ultrastructural appearance of both PC and IC in donkeys appeared similar to the pattern of caries in horses for all three calcified dental tissues (du Toit et al., 2008c). PC was found at low levels in donkeys fed hay, straw and grass and which never (or rarely) received silage or concentrates (Rodrigues et al., 2013). Many (55%) of these donkeys showed impaired jaw movement due to severe concurrent dental problems which could be a risk factor in the development of caries (Rodrigues et al., 2013).

When donkeys or horses suffer from concurrent dental problems the intra-oral movement of food and saliva decreases, so that plaque can accumulate and food material can stay in prolonged contact with teeth (Dixon et al., 2010).

1.5.3.2 Plaque and Bacteria

The microbial community that is formed in plaque has many advantages for the inhabiting micro-organisms (Marsh, 2005). Firstly, pioneer microbial colonisers create a micro-environment that is suitable for the attachment and growth of other micro-organisms. This process is also called coaggregation (Metwalli et al., 2013). Secondly, molecules that cannot be broken down by individual species of bacteria can sometimes be catabolised by the combination of micro-organisms living in the microbial community. Moreover, a pathogenic synergism may occasionally occur, causing the combination of organisms in the community to be more pathogenic than any individual micro-organism on its own. Additionally, because of the collaboration and gene transfer likely to occur in a microbial plaque community, the micro-organisms in a microbial consortium are more resistant to antimicrobial therapeutics, environmental stress and host defences than oral bacteria not living in a microbial plaque community (Marsh, 2005).

Another survival mechanism that oral bacteria are believed to use is a dormancy state during nutrient deprivation, when they show a state of metabolic arrest without any cell division or growth. This state is also known as a viable but nonculturable (VBNC) state (Oliver, 2010) when bacteria are less sensitive to antimicrobial agents and to changes in temperature and pH. When the bacteria regain access to nutrients after a period of deprivation, they return to their higher metabolic rates (metabolic reactivation), with resumption of cell growth and division. Chávez de Paz et al. (2008) investigated the metabolic reactivation of two oral bacteria (*Streptococcus anginosus* and *Lactobacillus salivarius*) in biofilms. A low reactivity of these nutrient-deprived oral bacteria after the introduction of nutrients was suggested to be part of their survival strategy. Additionally, the enhanced synthesis of certain proteins that could be regarded as stress proteins by oral bacteria such as *S. mutans* help bacteria to survive different suboptimal conditions (Svensäter et al., 2000).

1.5.3.3 Microorganisms Involved in Human Dental Caries

The microbiological flora in dental plaque varies between herbivorous, carnivorous and omnivorous mammalian species, while within the same dietary group the microflora appears to be quite similar (Dent, 1979). The Human Oral Microbiome Database (HOMD) includes approximately 700 prokaryote species that can be present in the human oral cavity (Chen et al., 2010). *Streptococcus species* (mainly *Streptococcus mutans* in humans) are usually the initiators of caries and can produce lactic acid, extracellular sticky glucans and intracellular polysaccharide. The extracellular sticky glucans adhere to enamel and thus allow bacteria to attach to its smooth surface. Intracellular bacterial polysaccharides can be converted to acidic end-products, even when dietary sugar is unavailable in the oral cavity. Lactobacillus species which are also acidogenic, play a role in the further progression of caries after demineralisation has occurred and when a niche for non-adhesive bacteria has been created (van Loveren et al., 2012).

However, caries has been found to be present in the absence of S. mutans. This finding led to a shift from the specific plaque hypothesis to the mixed/non-specific plaque hypothesis of caries development (Kleinberg, 2002; Kianoush et al., 2014). The latter hypothesis proposes that a small number of pathogens can cause caries or its progression, with more acidogenic bacteria included in the latter process. Viridans streptococci (*S. mutans, S. salivarius, S. sanguis and S. mitis)* are believed to be the main pathogens and first invaders in caries, followed by secondary invaders including Actinomyces, Bacteroids, Spirochetes and Lactobacillus species (Maripandi et al., 2011).

Ecological conditions are also important in the development of caries as noted in the ecological plaque hypothesis: wherein a biofilm is considered to consist of a normal resident bacterial community (eubiosis), whereas caries reflects an imbalance of plaque microflora (dysbiosis) (Kidd, 2005). A change in local environment can result in dysbiosis causing demineralization (Kidd, 2005). The heterogeneity of the local environments within a plaque leads to a wide range of bacteria making up its microbial community (Marsh, 2005).

Factors influencing the characteristics of the plaque microbiota include (1) oxygen concentration, (2) nutrient availability and (3) pH (Kianoush et al., 2014). (1) Because of close cell-to-cell contact there is a synergism between oxygen-consuming and oxygen-sensitive bacteria (Olsen, 2006). Most (72%) of the bacteria associated with human dental caries are facultative anaerobes and 28% are obligate anaerobes (Maripandi et al., 2011). (2) More nutrients become available when bacteria collaborate and thus benefit from the enzymes of each other in the catabolism of certain molecules. (3) Cariogenic and acidogenic bacteria can also be present in the healthy bacterial community at neutral pH, but only in small numbers as these bacteria are not competitive in an non-acidic environment (Olsen, 2006).

Frequent access to dietary fermentable carbohydrates or a decreased clearance of ingested carbohydrates by saliva (due to a lower saliva secretion rate), leads to more acid being produced with subsequent demineralisation of tooth substance (Kidd, 2005; Olsen, 2006). A low pH is beneficial for the growth of acidogenic and aciduric bacteria, thus enhancing their acidifying effect and predisposing the associated site of the tooth to caries. Kianoush et al. (2014) found that Lactobacillae were predominantly present in a pH range of 4.5-5.0 in carious dentine, but also could be found in more neutral environment. *Prevotella* and *Actinomyces* were found in a pH range of 5.0-5.5 (Kianoush et al., 2014).

While Firmicutes were the most dominant phylum across all pH groups, they were present in higher proportions (78%) in the most acidic conditions (pH 4.5–5.0), compared to less acidic conditions (36–53%, pH 5.0–>6.5). In communities from the most acidic lesion samples, the Firmicutes phylum was primarily (77%) represented by the *Lactobacillus* genus. In comparison, microbial populations from zones above pH 5.5 had lower frequencies (22–40%), of *Lactobacillus* and greater amounts of *Streptococcus*, *Pseudoramibacter* and *Dialister*.

The substantial core model was proposed after the finding that in a pH range of 4.5-7.8, 60% of the bacteria taxa associated with dental caries, including Leptotrichia, Prevotella and *Streptococcus salivarius* could be found in carious dentine lesions regardless of the pH (Kianoush et al., 2014). In acidic conditions, a low diversity in

microbiota was present, whereas in a neutral environment microbial populations were more variable.

Recent studies have shown that in addition to bacteria, high numbers of *Candida albicans* can be found in human dental plaque (Barbieri et al., 2007; Maripandi et al., 2011; Metwalli et al., 2013). *C. albicans* can grow as yeast, filamentous cells or pseudofilaments. Although *C. albicans* is normally a unicellular commensal of the oral cavity, it can switch to a pathogenic invasive, multiple filamentous form that can invade tissues.

Moreover, *C. albicans* and *S. mutans* appear to interact (Fig 1.10) and the presence of *C. albicans* can enhance the attachment of *S. mutans* to teeth and vice versa (Metwalli et al., 2013). *S. mutans* produces lactic acid (a source of carbon) which stimulates yeast growth. Yeast growth decreases oxygen levels and produces growth factors for bacteria (*Streptococcus*). The most common form in which *C. albicans* is present in combination with *S. mutans* is the yeast form with production of blastospores (Barbieri et al., 2007).

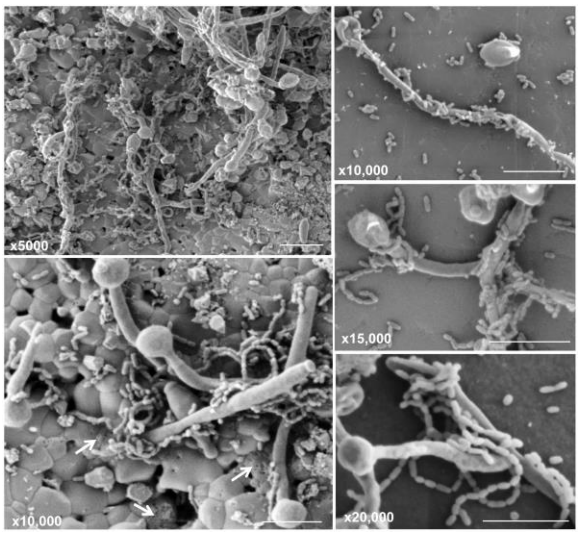


Fig 1.10. Scanning electron micrographs of mature mixed biofilms formed on discs of hydroxyapatite demonstrating the affinity of *S. mutans* to the hyphal elements of *C. albicans* (from Metwalli et al. [2013])

1.5.3.4 Microorganisms Involved in Equine Dental Caries

Recently, molecular bacteriological studies have investigated which bacteria are present in the subgingival plaque of orally healthy horses (Gao et al., 2016), and the

difference in subgingival plaque bacteria between horses with and without periodontal disease (Kennedy et al., 2016). However, little is known about which bacteria are involved in equine dental caries, although a recent study revealed the presence of a newly discovered bacteria species *Streptococcus devriesei* in infundibular caries (Lundström et al., 2007).

Baker (1979) reported that the healthy equine oral cavity often had high numbers of Streptococci and Micrococci spp., while Lactobacillus spp., Fusobacterium spp. and Coliforms were usually present in small numbers. Intermediate counts of Anaerobes, Veilonella spp. and H_2S producers were found. With progression of gingival inflammation the gram positive cocci and rods decreased in number, whilst the Gram- negative aerobes, anaerobes and spirochetes increased (Baker, 1979).

1.5.3.5 Caries in Brachydont (e.g. Human) Teeth

The first clinical sign of caries in a human tooth is a subsurface white spot caries lesion which appears white because of backscattering of light due to an increased porosity of enamel. The enamel surface layer covering a white spot lesion is more mineralised compared to the body of the lesion, but is less mineralised than sound enamel. As caries progresses, it tends follow the direction of the enamel rods, creating a cone-shaped lesion. Brown spot lesions can be caused by incorporation of exogenous food pigments in white spot lesions (Buchalla, 2013) although brown staining also has been linked to a bacterial-induced change in enamel proteins in affected areas before mineral loss (Miles and Grigson, 1990). Other possible causes for the brown staining in a caries lesion are the formation of melanins, lipofuscins or the uptake of metals or bacterial pigments, while the formation of pigments by the Maillard reaction (reaction between proteins and small aldehydes produced by bacteria) appears to be the major cause for this discoloration (Kleter, 1998; Kidd, 2005; Levallois et al., 2012).

1.5.3.5 Equine Infundibular Caries

Studies have described very diverse prevalences of equine cheek teeth IC, varying from 8% (Fitzgibbon et al., 2010) to 100% (Honma et al., 1962). This difference

could possibly be explained by cemental hypoplasia being classified as IC by Honma et al. (1962) and also to age-related differences, because the 100% caries prevalence found by Honma et al. (1962) were all in horses over 12 years of age.

Kilic et al. (1997c) examined 21 maxillary cheek teeth by light microscopy and ultrastructurally and found IC to be present in 5 teeth (23.8%): 4 involving the central infundibular cementum without involvement of the infundibular enamel and one involving the periphery of infundibular cementum. In the other 16 maxillary cheek teeth 64.7% contained 1 or 2 small channels located in the centre of infundibulae filled with shrunken connective tissue. These channels of variable depth continued occluso-apically and usually became narrower towards the apex of the infundibulae. In recently erupted teeth many lateral branches from the central vascular channels extended into the infundibular cementum and reduced in size towards the periphery of infundibular cementum and terminated adjacent to the cementoenamel junction.

Large areas of more apically located hypoplastic infundibular cementum often contained much residual connective tissue (Kilic et al., 1997c). It was suggested that when these areas eventually become exposed to the oral cavity with increasing age and occlusal wear, food and oral micro-organisms could then enter the infundibulum, causing degeneration of this connective tissue which could predispose to caries development (Kilic et al., 1997c).

Baker (1974) also proposed that teeth with cemental hypoplasia are predisposed to IC. This is supported by the finding that the maxillary 09s are usually most severely affected by infundibular cemental hypoplasia and also with IC. However, in a recent clinical survey in donkeys the 06s were the most commonly affected by IC (Rodrigues et al., 2013).

IC may lead to apical infection if caries proceeds through infundibular enamel and the adjacent dentine allowing pulp to become infected (Dacre et al., 2008c) or a pathological fracture (most often sagittal) can occur as a result of advanced caries, coalescence of the two carious infundibulae and mechanical weakening of the tooth (Dixon, 2002; Dacre et al., 2007).

The system that is most commonly used for grading IC is the modification of the Honma system described by Dacre (2005a) (Table 1.1).

Table1.1. Grading of infundibular caries by the modified Honma system (from Dacre [2005a]).

Grade	Description
0	Normal tooth i.e. no macroscopic infundibular caries visible; a small central defect at the occlusal surface of the infundibulae is considered normal.
1	Only cementum affected.
2	Cementum and underlying enamel are affected.
3	Cementum, enamel and dentine are affected.
4	Tooth integrity is affected (e.g. secondary dental fracture present).

1.5.3.6 Equine Peripheral Caries

PC can affect all layers of the tooth (cementum, enamel and dentine), so the term peripheral caries is preferred over the previously used term peripheral cemental caries (Dixon et al., 2010). The prevalence of equine PC appears to be increasing greatly in Europe. Wafa (1988) described a prevalence of 0.3% PCin 355 horse skulls in a post mortem study in Ireland and a 0.9% prevalence was found in two post-mortem dental surveys in Swedish horses Lundström and Pettersen (1988, 1990). A more recent post mortem study reported a prevalence of 6.1 per cent (31/510) PC in Swedish horses (Gere and Dixon, 2010). Likewise, a clinical survey of 800 donkeys from the Spanish-Portuguese border showed a similar prevalence of 5.9% (Rodrigues et al., 2013).

Cox et al. (2012) showed in a post mortem study that the prevalence of dental plaque overlying the interdental tooth surfaces was 100% (n=22) in the horse skulls examined. Erridge et al. (2012) found that 67% of (peripheral) caries lesions in

horses were covered by plaque that was usually thick (10-1000 µm), sometimes with food adherent to the plaque and teeth. The clinical crown surfaces of all control teeth contained pellicle (<10 µm in thickness) but no food material was present histologically in this plaque.

Gere and Dixon (2010) found that younger horses were more commonly affected by PC than older horses. This could be because the younger horses in the study were mostly Swedish Trotter horses (that had a higher prevalence of caries than the older, non-trotter horses) which were euthanised for intercurrent diseases or poor performance. PC most commonly affects the three caudal cheek teeth (Triadan 09-11). The salivary ducts drain rostrally in the mouth, therefore the buffering and lubricative effect of saliva may be less in the caudal aspect of the mouth (Gere and Dixon, 2010).

The prevalence of diastemata in a Swedish post-mortem study was significantly higher (64.5%) in horses with than without PC (45.7%) and the predilection sites for both lesions were the caudal cheek teeth interdental spaces (Gere and Dixon, 2010). This is in contrast with the clinical study of 108 horses by Ramzan and Palmer (2011) where diastemata were predominantly observed within the mandibular arcades affecting all interdental spaces and no significant association was found between diastemata and PC.

Periodontal disease can sometimes also be found adjacent to areas affected by PC (Gere and Dixon, 2010). However, Cox et al. (2012) showed that dental plaque often covered cemental “erosions”, but no statistically significant relationship was found between the amount of plaque or degree of peripheral cemental erosions and the presence and severity of periodontal disease. Rodrigues et al. (2013) found that periodontal disease was only associated with the presence of PC in 3.9 % of their cases. Concurrent IC was found in 13% (Erridge et al., 2012) and 32% (Gere and Dixon, 2010) of teeth affected by PC.

Grading of PCis generally carried out using Honma's grading system modified by Dacre (2005a) (Table 1.2).

Table 1.2. Grading of peripheral caries by the modified Honma system (from Dacre [2005a]).

Grade	Description
0	Normal tooth i.e. no macroscopic peripheral caries visible.
1.1	Only cementum affected: lesions appear as superficial erosions or pitting lesions or even as extensive erosions of the cementum surface, although there is still some underlying cementum left.
1.2	Only cementum affected: more severe peripheral caries where the cementum is completely lost in some areas of the tooth, exposing the underlying (but unaffected) enamel.
2	Cementum and underlying enamel are affected.
3	Cementum, enamel and dentine are affected.
4	Tooth integrity is affected (e.g. secondary dental fracture present).

PClesions macroscopically graded as grade 1.1 were histologically divided into two types by Erridge et al. (2012). In one type, thin layers of peripheral cementum became underrun by plaque and flaked off; in the second type, more focal, flask-like carious lesions developed and became filled with plaque. Grade 1.2 lesions showed extensive histological loss of peripheral cementum and the underlying enamel of the clinical crown became exposed in these regions. This exposure was often observed around the entire circumference of the tooth but the enamel seemed unaffected.

Because the enamel is dissolved during the histological decalcification process, it histologically appears as a blank space between the cementum and dentine. Whilst there was still a very small layer of intact cementum covering the enamel in grade 1.2 lesions, in grade 2 lesions plaque was found within the enamel space indicating that the cemental layer was fully penetrated and the enamel was now affected. Grade

3 lesions were not macroscopically found in this study, but Gram-staining showed bacteria within dentinal tubules in 63% of such sections, confirming its histological presence.

The above study of Erridge et al. (2012) showed that the macroscopic grading of carious lesions underestimated the severity of PC as compared to histopathological examination. Gram-and Picrosirius red staining of sections identified additional bacteria in dental tissues and thus revealed higher caries grades than H&E-stained slides. It was remarkable that even teeth which macroscopically and microscopically (H&E stained) appeared to have grade 1.1 lesions, turned out to be in fact to have grade 3 lesions after Gram-staining. This suggests that in equine (peripheral) cemental caries, just as in human cemental caries and donkey infundibular cemental caries, a simultaneous bacterial colonisation and demineralisation occurred (Frank, 1990; du Toit et al., 2008c), whereas in human enamel and (coronal and radicular) dentinal caries demineralisation appears to precede bacterial invasion (Frank, 1990).

1.6 Aims and Objectives

The aims of the epidemiological study are to determine the prevalence of equine PC and IC over a wide area of the UK and to assess the most common and/or most severe intraoral localisation/distribution of these dental diseases. The survey also aims to examine for possible risk factors for the development of PC and IC.

The aims of the bacteriological study are to identify which bacteria are most likely associated with PC and which ones are most likely to be associated with absence of PC (control group). The microbiota of the most commonly and severely affected cheek teeth positions of PC will be compared with less commonly and severely affected cheek teeth positions of versus the control group, in order to assess for a possible difference in microbiota between these sites. Additionally to a culture-independent study, some of the dental plaque samples will be cultured prior to performing DNA isolation, Polymerase Chain Reaction (PCR) and Next Generation Sequencing (NGS), which will be termed “culture-dependent microbiology”. These

combined procedures will be performed to ascertain if the bacteria identified by culture-independent bacteriology were alive and had the ability to grow and also to assess which bacteria would grow under standardised microbiology laboratory conditions. A further aim of the bacteriological study is to compare the microbiota of infundibulae with and without caries to assess for differences in the bacteria associated with IC and with the control group (no IC).

The aims of the pathological study are to examine PC (and IC) affected cheek teeth macroscopically, histologically and ultrastructurally to further investigate the pathological changes present in dental tissues with this disorder, and if possible, establishing the route of bacterial infection in cheek teeth affected by equine dental PC.

2 CHAPTER 2: EPIDEMIOLOGICAL SURVEY

2.1 Introduction

2.1.1 Peripheral Caries

Equine peripheral dental caries (PC) has been defined as destruction of the calcified dental tissue on non-occlusal aspects of the clinical crowns (Dacre, 2006). Limited numbers of studies have suggested that PC is a common equine disorder in the United Kingdom (Ramzan and Palmer, 2011) and also that it may be increasing in prevalence in Sweden (Gere and Dixon, 2010). Alternatively, PC may be increasingly recognised due to improved awareness of this disorder, along with the more thorough oral examinations currently being performed by many equine veterinarians and equine dental technicians.

The limited gross post-mortem (Gere and Dixon, 2010) and clinical (Ramzan and Palmer, 2011) surveys on equine PC performed to date have both shown that the caudal cheek teeth are more commonly affected with PC, suggesting localisation of the caries inducing, oral environmental changes in affected horses. Gross examination of equine teeth commonly underestimates the severity of PC in comparison to histological examination and caries that appears grossly to only affect the cementum often involves the underlying enamel or even dentine on histological examination (Erridge et al., 2012).

Honma *et al*. (1962) described grading for equine dental caries and Dacre's modification of this system (Dacre, 2005a), remains the standard grading system used to classify the severity of both infundibular caries (IC) and PC.

Equine PC is believed to be caused by acidogenic bacteria living in dental plaque (Borkent and Dixon, 2015), similar to the very well-studied dental caries of brachydont species. There is anecdotal evidence from Swedish post-mortem studies that horses fed haylage (silage) as opposed to hay, and those that receive high levels of dietary concentrates, are predisposed to PC (Gere and Dixon, 2010). Working donkeys in Mexico fed highly refined starch diet have also suffered severe PC of all teeth, including incisors (Dixon et al., 2010).

The aims of this study were to determine the prevalence of equine PC over a wide area of the UK in horses of different breeds, ages and workloads and to document the severity and intraoral distribution of these lesions by examination of detailed dental records of horses made by experienced operators. The survey also aimed to examine for possible associations between the presence of PC with diet, in particular with the feeding of haylage and concentrates, the presence of concurrent dental disorders including IC and diastemata, and frequency of routine dental care.

2.1.2 Infundibular Caries

Equine maxillary cheek tooth infundibular caries (IC) has been documented as an equine dental disorder for more than a century, including by J.F. Colyer (1906) and later by Honma et al. (1962) who, as noted, described an equine caries grading system. This system as modified by Dacre (2005a) remains the standard grading system used to classify the severity of IC as well as PC. Equine IC is believed to be caused by acidogenic bacteria present in food impacted in infundibular defects as recently reviewed (Borkent and Dixon, 2015), causing destruction of dental tissue similar to dental caries of brachydont species (Lundström et al., 2007). *Streptococcus devriesei* is a recently described bacteria isolated from equine cheek teeth IC lesions (Lundström et al., 2007) but its precise role in the aetiopathogenesis of IC remains unclear. Lundström et al. (2007) have proposed that feeding high levels of concentrates to horses provides a source of fermentable carbohydrates that predisposes to IC. A recent study (Suske et al., 2016a) has shown developmental defects of cementum to be commonly present deep in maxillary cheek teeth infundibulae, especially in the Triadan 09 positions and these can allow food impaction into infundibulae and likely predispose to IC development. Equine incisors have shallow infundibulae that have not been reported to suffer from clinical caries.

The main aims of this study were to clinically assess the prevalence of equine IC in the general equine population in different areas of the UK, and to determine the severity and the intraoral distribution of IC in affected horses. To achieve this, experienced operators were requested to use standardised criteria to complete dental charts of horses they examined. Other aims of this study were to assess for possible

associations between the presence of IC and diet, in particular with the feeding of higher levels of concentrates, the presence of concurrent dental disorders and age.

2.2 Materials and Methods

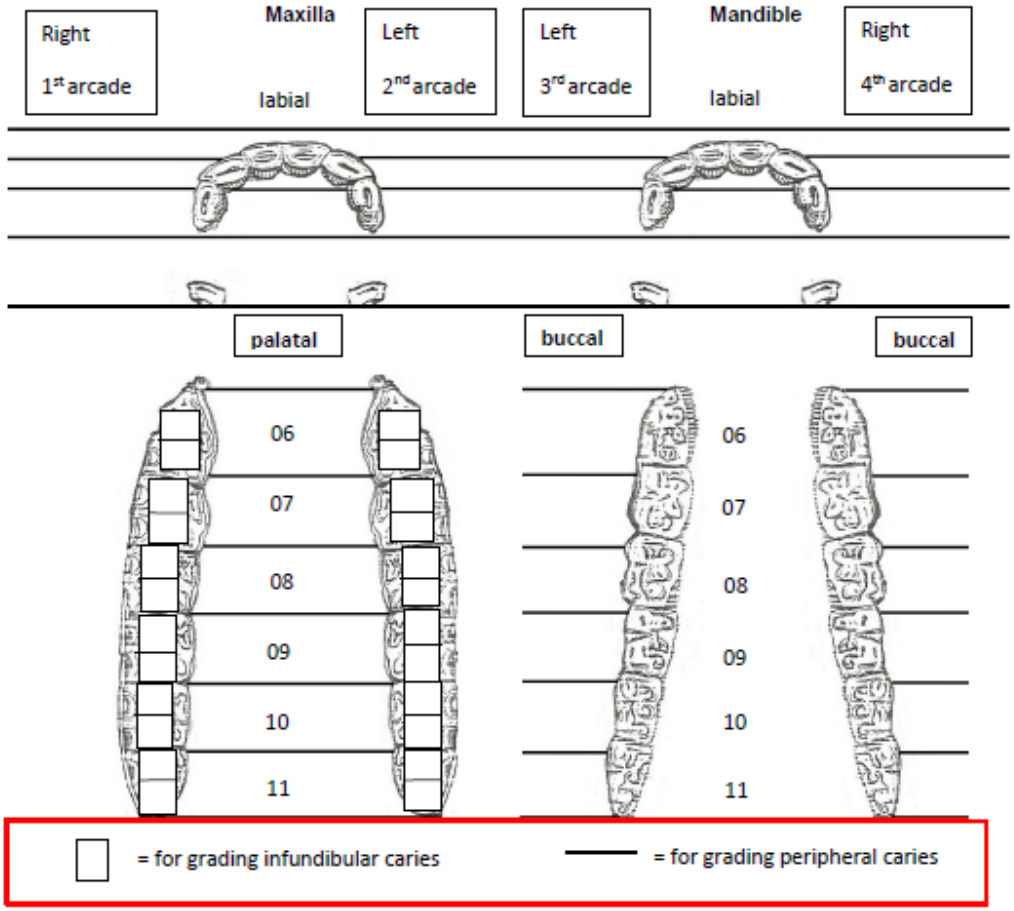
2.2.1 Selection of Survey Participants

Requests to participate in the survey were sent to local Scottish and Northern England veterinary surgeons who refer to the Dick Vet Equine Hospital and were recognised to have a high level of expertise in equine dentistry, to veterinary surgeons with specialist equine dental knowledge in other parts of the UK and to members of the British Association of Equine Dental Technicians (BAEDT).

2.2.2 Questionnaire and Dental Chart Design

Using the input of five European equine dentistry Diplomates, a dental chart and accompanying questionnaire were designed to record the presence, location and severity of PC and IC on individual teeth (Fig 2.1). Honma's grading system as modified by Dacre (2005a) was used to grade PC (Fig 2.2) and IC lesions (Fig 2.3). The guidelines allowed participants to differentiate between the presence of discolouration of the periphery of the teeth of some horses that is regarded as innocuous and PC. A balance was struck between the length of the questionnaire that would provide maximum information on possible risk factors and the likelihood of decreasing compliance with a very long and complex questionnaire.

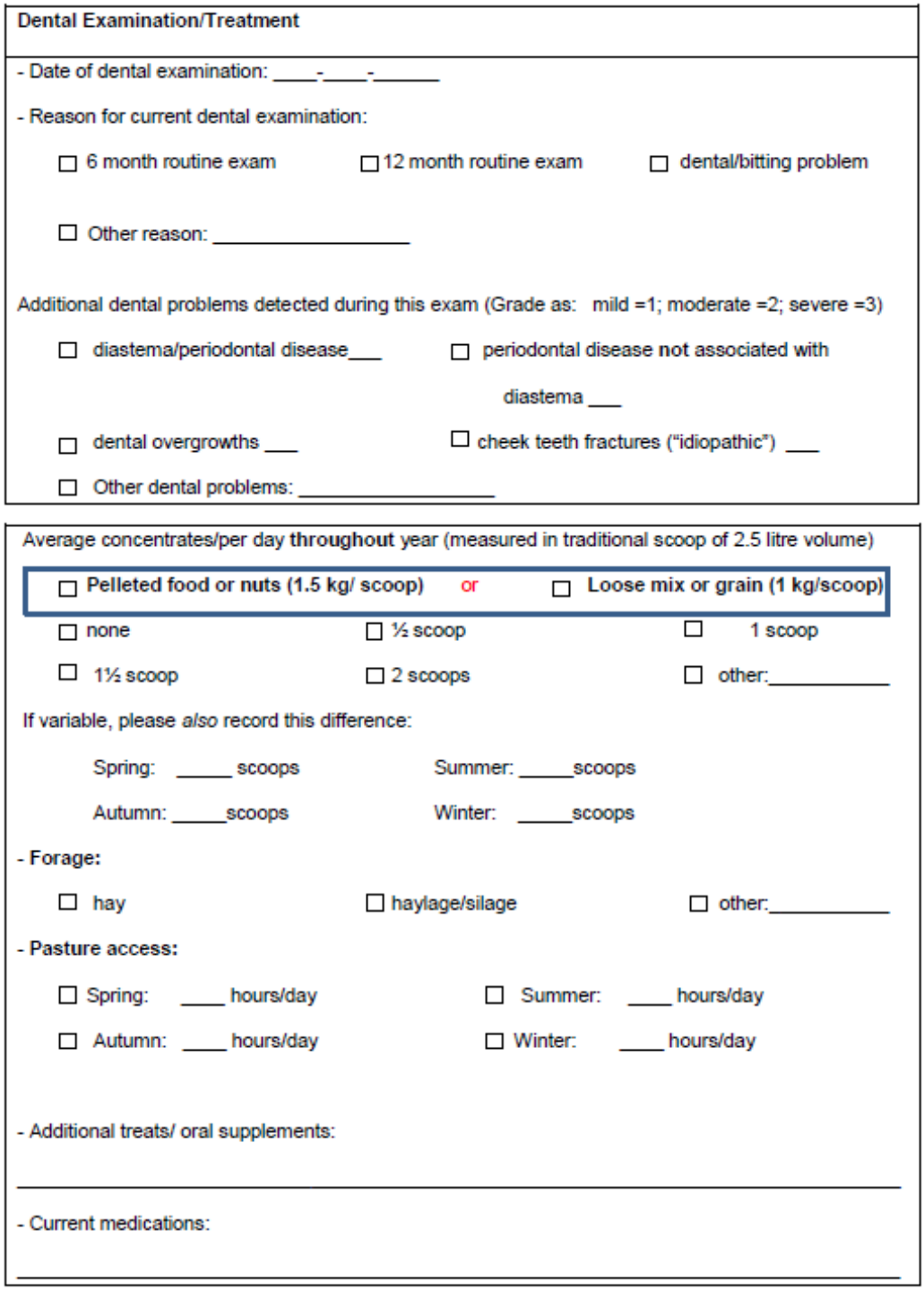
Dental Chart for Equine Dental Caries Survey



☐ = for grading infundibular caries — = for grading peripheral caries

Name of dental practitioner:_____

General Information on Horse		
- Breed: _____		
- Gender:		
☐ stallion	☐ gelding	☐ mare
- Age: ___ years		
- Work:		
☐ recreational	☐ sports/competition	☐ racing
- Owned for: ___ years / ___ months		
- Location (postal code) where horse is kept: _____		



Dental Examination/Treatment

- Date of dental examination: ____-____-_____

- Reason for current dental examination:

☐ 6 month routine exam ☐ 12 month routine exam ☐ dental/bitting problem

☐ Other reason: _____

Additional dental problems detected during this exam (Grade as: mild =1; moderate =2; severe =3)

☐ diastema/periodontal disease___ ☐ periodontal disease **not** associated with diastema ___

☐ dental overgrowths ___ ☐ cheek teeth fractures ("idiopathic") ___

☐ Other dental problems: _____

Average concentrates/per day **throughout** year (measured in traditional scoop of 2.5 litre volume)

☐ **Pelleted food or nuts (1.5 kg/ scoop)** or ☐ **Loose mix or grain (1 kg/scoop)**

☐ none ☐ ½ scoop ☐ 1 scoop

☐ 1½ scoop ☐ 2 scoops ☐ other:_____

If variable, please *also* record this difference:

Spring: _____ scoops Summer: _____scoops

Autumn: _____scoops Winter: _____scoops

- **Forage:**

☐ hay ☐ haylage/silage ☐ other:_____

- **Pasture access:**

☐ Spring: ____ hours/day ☐ Summer: ____ hours/day

☐ Autumn: ____ hours/day ☐ Winter: ____ hours/day

- Additional treats/ oral supplements:

_____

- Current medications:

_____

Fig 2.1. Dental chart and questionnaire designed to assess the prevalence of equine peripheral caries and infundibular caries in the United Kingdom and to examine for potential risk factors for the development of peripheral caries and infundibular caries.

Fig 2.2. Examples of different grades of peripheral caries. Grade 0, no caries; Grade 1.1, pitting or partial loss of peripheral cementum; Grade 1.2, total loss of peripheral cementum: Grade 2, also involves enamel; Grade 3, also involves dentine; Grade 4, loss of integrity of tooth; (adapted from Borkent and Dixon, 2015).

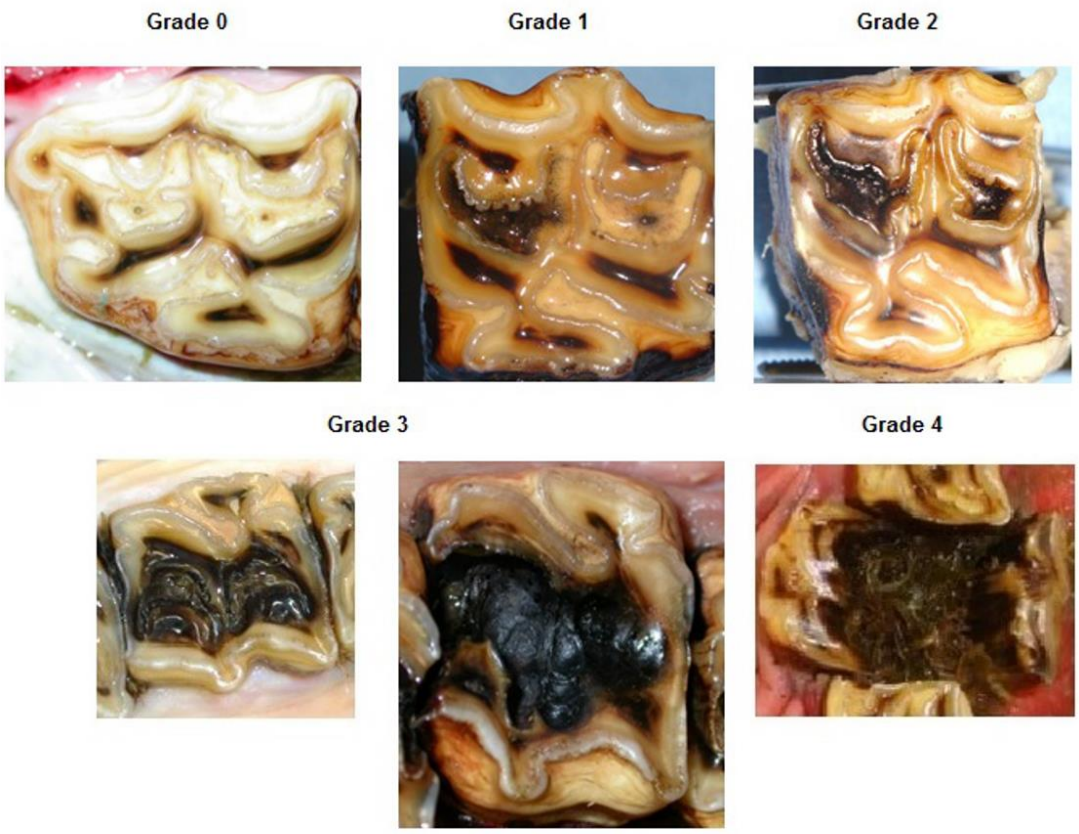


Fig 2.3. Examples of different grades of maxillary cheek tooth infundibular caries. Grade 0, no infundibular lesion or just small central "vascular" channel; Grade 1, involves infundibular cementum only; Grade 2, also involves enamel; Grade 3, also involves dentine (including coalescing carious infundibulae). Grade 4, loss of integrity of tooth (midline sagittal fracture) (adapted from Borkent and Dixon, 2015).

A recent UK post-mortem study showed that PC most severely affected the buccal aspects of the mandibular and the palatal aspects of the maxillary cheek teeth (Lee et al., 2017). Consequently, participants were asked to grade PC on each cheek tooth at these sites only. Guidelines, including one with images of different grades of PC and IC were distributed to participants, along with a shorter laminated similar guide for field use (Supplementary Item 1-3). Details of survey grading and recording systems were presented to BAEDT Annual Congress in 2015.

The questionnaire gathered general information on the examined horses including breed, age, sex, type of work, postcode, reason for current dental examination and presence of concurrent dental disorders, as well as detailed information on the amount and types of concentrates fed per day, type of forage fed and level of pasture access throughout the year. The survey was performed between 10 February and 21 September 2015. The participants were requested to grade consecutive dental examination cases without case selection, to minimise selection bias.

2.2.3 Evaluation of Participants' Caries Grading Standard

After completion of the survey, all participants received an email questionnaire in which they were asked to grade images of different grades of PC-affected, IC-affected and healthy teeth in order to confirm their diagnostic ability.

2.2.4 Potential Risk Factors

A total of 14 potential risk factors were chosen from *a priori* hypotheses and the literature on dental caries: the presence of concurrent IC; concurrent dental disorders other than IC (including: diastema/periodontal disease, periodontal disease not associated with diastema, dental overgrowths, cheek teeth fractures, 'other dental' disorders); breed (categorised as Warmblood types, Thoroughbred types, Saddlebred horses, Coldbloods, Arabian horses or ponies); sex; age; work type (categorised as recreational, sports/competition or racing use); location of horse (post code); fluoridation of drinking water; type and amounts of concentrates fed; type of forage

fed; duration of pasture access (mean hours per day over a full year) and additional treats/supplements fed.

The feeding of concentrates was hypothesised to be an important potential risk factor, so this variable was further scrutinised. Concentrate feeding was divided into two subcategories: pelleted food or nuts (assessed as 1.5 kg per standard 2.5 L scoop) or loose mix/grain (assessed at 1 kg/scoop). If there was a seasonal difference in concentrate feeding, the number of scoops per day/per season were recorded.

2.2.5 Data Analysis

The survey data were collated in Excel. Descriptive analysis including graphical representation of the data was then performed using R software (version 3.1.2) (R Core Team, 2014). For continuous data, a summary of the data was produced containing minimum, maximum, mean and median values. Plots with error bars were created providing an overview of possible association of prevalence of PC with the continuous or categorical variables.

The outcome 'presence of PC' was defined as PC 'yes' (Dacre grade ≥1.1 [Dacre, 2005a]) or 'no' (Dacre grade 0 [Dacre, 2005a]). The frequency of presence of PC was compared between the 12 rostral cheek teeth (Triadan 06–08) and the 12 caudal cheek teeth (Triadan 09–11) and between the mandibular and maxillary arcades using McNemar's tests.

If IC was present, this was defined as IC'yes' (Dacre grade ≥1) and if IC was absent, this was displayed by IC 'no' (Dacre grade 0) (Dacre, 2005a).

The frequency of presence of IC (in either rostral or caudal infundibulum; in either the 100 or 200 arcade) at each cheek tooth position (06-11) was calculated for all horses in the study. The prevalence of IC was compared between the cheek tooth position most commonly affected by IC and the other cheek teeth positions combined, using a McNemar test (to account for matching within a horse). A McNemar test was also used to compare the frequency of presence of IC between the 12 rostral and the 12 caudal infundibulae.

The log odds of the outcome vs. each continuous variable were examined graphically. If the relationship was nonlinear, categorical or alternative, binary, polytomous categorical (quartiles or quintiles) or quadratic and cubic terms were considered in the univariable and multivariable model to find the 'best fit' for the model (Dohoo et al., 2003). Nominal and ordinal categorical variables were numerically coded sequentially, with a 0 being assigned to the reference group. Univariable logistic regression was used to assess the relationships between the potential risk factors and outcome. Variables with P values <0.2, as well as any considered biologically plausible and any that had been reported as being significant in other studies, were considered for inclusion in the multivariable model. Variables were ordered by Akaike Information Criteria and log likelihood values, prior to sequential insertion into a single level multivariable regression model. Variables were retained in the multivariable model if P values were ≤0.05. The Wald test P value was used when comparing categories with the reference category.

Potential confounders were evaluated by resubmitting all of the variables from the univariable analyses not included in the final model after the forward stepwise process of model building. The effect of each potential confounder on the estimates for variables in the final model was assessed by adding each one, one at a time, into the final model. If addition of the potentially confounding variable altered odds ratios for variables in the final model by more than 20% (Dohoo et al., 2003), confounding was considered to be present, the confounder was retained in the final model and adjusted odds ratios were reported for variables in the final model. Correlation coefficients were produced between all quantitative variables in the final model. Variables with correlation coefficients of >0.4 and <–0.4 were further examined by investigating the effect of removing them individually from the model. The fit of the final multivariable model was assessed using the Hosmer-Lemeshow goodness-of-fit test (Hosmer et al., 2013). The predictive ability of the model was determined by generating a receiver operating characteristic (ROC) curve. All data analyses were performed using R software (version 3.1.2).

2.3 Results

2.3.1 Participants and Horses

A total of 25 participants took part in the survey including nine veterinarians who referred to the authors' clinic and 10 veterinarians and six BAEDT members who worked in England and Wales. Completed questionnaires and dental charts from 706 different horses were returned. The caries grading test that was performed following completion of the survey showed that all participants who completed this test could differentiate PC (grade 1.1 or higher) from no caries (grade 0) (Table 2.1) and could also differentiate between normal cheek teeth infundibulae and grade 1 or higher IC (Table 2.2).

Table 2.1. Results of the peripheral caries (PC) grading test performed by participants of the survey

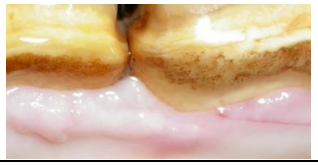
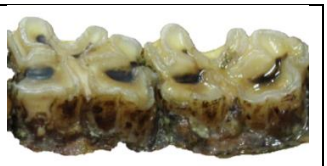
			
Observer	PC Grade	PC Grade	PC Grade
Observer 1	1.1	0	2
Observer 2	1.1	0	2
Observer 3	1.1	0	3
Observer 4	1.1	0	2
Observer 5	1.1/1.2	0	3
Observer 6	1.1	0	1.2
Observer 7	1.1	0	1.2
Observer 8	1.1	0	1.2
Observer 9	1.1	0	1.2
Observer 10	1.1	0	1.2

Table 2.2. Results of the infundibular caries (IC) grading test performed by participants of the survey

			
Observer	IC Grade	IC Grade	IC Grade
Observer 1	2	0	1
Observer 2	3	0	2
Observer 3	2	0	1
Observer 4	2	0	1
Observer 5	3	0	2
Observer 6	2	0	1
Observer 7	2	0	1
Observer 8	2	0	1
Observer 9	2	0	1
Observer 10	2	0	1

The mean age of horses in this study was 12.1 years (range 3–38 years) that was similar to the mean age of horses with PC (12.0 [range 3–30 years]) and the mean age of horses with IC (13.7 [range 3-30 years]). Sex was recorded in 673/706 horses and included 35% (236/673) females and 65% (437/673) males (63% geldings [423/673] and 2% [14/673] stallions). Horses were classified as Warmblood types (n = 366), ponies (n = 183), Thoroughbred types (n = 111), Arabian horses (n = 14), Coldbloods (n = 9) and Saddlebred horses (n = 1).

Details of geographical location (postcode) were available for 699 of the 706 horses and their distribution are shown graphically in Fig 2.4. No address was recorded for 5 horses and 47 horses lived in areas where mains water was partially fluoridated and so these 52 horses were excluded from this analysis. Of the 654 remaining horses, a prevalence of 51% PC (321/627) was found in horses currently residing in areas where water was not fluoridated whilst 48% of the remaining limited number of cases (13/27) lived in areas where water was fluoridated had PC.

There was no significant difference in prevalence of IC in horses living in areas with or without water fluoridation: 46% IC (286/627) in horses living in non-fluoridated water areas and 30% IC (8/27) in those living in fluoridated water areas.

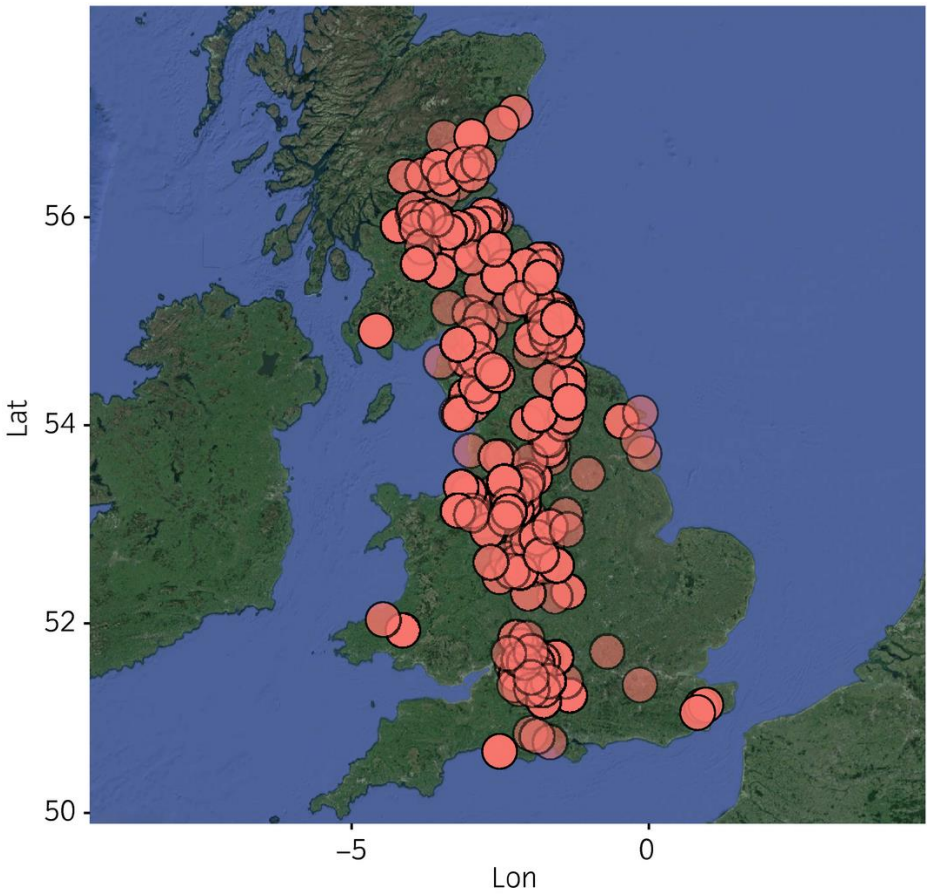


Fig 2.4. Map of the United Kingdom with red dots marking the location of 699 of the 706 horses examined in this survey.

2.3.2 Prevalence and Oral Distribution of Peripheral Caries

PCwas present in 51.7% (365/706) of horses, 23.2% (164/706) had PC only and 28.5% (201/706) had both IC and PC. PC primarily affected the cheek teeth, with only 6 incisors and no first premolar (‘wolf’) or canine teeth affected. PC was bilateral in 88.5% (n = 323/365) of affected horses and unilateral in 11.5% (42/365). The median of the maximum grade of PC per horse was 1.1 (range 0–4). The 12 caudal cheek teeth (Triadan 09–11) were significantly more commonly (odds ratio

[OR] 9.38, 95% confidence interval [C.I.] 6.0–15.5, P < 0.001) affected by PC than the 12 rostral cheek teeth (Triadan 06–08) (Fig 2.5). The mandibular cheek teeth were significantly more commonly (OR 3.0, 95%, C.I. 2.2–4.1, P < 0.001) affected by PC than the maxillary cheek teeth.

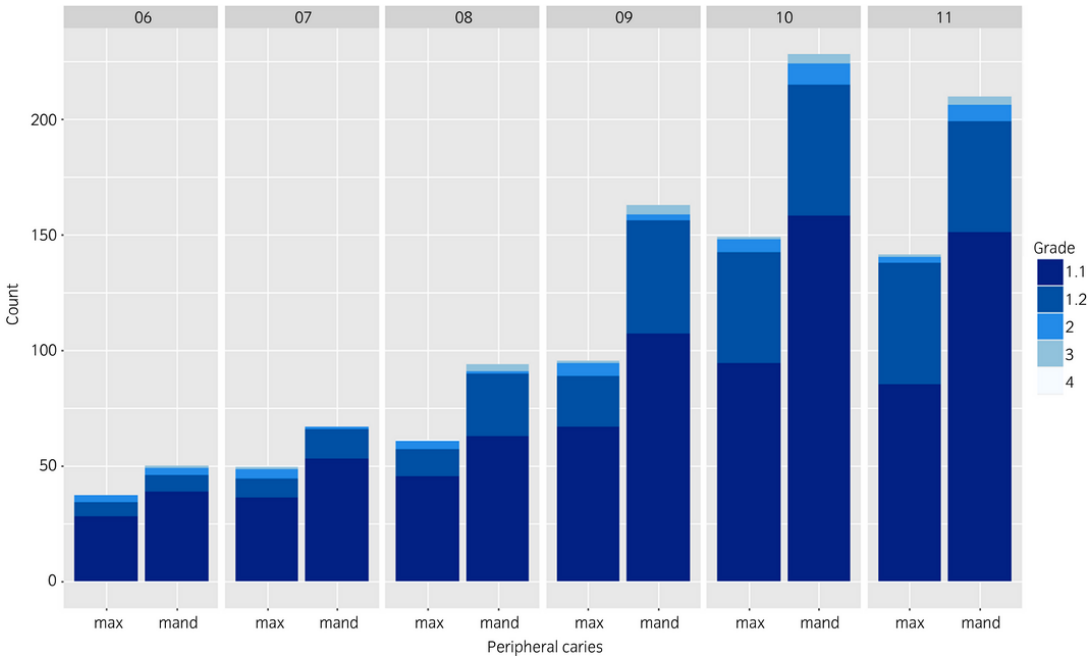


Fig 2.5. Bar plot showing frequency of different grades of peripheral caries on the buccal and palatal aspects of the mandibular and maxillary arcades respectively (mean of left- and right-sided values) (after Dacre [2005a]), subdivided by tooth (Triadan number) and maxillary or mandibular position. (max = maxillary cheek tooth; mand = mandibular cheek tooth).

2.3.3 Risk Factors for Peripheral Caries

Of the 14 variables screened at the univariable level, 9 were taken forward for consideration in the multivariable forward stepwise analysis. The results of univariable logistic regression analyses, including some examples of variable categorisation, are shown in Supplementary Item 4.

2.3.3.1 Final Multivariable Model with Only Fixed Effects

The final multivariable model of factors which increased the likelihood of PC in the surveyed horses is presented in Table 2.3. The presence of IC increased the risk of

PC compared with no concurrent dental disorder. Horses with diastemata and horses with multiple dental disorders other than IC (combination of dental fracture/diastema/overgrowths/'other' dental disorder) were also significantly more likely to have PC than horses without any dental disorder. The prevalence of PC varied between regions of the UK, with the highest prevalence observed in South East England and the lowest observed in the Midlands. The likelihood of having PC was significantly higher in South East England and South West England than in Scotland. The frequency of PC varied with amounts of concentrate fed, but the relationship was not linear (Supplementary Item 5). Only the group of horses fed between 2.1 and 3.0 kg concentrates per day had increased risk of PC compared with horses fed no concentrates.

2.3.3.2 Final Multivariable Model with Observer as Random Effect

Data of 662 horses (339 of which had PC) contained sufficient information and were included in the final multivariable model with observer as random effect (Table 2.1). The risk factors which remained significantly associated with the presence of PC were concentrates, if fed at a level of 2.1-3 kg per day (OR=1.92, 95% C.I. 1.05-3.49, P=0.033), and the concurrent dental disorders: diastema/periodontal disease (OR=3.46, 95% C.I. 1.41-8.49, P=0.007) and multiple dental disorders (OR=2.42, 95% C.I. 1.54-3.82, P<0.001). Dental fractures became significantly associated with the presence of PC (OR=13.03, 95% C.I. 1.45-116.52, P=0.022) but the association with IC did not remain significant.

2.3.3.3 Fit of the Models

The P-value of the Hosmer-Lemeshow goodness-of-fit test was 0.44 for the final multivariable model without observer as random effect and 0.46 for the model with observer as a random effect, showing that there is less evidence of lack of fit in the latter model. The area under the ROC curve was 0.70 for the final model without observer as random effect and 0.75 for the model with observer as random effect, indicating that although both models have a fair predictive ability, the model with observer as random effect has a slightly better predictive ability.

Table 2.3. Multivariable model showing variables significantly associated with the likelihood of having peripheral caries for horses in the UK.

Variable	Odds Ratio (95% C.I.)	P-value	Total (n=656)	Prevalence PC (%)	PC Frequency
IC		**< 0.001**			
No	referrent		363	43.8	159
Yes	1.89 (1.32, 2.71)	*< 0.001*	293	59.7	175
Concurrent dental disorder other than IC		**<0.001**			
No	referrent		199	42.2	84
Dental fracture	8 (0.94, 68.34)	*0.06*	8	87.5	7
Diastema/PD	2.72 (1.18, 6.27)	*0.02*	37	75.7	28
Multiple	2.42 (1.54, 3.82)	*< 0.001*	164	67.1	110
Other	0.97 (0.47, 2)	*0.9*	39	46.2	18
Overgrowths	0.9 (0.58, 1.38)	*0.6*	209	41.6	87
Region		**0.004**			
Scotland	referent		158	47.5	75
Midlands	0.62 (0.28, 1.41)	*0.3*	35	34.3	12
North England	1.3 (0.86, 1.98)	*0.2*	296	49.0	145
South East England	4.14 (1.63, 10.52)	*0.003*	36	80.6	29
South West England	2.04 (1.13, 3.68)	*0.02*	90	56.7	51
Wales	1.62 (0.76, 3.43)	*0.2*	41	53.7	22
Concentrates (kg/day)		**0.07**			
0	referent		130	43.1	56
0.1 to 2	1.1 (0.72, 1.68)	*0.7*	379	49.1	186
2.1 to 3	1.95 (1.11, 3.42)	*0.02*	114	65.8	75
3+	0.95 (0.41, 2.19)	*0.9*	33	51.5	17

Key: C.I. = confidence interval; PC = peripheral caries; IC = infundibular caries; PD = periodontal disease; Multiple concurrent dental disorders = more than one of the disorders listed in this category; P-values in bold are from the likelihood ratio test, while those in italics are from the Wald test.

Table 2.1. Multivariable logistic regression model with observer as random effect, showing variables significantly associated with the likelihood of UK horses having peripheral caries.

Variable	Odds Ratio (95% C.I.)	P-value	Total (n=662)	Prevalence PC (%)	PC Frequency
Concurrent dental disorder other than IC		**<0.001**			
No	Referent		200	42.0	84
Dental fracture	13.03 (1.45 – 116.52)	*0.02*	8	87.5	7
Diastema/Periodontal Disease	3.46 (1.41 – 8.49)	*0.01*	38	76.3	29
Multiple	2.42 (1.54,3.82)	*< 0.001*	168	67.9	114
Other	1.04 (0.44, 2.46)	*0.93*	39	46.2	18
Overgrowths	1.2 (0.68,2.12)	*0.53*	209	41.6	87
Concentrates (kg/day)		**0.11**			
0	referrent		131	43.5	57
0.1 to 2	1.16 (0.74,1.82)	*0.52*	383	49.3	189
2.1 to 3	1.92 (1.05, 3.49)	*0.03*	115	66.1	76
3+	0.78 (0.32, 3.49)	*0.60*	33	51.5	17

Key: C.I. = confidence interval; PC = peripheral caries; IC = infundibular caries; PD = periodontal disease; Multiple concurrent dental disorders=more than one of the disorders listed in this category; P-values in bold are from the likelihood ratio test, while those in italics are from the Wald test.

2.3.4 Prevalence and Oral Distribution of Infundibular Caries

ICwas recorded in 45.5% (321/706) of the surveyed horses, including in 28.5% (201/706) that had concurrent IC and PC and 17% (120/706) that only had IC, i.e.

within the IC affected horses, 37.4% (120/321) had only IC while 62.6% (201/321) had both IC and PC. When present, IC was bilateral in 85.7% (275/321) of cases and unilateral in 14% (46/321) (23 horses left side; 23 horses right side). No IC or PC was found in 31.3% (221/706) of horses. No incisor IC was recorded.

IC of either rostral, caudal or both infundibulae was most severe and most commonly present in the Triadan 09s, with at least one 09 infundibulum affected in 39% (276/706) of horses (Fig 2.6). The 09s were significantly more frequently affected than the other maxillary cheek teeth Triadan positions (OR=2.15, 95% C.I. 1.50-3.12, P<0.001), 06s 16% (110/706), 07s 9% (62/706), 08s 15% (105/706), 10s 19% (136/706), 11s 8% (55/706) of horses.

Considering all maxillary cheek teeth positions, the rostral infundibulum was significantly (P<0.001) more likely (OR=4.47, 95% C.I. 2.52-8.42) to have IC (13.4% [1139/8472]) than the caudal infundibulum (10% [877/8472]).

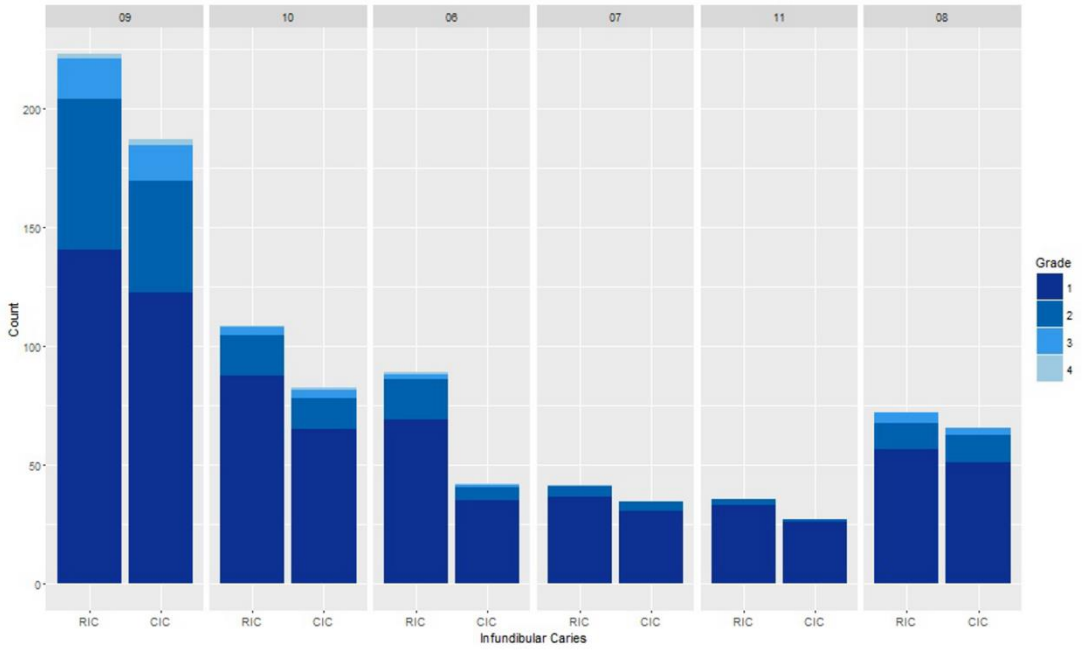


Fig 2.6. Barplot of the frequency and severity of infundibular caries (mean of left and right sided grades, after Dacre (2005a)) in each maxillary cheek tooth Triadan position in 706 horses in order of eruption time of the teeth (earlier eruption time is higher *dental* age). RIC= rostral infundibular caries; CIC= caudal infundibular caries.

2.3.5 Risk Factors for Infundibular Caries

Of the 14 variables which were analysed at the univariable level (Supplementary Item 6), four were considered for inclusion in the multivariable model.

2.3.5.1 Final Multivariable Model with Only Fixed Effects

Six hundred and sixty seven horses (297 of which had IC), with sufficient information were included in the final multivariable model (Table 2.5). Three variables were found to be associated with an increased likelihood of IC: the presence of PC; the presence of multiple concurrent dental disorders (other than PC) compared to absence of concurrent dental disorder; increasing age. Additionally, significant variations in likelihood of IC were observed between regions.

Table 2.5. Multivariable model showing variables significantly associated with the likelihood of having infundibular caries for UK horses

Category	Odds Ratio and 95% Confidence Intervals	P-value	Total (n=667)	Prevalence IC (%)	IC Frequency
PC		**< 0.001**			
No	1.00 (referent)		324	35.8	116
Yes	2.04 (1.42,2.92)	*< 0.001*	343	52.8	181
Concurrent		**0.028**			
No	1.00 (referent)		201	41.8	84
Dental fracture	2.10 (0.36,12.45)	*0.412*	8	75.0	6
Diastema/PD	1.88 (0.86,4.13)	*0.115*	39	61.5	24
Overgrowths	0.77 (0.49,1.21)	*0.257*	211	31.8	67
Other	0.88 (0.4,1.94)	*0.759*	40	40.0	16
Multiple	1.62 (1.01,2.6)	*0.045*	168	59.5	100
Age (years)	1.11 (1.08,1.15)	**< 0.001**			
Region		**< 0.001**			
Other	1.00 (referent)		275	61.5	169
North England	0.29 (0.2,0.42)	*< 0.001*	303	37.3	113
South West England	0.09 (0.05,0.18)	*< 0.001*	89	16.9	15

Key: IC= infundibular caries; PC = peripheral caries; PD = periodontal disease; Multiple concurrent dental disorders = more than one of the disorders listed in this category; Other = Midlands, Wales, Scotland and South East England; P-values in bold are from the likelihood ratio test, while those in italics are from the Wald test.

2.3.5.1.1 Peripheral Caries

Horses with PC (Dacre grade >0) were significantly (P<0.001) more likely (OR=2.04, 95% C.I.1.42-2.92) to have IC (52.8% IC, 181/343) than horses without PC(35.8% IC, 116/324).

2.3.5.1.2 Concurrent Dental Disorders

Concurrent dental disorders, other than PC, were recorded in 69.7% (465/667) of cases. Horses with multiple dental disorders other than PC (any combination of dental disorders: dental fracture/ diastema / overgrowths / "other" dental disorder) were significantly more likely to have IC (OR=1.62, 95% C.I. 1.01-2.6, P=0.045) than horses without any concurrent dental disorder.

2.3.5.1.3 Age

The median age of the complete horse population examined including horses with and without IC was 11.0 years (SD=5.9, IQR=9). The median age of horses with IC was 13.0 years (SD=5.8, IQR=9). The prevalence of IC increased significantly with increasing age (OR=1.11, 95% C.I. 1.08-1.15, P<0.001) (Fig 2.7).

2.3.5.1.4 Geographical Region

The prevalence of IC varied between regions (Fig 2.8). The lowest IC prevalence was observed in South West England (16.9%, 15/89) while the prevalence was highest in the Midlands (69.7 %, 23/33). The likelihood of having IC was significantly lower in North England (OR=0.29, 95% C.I. 0.2-0.42, P<0.001) and South West England (OR=0.09, 95% C.I. 0.05-0.18, P<0.001) than in the other regions (Midlands, Wales, Scotland and South East England).

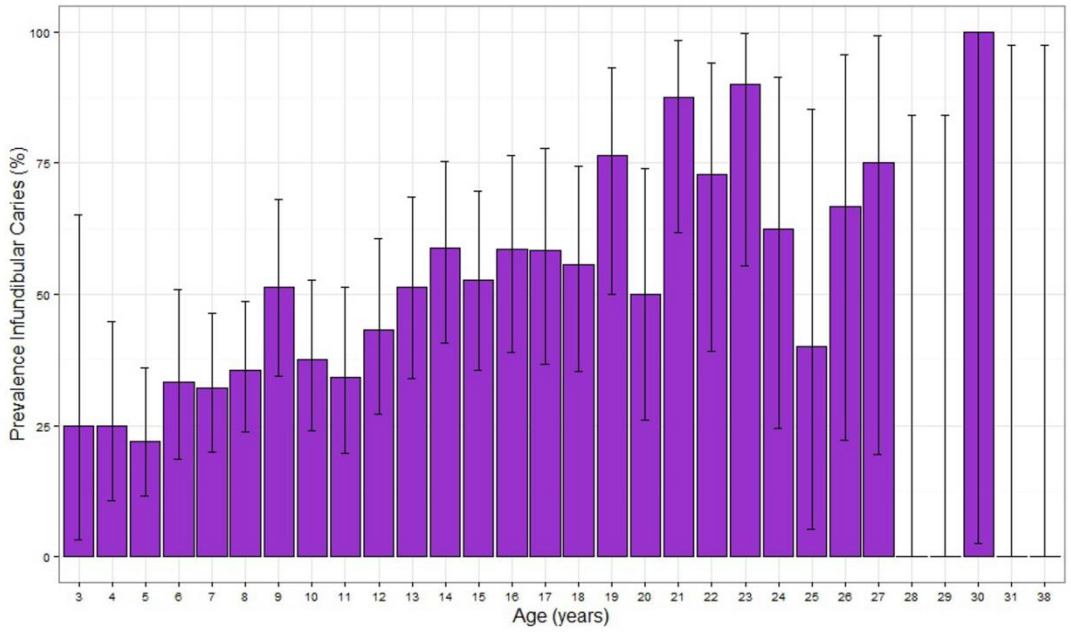


Fig 2.7. Prevalence of equine infundibular caries (IC) in 667 horses subdivided by age

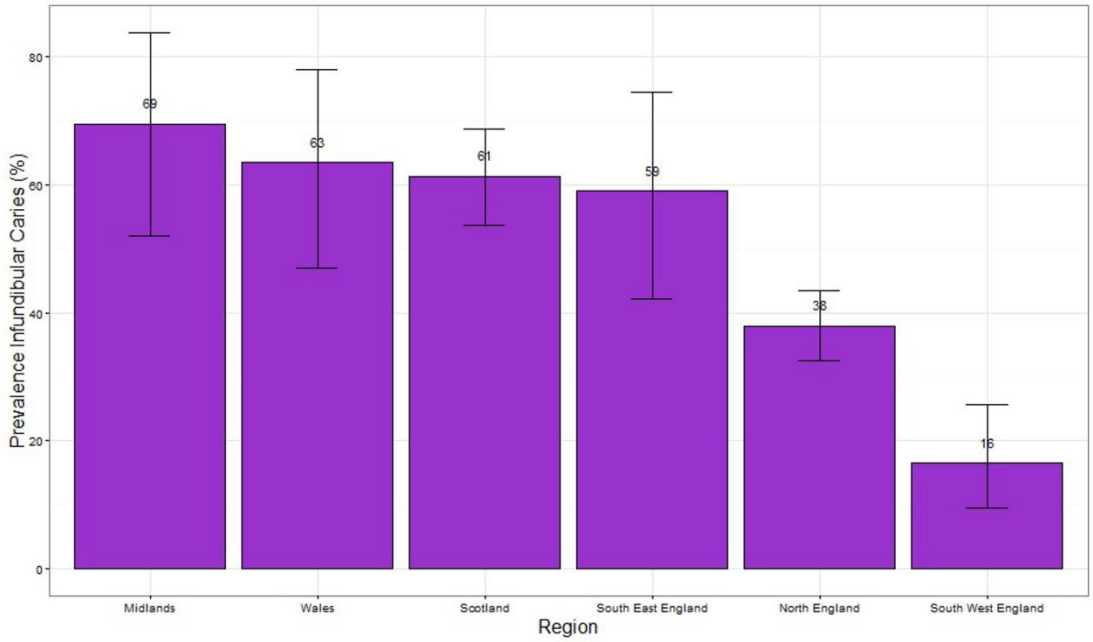


Fig 2.8. Prevalence of infundibular caries (IC) in different regions within the UK

2.3.5.2 Final Multivariable Model with Observer as Random Effect

Six hundred and seventy eight horses (302 of which had IC) with sufficient information were included in the final multivariable model with observer as random effect. The only risk factors remaining significantly associated with the presence of IC were: age (OR=1.16, 95% C.I. 1.11-1.20, P<0.001) and geographical region, with a significantly lower likelihood for horses to have IC in South West England (OR=0.31, 95% C.I. 0.12-0.80, P=0.016) than in the other regions (Midlands, Wales, Scotland and South East England) (Table 2.6).

2.3.5.3 Fit of the Models

The P-value of the Hosmer-Lemeshow goodness-of-fit test was 0.11 for the final model without observer as a random effect and 0.89 for the model with observer as a random effect, indicating less evidence of lack of fit for the latter model. The predictive ability of the final model without observer as a random effect was fair (area under ROC curve=0.77), but was good for the model including observer as a random effect (area under ROC curve=0.87).

Table 2.6. Multivariable logistic regression model with observer as random effect, showing variables significantly associated with the likelihood of UK horses having infundibular caries

Category	Odds Ratio and 95% Confidence Intervals	P-value	Total (n=678)	Prevalence IC (%)	IC Frequency
Age (years)	1.16 (1.11-1.20)	**<0.001**			
Region		**0.026**			
Other	1.00 (referent)		279	61.6	172
North England	0.59 (0.29-1.18)	*0.137*	310	37.1	115
South West England	0.31 (0.12-0.80)	*0.016*	89	16.9	15

Key: Other = Midlands, Wales, Scotland and South East England; P-values in bold are from the likelihood ratio test, while those in italics are from the Wald test.

2.4 Discussion

2.4.1 Peripheral Caries

The prevalence of PC (51.7%) found in this survey is much higher than the prevalence of 0.3% found in an Irish abattoir survey (Wafa, 1988), of 0.9% found in two earlier Swedish post-mortem dental surveys (Lundström and Pettersson, 1988; Lundström and Pettersson, 1990) and of 6.1% in a 2010 Swedish post-mortem study (Gere and Dixon, 2010). A recent clinical study of PC prevalence in donkeys in Portugal and Spain also reported a low prevalence (5.9%, 47/800) (Rodrigues et al., 2013). The lower prevalence of PC in post-mortem studies is unexpected because such examinations allow a more detailed examination of all teeth than clinical examinations. In contrast, a PC prevalence of 69.4% (75/108 horses) was recorded in a recent UK clinical study on referred cases (Ramzan and Palmer, 2011), which was higher than in the current study (51.7% PC). This difference could be explained by the use of an oral endoscope in the former study, which allows a more thorough oral examination than the current study, where most participants used a dental mirror and headlight to examine teeth. Additionally, tooth surface palpation can be used to differentiate between discoloration (smooth) and low grade PC (irregular/rough) and was also performed by some examiners in this study. Additionally, the horses in the former study were referred dental cases that would be expected to have a higher prevalence of dental disorders than the horses in the current study that were mainly examined during routine dental examinations, even though the examiners were specifically looking for this disorder. Overall, our results suggest that there is a high prevalence of PC in the UK that may be related to the relatively recent recognition and current increased awareness of this disorder, making many clinicians very adept at detecting it. For the very opposite reasons, a survey of 400 horses in a UK clinic in the 1990s did not record the presence of PC in any horse (Dixon et al., 2000). The current survey relied on the ability of participants to correctly identify the presence of PC, and a post-survey test confirmed that they could correctly identify PC.

Participants were asked to fill out the survey and questionnaire for consecutive dental cases and to record the reason for dental examination in order to minimise selection

bias and to assess if there would be variations in PC presence recorded with different reasons for dental examination. Although it could not be checked whether only consecutive cases were included in the study, no significant variations in PC presence were found between different reasons for dental examination (dental/bitting, routine or other reason).

No statistically significant breed predisposition to PC was found, in contrast to a recent Australian clinical study (Jackson et al., 2017) where PC was more likely to affect Thoroughbred horses than Warmbloods or Western breeds.

The most common and severely affected teeth, were the 12 caudal cheek teeth (Triadan 09–11) as previously recorded (Gere and Dixon, 2010), with mandibular cheek teeth being affected more often than maxillary cheek teeth. In the current study, the mean age of the full population and horses with PC (12 years for both) was greater than the age of PC-affected horses in a previous study (mean age 8.1 years) where it was proposed that high levels of concentrates and haylage were risk factors for PC (Gere and Dixon, 2010). The presence, site and grade of IC was also recorded in the current study and although both PC and IC were concurrently present in 28.5% of horses, these two forms of equine caries appear to be separate disorders.

PC preferentially affects the caudal upper and lower cheek teeth indicating an environmental change in the caudal aspect of the oral cavity that favours the growth of cariogenic bacteria. In contrast, IC can only affect the maxillary cheek teeth and most evidence would suggest the primary problem is caused by developmental defects in cemental filling of certain cheek teeth, especially the Triadan 09s (Windley et al., 2009; Fitzgibbon et al., 2010; Dixon et al., 2014; Suske et al., 2016a; Suske et al., 2016b). Nevertheless, changes in the oral environment is also likely to play a role and IC was positively associated with PC. That the association between PC and IC was not significant when observer was included in the model as a random effect in contrast to the model without observer as random effect, could be explained by that some observers were more likely to record PC and IC than other observers.

Age was not associated with PC prevalence in the current study, which is in contrast to IC where very distinct age-related increase in prevalence occurs (Honma et al., 1962; Baker, 1974; Crabill and Schumacher, 1998; Gere and Dixon, 2010; Rodrigues et al., 2013) and as was also recorded in this study. This might be because equine cheek teeth continue to erupt and wear with age so that peripheral aspects of clinical crowns of cheek teeth of young horses as well as of older horses "renew" every few years. PC mostly affects the clinical crown, whereas IC penetrates deeper into the tooth until deep into the reserve crown. This way the damage caused by IC accumulates with age because the IC lesion extends further and further, in contrast to PC.

Concurrent dental disorders, other than IC, were associated with an increased likelihood of PC, similar to observations in a study of donkeys in which it was hypothesised that impaired food movement creates an acidogenic bacterial environment in the mouth (Dixon et al., 2010; Rodrigues et al., 2013). A significant positive association between the presence of PC and diastemata was also found in a Swedish post-mortem study (Gere and Dixon, 2010) and in a recent Australian clinical study (Jackson et al., 2017). In contrast, Ramzan and Palmer (2011) found no association between presence of diastemata and of PC at either tooth or patient level. By removing interproximal cementum, PC could theoretically create or predispose to diastemata and PC may also play a role in the development of periodontal disease by interrupting the tight connection between cement and junctional epithelium (Dacre, 2005a). Conversely, diastemata and periodontal disease may change the local oral environment, creating a dysbiosis (microbial imbalance) favouring more acidogenic and aciduric micro-organisms which may have a role in the development of PC (Borkent and Dixon, 2015).

Additionally, in the final multivariable model with observer as random effect, a significant positive association between the presence of PC and dental fractures was found.

We observed regional differences in PC prevalence in the UK which could be due to geological, interobserver or interhorse variation in these regions, although the

observed associations between regions and likelihood of PC did not vary significantly when additional information, such as horse breed and use, were included in the model. However, in the model with observer as random effect there was no significant association between presence of PC and region. That might be because there is an effect of observers in certain regions who found PC more/less often than observers in other regions.

Fluoridation of the water supply results in significant improvement in oral health in man (Dean et al., 1950; Brunelle and Carlos, 1990). However, in the current study there were insufficient horses from areas with fluoridated water to make the comparison worthwhile. Some relationship between the prevalence of PC and the level of concentrate feeding was shown, but there was no linear trend in this regard.

It is possible that the lack of significance to the hypothesised risk factors in all groups is related to the sample size, but the association between feeding of concentrates and likelihood of PC was not as strong as expected. This might also be because of the chosen data stratification method which included relatively fewer horses in the group which were fed high levels of concentrates. The feeding of haylage has been hypothesised to be a risk factor for PC (Gere and Dixon, 2010) and may be a contributor to the increasing prevalence of PC over the past three decades that has coincided with increased use of haylage instead of hay. However, we found no significant difference in prevalence of PC between horses fed haylage and those fed hay or chaff. No relationship was found between PC prevalence and mean time spent on pasture throughout the year. It is not known if a natural diet might be protective against caries, or alternatively that there would be an increase in PC prevalence because of the presence of fructans, a fermentable carbohydrate in grass. However, this was not examined in the current study and no horses in this survey were permanently at pasture without supplementary feeding.

Jackson et al. (2017) found a positive association between PC and oaten hay. Protective factors found in this Australian study were feeding of meadow hay and all year round pasture access (compared to no pasture access). Horses with access to groundwater were less likely to have PC compared to horses drinking rainwater, drinking water or dam water.

The model fit was good and predictive ability was fair for both models, but the model which included the observer as a random effect had less evidence of lack of fit and had a slightly better predictive ability than the model without observer as random effect. The area under the ROC curve measures the discrimination of the test with considerable numbers of PC-affected horses in every risk factor subgroup. Consequently, it is more difficult to predict which horses would or would not have PC than would be the case if there were clear differences in the prevalence of PC between the subgroups. The models show that certain risk factors are associated with PC but this does not mean that horses without these risk factors cannot have PC.

In conclusion, PC is a common dental disorder in horses the UK, predominantly affecting the caudal cheek teeth. In contrast to expectations, no major link with diet was observed except for concentrates if fed to horses at a level of 2.1-3.0 kg per day. Positive associations were found between the presence of PC and cheek teeth diastemata, dental fractures and the presence multiple dental disorders. PC and IC were not associated when observer was included as a random effect. Some regional variations in PC prevalence which were found in the model without a random effect did not remain significant when observer was included in the model as a random effect. Further epidemiological, microbiological and pathological studies are needed to examine the aetiopathogenesis of this common disorder.

2.4.2 Infundibular Caries

Using clinical examination of the occlusal surface of cheek teeth, a 45.5% prevalence of IC was found in this study of UK horses. Major differences in the prevalence of IC have been reported in different studies varying from 8% (Fitzgibbon et al., 2010) to 90% (Windley et al., 2009). Some of this variation is likely due to inter-study differences in IC classification, with the central infundibular cemental defect (a remnant of a developmental vascular channel) that can be dark-stained in some teeth (Suske et al., 2016a), being classified as IC by some authors. The other major cause of inter-study differences in IC prevalence is related to the method of study, with imaging studies (Veraa et al., 2009; Windley et al., 2009) and pathological studies (Kilic et al., 1997c; Fitzgibbon et al., 2010; Bühler et al., 2014; Suske et al., 2016a) able to identify subocclusal lesions and thus not surprisingly reporting higher IC

prevalences than studies (such as this) that used clinical examination of the occlusal surface only (Colyer, 1906). For example Windley et al. (2009) found evidence of infundibular abnormalities in 90% of maxillary cheek teeth using computed tomography but found occlusal infundibular lesions in only 65% of these teeth. It is apparent that clinical surveys of IC prevalence should only be compared with other clinical surveys. Unlike some previous studies, the current survey had well-defined guidelines on what constituted an IC lesion and was performed by experienced operators and thus should provide more accurate results than some other clinical studies. Finally, the age of the study population will also influence prevalence, due to the age-related increase in IC prevalence present in this disorder.

In this survey, IC was most severe and most commonly present in the Triadan 09s as also found in other studies (Fitzgibbon et al., 2010; Suske et al., 2016a). It has been suggested that the 09s are more prone to IC due to their older dental age (time since eruption) compared to other cheek teeth (Dacre et al., 2007). More recently Suske et al. (2016a) proposed that the 09 tooth is more commonly affected by IC because it has less time (11.5 months) to develop than the remaining cheek teeth, that have 16 to 21 months development time. The lowest prevalence of IC was found in the Triadan 11s, which could be explained by the late eruption time of these teeth, but also could be related to difficulties in visualising these infundibulae, particularly in unsedated animals and without use of an oral endoscope.

In this study, the rostral infundibulae were more commonly affected by caries than the caudal infundibulae in all Triadan positions. Suske et al. (2016b) proposed that this difference is due to earlier loss of the rostral infundibulum's subgingival, accessory blood supply than the caudal accessory blood supply to the caudal infundibulum. This proposal is supported by a computed tomographic study where infundibular changes were shown to be more frequently present in the rostral than in the caudal infundibula in horses with and without clinical signs of an apical infection (Bühler et al., 2014). Fitzgibbon et al. (2010) also found the rostral infundibulae of longitudinally sectioned teeth to be more commonly affected with areas of total

cemental hypoplasia (aplasia) (24.4% prevalence) compared to caudal infundibulae (20.8%). However they found complete IC more common in the caudal (9%) than the rostral infundibula (7.2%) (Fitzgibbon et al., 2010).

Although 62.6% of horses with IC had both IC and PC, IC was significantly associated with PC in a multivariable model with only fixed effects. However, when observer was included as random effect, there was no longer any significant association between the presence of IC and PC. Additionally the association between the presence of IC and multiple dental disorders did not remain significant when observer was included as a random effect. This difference could be explained by an observer effect in which some observers recorded more IC, PC and multiple concurrent dental disorders than others. The inclusion of observer as a random effect improved the fit of the model, suggesting that the risk factors in that (multilevel) model may be more reliable than those from the model without the random effect. However, because the reliability of the observers in grading IC had been confirmed (although practically there is a difference between grading IC from a picture and grading the infundibulae of teeth in the oral cavity of a live horse, even when sedated) and the true associations between risk factors and IC are unknown, the results of both models have been reported. Further studies with larger numbers of observers and/or increased numbers of horses per observer would help to evaluate this further.

The prevalence of IC was significantly associated with increasing age. This is likely because many areas of infundibular cemental hypoplasia that lie deep in infundibulae progressively become exposed on the occlusal surface with normal wear. Subsequent food impaction in these defects predispose these sites to develop IC, which weakens the tooth and may even lead to a midline sagittal fracture or apical infection (Bühler et al., 2014). There were insufficient horses in this study with dental fractures alone to accurately assess their association with IC frequency. Some of the categorisations of risk factors resulted in small group sizes, which may explain lack of observed significance when considering the results of the power calculation. Recruitment of a larger sample population would help to clarify associations.

The region with the highest prevalence of IC was the Midlands (69.7%) while the lowest prevalence was found in South West England (16.9%), but it is unclear what causes these regional differences. It may have been due to horse-related factors, although the association between IC and region did not change significantly when horse-related factors such as breed and use were included in the model. Instead these regional differences in prevalence may have been due to differences in observer skill and accuracy, but these regional differences remained when observer was included in the model as a random effect. Additionally, horse management may vary between regions and regional geographical differences in soil and climate could play a role in pasture quality, including differences in grass species and content. The prevalence of PC also varied between different geographical regions (Borkent et al., 2016) but this regional variation was dissimilar between the two studies. For example, in this study the highest prevalence of IC was found in the Midlands, whilst the lowest prevalence of PC was recorded in that region (Borkent et al., 2016).

It has been proposed that the high prevalence of IC in European horses is due to high levels of concentrate feeding that provides a substrate for cariogenic bacteria (Dacre et al., 2007). However this study found no relationship between IC prevalence and levels of concentrate feeding, or indeed between different types of forage feeding, i.e. haylage, hay or chaff or surprisingly in the duration of time spent at pasture throughout the year. This might be related to the size of this study but it may also indicate that IC development does not require any specific type or amount of food as long as there is enough dietary substrate (i.e. fermentable carbohydrates) for cariogenic bacteria in the infundibulae. In the absence of detecting any significant environmental factor that could be modified to reduce the prevalence of IC, it appears that directing attention to restoratively treating susceptible maxillary cheek teeth, i.e. those with large developmental cemental defects or significant IC would be more rewarding. Equine oral bacteriology has been a neglected field and further research in that area would also be helpful.

Conclusion

In conclusion, this clinical survey showed that IC affected the occlusal surface of maxillary cheek teeth in 45.5% of UK horses and that its prevalence significantly increased with age. It was most commonly observed in the Triadan 09 teeth and more commonly in the rostral than in the caudal infundibulae. The prevalence of IC varied in different regions and in contrast to expectations, no links with diet or management were observed.

3 CHAPTER 3: MICROBIOLOGY

3.1 Introduction

Dental caries is believed to be caused by acidogenic oral micro-organisms which can convert fermentable carbohydrates to acids. These acids can then damage the tooth by causing a demineralisation and disintegration of the inorganic and organic substances of the tooth, respectively (Soames and Southam, 2005). As discussed earlier, two variants of equine dental caries are known to occur: equine dental peripheral caries (PC), which is caries at the peripheral sites of the teeth, and infundibular caries (IC), which is caries of the infundibulum of the maxillary cheek teeth.

The postulates of Koch (1890) are not fully applicable to dental caries because one of the postulates states that the disease-causing micro-organism must be present in abundance in all subjects affected by the disease, and this micro-organism should not be present in subjects without the disease. Several bacteria have been associated with dental caries, but often these bacteria are found both in subjects with caries, as well as in subjects without caries. Moreover, although *Streptococcus mutans* is still thought to be one of the most important cariogenic bacteria in human caries, caries can develop in the absence of *Streptococcus mutans* (Loesche et al., 1975; Aas et al., 2008; Peterson et al., 2011; Gross et al., 2012). This supports the *ecological plaque hypothesis* which states that dental caries is a complex disease and is thought to be caused by a dysbiosis, i.e. an imbalance of the resident oral bacteria community following a change in the local environment (Kidd, 2005; Takahashi and Nyvad, 2011; Borkent and Dixon, 2015; Takahashi and Nyvad, 2016).

The persistence and variety of the resident oral microflora is mainly determined by endogenous host factors including the individual characteristics of the host saliva and gingival crevicular fluid (Marsh et al., 2009). This was confirmed by the finding that in humans and animals fed through a naso-gastric tube, the diversity of the oral microbiota remained as it was during normal eating. Interestingly, the dental plaque of insectivore, herbivore and carnivore mammals consists of similar bacterial genera, but differences can be found at the species level. The only dietary compounds found to significantly change the ecology of oral microbiota are fermentable carbohydrates

(Marsh et al., 2009). Studies have examined the oral microbiome of horses in health and with periodontal disease (Kennedy et al., 2016) but no study has examined the microbiome of horses with dental caries.

Baker (1979) collected gingival crevicular fluid and dental plaque from the buccal aspects of the 06 maxillary cheek teeth and cultured them aerobically and anaerobically. Bacteria found in high numbers included Gram positive cocci, mainly Streptococci, Micrococci and starch hydrolysers. Bacteria found in intermediate numbers included various anaerobes, Veillonella spp. and H_2S producers. Bacteria found in low amounts included Lactobacillus spp., Fusobacteria spp., and coliforms. Cultures of infundibular hypoplasia/caries lesions were unsatisfactory as were infected gingival cultures, with only a few Streptococci or Micrococci identified or, alternatively, bacterial overgrowth on the plates, despite the preparation of serial dilutions.

Lundström et al. (2007) isolated *Streptococcus devriesei* from IC lesions of 50 teeth and also (in lower numbers) from 4 control teeth. They also investigated the normal anaerobic bacterial flora of the pharyngeal and laryngeal surfaces and the possible associations of this flora with lower respiratory tract and paraoral infections. A molecular bacteriological study by Gao et al. (2016) used 16S rRNA gene pyrosequencing to identify the bacteria present in the subgingival plaque of two healthy horses. Recently, Kennedy et al. (2016) described the differences in microbiota between equine periodontitis and healthy gingiva using high-throughput sequencing of the V3-V4 region of the 16S rRNA gene. Prevotella and Veillonella species bacteria were found to be most commonly associated with periodontitis and Gemella and Actinobacillus species were most commonly associated with health.

The current study will also use high-throughput sequencing of the V4 region to investigate the bacteria involved in equine dental caries. One of the aims of this study is to identify which bacteria are most likely associated with PC and which ones are most likely to be associated with absence of PC (control group). Because PC is more commonly found in the caudal compared to the rostral cheek teeth (Gere and Dixon, 2010; Ramzan and Palmer, 2011; Borkent et al., 2016), PC of the rostral cheek teeth

will also be compared to PC of the caudal cheek teeth versus the control group, in order to assess for a possible difference in microbiota between these sites.

Additionally, some of the dental plaque samples of the palatal aspect of the maxillary cheek teeth will be cultured prior to performing molecular microbiology on the latter samples, will be termed "culture-dependent microbiology". These combined procedures will be performed to ascertain if the bacteria identified by culture-independent bacteriology were alive and had the ability to grow and also to assess which bacteria would grow under standardised microbiology laboratory conditions.

A further aim of this study is to compare the microbiota of infundibulae with and without caries to assess for differences in the bacteria associated with IC and with the control group (no IC).

3.2. Materials and Methods

3.2.1 Collection of Supragingival Equine Dental Plaque

After intravenous sedation of clinical cases referred to the University of Edinburgh for dental examination, with a combination of 44 µg/kg romifidine HCl (Sedivet [10 mg/mL], Boehringer Ingelheim, Bracknell, UK) and 22 µg butorphanol/kg butorphanol tartrate (Torbugesic [10 mg/mL], Zoetis, London, UK), the oral speculum was placed and the oral cavity was opened and rinsed with lukewarm water without disinfectant. A head light and mirror or an oral endoscope were used to examine the teeth for the presence of PC and the dental examination findings were recorded on a dental chart. Supragingival dental plaque was collected by rubbing a sterile, plain rayon-tipped microbiology swab (Copan; VWR International, Lutterworth, UK) firmly over the palatal aspects of the maxillary cheek teeth. Initially, samples were taken from multiple or even from all maxillary cheek teeth whilst recording whether these teeth were affected by PC or not (unpaired data, only one sample per horse). Later, following initial microbiological screening, it was decided to take two samples per horse: one sample from the rostral cheek teeth (Triadan 106-108, 206-208) and one from the caudal cheek teeth (Triadan 109-111, 209-211), recording the presence or absence of PC at these sampled sites (paired data, always one rostral and one caudal sample from same horse. Possible sample

combinations per horse were: one sample with PC, the other without PC (i.e. rostral sample with PC and caudal sample without PC; or rostral sample without PC and caudal sample with PC); both samples with PC; both samples without PC.

Infundibulae were sampled in seven horses that had at least one infundibulum with and one infundibulum without caries each, and in one horse where only a carious infundibulum was sampled.

Immediately after collection, swabs were placed into 2 mL SafeSeal micro tubes (Sarstedt, Nümbrecht, Germany) containing reduced transfer fluid (RTF) (Syed and Loesche, 1972) to decrease the oxygen concentration in the sample and optimise the survival of obligate anaerobic bacteria. To maximise its efficacy, RTF was prepared less than one hour prior to sample collection by adding 1 mL of freshly prepared dithiothreitol (DTT) solution to 1 mL of a premade stock solution of membrane sterilised water with K_2HPO_4, NaCl, $(NH_4)_2SO4$, KH_2PO_4, $MgSO_4$ x 7 H_20, Na_2CO_3, EDTA and resazurin and stored at 4°C until required.

For the direct microbiological method (i.e. culture-independent microbiology, using molecular microbiology directly without prior culturing of the samples) the samples were centrifuged for 10 minutes (13,000 rpm, 4°C), the supernatant was removed and replaced with TE buffer. Thereafter these samples were stored at -80°C until the samples were required for DNA extraction.

3.2.2 Conventional Microbiology

The swab containing dental plaque was stirred in a tube containing 1 mL of RTF. Serial ten-fold dilutions of the sample were prepared and then vortexed for 20 seconds, after which 100 ul of each sample dilution was placed on a Columbia agar plate containing 5% v/v defibrinated horse blood (Thermo Scientific Oxoid, Loughborough, UK). This fluid was then streaked out with a flame-sterilised loop. The plates were incubated both aerobically and anaerobically at 37°C with the anaerobic cultures placed in an anaerobic jar with an Oxoid AnaeroGen sachet

(Thermo Scientific Oxoid, Loughborough, UK). After 72 hours all visible colonies were picked off, placed in TE buffer and stored at -80°C.

3.2.3 Molecular Microbiology

3.2.3.1 DNA Extraction

Cell Lysis, DNA Purification and Elimination of Proteins and Lipids

DNA was extracted using phenol (Tris-buffered water saturated phenol, pH 8.0; Severn Biotech Ltd., Kidderminster, UK), chloroform (stabilized with amylene; Scientific Laboratory Supplies, Newhouse, UK), isoamylalcohol (reagent grade 98%; Sigma-Aldrich, Gillingham, UK) and a Mag Mini DNA extraction kit™ (LGC Genomics GmbH, Berlin, Germany). The samples were taken out of the -80°C freezer and centrifuged for 10 minutes at 4°C. To the thawed samples, each containing 50 microliter dental plaque and TE buffer, 50 µl *lysis buffer BL* (Mag Mini DNA extraction kit™) was added as well as 5 µl proteinase K to eliminate contaminating protein. A DNA extraction kit negative control, which consisted of 50 µl TE buffer without dental plaque, was included in each DNA extraction and processed in parallel with the samples. After gently mixing the mixture in all sample tubes 10 times with a pipette, samples were placed in a thermoblock (Dri-block®; Techne, Stone, UK) for one hour at 56°C. This temperature was chosen because it is the optimal temperature for enzymatic activity of proteinase K (Kottke-Marchant and Davis, 2012).

The samples were taken out of the thermoblock and the content of each microtube (2 mL DNA-free, DNase- and RNase-, PCR inhibitor-free SafeSeal micro tube, Sarstedt, Nümbrecht, Germany) was transferred to a bead beating tube containing 0.1 mm Zirconium beads (Beadbug™ prefilled tubes, 2.0 mL capacity; Sigma-Aldrich, Gillingham, UK). One hundred µl of TE buffer, 100 µl phenol, 96 µl chloroform and 4 µl isoamylalcohol (phenol/chloroform/isoamylalcohol, ratio of 25:24:1) were added to each bead beating tube. To disrupt bacterial cells mechanically, the bead beating tubes were placed in a bead beater (MP Biomedicals FastPrep-24™ Instrument; Thermo Fisher Scientific, Loughborough, UK) and the instrument was operated at speed setting 5 for 45 seconds. The bead beating tubes were cooled down

on ice for 2 minutes, then replaced in the bead beater machine and shaken at the same speed for the same length of time. They were again cooled down on ice for 2 minutes, before being placed in a centrifuge (Biofuge fresco; Heraeus Instruments GmbH, Hanau, Germany) for 10 minutes (13,000 rpm, 18°C). This resulted in formation of three different layers in the tube, including the lower layer of beads with cellular debris and lipids in phenol/chloroform/isoamylalcohol solution (organic phase), a white flocculent layer of denatured precipitated proteins and carbohydrates (interphase), and a top layer of TE buffer (aqueous phase) containing DNA (and RNA). As much of the top layer as possible was collected and placed into a new tube, "tube 2" (STARLAB International GmbH, Hamburg, Germany), taking care to avoid adding any of the two underlying layers.

A back extraction was also performed in order to collect as much of the remaining DNA as possible that otherwise might be left behind in the phenol phase (Towner, 1991; Henry, 2008). A volume of TE buffer was added to the bead beating tube similar to the volume of supernatant that had been removed. The bead beating tubes were shaken in the bead beater again at the same settings, but for a single cycle. They were then placed on ice for 2 minutes and placed in the centrifuge for 10 minutes (13,000 rpm, 18°C). As much supernatant as possible was collected without disturbing the underlying layers and was added to tube 2.

In order to remove phenol traces, equal volumes of a chloroform/ isoamylalcohol mixture (48:2 ratio of chloroform:isoamylalcohol) were added to tube 2. For example if 300 µl supernatant was collected in total, then 288 µl chloroform and 12 µl isoamylalcohol were added to tube 2. This mixture was vortexed vigorously for one minute and then centrifuged for 10 minutes (13,000 rpm, 18°C). Subsequently, as much supernatant as possible was collected (while avoiding collecting any of the chloroform / isoamylalcohol phase) and placed into a new tube (tube 3).

DNA Binding, Washing and Elution

Magnetic bead suspension (20µl *mag particle suspension BL*, Mag Mini DNA extraction kit^{TM}) and two volumes of *binding buffer BL* (Mag Mini DNA extraction

kit™) were added to tube 3. The solution was mixed gently by pipetting it up and down five times, followed by an incubation for 20 minutes at room temperature to allow enough time for binding of DNA to the magnetic beads.

The tubes were then placed on a 96 side magnet (DynaMag™, Life Technologies, Thermo Fisher Scientific, Loughborough, UK) for one minute and a pellet of magnetic beads (with bound nucleic acids) was formed at the side of each tube wall. Subsequently as much supernatant as possible was removed and discarded without dislodging the pellet.

0,2 M sodium chloride solution (65 µl) and 65 µl of ice cold isopropanol (molecular biology grade; Thermo Fisher Scientific, Loughborough, UK) were added to the tubes and were then placed in a freezer for 1 hour and 45 minutes at -20°C.

After the tubes were removed from the freezer, they were placed in a centrifuge for 5 minutes at 13,000 rpm and then placed on the magnet for one minute to allow removal of the supernatant fluid without aspirating the magnetic beads with their attached nucleic acids.

Pure ethanol (250µl, 70%; Thermo Fisher Scientific, Loughborough, UK) was added and the solution thoroughly mixed by pipetting up and down five times. After five minutes, the tubes were placed on a magnet for one minute and the supernatant removed. The tubes were air dried for five minutes and 70 µl of *wash buffer BL 2* (Mag Mini DNA extraction kit™) was added to each tube. The solution was mixed thoroughly by pipetting up and down five times or until the beads were fully resuspended. After five minutes incubation at room temperature, the tubes were placed on a magnet for one minute and the supernatant was aspirated and discarded.

Subsequently, all tubes were placed in a thermoblock for 10 minutes at 55°C. The lids of the tubes were left open to allow evaporation of ethanol and acetone to occur. The *elution buffer BL* (Mag Mini DNA extraction kit™) was preheated for the same length of time at the same temperature. The tubes were removed from the thermoblock and 63 µl of elution buffer BL was added to each tube and the pellet of magnetic beads and DNA was resuspended by pipetting up and down five times. The tubes were incubated for 10 minutes at 55°C while mixing the solution (pipetting it 5 times) during this incubation. The tubes were placed on a magnet for three minutes to

allow the beads to form a pellet and the eluate was transferred to 0.2 mL PCR tubes (Thermo Fisher Scientific, Loughborough, UK).

3.2.3.2 Concentration and Purity of Extracted DNA

A Nanodrop® spectrophotometer ND-1000 (LabTech International Ltd, Heathfield, UK) was used to assess the concentration and purity of extracted DNA. After cleaning the pedestal with 5 µl sterilised water, and using 1µl of elution buffer as a blank, each sample was analysed by placing 1µl from one of the PCR tubes onto the pedestal; the arm was closed and this instrument measured absorbance of the sample at a variety of wavelengths (220 nm - 350 nm). In between each sample, the pedestal was dried with a paper tissue.

The recorded peak wavelength of DNA containing samples should be 260nm, i.e. the wavelength at which DNA absorbs UV light. The 260/280 ratio should be >1.8 and 260/230 ratio around 2.0-2.2. Contamination can invariably cause altered wavelength ratios. For example, phenol contamination produces a 270nm peak in addition to a 220-230 nm peak (visible in the plot). Consequently, an overestimation of the DNA concentration can occur while the 260/280 and 260/230 ratios could be within normal ranges (making it look like pure DNA) or outside the ranges (reflecting the contamination). By only checking the wavelength report and not the plot, the DNA could erroneously appear to be very pure, while in fact phenol contamination is present. Therefore both the report with the concentrations, 260/280 and 260/230 ratios as well as the plot (for 220-230 nm and 270 nm peaks) were closely examined to assess the purity of DNA in all samples.

After the nanodrop measurements the PCR tubes with extracted DNA were stored at -80°C until required.

3.2.3.3 PCR

Barcoded Primers

The primers used were the 515F primer (**GTGCCAGCMGCCGCGGTAA**; 19bp**)** and 806R primer (**GGACTACHVGGGTWTCTAAT**; 20bp) designed by Caporaso et al. (2011). The barcoded primers (4nmole Ultramers with TruGrade processing)

included standard Illumina TruSeq i5 and i7 barcodes (Integrated DNA Technologies, Leuven, Belgium)

PCR Protocol

A Master Mix was prepared containing Phusion Hot Start II high-fidelity DNA polymerase to minimize PCR errors (Thermo Fisher Scientific, Loughborough, UK), Phusion HF Buffer, a dNTP mix (Thermo Fisher Scientific, Loughborough, UK) and PCR-grade water (Roche Diagnostics GmbH, Mannheim, Germany). To each 0.2 mL PCR tube, which was part of a strip of 8 PCR tubes (Thermo Fisher Scientific, Loughborough, UK), 15 µl Master Mix, 5 µl sample and 2.5 µl of the Forward and Reverse primers were added (Table 3.1).

Table 3.1. The volume of Master Mix, primers and sample added to PCR tube (Thermo Fisher Scientific, Loughborough, UK). The volume of each Master Mix component was multiplied by the number of samples to create the Master Mix using a 2 mL DNA-, DNase-, RNase- and PCR inhibitor-free microtube (Sarstedt, Leicester, UK). Then 15 µl of Master Mix was added to each PCR tube.

	Component	**Volume per well (µl)**
Mastermix (15 µl per well)	Phusion HF buffer 5x	5
	dNTPs mix 10 mM each	0.5
	H20	9
	Phusion II HS HF Dna Polymerase	0.5
	Component	**Volume per well (µl)**
Individual (5 µl in total)	Forward primer 5uM	2.5
	Reverse primer 5 uM	2.5
Individual (5µl)	Sample	5

A negative control was included in each PCR run (and named "NEG" + a code relating to the samples in that run, for example NEG2, NEGofF) which consisted of 5 µl PCR grade water, instead of a 5 µl sample. The same amount of Master Mix and primers were added to the negative control as to each sample. DNA of a known concentration (21.7 ng/µl) that was extracted from a subculture of aerobically

cultured bacteria isolated from dental plaque was used as a positive control in each PCR run. It was added to the PCR tube instead of the 5 µl sample with the same amount of Master Mix and primers as described above.

PCR conditions were as follows. A denaturation step of 98°C for 30 seconds followed by 30 cycles of 98°C for 10 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (extension) and one final cycle of 72°C for 300 seconds, after which the PCR instrument (Mycycler^{TM}; BioRad, Watford, UK) was cooled down to 15°C (Table 3.2). In PCR terms, a cycle is defined as a series of repeated temperature changes. The (initial) denaturation step at 98°C when single strands are formed by disrupting hydrogen bonds between complementary bases. An annealing step takes place at 55°C, when the primers anneal to the single-stranded DNA template. The elongation step occurs at 72°C. During this step each DNA polymerase synthesises a new DNA strand complementary to the DNA template strand. The final elongation step serves to fully elongate any remaining single DNA strand. The final hold step is used to cool down the reaction chamber of the PCR instrument, as well as the PCR tubes and contents and can be used for short-term storage.

PCR of a mock community was also performed (HM-783D: staggered low mock community 16S PCR for Sanger or amplicon sequencing, BEI resources, ATCC, Manassas, VA, USA) using the same protocol in order to check whether all bacteria present in this mock community could be identified.

Table 3.2. PCR protocol used to attach the barcoded primers to the DNA strands and to amplify the V4 region of each 16S rRNA gene.

(s)	temp (°C)	Cycles
30	98	1x
10	98	30x
30	55	
30	72	
300	72	1x
forever	15	

3.2.3.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyse PCR products before and after ampure bead purification. To a 500 mL laboratory bottle, 1.2 gram Agarose powder (Ultrapure™ Agarose; Thermo Fisher Scientific, Loughborough, UK), 100 mL of 1X Tris-acetate-EDTA (TAE) buffer, and 10 µl of SYBR™ Safe DNA gel stain (Thermo Fisher Scientific, Loughborough, UK) were added. The bottle was then placed into a microwave oven for two minutes at full power until all agarose powder was dissolved and bubbles were seen in the solution. After the solution had cooled down to approximately 60°C, it was poured into a gel casting tray. After 30 minutes, when a gel had formed, the gel and tray were placed into the electrophoresis chamber. The sample combs were removed and, if necessary, some extra TAE buffer was added to ensure the gel was fully covered by this buffer.

The first and final well were loaded with 5 µl of 1kb DNA ladder (Promega, Southampton, UK) along with 2 µl of loading dye (Promega, Southampton, UK). The other wells were filled with 5 µl of sample (PCR product) and 2 µl of loading dye.

The gel was run at 140 volts for 30 minutes in an agarose gel electrophoresis instrument (Horizon™ 11.14, Gibco BRL Gel electrophoresis apparatus by Life Technologies; Thermo Fisher Scientific, Loughborough, UK). The gel was then analysed using a Molecular Imager® Gel Doc™ XR+ instrument (BioRad, Watford, UK).

3.2.3.5 Purification of PCR Products with Ampure Beads

In a 96 well PCR plate (0.2 mL ultra-rigid semi-skirted 96-well PCR plates, Thermo Fisher Scientific, Loughborough, UK) 16 µl PCR product and 16 µl beads (shaken before use) were added to each well and this solution was gently mixed with a pipette 10 times. After 5 minutes incubation at room temperature, the PCR plate was placed on a magnet for 2 minutes, ensuring the solution was clear. The supernatant was aspirated and discarded. While keeping the PCR plate on the magnet, 200 µl of 80% ethanol was added for 30 seconds and then aspirated and discarded. The ethanol step

was repeated once and the PCR plate was then left to dry for 5 minutes to allow the remaining ethanol to evaporate. Subsequently, the PCR plate was removed from the magnet and 40 µl elution buffer (buffer EB; Qiagen, Manchester, UK) with 0.1% Tween™-20 (Thermo Fisher Scientific, Loughborough, UK) was added to each well. This solution was mixed with a pipette 10 times and, after 2 minutes incubation at room temperature, the PCR plate was placed on the magnet again for 1 minute. The eluate was then transferred to a new PCR plate.

3.2.3.6 Qubit

The Qubit® dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific, Loughborough, UK) was used to measure the concentration of the PCR products in a Qubit® Fluorometer (Thermo Fisher Scientific, Loughborough, UK). This assay is claimed to be accurate for initial sample concentrations of 10 pg/µL to 100 ng/µL.

Sterile 0.5 ml PCR tubes (Fisherbrand™; Thermo Fisher Scientific, Loughborough, UK) were labelled and placed in the ultraviolet crosslinker instrument (Hoefer™ UVC 500 crosslinker, Hoefer, San Francisco, CA, USA). A working solution was prepared in a 2 mL microtube by diluting *Qubit® dsDNA HS Reagent* 1:200 in *Qubit® dsDNA HS Buffer*. Purified PCR product (2µl) was transferred from each well of the PCR plate to 0.5 mL PCR tubes and 198 µl working solution was added to each tube. For the standards; 10 µl *Standard 1* and 190 µl working solution was added to a 0.5 mL PCR tube, and 10 µl *Standard 2* and 190 µl working solution was added to another 0.5 mL PCR tube.

Each 0.5 mL PCR tube was vortexed for 3 minutes and then allowed to stand for 2 minutes before measuring the concentrations of the samples in a Qubit® Fluorometer.

3.2.3.7 Pooled Library, Quality Control and Next Generation Sequencing

Concentration measurements from the Qubit® Fluorometer were included in a table and the volume of sample that needed to be added to the pooled library was calculated (Table 3.3).

A pooled library was created by adding equimolar amounts of each amplicon to a micro tube. Quality control was performed using an *Agilent 2100 Bioanalyzer* (Santa Clara, CA, USA), and paired-end Next Generation Sequencing (NGS) of amplicons was carried out using Illumina MiSeq (San Diego, CA, U.S.) by Edinburgh Genomics using the MiSeq Reagent Kit v2.

Table 3.3. Calculation of the volume of each sample needed to be added to the pooled library to make the final pooled library equimolar.

Concentration ng/µl	Base Pair Length	MW calculation (g/mol)	nmol/µl	nM	Volume added to library (ul)
	427	(bp length* 607.4) +157.9	concentration/ bp length	nmol/µl* 1000000	(concentration/ median nM)*2

3.2.4 Bioinformatics

Primers were removed from both the forward and reverse sequencing reads using cutadapt version 1.4 (Martin, 2011). The level of error tolerance was 0.1 for the forward reads and 0.2 for the reverse reads, meaning that sequences containing more than one base or two base errors, respectively, per 10 primer bases were removed.

The MiSeq Standard Operating Procedure (Kozich et al., 2013) was used as a protocol to further process and analyse the data in MOTHUR version 1.33.3 (Schloss et al., 2009).

The forward and reverse reads were then aligned. Sequences that failed to align; had ambiguous bases, contained more than 275 base pairs or had a homopolymer length of more than 9 were removed to minimize sequencing and PCR errors. Duplicates (identical sequences) were identified and merged so that only unique sequences were left. These unique sequences were then aligned to the V4 region of the SILVA reference database. Chimeras were detected and removed with the UCHIME algorithm within MOTHUR. Undesirable sequences (i.e. sequences other than bacterial 16S rRNA, such as 18S rRNA gene fragments or 16S rRNA from Archaea, chloroplasts, and mitochondria) that were amplified by the primers were identified using the Bayesian classifier against the Greengenes reference database and then

removed. After the sequences of the mock samples were removed from the dataset and the error rate of the remaining samples determined, the sequences of the remaining samples were clustered into operational taxonomic units (OTUs). These OTUs were clustered using taxonomic information from the Greengenes reference database, and the number of reads per OTU per sample as well as the taxonomy of each OTU determined.

3.2.5 Statistical Analysis

To standardise the data obtained, subsamples containing the same number (2,242) of reads per sample were created, which were then used for further analysis. A Good's coverage estimator (Esty, 1986) was calculated for each sample to check if the sequencing depth of the subsamples was sufficient. Alpha-diversity metrics such as Observed Species Richness (Sobs) which reflects the number of OTUs/sample, Chao-1 (Chao, 1984; Colwell and Coddington, 1994; Gotelli and Colwell, 2011), Shannon Diversity Index, and Inverse Simpson's Index per group were used to measure the diversity within samples. Mann-Whitney tests were applied to the alpha-diversity metrics to assess whether there were statistically significant differences in diversity within samples between groups. Beta-diversity analysis was performed to compare samples to each other. Distance matrices were created using the Yue and Clayton theta similarity coefficient (Yue and Clayton, 2005) which represented a measure of similarity between structures of different communities. These distance matrices were used to perform a Principal Component Analysis (PCA), Analysis of MOlecular VAriance (AMOVA) and HOmogeneity of MOlecular Variance (HOMOVA). A PCA was performed to visualise similarities and dissimilarities in the data. The microbiological profiles of the groups were compared using AMOVA and the intrapopulational genetic diversity between communities was analysed using HOMOVA. Additionally a Bray Curtis dissimilarity percentage between groups was calculated. Also PERmutational Multivariate ANalysis Of VAriance (PERMANOVA) was performed using Bray Curtis dissimilarities to test whether the centroid and/or spread of the objects was different between groups.

In order to identify OTUs or taxa which were most likely to be responsible for differences between groups, linear discriminant analysis (LDA) effect size (LEfSe)

was performed at OTU level, family level and/or genus level. LefSe plots were created in Galaxy (Segata et al., 2011; Afgan et al., 2016). In Galaxy the groups (i.e. caries and healthy) were filled in in the "class" field and the horses were assigned a separate code which was filled in in the "subjects" field. All other graphical representations were performed in R (R Core Team, 2014).

3.3. Results

3.3.1 Study Population

Samples were taken from 63 horses of mean age 12.2 years (range: 4-24 years; SD=5.21), of which 71.4% (45/63) had PC. Rostral and caudal cheek tooth peripheral samples were obtained in 38 of the 63 horses and infundibular samples were collected from 8 of these 63 horses. The sampled population included 25 Warmblood horses (10 unspecified Warmblood horses, 15 specified Warmblood horses: 3 Appaloosas, 3 Irish Draughts, 2 Cobs and 2 Cob-cross breeds, 2 Irish sports horses, 1 Andalusian, 1 Friesian, 1 Irish Draught cross); 19 ponies (8 Exmoor, 3 Highland, 2 Connemara, 2 Welsh ponies, 2 unspecified breeds, 1 Shetland pony, 1 Welsh Cob), 4 Thoroughbreds, 4 Thoroughbred-cross breeds, 3 Arabian horses, 2 Clydesdale-cross, 1 Clydesdale and 5 unknown breeds. If smaller groups (<n=10) were excluded from analysis or if these less common breeds were combined into one group named "other", then Warmblood types were more likely to have PC (OR= 8.15, 95% C.I. 1.81 - 36.71, P=0.006) than ponies.

There was no statistical significant difference in the presence of PC between male (n=37) and female horses (n=26) and the presence of PC did not differ significantly with age.

In a multivariable logistic regression model that included sex, breed and age, only breed remained significantly different (Likelihood Ratio test P = 0.013), with Warmblood horses more likely to have PC (OR=10.89, 95% C.I. 1.8, 65.86, P=0.009) than ponies.

There was no statistical difference in the presence of PC if Candida was present (n=20) or absent (n=20) on the palatal aspect of maxillary cheek teeth (23 horses not tested for Candida).

3.3.2 Sequencing Output

The electropherogram had only one clear peak showing that the quality of the pooled library was good.

The total number of sequences before quality processing was 13,978,816. After quality processing and removing mock communities, 9,901,855 reads remained (29% loss of sequences). These reads were clustered in an OTU table and consisted of 14,490 OTUs. The sequencing error rate was 0.02%. The minimum number of reads per sample was 1, with a maximum of 1,277,756 (median 29,230; mean 34,743; standard error of the mean 1,438).

Subsampling was performed with 2,242 reads per sample. This resulted in a loss of 3 samples and 3 negative controls, each which contained less than 2,242 reads. After subsampling, 3,217 OTUs remained and were used for further analysis. These OTUs corresponded to 21 phyla, 41 classes, 69 orders, 141 families, 243 genera and 122 species.

The minimum Good's coverage was 0.94 (median 0.99; mean 0.99; maximum 1.00), meaning that a minimum of 94% of bacteria of the original sample were likely to be found. This indicated that the sequencing depth of the subsamples was sufficient.

The negative controls which showed a band on the gel after performing gel agarose electrophoresis, were selected for NGS to identify which bacteria were present in these negative controls. Most of the negative controls were extraction kit controls, except for NEG2 and NEGofF which were PCR controls. The OTUs of the extraction kit controls and PCR controls were not removed from the OTU table because these OTUs belonged to bacteria including *Streptococcus* and *Veillonella* which were often found in the oral cavity. It is possible that some of the negative controls were contaminated with samples and therefore showed a band indicating

presence of bacteria on a picture of a gel after agarose gel electrophoresis. The most common OTUs in the negative controls are shown in Fig 3.1.

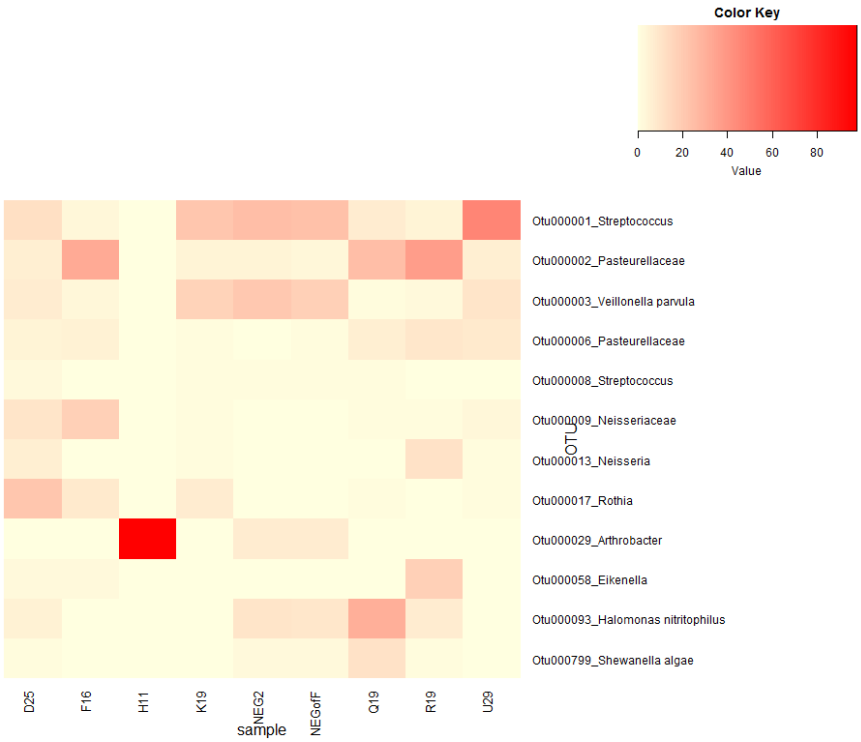


Fig 3.1. Heatmap showing the relative abundance of OTUs which were most abundant (relative abundance >1%) in the extraction kit controls (D25, F16, H11, K19, Q19, R19, U29) and PCR controls (NEG2, NEGofF).

The compositions of bacterial communities of all sample groups (culture-independent IC and control group and culture-independent and culture-dependent (aerobically and anaerobically cultured) PC and control groups) differed significantly (AMOVA: $p=0.03$ for anaerobic caries, $p<0.001$ for all other groups) from the negative controls of the extraction kit and PCR kit.

A mock community was used to check whether all the known bacteria it contained could be identified. All bacteria were correctly identified up to genus level, however at species level, 11 out of 20 species were marked as unclassified; only 6 out of 20

species were completely and correctly identified, and 3 out of 20 species were wrongly assigned.

3.3.3 Culture-independent Bacteriology: Peripheral Caries versus Control Group

Examination of 56 samples from teeth with PC (PC group) and 44 samples from teeth without PC (control group) revealed a total of 1,854 OTUs in the samples collected for culture-independent bacteriology. These corresponded to 21 phyla, 40 classes, 64 orders, 125 families, 204 genera, 102 species. These results included one unclassified category for each taxon.

3.3.3.1 Alpha-Diversity: Diversity within a Sample: Within-Habitat Diversity

The mean number of OTUs per sample in the PC group was 91 (SD = 43; range = 40-283 OTUs) and in control samples 97 OTUs (SD = 42; range = 40-221 OTUs). The diversity of the paired (rostral, caudal) and unpaired samples within the PC group was not statistically significantly different from the diversity of the paired (rostral, caudal) and unpaired samples within the control group.

3.3.3.2 Beta-diversity: Comparison of Samples to Each Other: Between-Habitat Diversity

Although the clustering of the microbiomes of the PC group and control group was not easy to distinguish in a PCA plot (Fig 3.2), compositions of bacterial communities differed significantly between the PC group and control group (AMOVA p-value <0.001) and the genetic diversity between the communities from these groups was significantly different (HOMOVA p-value=0.01), with a larger variation in genetic diversity within the control group (0.23) than in the PC group (0.16). The difference between microbial profiles of the PC group and control group was not statistically different (p = 0.371, F = 1.0482, PERMANOVA).

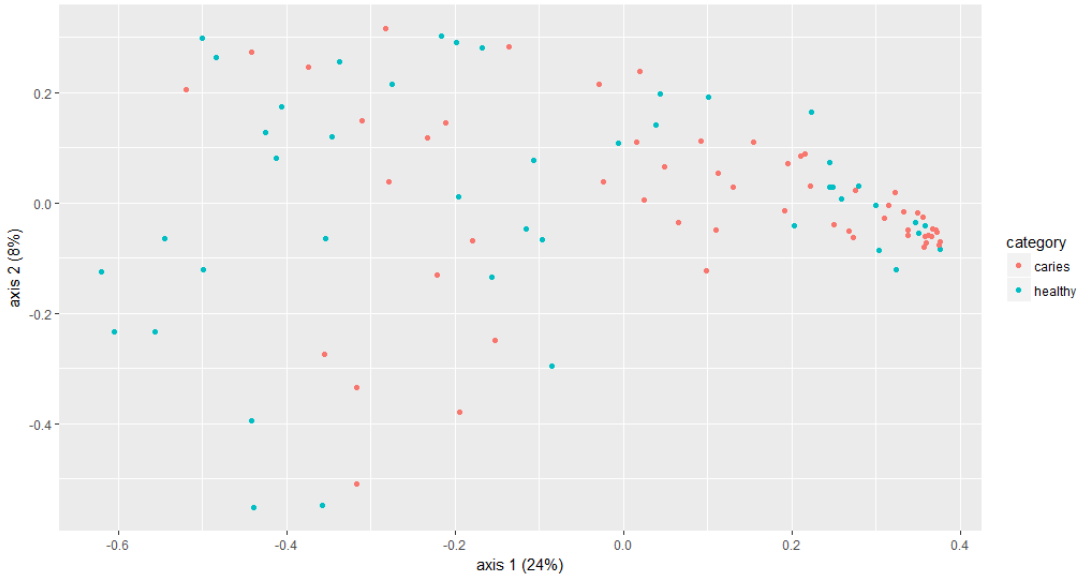


Fig 3.2. PCA plot of the culture-independent samples of the PC versus control group. Axes 1 and 2 show 24% + 8% = 32% variation for the thetaYC distances. A dissimilarity percentage of 57% was found between the PC and control group.

3.3.3.3 Comparison of Bacterial Composition of Peripheral Caries and Control Groups

The relative abundance of Firmicutes in the PC group was higher (76.8%) than in the control group (70.7%) (Fig 3.3). However, Proteobacteria were lower in relative abundance in the PC group (8.8%) compared to the control group (15.5%).

For paired data (including 28 samples of 14 horses) the relative abundance of Firmicutes was significantly higher (78.7%) in the PC group than in the control group (69.8%) (p-value of paired t-test= 0.01). Proteobacteria in the PC group (7.3%) were significantly lower in abundance (p-value of paired t-test<0.001) than in the control group (15.1%).

For unpaired data (which included only 6 healthy samples and 18 PC samples), the relative abundance of Firmicutes was slightly lower in the PC group (70.1%) compared to the control group (74.0%), but this was not significant (p-value of 2 sample t-test=0.52). The difference between Proteobacteria was also not significant (PC group 13.8%, control group= 11.6%, p-value of 2 sample t-test=0.56)

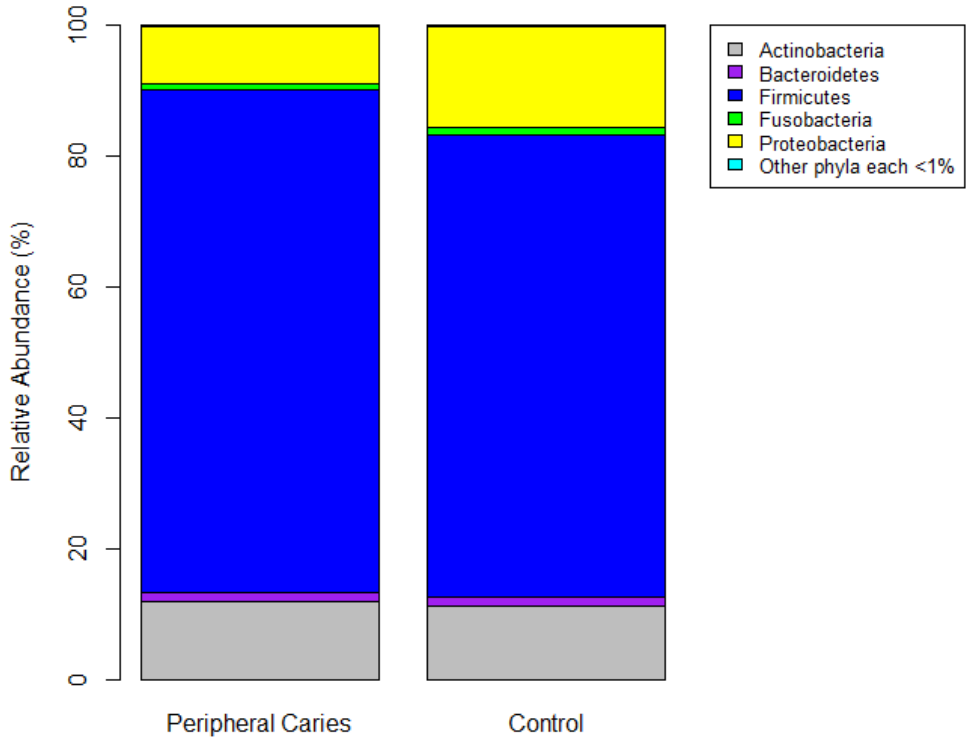


Fig 3.3. Relative abundance of phyla (in percentage) present in samples of peripheral caries (n=56) and control groups (n=44). "Other phyla each <1%" included: Acidobacteria, Chlamydiae, Chlorobi, Chloroflexi, Cyanobacteria, Fibrobacteres, GN02, Planctomycetes, Spirochaetes, SR1, Synergistetes, Tenericutes, TM7, Verrucomicrobia, [Thermi] and unclassified phyla.

Using the culture-independent microbiology method, the relative abundance of the combined unclassified genera of the control group was 11.8% and for the PC group 8.2%

Within the PC group, 156 classified genera were identified and within the control group 164 classified genera were identified. A total of 203 classified genera were identified between the PC group and control group, including 117 shared genera.

Of the 203 genera present in the PC and/or control group, 25 classified genera had an abundance larger than 0.1% and are displayed in

Table 3.4.

The most common identified genera (>1%) in the caries group were respectively Streptococcus, Gemella, Actinomyces, Arthrobacter, Lactobacillus, Veillonella and Actinobacillus. In the control group, the most common identified genera (>1%) were respectively: Streptococcus, Gemella, Actinomyces, Actinobacillus, Arthrobacter, Veillonella and Eikenella.

When assessed at OTU-level, LEfSe showed statically significant differences between the culture-independent PC group and control group for 70 out of 1854 OTUs with an LDA score of >2 and a p-value of <0.05. Twenty-four OTUs representing bacterial taxa were found to be associated with PC and 46 OTUs representing bacterial taxa were found to be associated with the control group (Table 3.5).

From 303 taxa identified at the genus or higher level, 51 discriminant taxa were found between the PC and control group (LDA score >2, p-value<0.05). Fourteen taxa were associated with PC and 37 taxa with control group (Fig 3.4). Gemella and Actinobacillus were the genera most associated with the control group. The genera most associated with PC were Streptococcus, Olsenella and Scardovia. Additionally, if LEfSe was performed at genus level only, then Mitsuokella was also shown to be associated with PC.

From 161 taxa at family or higher level, 29 taxa were found to be significantly different between the PC (n=10) and the control group (n=23) (Fig 3.5).

Table 3.4. Relative abundance (%) of the most common classified genera (at least in one group >0.1%) present in the peripheral caries and control groups.

Genus	Relative Abundance (%) in Peripheral Caries Group (n =56)	Relative Abundance (%) in Control Group (n=44)
Acinetobacter	0.13	0.02
Actinobacillus	1.41	3.54
Actinomyces	6.39	6.29
Alysiella	0.14	0.18
Arthrobacter	2.65	1.70
Corynebacterium	0.06	0.38
Eikenella	0.49	1.42
Fusobacterium	0.25	0.37
Gemella	6.75	10.95
Kingella	0.27	0.34
Lactobacillus	2.06	0.73
Lautropia	0.49	0.90
Leptotrichia	0.75	0.67
Megasphaera	0.16	0.04
Moraxella	0.34	0.52
Neisseria	0.06	0.73
Olsenella	0.81	0.34
Porphyromonas	0.10	0.09
Prevotella	0.90	0.75
Pseudomonas	0.44	0.01
Rothia	0.17	0.36
Scardovia	0.14	0.06
Sharpea	0.17	0.12
Streptococcus	63.32	54.74
Veillonella	1.99	1.62

Key: Red=Genus more abundant in Peripheral Caries group; Green=Genus more abundant in Control Group.

Table 3.5. Results of linear discriminant analysis effect size (LEfSe) demonstrate which OTUs are statistically significantly different between the peripheral caries and control group (LDA score>2, p<0.05).

OTU_ID and taxon	Log of the highest class average	Class	LDA effect size	p-value
Otu000659_*Eubacterium_cylindroides*	2.047304	healthy	2.125166	0.01933
Otu000240_Weeksellaceae	2.63938	healthy	2.147084	0.0149087
Otu000248_*Mogibacterium*	2.328131	healthy	2.170491	0.0475354
Otu000415_*Sharpea_azabuensis*	2.119855	healthy	2.181834	0.0455965
Otu000640_Leptotrichiaceae	2.468309	healthy	2.198146	0.0133146
Otu000178_*Oribacterium*	2.348334	healthy	2.201494	0.0162098
Otu000581_*Butyrivibrio*	2.348334	healthy	2.226966	0.0485558
Otu000395_*Leptotrichia*	2.668669	healthy	2.245574	0.024569
Otu000275_Neisseriaceae	1.85101	healthy	2.248734	0.0484059
Otu001972_*Leptotrichia*	2.403852	healthy	2.269446	0.0010365
Otu000674_Lactobacillales	2.284665	healthy	2.271497	0.0228274
Otu001577_Ruminococcaceae	1.704881	healthy	2.284537	0.0484059
Otu001915_Ruminococcaceae	1.851009	healthy	2.291288	0.0484059
Otu001077_*Suttonella*	1.784063	healthy	2.326568	0.0493715
Otu000383_*Prevotella*	1.85101	healthy	2.330386	0.0210154
Otu000823_*Fusobacterium*	1.85101	healthy	2.337522	0.01933
Otu000445_*Pantoea_agglomerans*	1.909001	healthy	2.359275	0.0438021
Otu001353_Leptotrichiaceae	1.704881	healthy	2.378932	0.0474524
Otu000121_*Alysiella_crassa*	3.238908	healthy	2.427739	0.0067927
Otu002473_Lachnospiraceae	1.607972	healthy	2.441017	0.0484059
Otu000360_*Streptococcus*	1.909001	healthy	2.466362	0.0020913
Otu000161_*Streptococcus*	1.784063	healthy	2.487398	0.0219813
Otu002434_*Capnocytophaga_canimorsus*	1.607972	healthy	2.511122	0.0484059
Otu000380_Moraxellaceae	1.704882	healthy	2.511707	0.0484059
Otu004638_*Odoribacter*	1.483033	healthy	2.528382	0.0484059
Otu000545_*Bosea_genosp_*	1.483033	healthy	2.531465	0.0484059
Otu000191_Pasteurellaceae	3.055129	healthy	2.577837	0.0386693
Otu000093_*Halomonas_nitritophilus*	2.898006	healthy	2.637369	0.0143639
Otu000570_*Prevotella*	1.704881	healthy	2.638104	0.0100501
Otu000172_*Corynebacterium*	3.022945	healthy	2.707606	0.0176147
Otu000133_*Kingella*	3.466809	healthy	2.708468	0.0338681
Otu011044__Tissierellaceae_	1.607971	healthy	2.745829	0.0219813
Otu000462_*Streptococcus*	1.483033	healthy	2.749221	0.0484059

Otu000073_Microbacteriaceae	3.251425	healthy	2.834486	0.0341582
Otu000569_*Olsenella*	1.607972	healthy	2.949872	0.0484059
Otu000006_Pasteurellaceae	3.810732	healthy	3.000815	0.0425434
Otu000090_*Peptostreptococcus_anaerobius*	3.412452	healthy	3.08809	0.0437203
Otu000056_*Lautropia*	3.954325	healthy	3.362971	0.0109872
Otu000023_*Streptococcus*	3.960637	healthy	3.481053	0.0001412
Otu000009_Neisseriaceae	3.906825	healthy	3.495488	0.0011223
Otu000013_*Neisseria*	3.862036	healthy	3.585076	0.0480475
Otu000058_*Eikenella*	4.15266	healthy	3.704824	0.036172
Otu000020_Neisseriaceae	4.465907	healthy	3.909271	0.0004235
Otu000057_*Actinobacillus_porcinus*	4.548861	healthy	3.969596	0.0040698
Otu000018_*Rothia*	4.722582	healthy	4.286744	0.0013814
Otu000012_*Gemella*	5.039014	healthy	4.312638	0.0081531
Otu000213_*Capnocytophaga_ochracea*	2.01512	caries	2.133082	0.049007
Otu000036_*Streptococcus*	2.262904	caries	2.133178	0.0339958
Otu001010_*Prevotella*	2.047304	caries	2.143229	0.0431239
Otu000434_*Atopobium*	2.33254	caries	2.219624	0.015156
Otu000108_*Lactobacillus_delbrueckii*	2.33254	caries	2.257934	0.015156
Otu000202_*Leptotrichia*	2.950394	caries	2.295879	0.0129056
Otu000922_*Sharpea_p_3329_23G2*	1.804266	caries	2.333436	0.0431239
Otu000411_*Selenomonas*	2.544629	caries	2.38832	0.0156233
Otu000037_*Streptococcus*	2.657051	caries	2.39754	0.0007939
Otu000852_*Bulleidia_p_1630_c5*	2.015119	caries	2.417355	0.0259569
Otu000277_*Veillonellaceae*	2.770408	caries	2.41937	0.0073007
Otu001000_*Lachnospiraceae*	1.746274	caries	2.423267	0.0431239
Otu000273_*Mitsuokella*	1.855419	caries	2.434647	0.0272761
Otu000280_*Olsenella_profusa*	2.71409	caries	2.492726	0.0259569
Otu000030_*Streptococcus*	2.973058	caries	2.498356	0.0050307
Otu000639_*Streptococcus*	1.600146	caries	2.516048	0.0431239
Otu000381_*Sharpea_p_3329_23G2*	2.892402	caries	2.557891	0.0136142
Otu000025_*Scardovia*	3.139222	caries	2.667925	0.0006945
Otu000138_*Leptotrichia*	3.206528	caries	2.846185	0.0000724
Otu000041_Propionibacteriaceae	3.369524	caries	2.885182	5.26E-06
Otu000028_*Olsenella_profusa*	3.754874	caries	3.12792	0.0096623
Otu000072_*Lactobacillus*	3.690052	caries	3.225969	0.0148045
Otu000008_*Streptococcus*	4.844523	caries	4.189506	0.0002459
Otu000001_*Streptococcus*	5.605627	caries	4.833979	0.0027637

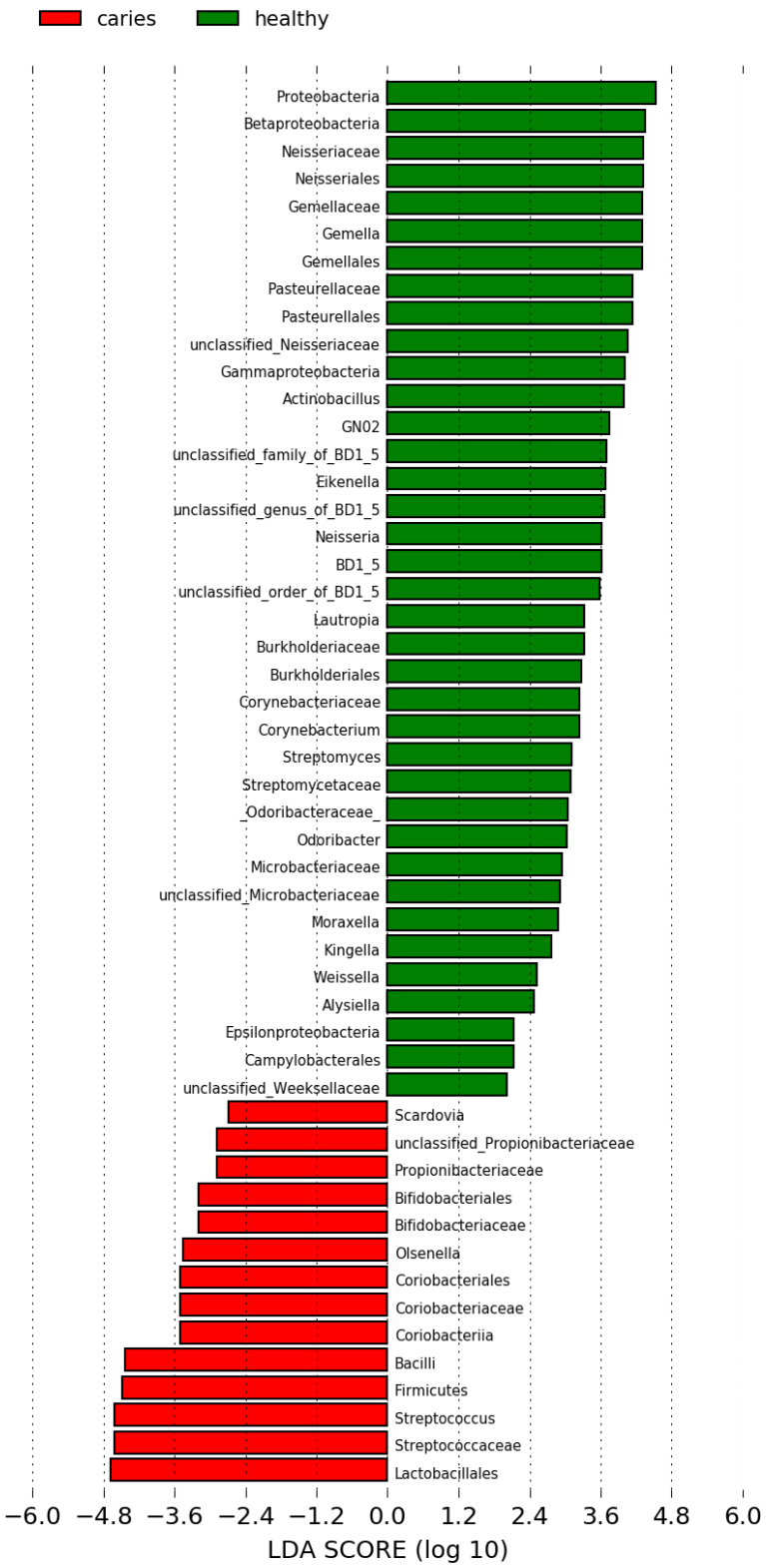


Fig 3.4. Results of linear discriminant analysis effect size (LEfSe) at the genus or higher level, showing which taxa are statistically significantly more associated with the peripheral caries or control group (LDA score>2, p<0.05).

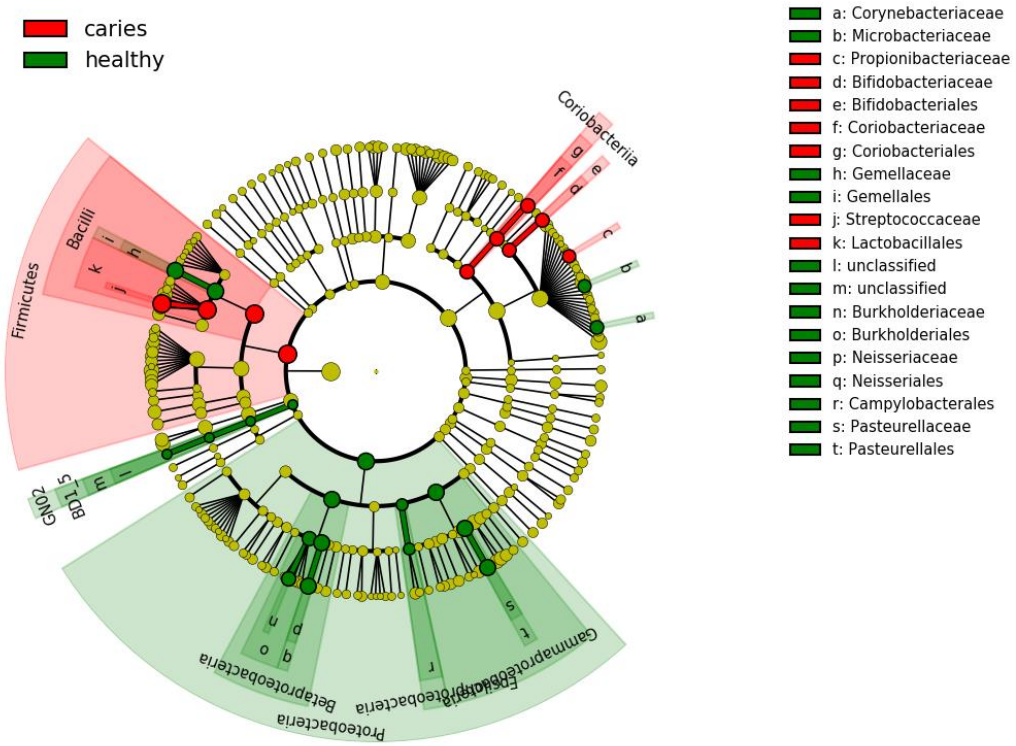


Fig 3.5. Cladogram depicting the results of linear discriminant analysis effect size (LEfSe) at the family or higher level, showing which taxa are statistically significantly more associated with the PC or control group (LDA score>2, p<0.05). The size of each circle is proportional to the abundance of the taxon it represents.

3.3.3.4 Comparison of Bacterial Composition of Rostral and Caudal Cheek Teeth PC and Control Groups

Both rostral and caudal cheek teeth samples were collected in 38 horses. Of the rostral cheek teeth samples, 24 belonged to the control group (63.2%) and 14 to the PC group (36.8%). Of the caudal cheek teeth samples, 14 belonged to the control group (36.8%) and 24 to the PC group (63.2%). Twelve of the 38 horses (31.6%), had no PC rostrally or caudally; 12 of the 38 horses (31.6%), had PC on both rostral and caudal cheek teeth; 2 of 38 (5.3%) had PC only on rostral cheek teeth and 12 of 38 (31.6%) had PC only on caudal cheek teeth. Using a Generalised Linear Mixed-Effects Model with horse taken into account as a random effect, the caudal maxillary cheek teeth (09s-11s) were significantly more likely to be affected by PC (OR=5.07, 95% C.I. 1.28-20.04, P=0.02) than the rostral maxillary cheek teeth (06s-08s).

Out of 1573 OTUs, 45 OTUs were shown to be statistically significantly different between the sample groups rostral cheek teeth PC, caudal cheek teeth PC and control group using a LEfSe at OTU-level (LDA score >2, p-value<0.05) (Supplementary Item 7).

From 262 taxa identified at the genus or higher level, 42 discriminant taxa were found between the rostral and caudal PC and control groups (LDA score >2, p-value<0.05). Three taxa were associated with rostral cheek teeth PC, ten taxa were associated with caudal cheek teeth PC and 29 taxa with the control group (Fig 3.6). The genera found to be most commonly associated with PC of the rostral and caudal cheek teeth and the control group (genera with highest LDA score) were respectively *Veillonella*, *Streptococcus* and *Corynebacterium*.

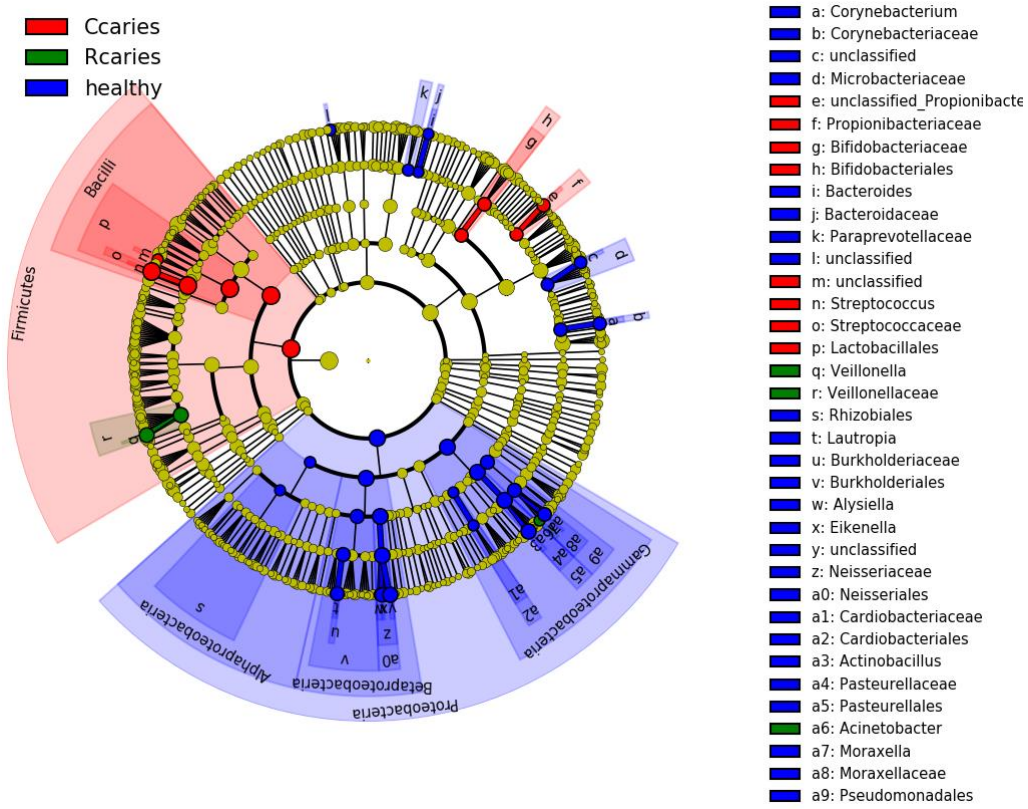


Fig 3.6. Cladogram depicting the results of linear discriminant analysis effect size (LEfSe) at the genus or higher level, showing which taxa are statistically significantly more associated with rostral cheek teeth PC, caudal cheek teeth PC or control group (LDA score>2, p<0.05). The size of each circle is proportional to the abundance of the taxon it represents.

3.3.4 Culture-dependent Bacteriology: Peripheral Caries Group versus Control Group

Molecular bacteriology of the anaerobic and aerobic cultures identified 1614 OTUs, of which 189 overlapped.

This corresponded to identification of 120 classified genera in the anaerobic and aerobic cultures. The anaerobic culture had 53 genera in common with the aerobic culture, indicating that 44.2% (53/120) of cultivable genera under current culture conditions (i.e. 3 days growth at 37°C on horse blood agar plates) allows both aerobic and anaerobic bacterial growth (facultative anaerobes or micro-aerotolerant bacteria). Culture-dependent bacteriology identified 59.1% (120/203) of the classified genera that were identified by culture-independent bacteriology. If only the culture-independent samples which were also aerobically and anaerobically cultured were compared, then the percentage of classified genera identified with culture-dependent bacteriology was 67.8% (120/177) of the genera identified with culture-independent bacteriology.

3.3.4.1 Molecular Bacteriology of Aerobic Cultures

The 709 OTUs present in the aerobic cultures of the culture-dependent bacteriology group containing PC samples and control samples corresponded to 7 phyla, 14 classes, 27 orders, 57 families, 88 genera, 44 species. This included one unclassified category for each taxon.

Of the aerobic cultures 46 samples belonged to the PC group and 27 samples to the control group.

3.3.4.1.1 Alpha-Diversity: Diversity within a Sample: Within-Habitat Diversity

The mean number of OTUs per sample in the aerobically cultured PC group was 40.4 (SD = 13.9; range = 17-69 OTUs) and the control group was 39.1 OTUs (SD = 12.8; range= 16-65 OTUs). The within-sample diversity of the paired and unpaired samples was not statistically significantly different between the aerobically cultured PC group and the aerobically cultured control group.

3.3.4.1.2. Beta-Diversity: Comparison of Samples to Each Other: Between-Habitat Diversity

A PCA plot shows the (dis)similarities between the PC samples of aerobic cultures and the samples of the control group of aerobic cultures (Fig 3.7). Compositions of bacterial communities did not differ significantly between the aerobic cultures of the PC group and control group (AMOVA p-value= 0.678) and the genetic diversity between the communities from these groups was also not significantly different (HOMOVA p-value=0.487). The difference between microbial profiles of the aerobic cultures of the PC group and control group was not statistically different (p = 0.322, F = 1,0925, PERMANOVA).

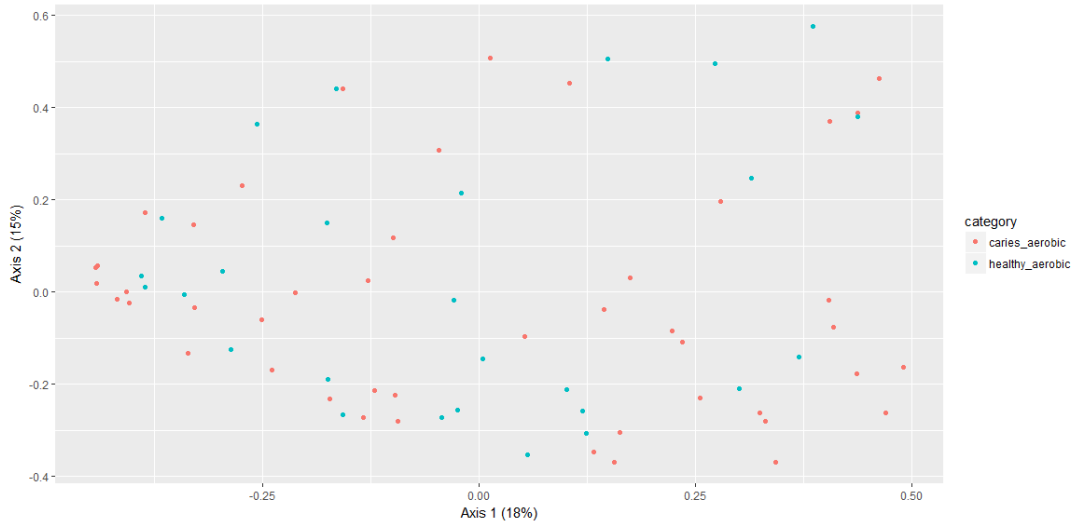


Fig 3.7. PCA plot of the aerobic cultures of the peripheral caries group (caries_aerobic) versus control group (healthy_aerobic). Axes 1 and 2 show 18% + 15% = 33% variation for the thetaYC distances. A dissimilarity percentage of 57% was found between the aerobic cultures of the PC and control group.

3.3.4.2 Molecular Bacteriology of Anaerobic Cultures

The 1094 OTUs present in the anaerobic cultures of the culture-dependent bacteriology group comparing the PC group and control group corresponded to 8 phyla, 16 classes, 26 orders, 53 families, 86 genera, 46 species. This included one unclassified category for each taxon.

Of the anaerobic cultures, 46 samples were from the PC and 27 samples from the control group.

3.3.4.2.1 Alpha-Diversity: Diversity Within a Sample: Within-Habitat Diversity

The mean number of OTUs per sample in the anaerobically cultured PC group was 60.57 (SD =30.59; range = 20-163 OTUs) and the control group was 73.24 OTUs (SD=27.55; range= 30-156 OTUs). Paired samples and unpaired samples within the anaerobically cultured PC group were not significantly different in diversity than the control group.

3.3.4.2.2. Beta-Diversity: Comparison of Samples to Each Other: Between-Habitat Diversity

A PCA plot shows the (dis)similarities between the PC samples of anaerobic cultures and the control samples of the anaerobic cultures (Fig 3.8). Compositions of bacterial communities differed significantly between the anaerobic cultures of the PC and control group (AMOVA p-value 0.007) and the genetic diversity between the communities from these groups significantly differed (HOMOVA p-value<0.001). The variation within the PC group was larger (0.22) than in the control group (0.07). The difference between microbial profiles of the anaerobic cultures of the PC group and control group was statistically different ($p = 0.007$, $F = 2{,}7918$ PERMANOVA).

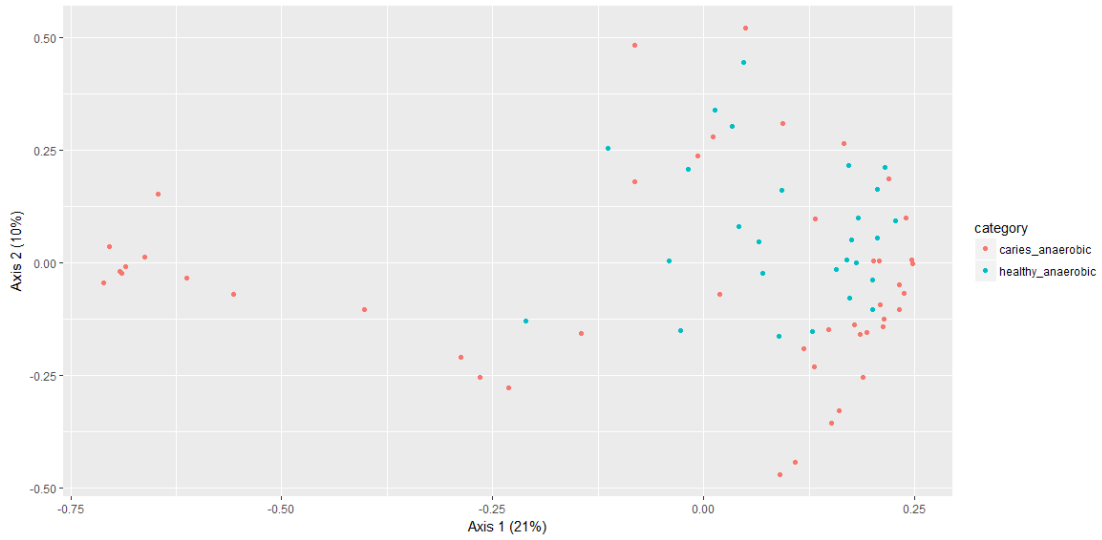


Fig 3.8. PCA plot of the anaerobic cultures of the PC group versus control group. Axes 1 and 2 show 21% + 10% = 31% variation for the thetaYC distances. A dissimilarity percentage of 45% was found between the anaerobic cultures of the PC and control group.

3.3.4.3 Comparison of Bacterial Composition of Aerobic and Anaerobic Cultures of the PC and Control Groups

Phyla

The anaerobic cultures contained one more classified phylum than the aerobic cultures, and the aerobic and anaerobic cultures contained two different classified phyla: Cyanobacteria were only present in aerobic cultures and Spirochaetes and Synergistetes were only present in anaerobic cultures, but all of these phyla were only present in low abundance (<1%).

Firmicutes, Fusobacteria and Bacteroides were more commonly present in anaerobic as compared to aerobic cultures (Fig 3.9). Proteobacteria and Actinobacteria had a higher relative abundance in the aerobic than in the anaerobic cultures. There were also differences between the phyla of PC and control group within each (aerobic or anaerobic) culture group, but these differences were not as pronounced as the differences between aerobic and anaerobic cultures.

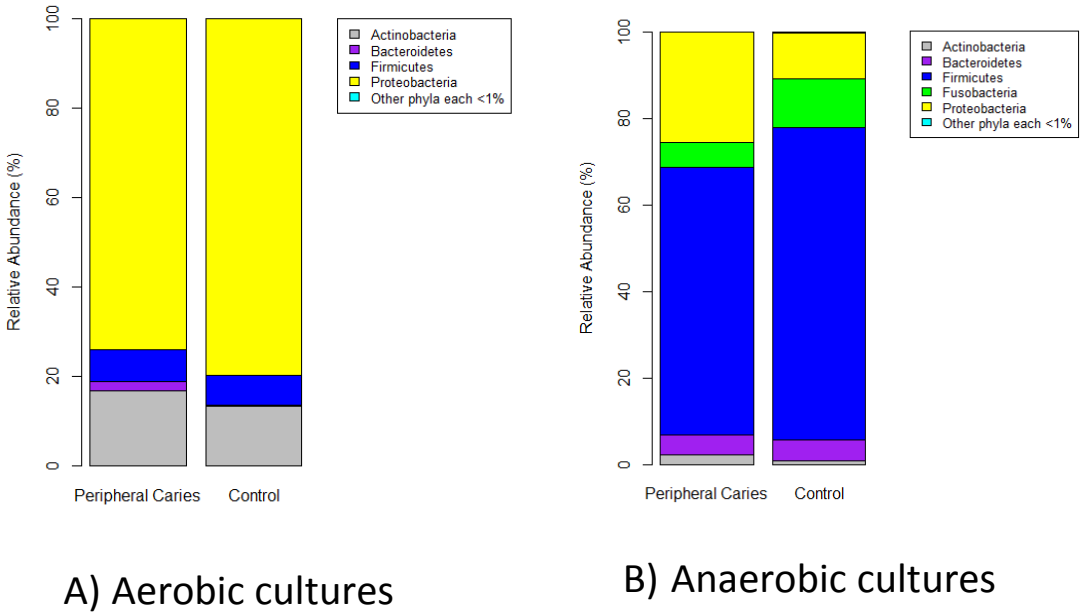


Fig 3.9. A) Aerobic cultures: Relative abundance of phyla (in percentage) present in aerobically cultured samples of the PC group (n=46) and control group (n=27). "Other phyla each <1%" included: Cyanobacteria, Fusobacteria, and unclassified phyla. B) Anaerobic cultures: Relative abundance of phyla (in percentage) present in anaerobically cultured samples of the PC (n=46) and control group (n=27). "Other phyla each <1%" included: Spirochaetes, Synergistetes, and unclassified phyla.

Genera

Many genera of the aerobically cultured samples could not be classified. The relative abundance of the unclassified genera present in the aerobic cultures of the PC group and control group were, 56.2% and 57.8%, respectively. There were 75 classified genera identified in the aerobically cultured PC group and 50 classified genera in the control group. A total of 87 different genera were identified between the aerobically cultured PC and control groups, including 38 shared genera. Of the 87 genera present in the PC group and/or control group, 29 had an abundance greater than 0.1% and these are displayed in Table 3.6.

The most commonly identified genera (>1%) in the aerobically cultured samples of the PC group were, respectively: Rothia, Neisseria, Streptococcus, Eikenella, Wautersiella, Moraxella. In the control group they were Neisseria, Rothia, Streptococcus, Eikenella, Kingella and Bacillus.

Olsenella, Gemella and Actinobacillus were also cultured, but had a low relative abundance in the aerobic cultures of both the PC and control groups (Olsenella: PC: 0.001%, control: 0.007%; Gemella: PC: 0.037%, control: 0.023%; Actinobacillus: PC: 0.023%, control: 0.018%) (Supplementary Item 8).

The relative abundance of the unclassified genera present in the anaerobic cultures of the PC and control groups were 23.3% and 14.1%, respectively. There were 80 classified genera identified in the anaerobically cultured PC group, and 63 classified genera identified within the anaerobically cultured control group. A total of 85 genera were identified between the anaerobically cultured PC group and control group, including 58 shared genera. Of the 85 genera present in the PC and/or control group, 22 classified genera had an abundance greater than 0.1% and are displayed in Table 3.7.

The most commonly identified genera (>1%) in the anaerobic cultures of the PC group were, respectively, Streptococcus, Veillonella, Fusobacterium, Neisseria, Prevotella, Peptostreptococcus, Acidaminococcus and Megasphaera. In the control group, the most commonly identified genera (>1%) were, respectively, Streptococcus, Veillonella, Fusobacterium, Prevotella and Peptostreptococcus. When the anaerobically cultured PC group was compared to the control group, the difference in growth of Streptococcus was small. The same applied to Olsenella growth. Gemella did grow but made up only a small percentage (0.013%) of the total growth for both anaerobically cultured PC group and control group (0.012%). Actinobacillus was present at only 0.005% in anaerobically cultured PC group and 0.003% in the control group.

No growth of Scardovia was identified in either the aerobic or anaerobic cultures (Supplementary Item 8).

Table 3.6. Relative abundance (%) of the most commonly classified genera (at least in one group >0.1%) in aerobic cultures of the peripheral caries (PC) group and control group

Genus	Relative Abundance (%) in Aerobically Cultured PC Group (n =46)	Relative Abundance (%) in Aerobically Cultured Control Group (n=27)
Acidaminococcus	0.012605196	0.302309522
Acinetobacter	0.208470833	0.085902
Actinomyces	0.229802461	0.300657541
Alcaligenes	0	0.110681481
Bacillus	0.018422978	1.478506148
Brevibacillus	0.108598761	0
Brevundimonas	0	0.997787778
Comamonas	0.181321087	0
Corynebacterium	0.223015109	0.109029563
Dermacoccus	0.518752985	0.127201148
Eikenella	4.083118135	3.518680633
Enhydrobacter	0.528449676	0
Escherichia	0.836791435	0.052862704
Kingella	0.964782374	1.797338889
Kocuria	0.152232133	0
Lactobacillus	0.152232074	0.029735352
Lautropia	0.118294961	0.132157304
Mannheimia	0.21137963	0.004955889
Megasphaera	0.009696304	0.115637374
Moraxella	1.165497093	0.644266263
Neisseria	9.379241346	14.83959106
Pantoea	0.07854013	0.464201963
Prevotella	0.015514091	0.127201296
Pseudomonas	0.569173263	0.158588593
Rothia	14.58519557	12.20636491
Stenotrophomonas	0.32870472	0.337000852
Streptococcus	6.481983435	3.9316777
Streptomyces	0.180351435	0.01651963
Wautersiella	1.767639261	0.004955893

Key: Red=Genus more abundant in peripheral caries group; Green=Genus more abundant in control group.

Table 3.7. Relative abundance (%) of most common classified genera (at least in one group >0.1%) found in anaerobic cultures of the peripheral caries (PC) group and control group

Genus	Relative Abundance (%) in PC Group Anaerobic Culture (n =46)	Relative Abundance (%) in Control Group Anaerobic culture (n=27)
Acidaminococcus	1.5455918	0.885452381
Actinomyces	0.666136639	0.528628641
Bacteroides	0.121203983	0.469157633
Capnocytophaga	0.55365968	0.028083378
Dialister	0.105689748	0.16684833
Eikenella	0.386882157	0.008259815
Escherichia	0.471240652	0.006607852
Fusobacterium	5.856573537	11.14910555
Kocuria	0.567235065	0
Lactobacillus	0.744676672	0.619486315
Megasphaera	1.181010361	0.698780867
Mogibacterium	0.128960878	0.221363311
Neisseria	3.271534785	0.460899074
Olsenella	0.100841576	0.112333485
Oribacterium	0.080479352	0.132157122
Parvimonas	0.10375048	0.155284719
Peptostreptococcus	2.064344546	2.968581522
Porphyromonas	0.345188748	0.193279926
Prevotella	2.992280989	3.137079411
Rothia	0.565295635	0.013215704
Streptococcus	36.39025574	36.29530564
Veillonella	17.77721899	27.02117841

Key: Red=Genus more abundant in peripheral caries group; Green=Genus more abundant in control group.

3.3.5 Culture-Independent Bacteriology: Infundibular Caries versus Infundibulae without Caries

The 691 OTUs present in the culture-independent bacteriology group comparing infundibular caries (IC group) versus no IC (control group) corresponded to 16 phyla, 30 classes, 50 orders, 100 families, 134 genera, 65 species. This included one unclassified category for each taxon. There were eight IC samples and seven control samples.

3.3.5.1 Alpha-Diversity: Diversity within a Sample: Within-Habitat Diversity

The mean number of OTUs per sample in the IC group was 108.8 (SD =38.7; range = 64-171 OTUs) and the control group consisted of a mean of 118.4 OTUs (SD=40.4; range= 62-171 OTUs). The samples within the IC group were slightly less diverse than the control group, although this was not statistically significant.

3.3.5.2 Beta-Diversity: Comparison of Samples to Each Other: Between-Habitat Diversity

A PCA plot shows the (dis)similarities between samples of the IC group and control group (Fig 3.10). Compositions of bacterial communities did not differ significantly between the IC group and control group (AMOVA p-value <0.071) but the genetic diversity between the communities from these groups was significantly different (HOMOVA p-value=0.003). The variation within the IC group was larger (0.29) than in the control group (0.13). The difference between microbial profiles of the IC group and control group was not statistically different (p = 0.107, F = 1,3968, PERMANOVA).

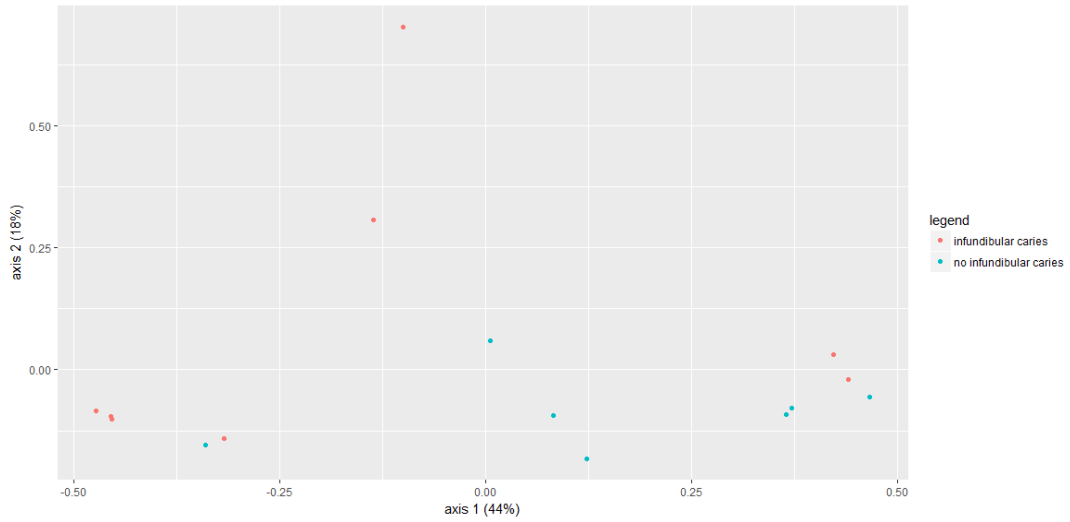


Fig 3.10. PCA plot of the IC group versus control group. Axes 1 and 2 show 44% + 18% = 62% variation for the thetaYC distances. A dissimilarity percentage of 60% was found between the IC and control group.

3.3.5.3 Comparison of Bacterial Composition between the IC and Control Group

Ten classified phyla were identified in the IC group, and 13 were identified in the control group. Of the total of 15 classified phyla in the IC group and control group, 8 were common to both groups. The phyla with an abundance of >1% are shown in Fig 3.11.

Within the IC group, 97 classified genera were identified and 105 classified genera were identified in the control group. A total of 133 classified genera were identified between the IC group and control group, including 69 shared genera. Of the 133 classified genera present in the IC and/or control group, 32 classified genera had an abundance larger than 0.1% and are displayed in Table 3.8.

The most common identified genera (>1%) in the IC group were, respectively, Streptococcus, Gemella, Olsenella, Actinobacillus, Lactobacillus and Actinomyces.

The most common identified genera (>1%) in the control group were, respectively, Streptococcus, Gemella, Actinobacillus, Actinomyces, Rothia, Eikenella, Moraxella and Prevotella.

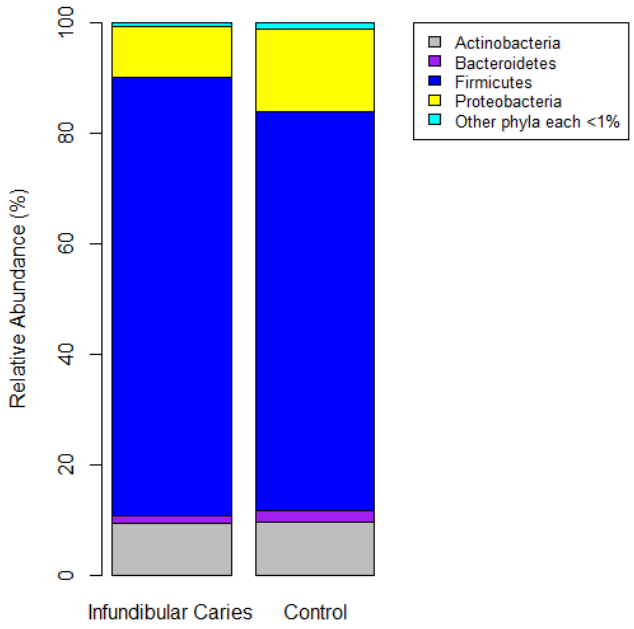


Fig 3.11. Relative abundance of phyla (in percentage) present in the IC group (n=8) and control group (n=7). "Other phyla each <1%" included: Chloroflexi, Cyanobacteria, Fibrobacteres, Fusobacteria, GN02, Planctomycetes, Spirochaetes, Synergistetes, Tenericutes, Verrucomicrobia, [Thermi] and unclassified phyla.

Table 3.8. Relative abundance (%) of most common genera (at least in one group >0.1%) present in the infundibular caries and control groups.

Genus	Relative Abundance (%) in IC Group (n =8)	Relative Abundance (%) in Control Group (n=7)
Eubacterium	0.027877	0.108322
Actinobacillus	4.237287	3.931439
Actinomyces	1.32694	3.612846
Alysiella	0.133809	0.324965
Arthrobacter	0.239742	0.108322
Bulleidia	0.122658	0.031859
Butyrivibrio	0.167261	0.038231
Corynebacterium	0.105932	0.127437
Dialister	0.100357	0.191156
Eikenella	0.908786	1.637569
Fusobacterium	0.027877	0.223015
Gemella	15.73372	23.34012
Halomonas	0.094781	0.140181
Kingella	0.078055	0.293105
Lactobacillus	3.936217	0.643558
Lautropia	0.473908	0.407799
Leptotrichia	0.485058	0.592583
Leucobacter	0.055754	0.809227
Megasphaera	0.139385	0.012744
Mogibacterium	0.094781	0.10195
Moraxella	0.37355	1.414553
Neisseria	0.022302	0.165668
Olsenella	5.218559	0.331336
Porphyromonas	0.195138	0.197528
Prevotella	0.953389	1.357206
Pseudoramibacter	0.747101	0.019116
Rothia	0.507359	1.688544
Scardovia	0.172837	0.019116
Sharpea	0.245317	0.07009
Streptococcus	52.69848	44.91525
Syntrophomonas	0.172836	0
Veillonella	0.418153	0.592583

Key: Red=Genus more abundant in IC group; Green=Genus more abundant in Control Group.

For fourteen out of 691 OTUs, statistically significantly differences in abundance were found between the IC group and control group in a LEfSe performed at OTU level (LDA score >2, p-value<0.05). Only one OTU was a discriminative feature for the IC group and 13 OTUs were discriminative features for the control group (Table 3.9).

From 205 taxa identified at the genus or higher level, 15 discriminant taxa were found between the IC and healthy group (LDA score >2, p-value<0.05). One taxon was associated with IC and 14 taxa with the control group (Fig 3.12).

Table 3.9. Results of linear discriminant analysis effect size (LEfSe) demonstrate which OTUs are statistically significantly different between the IC and control groups (LDA score>2, p<0.05).

OTU_ID and taxon	Log of the highest class average	Class	LDA effect size	p-value
Otu000149_Mogibacteriaceae	2.804266	healthy	2.992836	0.047106
Otu000306_Veillonellaceae	2.406326	healthy	2.994281	0.047106
Otu000693_Lachnospiraceae	2.582417	healthy	3.05156	0.047106
Otu000172_Corynebacterium	2.582418	healthy	3.133166	0.047106
Otu000015_Fusobacterium	2.980358	healthy	3.15019	0.045714
Otu000708_Atopobium	2.582418	healthy	3.178018	0.047106
Otu000608_Lachnospiraceae	2.406326	healthy	3.323734	0.017485
Otu000803_Bacteroidales	2.582417	healthy	3.338835	0.047106
Otu000269_Parvimonas	2.649364	healthy	3.36027	0.017485
Otu001336_Campylobacter	2.406326	healthy	3.384272	0.047106
Otu000089_Peptostreptococcus anaerobius	2.281387	healthy	3.384674	0.047106
Otu001972_Leptotrichia	2.406326	healthy	3.4495	0.047106
Otu000004_Streptococcus minor	5.296048	healthy	4.664047	0.049141
Otu000415_Sharpea azabuensis	2.445244	caries	2.724109	0.015891

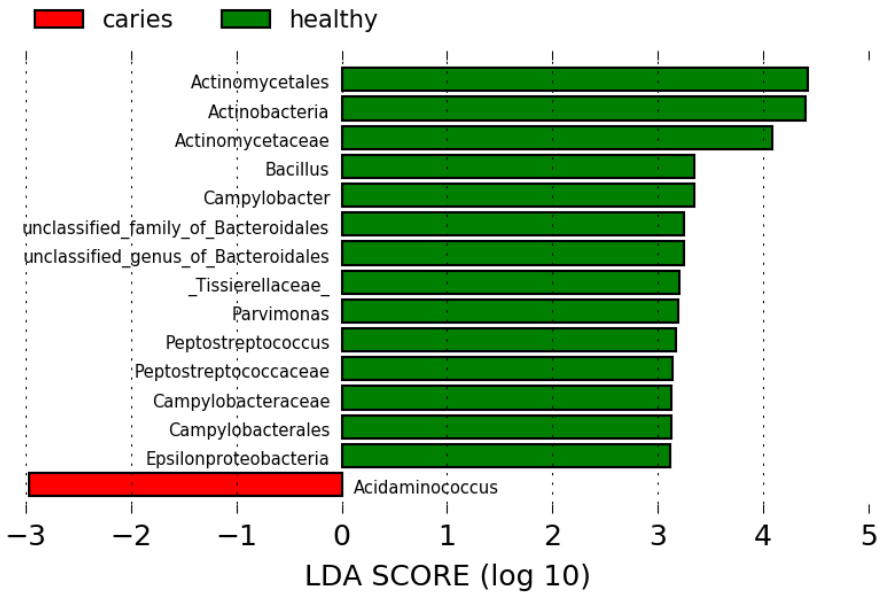


Fig 3.12. Results of linear discriminant analysis effect size (LEfSe) at the genus or higher level, showing which taxa are statistically significantly more associated with the IC or control groups (LDA score>2, p<0.05).

3.4 Discussion

In this study 71.4% (45/63) of all horses examined (all cases referred for dental examination or treatment) were affected by PC. This prevalence is higher than reported in our UK-wide survey (51.7%) (Chapter 2; Borkent et al., 2016), which is likely due to the high prevalence of concurrent dental problems in this study population. The presence of multiple concurrent dental problems and diastemata were shown to be risk factors for PC (Borkent et al., 2016). In this study, a dental mirror and oral endoscope were used, and the latter can increase the recognition of PC compared to using a mirror only. There was no statistically significant difference in diversity within-sample when the PC group and control group were compared. However, the overall bacterial composition between groups as well as the overall genetic diversity in community structure between the groups was statistically significantly different. The variation within the control group was larger (0.23) than in the PC group (0.16).

Firmicutes, Actinobacteria, Proteobacteria, Bacteriodetes and Fusobacteria were the most common phyla (>1%) present in supragingival plaque in this study, in both PC and control groups. These were also the most common phyla reported to be present in human dental biofilm. (Peterson et al., 2011; Chen et al., 2017) and in the subgingival plaque of healthy subgingival sites of two horses, using 16S rRNA gene amplicon pyrosequencing (Gao et al. (2016). Using the culture-independent bacteriology approach, the phylum predominantly associated with PC as well as IC was Firmicutes. In contrast, bacteria belonging to the phylum Proteobacteria were mainly present in the control group.

In a study investigating the microbiota of dental plaque of the primary dentition of young children with and without caries lesions, Gross et al. (2012) also found that the relative abundance of Firmicutes increased with advancing caries stage (from healthy, intact enamel, white spot lesion to cavitated lesion). Additionally, Proteobacteria, Actinobacteria and Bacteroidetes significantly decreased in relative abundance with increasing caries stage (Gross et al., 2012). Chen et al. (2017) showed that in human caries the relative abundance of bacteria of the phylum Firmicutes was increased and the relative abundance of the phylum Proteobacteria was reduced in salivary and oropharyngeal samples, but only for the youth and adult age groups, because the microbial profiles of children showed the opposite trend.

Because the species of some of the mock community bacteria were not assigned correctly to the corresponding OTUs, in contrast to assignations to the genus or higher taxonomic level, the latter level was considered to be more reliable for subsequent analyses. However, it is important to bear in mind that bacteria of the same genus may play different roles in the dental caries/dental health balance. If two bacteria of the same genus but belonging to different species were present in a sample, one could be cariogenic, while the other one could be non-cariogenic (Schloss and Westcott, 2011). If, for example, there are relatively many more cariogenic Streptococci than non-cariogenic Streptococci present at a site, then in a LEfSe analysis performed at genus or higher level a sample of this site will show that Streptococci are more commonly associated with caries than with health. However,

the LEfSe at the OTU-level may show health-associated as well as caries-associated Streptococci. This phenomena was observed using the culture-independent bacteriology method, when the PC group was compared with the control group.

When the PC group and control group were compared using LEfSe at the genus or higher level, *Streptococcus* and *Olsenella* were the most discriminative genera of the PC group.

Streptococci bacteria are facultative anaerobic, gram positive cocci (Samaranayake, 2012). Members of the group of mutans Streptococci include *S. mutans*, *S. sobrinus*, *S. criceti*, *S. ferus*, *S. ratti*, *S. macacae, S. downei* and *S. devriesei* and these bacteria are often associated with caries. They are highly acidogenic and aciduric and, additionally, they can metabolise sucrose to produce extracellular polysaccharides which increases their ability to adhere to the tooth surface (Alam et al., 2000; Takahashi and Nyvad, 2011; Karpiński and Szkaradkiewicz, 2013). In humans, *S. mutans* is still considered to be one of the most important cariogenic bacteria (Loesche et al., 1975; Peterson et al., 2011), although *S. mutans* is sometimes absent or present in low levels in caries lesions, where the caries is mainly associated with *S. sobrinus* and non-mutans Streptococci (*S. salivarius* and *S. parasanguinis*) (Gross et al. (2012). Aas et al. (2008) showed in subjects without detectable *S. mutans* that low-pH non-*S. mutans* streptococci, Lactobacillus *spp*. and *Bifidobacterium dentium* were present in high levels. Additionally in subjects with *S. mutans*, other species of the genera Atopobium, Propionibacterium and Lactobacillus were present in significantly higher abundance than *S. mutans* (Aas et al., 2008). These findings confirm that multiple bacteria are likely to play a role in the development of caries.

Another bacterium that was associated with PC in our study and which is also associated with human caries is Olsenella, a small, rod-shaped microaerotolerant (moderately obligate) anaerobic bacterium (Dewhirst et al., 2001; Kraatz et al., 2011) which can ferment carbohydrates, predominantly to lactic acid but also to formic and acetic acids. Olsenella spp (including *O. profusa*) have been shown to be associated with human dentine caries (Chhour et al., 2005; Obata et al., 2014), as well as root

(cemental) caries, in a study where supragingival plaque samples of root caries were compared to samples of healthy subjects (Chen et al., 2015).

When the caudal cheek teeth PC group, rostral cheek teeth PC group and healthy group were compared using LEfSe at genus or higher level, the genus most associated with rostral cheek teeth PC was Veillonella and the genus most associated with caudal cheek teeth PC was Streptococcus. In human studies, Veillonella was also associated with caries and was highly correlated with total acid producing species (Aas et al., 2008; Gross et al., 2012). Veillonella are gram negative, small anaerobic cocci which are asaccharolytic and convert lactic acid into weaker (acetic and propionic) acids so that the pH of the dental plaque rises (Samaranayake, 2012; Peterson et al., 2014). Veillonella has therefore been described as an acid sink. On the one hand, this feature seems to be a health-promoting feature in the dental caries/dental health balance but it also can help other fermentative bacteria to survive, grow and have an active metabolism. Some acidogenic Streptococcus and Granulicatella bacteria might even reduce the acidity of their environment because they possess the L-lactate-dehydrogenase gene (McLean et al., 2012; Peterson et al., 2014).

Gemella and Actinobacillus were the most two discriminative genera for the control group, when the PC and control groups were compared at the genus level. Interestingly, Kennedy et al. (2016) also found Gemella and Actinobacillus to be the genera which were most associated with their control group (periodontal health). Other genera found to be associated with controls in this study were Eikenella, Neisseria, an unclassified genus of the Candidate phylum BD1-5 (also known as candidate phylum GN02 and recently renamed Gracilibacteria (Rinke et al., 2013)]), Lautropia, Corynebacterium, Streptomyces, Odoribacter, Moraxella, Kingella, Weisella and Alysiella.

Using culture-independent bacteriology will give a more accurate representation of the microbiota present in the supragingival dental plaque compared to the indirect approach (culture-dependent bacteriology, using cultures of bacteria prior to carrying out molecular bacteriology) because the environment (including nutrients, pH,

oxygen concentration) is different on culture plates than in the oral cavity. Consequently, the relative abundance of oral bacteria is likely to change if cultures are used first. However, it was interesting to discover which bacteria grew best under standardised conditions on aerobic culture, anaerobic culture and under both conditions. Culture-dependent bacteriology also showed that the bacteria identified using this method are alive and retain the ability to grow; features which are not certain if only culture-independent bacteriology is performed.

Differences between caries and control samples are likely to change and possibly reduce because the bacteria are brought into similar environments. Consequently, it is better to compare the caries group and control group using culture-independent bacteriology.

If the relative abundance of the phyla of bacteria of the culture-independent method was compared to the culture-dependent (aerobic and anaerobic) methods, then in the anaerobic culture-dependent method, the relative abundance of the phyla of bacteria was more similar to the culture-independent method (both mainly identifying Firmicutes) than the aerobic culture-dependent method (mainly identified Proteobacteria).

Although most of the bacteria which were found to be significantly more associated with PC or with controls could be cultured aerobically, anaerobically or both, Scardovia was an exception. Scardovia was found to be associated with PC using culture-independent bacteriology, but was not identified in any aerobic or anaerobic cultures of these same samples. Scardovia, an anaerobic Gram positive bacillus should be able to grow on an anaerobically cultured blood agar plate, although it preferentially grew on acid agar in a study using anaerobic cultures that evaluated the oral bacteria of children with severe early childhood caries (Tanner et al., 2011).

When the IC group was compared with the control group using LEfSe at the genus or higher level, Acidaminococcus was a more discriminative feature for IC than for the control group. Acidaminococcus is a Gram negative, anaerobic coccus which utilises amino acids (mainly glutamic acid) for its growth, degrading them to acetate and butyrate (Rogosa, 1969; Cook et al., 1994; Jumas-Bilak et al., 2007; Chang et al.,

2010). Glutamic acid was found as a free amino acid in human incisor dental plaque, making up at least 50% of all free amino acids in this plaque (Singer and Kleinberg, 1983). Additionally, Acidaminococcus bacteria can use citrate as a source of energy, producing hydrogen and hydrogen sulphide as metabolites (Cook et al., 1994). Two Acidaminococcus species haven been described: *Acidaminococcus fermentans* and *Acidaminococcus intestini*.

Acidaminococci have also been found in the intestinal tract of pigs (Fuller, 1966; Rogosa, 1969) and humans (Jumas-Bilak et al., 2007), in the rumen of cattle (Cook et al., 1994; Callaway et al., 2010) and in a perianal abscess of a diabetic human patient (Galan et al., 2000). These bacteria were also isolated from other human abscesses, abdominal fluid, peritoneal fluid, infected parietal haematoma, a pressure ulcer at the sacrum and from a necrotic mandible (Jumas-Bilak et al., 2007).

To date, Acidaminococci have not been reported in dental plaque, although it was proposed as a possible plaque bacterium because it could use energy from amino acids present in the subgingival biofilm (Carlsson, 1997). In this study Acidaminococci were present in supragingival equine dental plaque of the IC group and IC control group, the PC group and PC control group in culture-independent bacteriology as well as in the culture-dependent bacteriology of the cultures of PC group and PC control group. It could be both aerobically and anaerobically cultured, although its relative abundance was higher in the anaerobic cultures.

Increased numbers of IC samples are needed to identify more biomarkers for the IC and control groups. Increased sample size would also increase the power of the study and assist the identification of bacterial taxa that differ significantly between the IC and control groups. Because of the limited number of IC samples compared to the PC samples, these two different types of caries were not compared directly in this study. A future study with more IC samples could make this direct comparison possible.

Further, in addition to metagenome analysis, metatranscriptome analysis could be performed using the same dental plaque samples for both analyses, to identify not only which bacteria are present and their relative abundance, but also to determine which bacteria are active and then assess whether there is a correlation between the

presence of bacteria and their activity by comparing the relative abundance of DNA and RNA of the microbiota. These metagenome and metatranscriptome findings could be compared between caries and control samples. This potential study may help identify which microbiota are important for caries development since there is a variable correlation between microbial presence and microbial activity (Benítez-Páez et al., 2014; Nascimento et al., 2017).

A disadvantage of using the 16S rRNA gene for metagenomics studies is that some bacteria can have multiple heterogeneous copies of this gene, which can affect the estimation of relative abundance of bacteria (Kang et al., 2010; Větrovský and Baldrian, 2013). There can be a large variation of 16S rRNA copy numbers in the phylum Firmicutes and the class Gammaproteobacteria and, additionally, a large variation of 16S rRNA sequences within the genome can occur (Větrovský and Baldrian, 2013). Because shotgun metagenomics sequencing is not immediately affected by the amount of copy numbers (although limited by the variation in sizes of the genomes), it would be interesting to use this method on caries and control samples. Moreover, shotgun metagenomics sequencing will not only provide metagenomics data but also will allow a functional characterisation of the microbiota. Additionally, shotgun metagenomics sequencing can be used to identify new taxa or genomes (Větrovský and Baldrian, 2013; Sharpton, 2014) or contributions of pathogens other than bacteria, such as viruses, parasites and fungi.

Because host genes may be involved in a genetic predisposition for equine dental caries, possible genetic predisposition could be studied using whole-genome association (WGA) studies. In human studies, multiple genes associated with caries predisposition were revealed using this method (Shaffer et al., 2013; Morrison et al., 2016).

4 CHAPTER 4: QUALITATIVE HISTOLOGICAL AND ULTRASTRUCTURAL STUDY OF EQUINE DENTAL CARIES

4.1 Introduction

4.1.1 Cementum

In brachydont (short-crowned) teeth, root cementum covers the subgingival dentine, whilst the crown dentine is covered by enamel. In brachydont teeth, the anatomical crown (i.e. part of the tooth covered by enamel) is usually the same as the clinical crown (i.e. erupted part of the tooth). The transition between root and crown in brachydont teeth is distinct and is termed the neck of the tooth (Wiggs and Lobprise, 1997; Verstraete, 1999; Dixon and du Toit, 2010). Equine cheek teeth are hypsodont, which means they have a long crown, that can be divided into a clinical crown (i.e. erupted or supragingival part) and a reserve crown (i.e. subgingival part) (Dixon and du Toit, 2010). Equine cheek teeth do not have a distinct neck and young horses have short roots in comparison to crown length. In (equine) hypsodont teeth, cementum covers the root dentine, but also the enamel on the clinical and reserve crown, which in turn covers dentine at the peripheral aspects of the teeth. On the occlusal surface, all three hard dental tissues become exposed once the cementum and enamel overlying the occlusal surface are worn away after eruption of the equine tooth (Dellmann and Brown, 1976). Equine cementum can be found on the periphery of cheek teeth (including in their infoldings), canines and incisors, but also inside occlusal enamel infoldings in the incisors and maxillary cheek teeth (infundibulae).

Havers (1691) first described the peripheral cementum in equine teeth as a “bony crust” covering the “stony case” or “cortex” (i.e. enamel) at the peripheral aspects of the teeth. Blake (1798) investigated elephant teeth and termed the cementum present in these teeth “crusta petrosa”. Owen (1845) described the microscopic structure of teeth of many animals, including horses, and he introduced the term “cementum” which has been used since then.

Two types of collagen fibres can be observed in cementum. Sharpey’s fibres, produced by fibroblasts, are continuous with the extrinsic fibres of the periodontal ligament (PDL) (which connect the alveolar bone with the tooth) and run

perpendicular to the cementoenamel junction and the peripheral aspect of the tooth. The second type are the smaller intrinsic fibres, which are produced by cementoblasts and are directed longitudinal to the cementoenamel junction and the peripheral aspect of the tooth (Wang et al., 2006). Sharpey's fibres form bundles ranging from 0.9 to 55 µm wide (Kilic et al., 1997c) that are woven between the intrinsic fibres.

Cementum is deposited in an irregular pattern and incremental growth lines (also named incremental lines of Salter in brachydont teeth) can be observed in cementum that reflect the periodicity of cementum formation (Berkovitz et al., 2009). Although the term "incremental growth line" indicates that this line is caused by appositional growth, this is misleading because these lines represent an area of slower growing cementum, while the cementum in areas in between these lines develops at a faster rate. The term line of arrested growth (LAG) could be more accurately used for these lines (Burke and Castanet, 1995). The cementum in a line of arrested growth is more heavily mineralised, but is less dense in cells and fibres, i.e. contains fewer cementocytes, Sharpey's fibres and intrinisic fibres relative to ground substance as compared to areas between lines of arrested growth (Hillson, 2005b).

In contrast to brachydont teeth, small vascular channels have been described in the cementum of equine (Owen, 1845; Mitchell et al., 2003) and other hypsodont teeth. This means that equine cementum is a living tissue (to at least up to a few millimeters above the gingival margin for peripheral cementum and for a period after eruption in infundibular cementum while its accessory vascular supply remains) when new cementum is still being formed. Additionally, existing subgingival equine cementum is the most flexible dental hard tissue and can readily undergo resorption, deposition and repair. Nerve fibres also have been described in peripheral cementum adjacent to the periodontium (Mitchell et al., 2003).

Most equine cemental research to date has been on infundibular rather than peripheral cementum, because infundibular caries (IC), which starts in infundibular cementum can extend into adjacent enamel and dentine. IC has long been recognised as a potential cause of midline fracture or apical infection of the affected tooth with possible extension into the overlying sinus or maxillary bone as discussed in Chapter

2 (J.F. Colyer, 1906; Honma et al., 1962; Kilic et al., 1997c; Windley et al., 2009; Fitzgibbon et al., 2010).

Recently there has been an increased research interest in equine peripheral cementum (Kilic et al., 1997c; Mitchell et al., 2003), especially since the recognition and/or prevalence of peripheral caries (PC) has increased (Ramzan and Palmer, 2011; Erridge et al., 2012; Borkent et al., 2016; Jackson et al., 2017; Lee et al., 2017). This disorder starts in the peripheral cementum but may extend into enamel and dentine, and can lead to abnormal dental wear, dental fracture and apical infection. It is also associated with diastema formation and/or periodontal disease. A pilot study by Erridge et al. (2012) described two histological patterns of cemental PC: one where the superficial layers of peripheral cementum flake off and another with more focal flask-like lesions in affected cementum.

The aim of this study was to examine PC affected teeth histologically and ultrastructurally to further investigate the pathological changes present in all dental tissues with this disorder.

4.1.2 Enamel

Enamel, with a 98% mineral content, is the hardest substance in the body (Bhaskar, 1991) and studies in human teeth have revealed that it is the calcified dental tissue most resistant to acids (and thus to dental caries), with a lower critical pH (5.5) compared to dentine (6.0) and cementum (6.7), making cementum very susceptible to damage by acids (Tanzer, 1992; Vanuspong et al., 2002). Caries of the crown of brachydont teeth first has to affect the overlying enamel before it can affect the underlying dentine. However, in equine (hypsodont) teeth, if ingested acidic substances (causing dental erosion) or caries (caused by acidogenic bacteria) affect the occlusal surface, they can affect all three exposed calcified dental tissues. It is also possible that severe PC could eventually affect all layers of the peripheral aspect of teeth, initially affecting cementum, then the underlying enamel and finally dentine.

Histologically (on undecalcified sections), some normal enamel features such as enamel spindles and lamellae could be mistaken for defects. Enamel spindles are

formed by developing odontoblast processes which extend into the ameloblast layer and become embedded in enamel when enamel formation starts (Nanci, 2008). Enamel lamellae are linear, longitudinally orientated, fissure-like, hypomineralised structures which extend into the enamel to varying depths from the enamel surface in brachydont teeth or from the cemento-enamel junction in equine and other hypsodont teeth. They extend to the dentino-enamel junction and are filled with organic material (Nanci, 2008). The difference between enamel lamellae and fissure fractures (hairline cracks) is that, after decalcification of a ground section, lamellae are still visible, whereas fissure fractures disappear (Kumar, 2014). In contrast to the well-described enamel caries in brachydont teeth, little is known about PC of equine enamel.

The aim of this study was to examine the normal histological and ultrastructural appearance of equine cheek teeth enamel and to compare this to the ultrastructure of PC-affected (grade 2 PC) and macroscopically normal enamel (grade 1.2 PC) that has been exposed by cemental PC.

4.1.3 Dentine

As described in the general introduction, dentine can be categorised as primary, secondary (regular and irregular), and tertiary. Primary dentine is laid down first and its dentinal tubules are lined by intratubular dentine (Kilic et al., 1997b). Later, regular secondary dentine is produced by odontoblasts which line the periphery of the pulp horns, including subocclusally, which prevents pulpar exposure by normal dental wear. Finally, irregular secondary dentine is laid down in the centre of the pulp horn. Denticles (pulp stones - spherical areas of dentine) can also form in normal pulp. Tertiary dentine is produced in response to local pulpar insults. Dentinal tubules near-horizontally run from the pulp to the dentino-enamel junction or vertically, from the occlusal tip of the pulp towards the occlusal surface, and contain odontoblast processes whose viability in subocclusal dentine is unknown (Muylle et al., 2001). Intertubular dentine can be identified lying between dentinal tubules.

A small histological study by Erridge et al. (2012) recognised PC of equine dentine on Gram stained sections of PC-affected teeth which appeared to have sound dentine on gross examination, A ccomputed tomographic (CT), histological and ultrastructural pathological study of caries and occlusal pulpar exposure in donkey cheek teeth by du Toit et al. (2008c) also briefly recorded some characteristics of equine dentinal PC, but no other such studies appear to have been described. The aim of this study was to further investigate the pathological changes in PC-affected dentine in PC affected teeth including, if possible, establishing the route of bacterial infection in equine dentinal PC.

4.2 Materials and Methods

Approval for this study was obtained from the Veterinary Ethical Review Committee of the Royal Dick School of Veterinary Studies (RDSVS).

Twenty cheek teeth including mandibular (n=8) maxillary cheek teeth (n=12) from horses that had undergone post-mortem examination at the RDSVS Pathology Department following death from disease or euthanasia on humane grounds were extracted using a stainless steel chisel and mallet. The maximum grade of PC in each tooth was assessed macroscopically: four cheek teeth with grade 0 PC served as controls; in 11 cheek teeth PC-affected only cementum i.e. grade 1.1 PC (n = 8); grade 1.2 PC (n = 3), in three teeth cementum and enamel were affected (grade 2 PC), and in two cheek teeth cementum, enamel and dentine were affected (grade 3 PC).

Cheek teeth were sectioned into 2-5 mm thick longitudinal or transverse sections using a water-cooled tile saw with a 0.8-mm thick, continuous-rim, diamond tipped blade (Malvern Lapidary, Worcester, UK). Ten cheek teeth were examined histologically, using routine haematoxylin and eosin (H&E) staining and also Gram staining to help identify bacteria or Masson's trichrome stain to further characterise the dental tissue matrix. Seven cheek teeth were examined using Scanning Electron Microscopy (SEM) and four cheek teeth were examined using Transmission Electron Microscopy (TEM), including one tooth that had both SEM and TEM examinations: the buccal aspect was examined using SEM and the lingual aspect using TEM.

The examined maxillary cheek teeth infundibulae were also examined for IC: one control tooth (rostral infundibular caries (RIC) and caudal infundibular caries (CIC) grade 0); seven cheek teeth had IC of one infundibulum (grade 1 RIC, grade 0 CIC), one cheek tooth had cementum and enamel affected in both infundibula (grade 2 RIC and CIC); two cheek teeth had cementum, enamel and dentine affected (grade 3 RIC and CIC). One maxillary cheek tooth had its infundibulae fully worn away.

Additionally, two undecalcified transverse histological sections, one of a maxillary and one of a mandibular cheek tooth prepared for a study by Dacre and Dixon (2005) were used to examine enamel.

4.2.1 Histology

For histology, 5-10 mm thick transverse sections of teeth were immersed in 10% neutral-buffered formalin for a minimum of 72 hours for fixation. All further histological preparation steps were performed by the Histology Technicians at the RDSVS, University of Edinburgh.

After rinsing with cold tap water for 20 minutes, the fixed sections were placed in decalcifying fluid Surgipath® Decalcifier II (Leica Microsystems Ltd., Linford Wood, UK) for 4 days. They were then placed in decalcifying fluid Surgipath® Decalcifier I (Leica Microsystems Ltd., Linford Wood, UK) and were examined each week by palpation, to determine the softness of the dental tissues and thus the level of decalcification. Full decalcification can take up to a month. Once decalcified, the sections were rinsed with cold tap water for 20 minutes and placed in an automated tissue processor (Thermo Shandon, Runcorn, UK) where they were dehydrated by immersion in a series of graded ethanol and xylene baths and finally impregnated with Histoplast^{TM} paraffin wax (Thermo Fisher Scientific, Loughborough, UK) and cast into paraffin wax blocks. After placing these paraffin wax blocks on ice for one hour, they were cut into 5 um thick sections and mounted onto Biobond coated glass slides (BB International, Golden Gate, Cardiff, UK). The mounted sections were dried in an incubator (at 37°C, for 48 hours) and stained with H&E, Gram stain, or Masson's Trichrome (Culling, 1974).

These slides were later examined using a light microscope (Nikon Eclipse Ni, Nikon Instruments Europe B.V., Kingston-upon-Thames, UK).

4.2.2 Scanning Electron Microscopy

For SEM examination, the samples were fixed in a solution of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 hours. Further processing took place at the Electron Microscope facility of the School of Biological Sciences (University of Edinburgh). The samples were washed for 3 x 10 minute in 0.1 M sodium cacodylate buffer. Samples were then postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 45 minutes. A further 3 x 10 minute washes were performed in 0.1 M sodium cacodylate buffer. Dehydration in graded concentrations of acetone (50%, 70%, 90%, and 3 x 100%) for 10 minutes each, was followed by critical point drying using liquid carbon dioxide. After mounting on aluminium stubs with carbon tabs attached, the specimens were sputter coated with 20 nm gold palladium and viewed using a Hitachi S-4700 scanning electron microscope (Hitachi, Maidenhead, UK).

4.2.3 Transmission Electron Microscopy

For TEM, samples were fixed in 3% glutaraldehyde in 0.1M Sodium cacodylate buffer, pH 7.3, for 2 hours. The samples were then decalcified by the Histology Service at the RDSV, University of Edinburgh, as described above. Further processing was undertaken by the Electron Microscope facility of the School of Biological Sciences (University of Edinburgh), where the samples were washed three times for 10 minute with 0.1M sodium cacodylate. Specimens were post-fixed in 1% Osmium Tetroxide in 0.1M sodium cacodylate for 45 minutes, then washed again in 0.1M Sodium cacodylate buffer three times for 10 minutes. Samples were dehydrated in 50%, 70%, 90% and 100% ethanol (x3) for 15 minutes each, then for 2x 10 minutes in propylene oxide. Samples were embedded in TAAB 812 resin. Sections were cut at 1 μm thickness on a Leica Ultracut ultramicrotome, stained with Toluidine Blue, and viewed in a light microscope to select suitable areas for investigation. Ultrathin sections (60 nm thick) were cut from selected areas, stained in uranyl acetate and lead citrate then viewed in a JEOL JEM-1400 Plus TEM (JEOL

UK Ltd., Welwyn Garden City, UK). Representative images were collected on a GATAN OneView camera. Scale bars were added to TEM and SEM images using ImageJ 1.51p Software (Schneider et al., 2012)

4.3 Results

4.3.1 Cementum

4.3.1.1 General Histological and Ultrastructural Findings in Equine Cheek Teeth Cementum

All three layers (primary, secondary and tertiary) of cementum could not always be identified in all sections (in fact, tertiary cementum was not present in some transverse sections). However, when histology was performed on longitudinal sections, all three layers of cementum could be clearly identified in many sections (Figs 4.1 and 4.2). Histological examination with H&E staining showed extrinsic PDL fibres to continue their course as Sharpey's fibres, after they became embedded in cementum (Fig 4.3).

Using TEM in areas with lines of arrested growth, Sharpey's fibres were easier to distinguish from the intrinsic fibres (Figs 4.4 and 4.5) than in areas without lines of arrested growth, where the density of both fibres was greater. For TEM, sections were obtained from the periphery of teeth so that the Sharpey's fibres could be recognised by their circular shape (and larger size), and the intrinsic fibres by their thin and sometimes long collagen fibres containing transverse stripes (Fig 4.6). The intrinsic fibres surrounded and were oriented perpendicular to Sharpey's fibres. Using SEM (of the periphery of cementum), the difference between Sharpey's fibres and intrinsic fibres was also clearer in the lines of arrested growth than in areas without lines of arrested growth, although individual intrinsic fibres could not be observed because, similar to Sharpey's fibres, they were covered by dental plaque on the cemental surface (Figs 4.7 and 4.8).

Using Masson's trichrome staining, some areas of PC-affected cementum were coloured red, while other areas were blue (Fig 4.9). The blue colour was mainly present in the PDL, both surrounding the lacunae and at the level of the intrinsic growth lines. Sharpey's fibres and the outer layer of cementum were usually stained red.

Other structures histologically identified in cementum included lacunae and canaliculi, with some lacunae containing shrunken cementocytes with or without visible cell processes (Fig 4.3).

Cementocytes were most obvious and most numerous in (sub)gingival sections, and the closer the sections were to the occlusal surface, the fewer cementocytes were present. On TEM examination, lacunae and canaliculi, but no cementocytes, were observed, probably because these sections were obtained from the clinical crown (supragingival sections) where cementocytes would have died off, although shrunken cementocytes were still sometimes observed in supragingival histological sections.

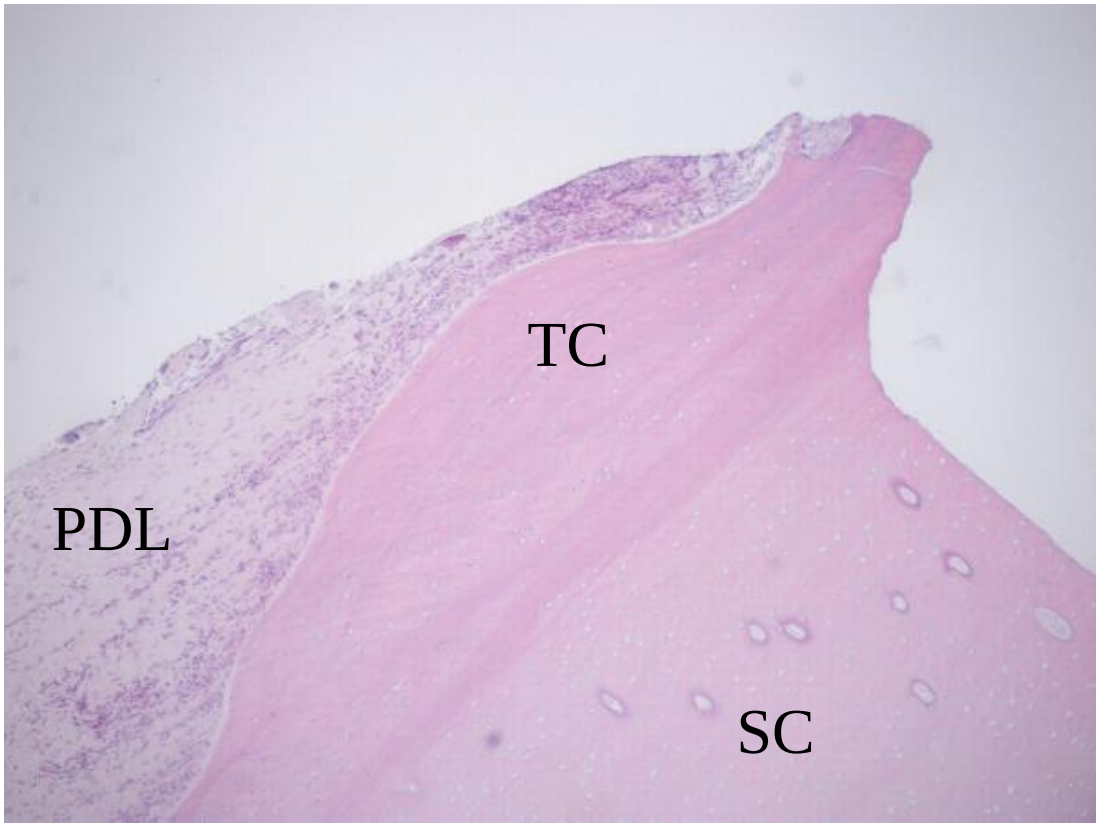


Fig 4.1. Histomicrograph of a decalcified longitudinal section of PDL and interproximal cementum of a maxillary cheek tooth (107). PDL = periodontal ligament; SC = secondary cementum; TC = tertiary cementum [Original magnification X 40, H&E]

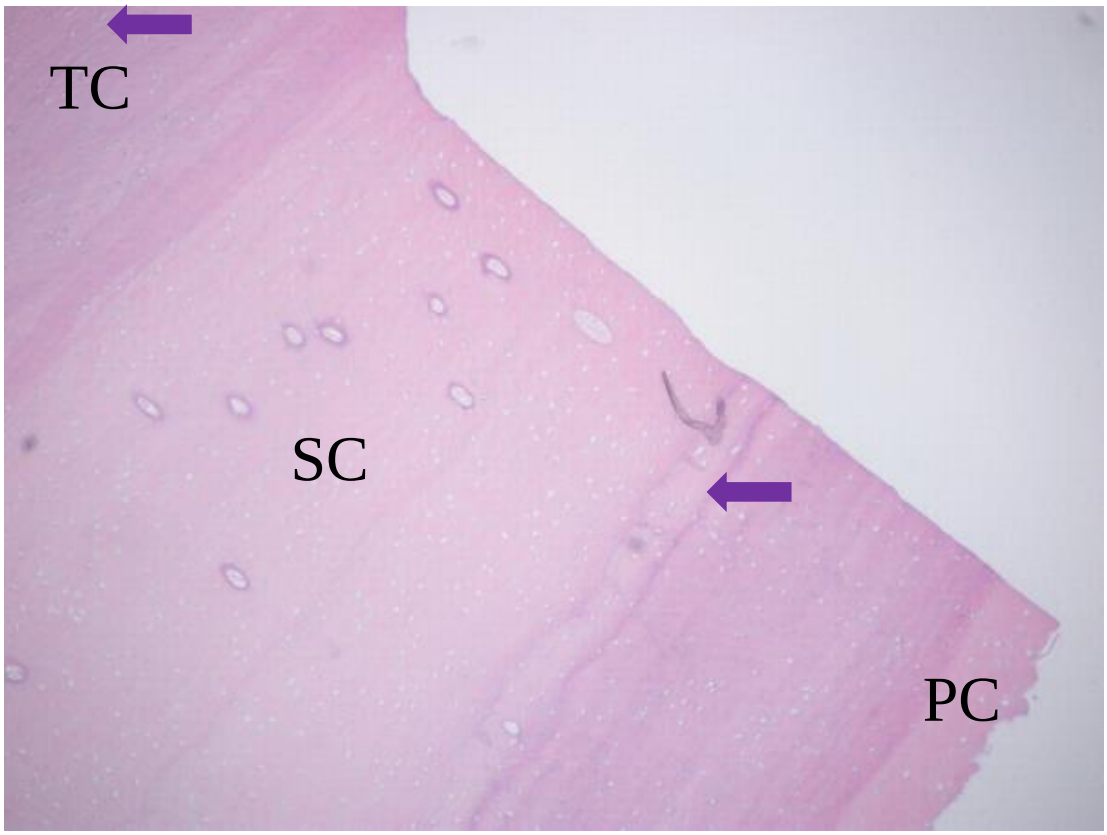


Fig 4.2. Histomicrograph of a decalcified longitudinal section of interproximal cementum of a maxillary cheek tooth (107). TC=tertiary cementum; SC=secondary cementum; PC= primary cementum; arrows = lines of arrested growth. [Original magnification X 40, H&E]

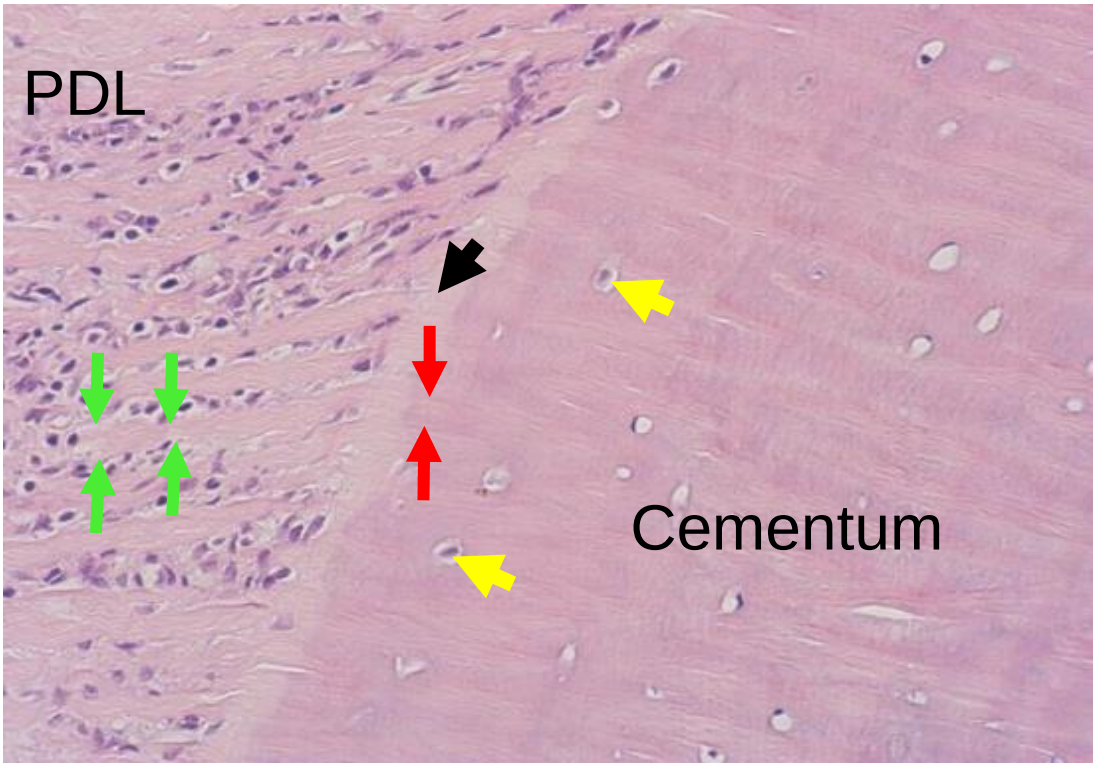


Fig 4.3. Histomicrograph of a decalcified transverse section of a maxillary cheek tooth (108). Bundles of extrinsic periodontal ligament (PDL) fibres (one bundle is outlined by green arrows) become embedded in cementum and are then termed Sharpey's fibres (outlined by red arrows). Black arrowhead = pre-cemental layer; yellow arrowheads = cementocytes within lacunae [Original magnification X 200, H&E]

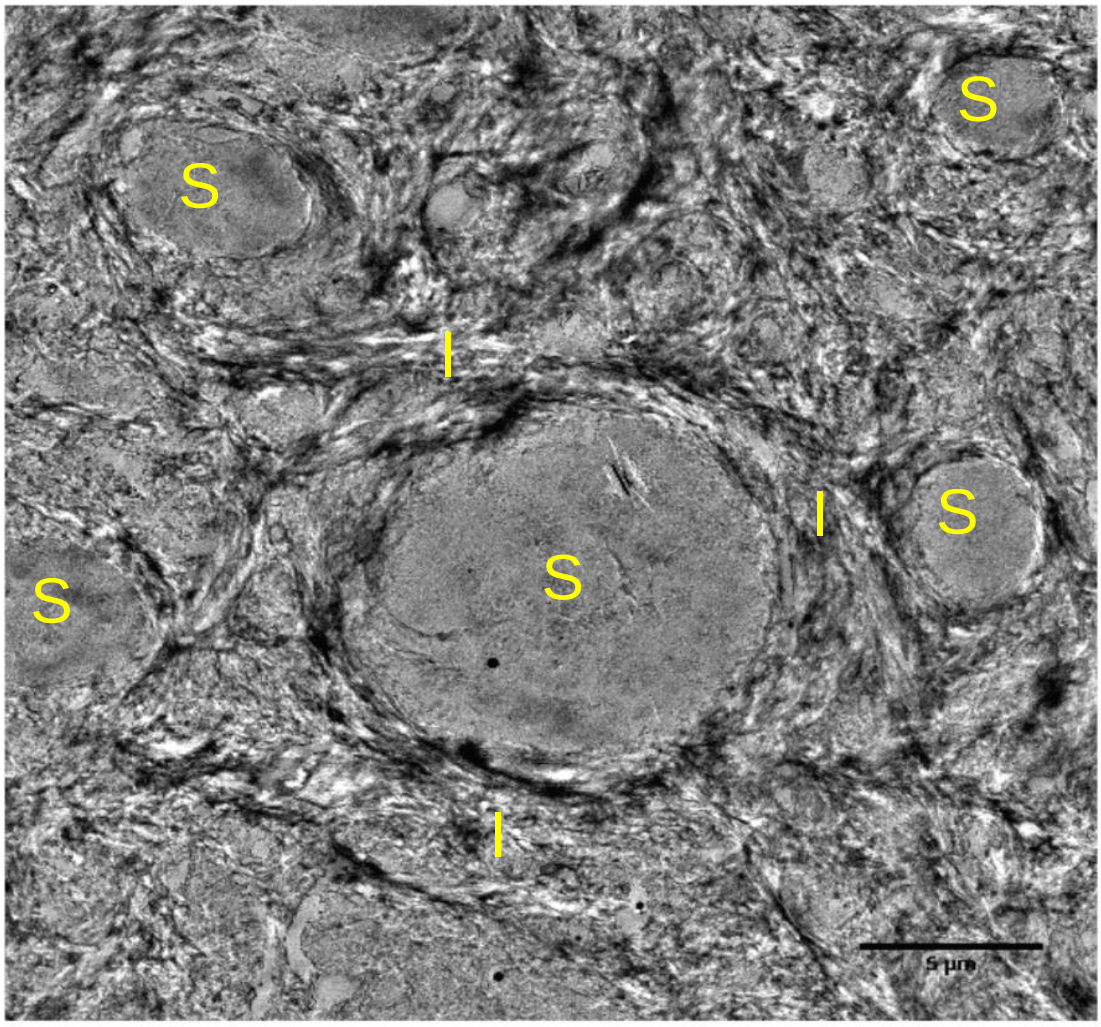


Fig 4.4. TEM image of the buccal aspect of a maxillary cheek tooth (210) showing the large difference in size, orientation and apearance between intrinisic fibres (I) and Sharpey's fibres (S) in an peripheral cementum incremetal growth line.

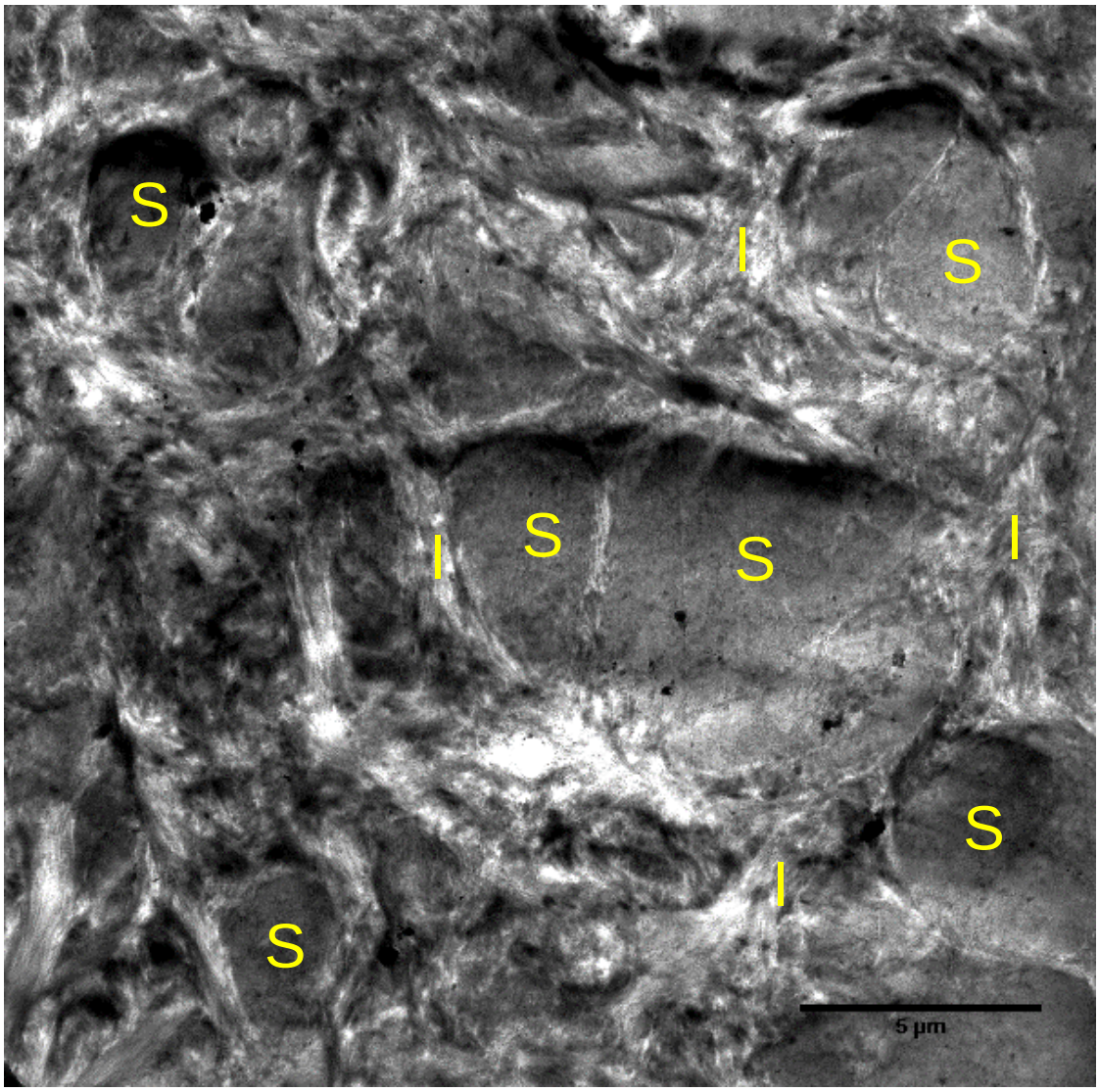


Fig 4.5. TEM image of the palatal aspect of a maxillary cheek tooth (206) showing the clear difference between intrinisic fibres (I) and Sharpey's fibres (S) in a line of arrested growth.

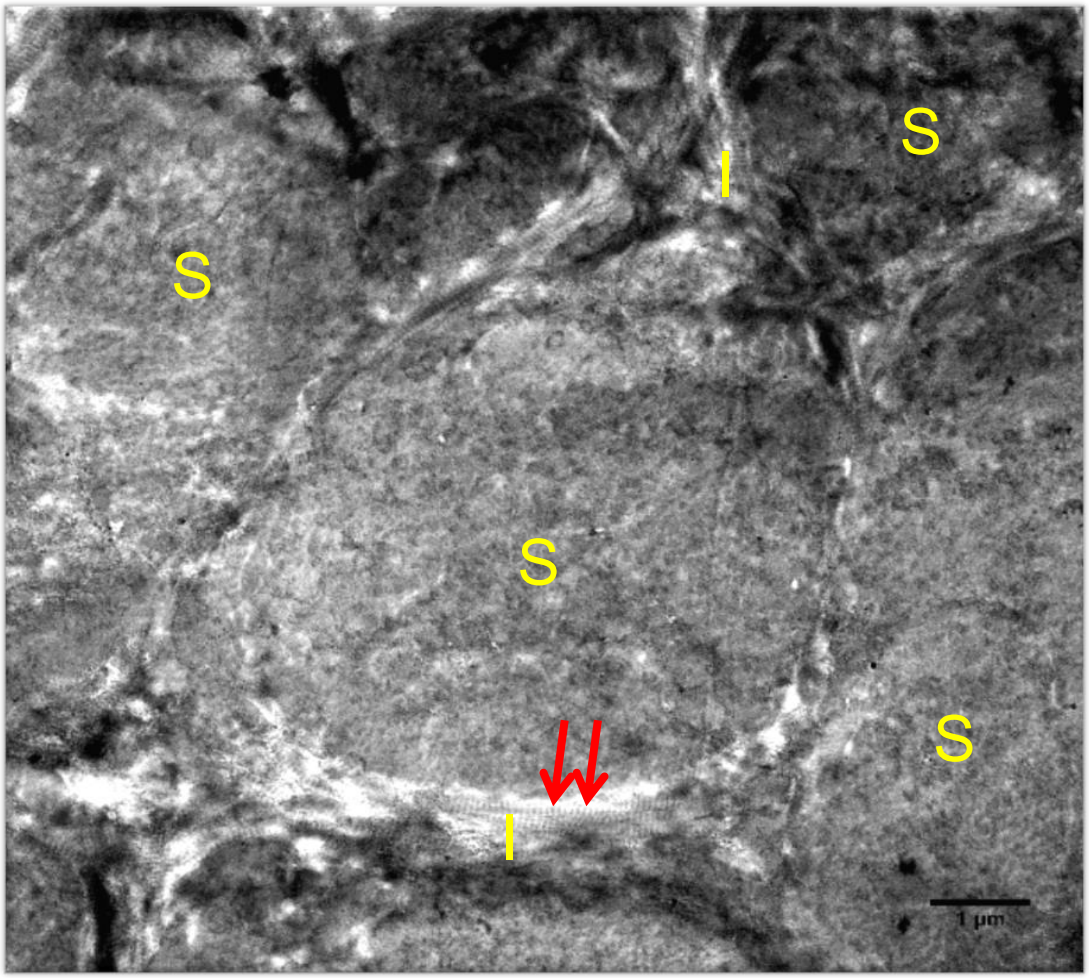


Fig 4.6. TEM image of the palatal aspect of a maxillary cheek tooth (206) showing the clear difference in size, appearance and orientation between intrinisic fibres (I) and Sharpey's fibres (S) in a line of arrested growth. Note the transverse stripes (i.e. periodicity) in the collagenous intrinsic fibres (arrows).

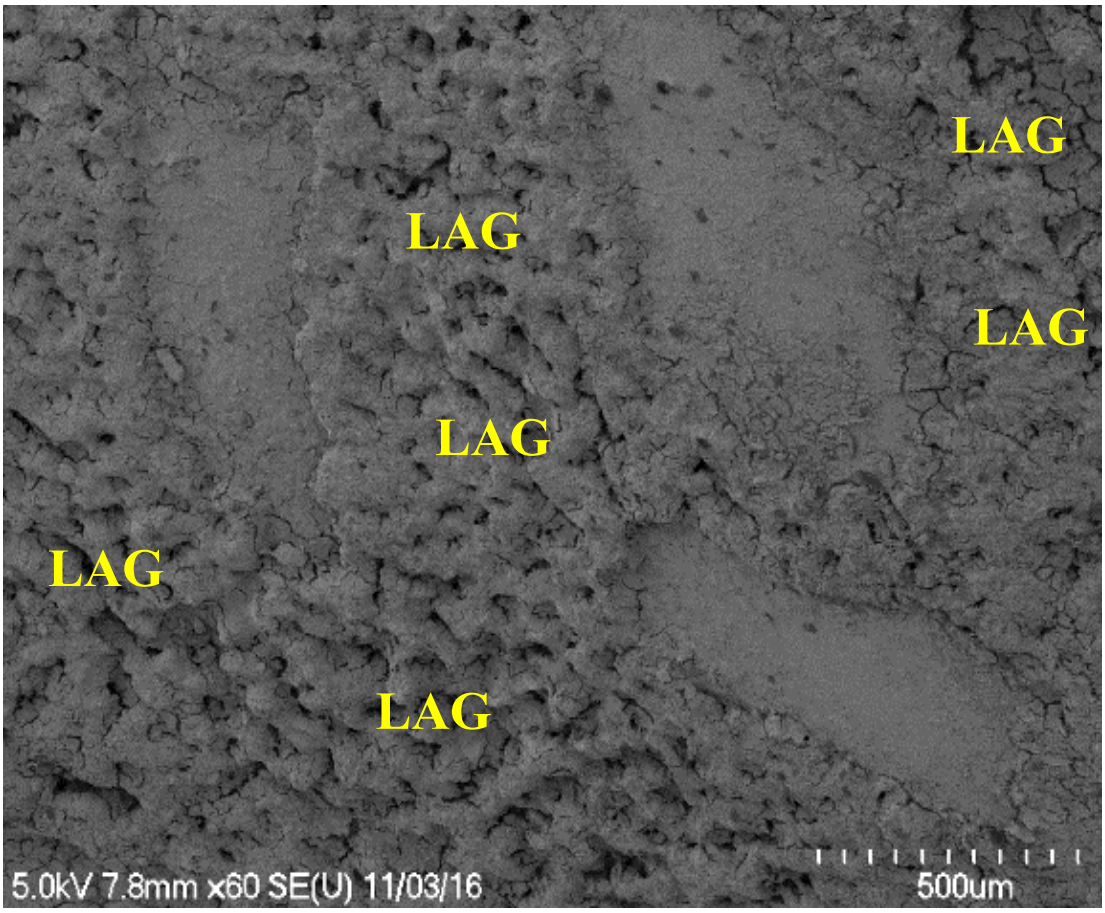


Fig 4.7. SEM image of the buccal aspect of a mandibular cheek tooth (306) showing a line of arrested growth (LAG) in peripheral cementum.

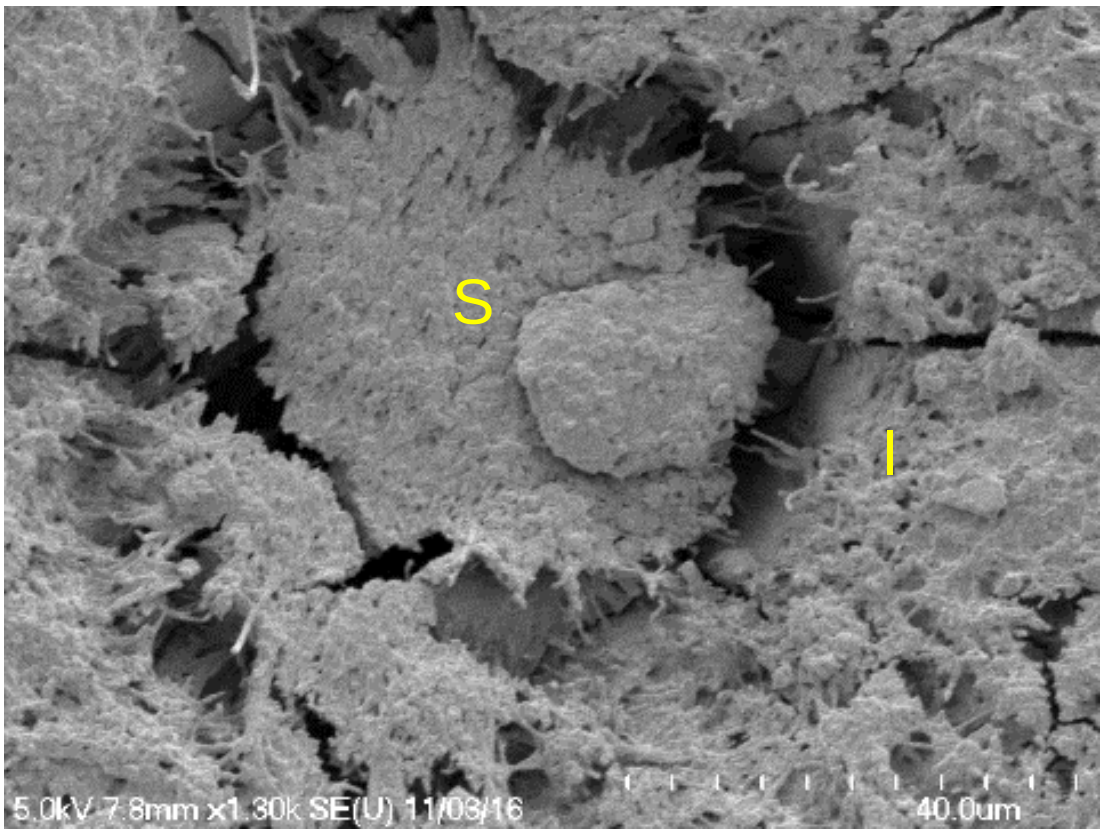


Fig 4.8. SEM image of the buccal aspect of a mandibular cheek tooth (306). Magnification of a line of arrested growth (previous figure: Fig 4.7), showing a Sharpey's fibre (S) in the centre, surrounded by protruding intrinsic fibres (I) and covered by a layer of dental plaque and its micro-organisms. Diameter of this Sharpey's fibre= 40 µm; Sharpey's fibres can be up to 55 µm diameter.

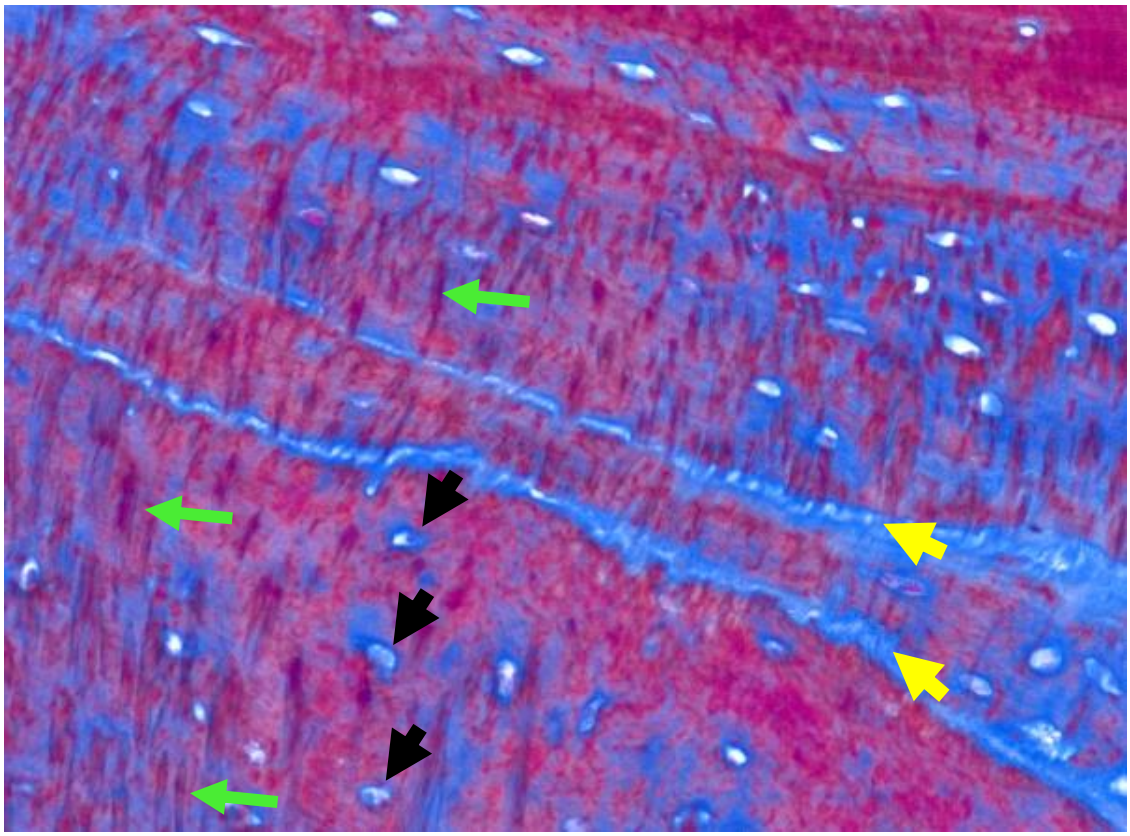


Fig 4.9. Histomicrograph of a decalcified section of a maxillary cheek tooth (209) showing cementum with lines of arrested growth (yellow arrows), lacunae (black arrows) and Sharpey's fibres (green arrows). Just like bone, cementum stains blue/red. [Original magnification X 200, Masson's trichrome]

Blood vessels or their remnants were present in the peripheral cementum of all cheek teeth and in the infundibular cementum of all maxillary cheek teeth examined, in both decalcified and undecalcified sections. In an undecalcified section of a maxillary cheek tooth, blood vessels were observed to enter the preprotoconal groove and postprotoconal valley, which are the cemental infoldings at the rostro-palatal and caudo-palatal aspects, respectively, of a maxillary cheek tooth (Fig 4.10). Although this feature was also observed using SEM (Fig 4.11) and in decalcified sections of maxillary cheek teeth (Fig 4.12), this undecalcified section showed more clearly than SEM or decalcified sections that the rostral and caudal infundibulae each had a large blood vessel in their centre, with radiating horizontal branches extending towards the cementoenamel junction (Figs 4.13 and 4.14). Large blood vessels and their branches were observed to penetrate into the peripheral cementum in an undecalcified section in the entoflexid and metaflexid of a mandibular cheek tooth. On decalcified H&E stained sections, some blood vessels appeared empty while the lumina of others of were obliterated.

Subgingivally, protrusions of vascularised PDL containing fibroblasts and multiple Sharpey's fibres penetrated the cementum perpendicular to the peripheral aspect of the tooth (Figs 4.15 and 4.16). Some protrusions, often with basophilic outlines, were found in the peripheral cementum as inclusions, fully surrounded by cementum (Figs 4.17, 4.18 and 4.19). Red blood cells were present in some, indicating that these inclusions consisted of, or contained, viable blood vessels, although a distinct vascular endothelium was not observed. Cementum, which appeared newly formed (because concentric lines of the same shape as the inclusions were observed), was present in some inclusions (Fig 4.20).

Remodeling was observed in alveolar bone surrounding a cheek tooth, where a single osteoclast was observed associated with mildly resorbed bone (Fig 4.21). Similarly to osteoclastic resorption of bone, resorption of cementum occurs by cementoclasts (also called odontoclasts).

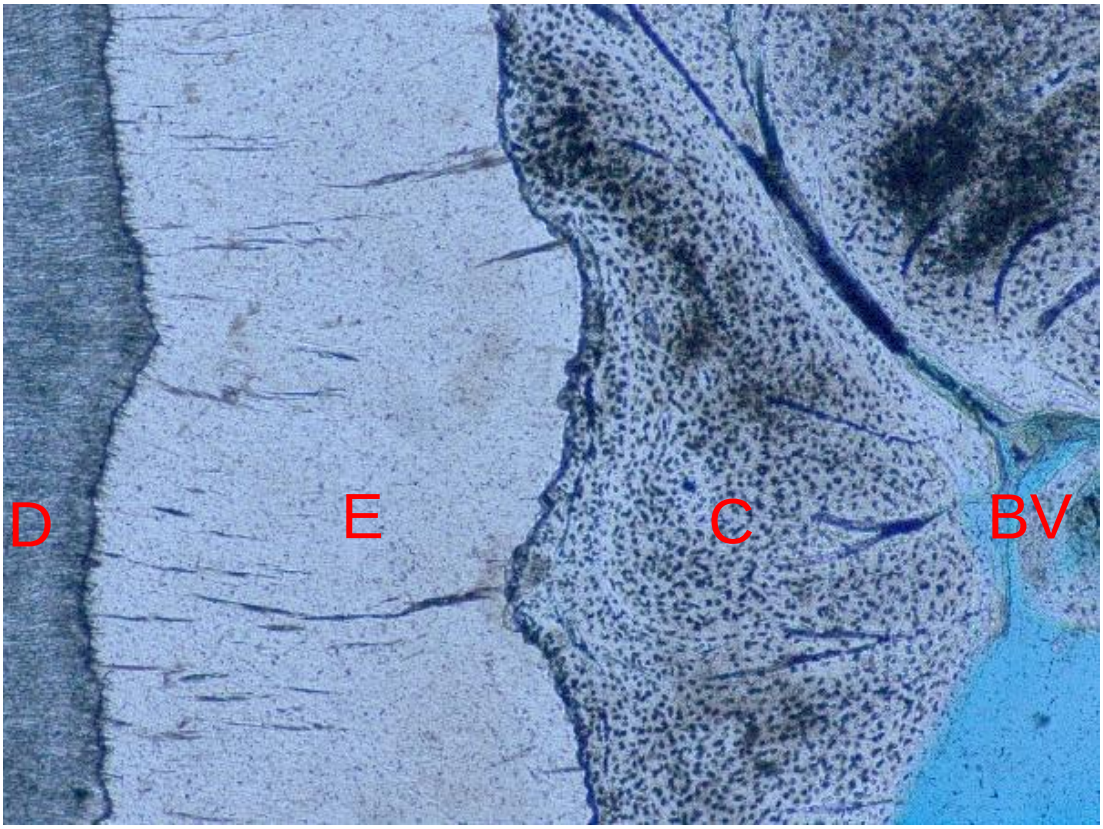


Fig 4.10. Histomicrograph of an undecalcified transverse section of a maxillary cheek tooth showing a blood vessel entering and branching in the preprotoconal groove. (Courtesy of I. Dacre and P.M. Dixon). D = dentine; E = enamel; C = cementum; BV = blood vessel [Original magnification X 40]

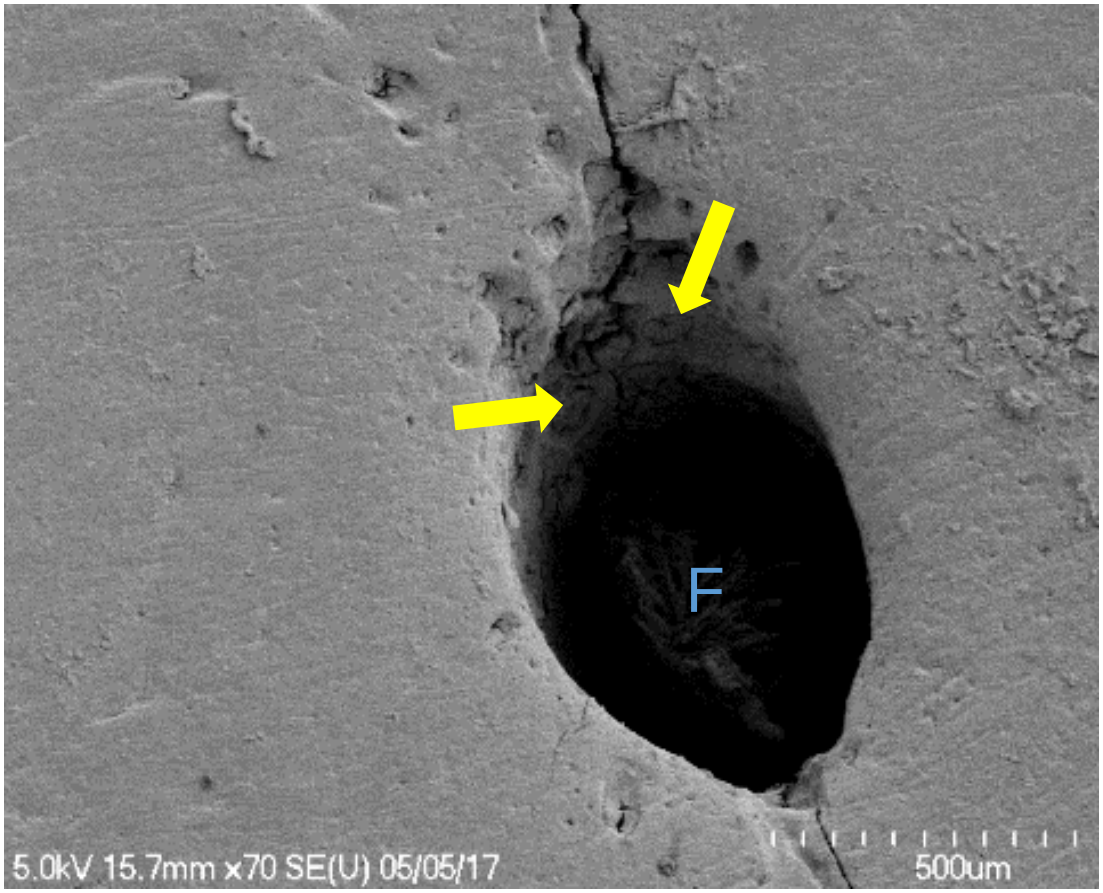


Fig 4.11. SEM of a normal infundibulum from a maxillary cheek tooth (208), with entrapped food material (F). The opening is actually a channel where a central blood vessel previously entered the tooth. The circular structures (arrows) in the wall of the channel could be occluded branches of the former central blood vessel.

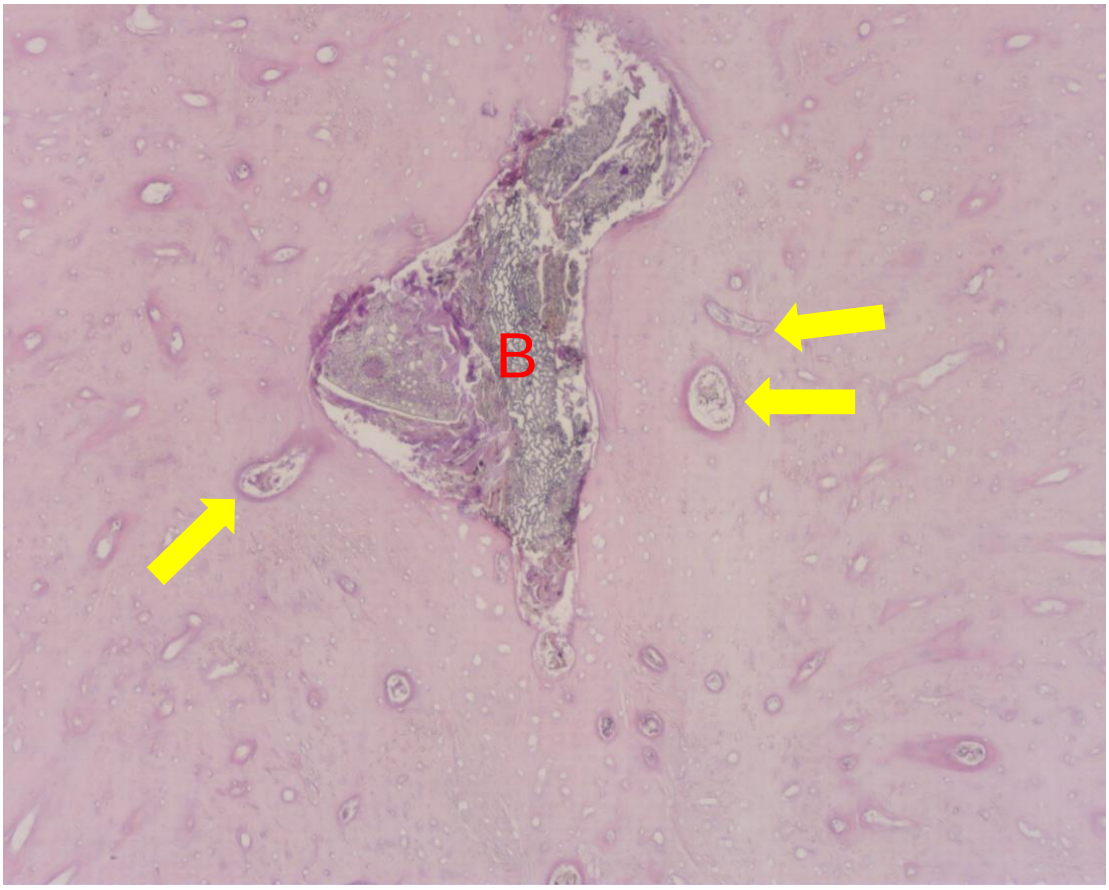


Fig 4.12. Histomicrograph of a decalcified section of a maxillary cheek tooth (108) of a caudal infundibulum without grossly visible infundibular caries. A central blood vessel (BV) and its branches (arrows) can be observed and contain food material. [Original magnification X 40, H&E]

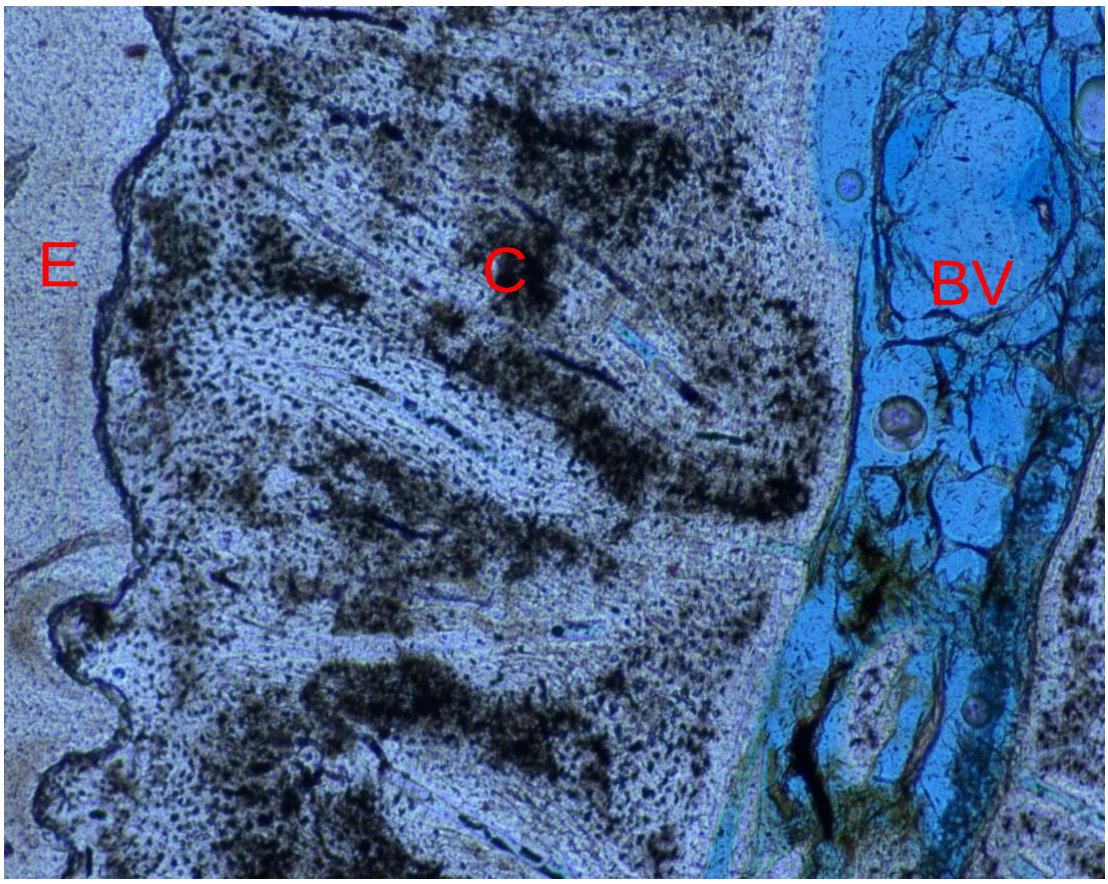


Fig 4.13. Undecalcified transverse section of a maxillary cheek tooth showing a central blood vessel in the infundibular cementum. Its branches radiate towards the cemento-enamel junction. (Courtesy of I. Dacre and P.M. Dixon). E = enamel; C = cementum; BV= blood vessel [Original magnification X 40]

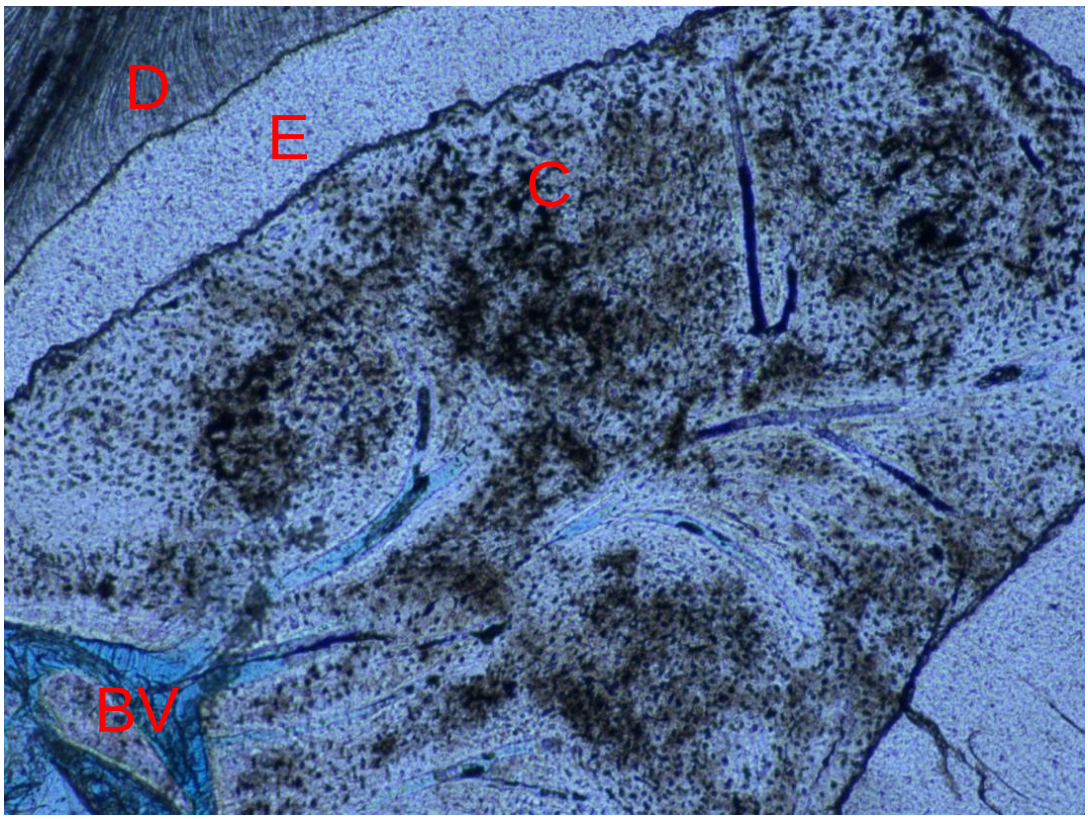


Fig 4.14. Undecalcified transverse section of a maxillary cheek tooth showing a central blood vessel in infundibular cementum and its branches radiating towards the cemento-enamel junction. (Courtesy of I. Dacre and P.M. Dixon). D= dentine; E = enamel; C = cementum; BV = blood vessel [Original magnification X 40]

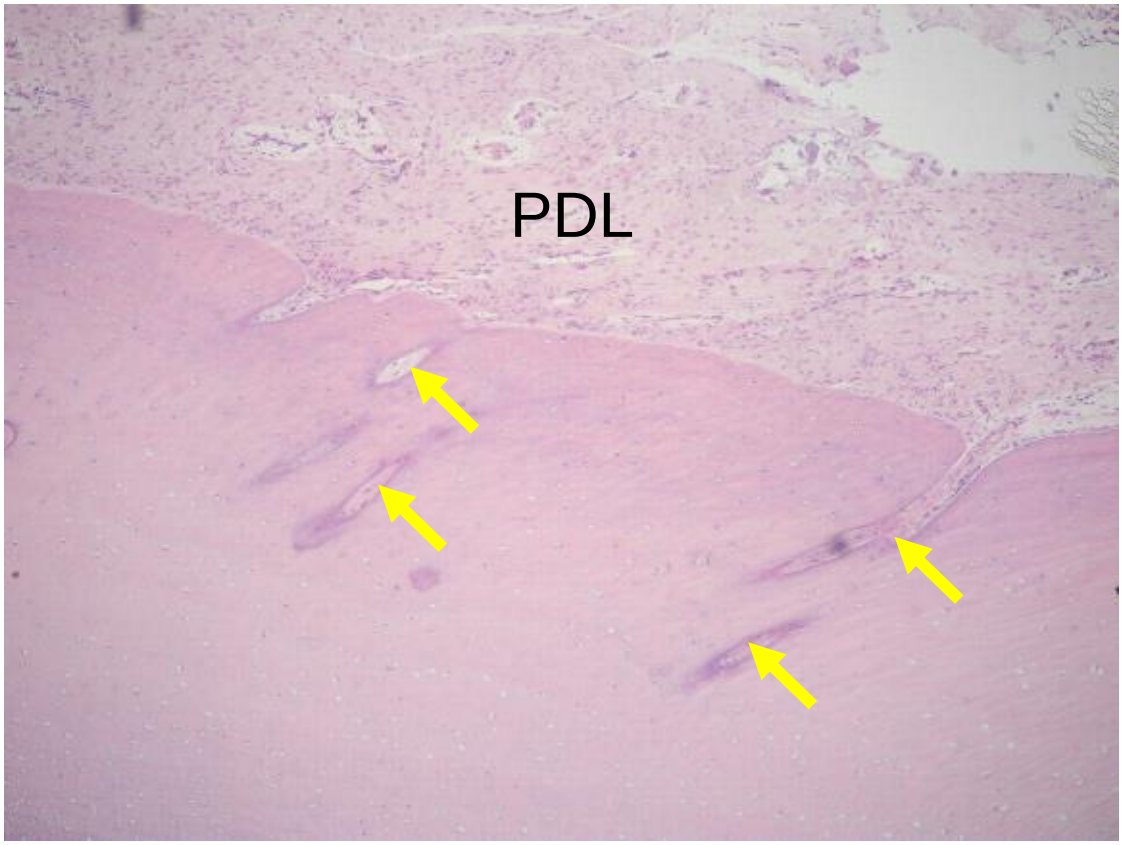


Fig 4.15. Histomicrograph of a decalcified, transverse section of a maxillary cheek tooth (108) at the level of the gingiva (i.e. occlusal aspect of the reserve crown). Bundles of extrinsic periodontal ligament (PDL) fibres have entered the cementum and are now termed Sharpey's fibres (yellow arrows). C: Cementum [Original magnification X 40, H&E]

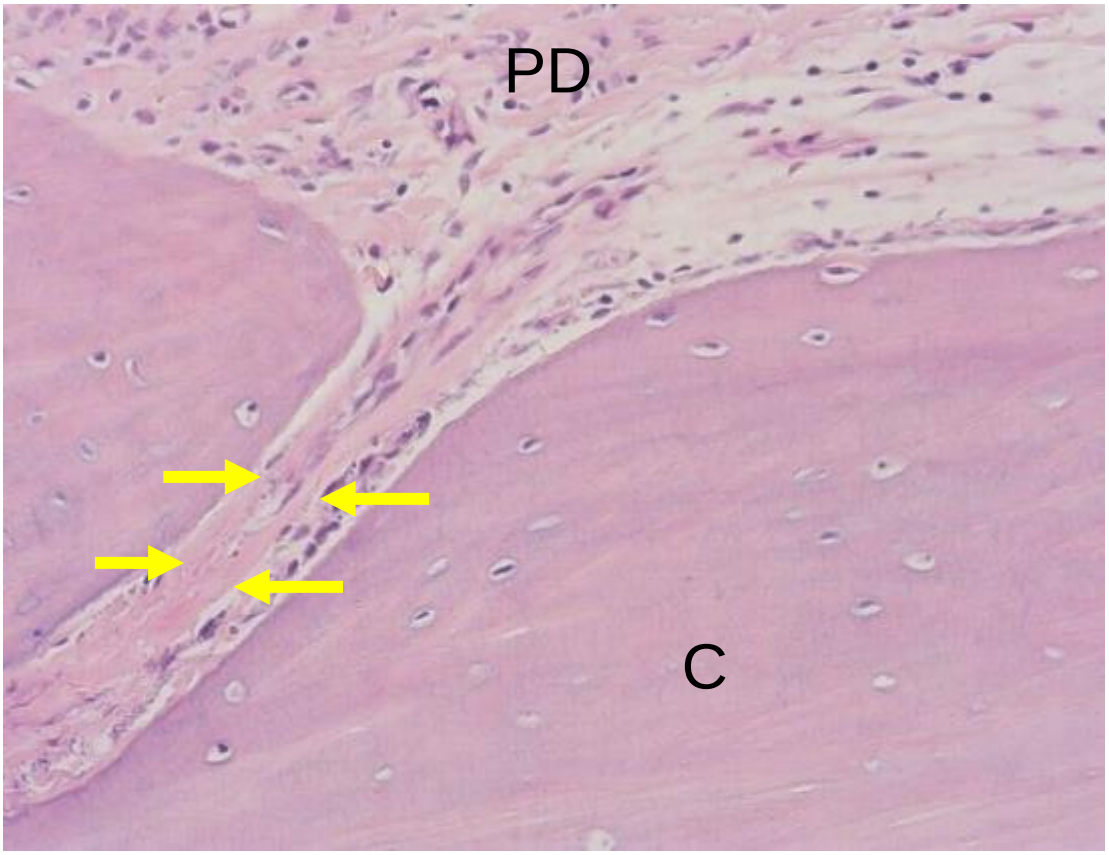


Fig 4.16. Higher magnification of previous image (Fig 4.15), a histomicrograph of a decalcified, transverse section of a maxillary cheek tooth (108) at the level of the gingiva (i.e. reserve crown) showing Sharpey's fibres (yellow arrows) entering the cementum (C) of the reserve crown. [Original magnification X 200, H&E]

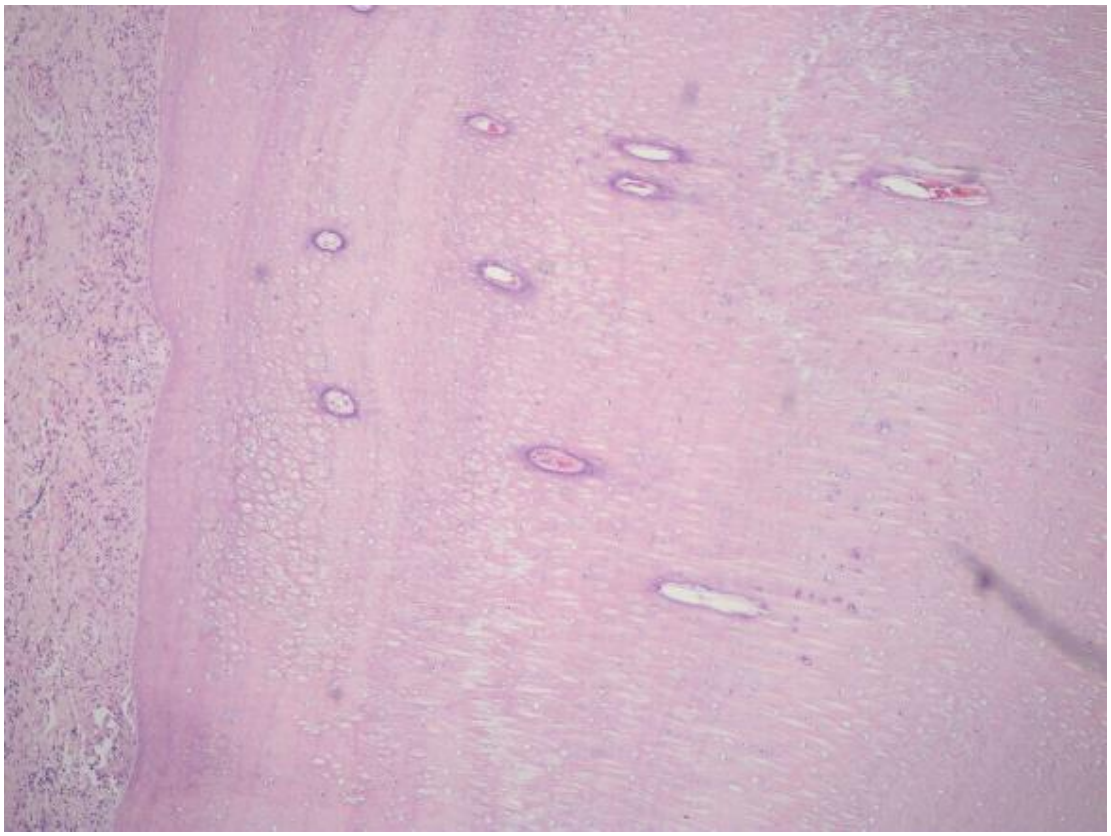


Fig 4.17. Histomicrograph of a decalcified, transverse section of a maxillary cheek tooth (108) at the level of the gingiva (i.e. reserve crown). Inclusions of connective tissue comprising bundles of Sharpey's fibres and capillaries, all with a basophilic outline, are embedded in the cementum. [Original magnification X 40, H&E]

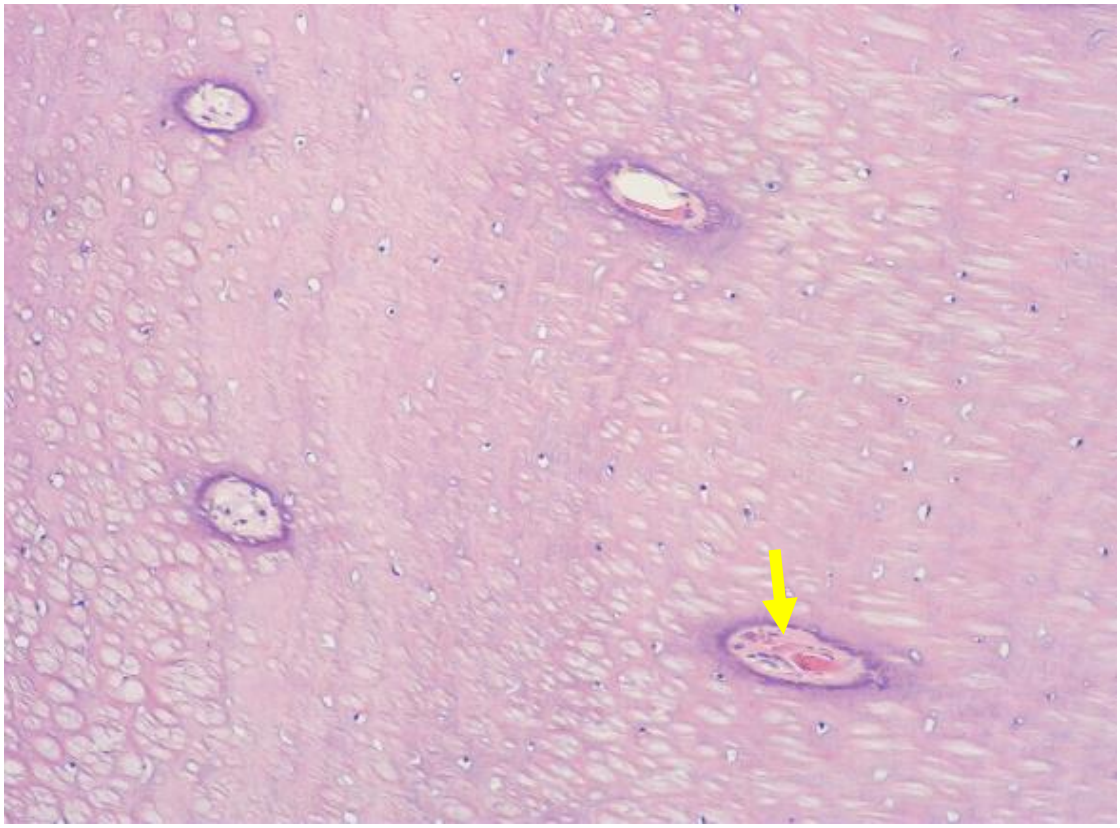


Fig 4.18. Higher magnification of previous image (Fig 4.17), Histomicrograph of a decalcified, transverse section of a maxillary cheek tooth (108) at the level of the gingiva (i.e. reserve crown). Bundles of Sharpey's fibres and capillaries are embedded in the cementum and these inclusions have a basophilic outline. Yellow arrow = red blood cells in capillary. [Original magnification X 100, H&E]

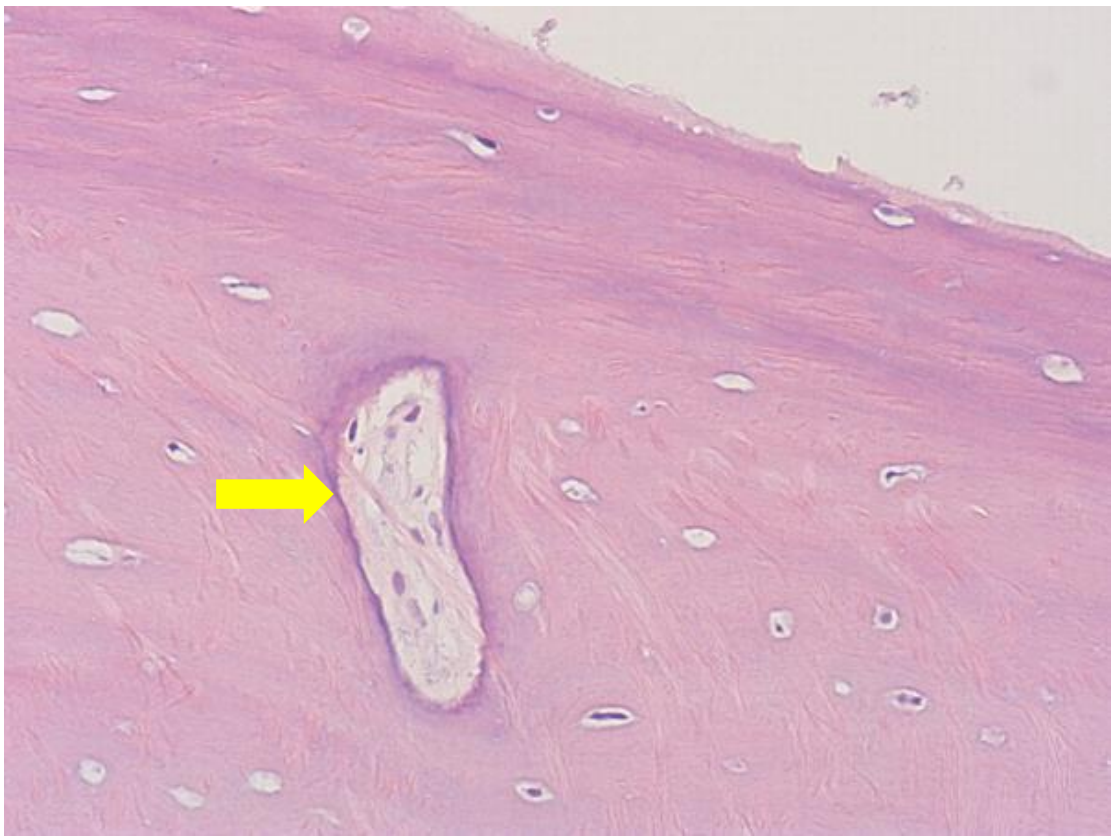


Fig 4.19. Histomicrograph of a decalcified transverse section of a maxillary cheek tooth (108) at the level of the gingiva (i.e. reserve crown). An ellipsoid inclusion containing connective tissue (arrow), including bundles of Sharpey's fibres, and mesenchymal (fibroblast-like) cells is present. [Original magnification X 200, H&E]

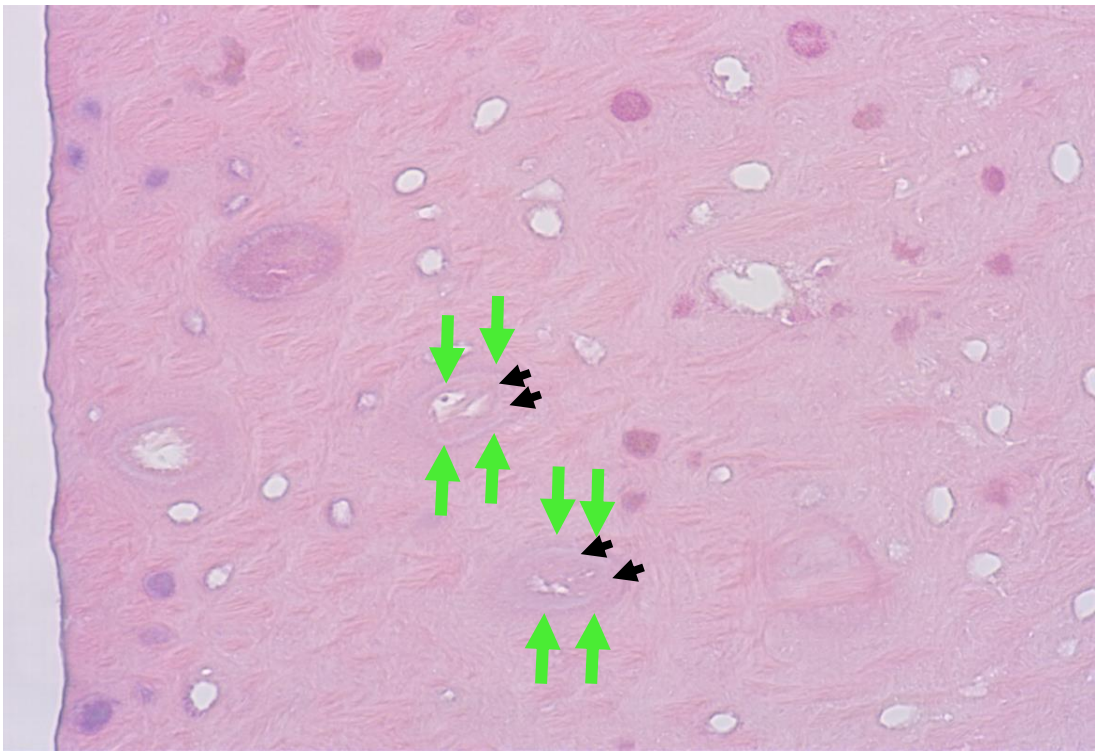


Fig 4.20. Histomicrograph of a decalcified, longitudinal section of a maxillary cheek tooth (107). The previous sites of soft tissue inclusions (green arrows) are partially filled with what appears to be new cementum (black arrows). [Original magnification X 200, H&E]

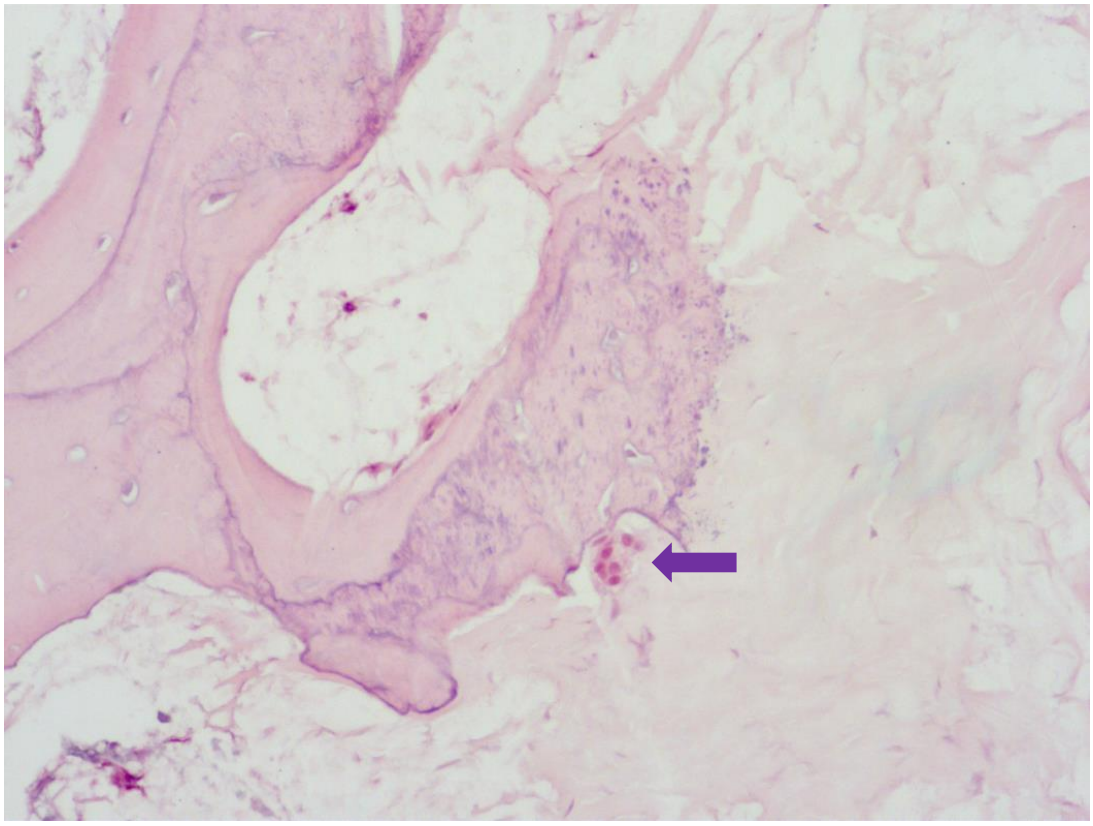


Fig 4.21. Osteoclast (arrow) resorbs the alveolar bone of a maxillary cheek tooth (209) [Original magnification X 200, H&E]

4.3.1.2 Histological and Ultrastructural Findings in Equine Cheek Teeth Cementum Affected by Peripheral Caries

Dental plaque was observed overlying caries lesions using histology, SEM and TEM. Using SEM, dental plaque covering PC lesions contained micro-organisms of different shapes and sizes (Fig 4.22). Using TEM, clusters of micro-organisms within the dental plaque sometimes had specific configurations, i.e. were sometimes arranged in long rows perpendicular to the tooth surface as strands of fibrillar material (Fig 4.23). This fibrillary material surrounded individual and groups of micro-organisms (Fig 4.24).

Histology of transverse sections of PC-affected equine cheek teeth showed three types of PC lesions (Table 4.1): flake-like lesions (Type A1 and A2), flask-like lesions (Type B) and a previously undescribed ellipsoid type lesion (Type C), or a combination of these three types. Micro-organisms present in these PC lesions could have entered the tooth either from the occlusal surface or from the peripheral aspect of the tooth, then progressed parallel and/or perpendicular to the peripheral aspect of the tooth. The PC lesions varied in size but some flake-like lesions at or parallel to lines of arrested growth and the peripheral aspect of the tooth were substantial in size.

Dental plaque containing bacteria penetrated the cementum perpendicular to the peripheral aspect of affected teeth between the Sharpey's fibres (Type A1), creating flake-like lesions and also penetrated into lacunae (where cementocytes normally reside) and into the canaliculi (which connect lacunae containing cementocytes with each other) (Figs 4.25 and 4.26). Micro-organisms were also apparent in these two locations using TEM (Figs 4.27 and 4.28, respectively). Bacteria were also found in ellipsoid type lesions in the outer cemental layer (Figs 4.29, 4.30, 4.31).

In flake-like lesions, in which micro-organisms were spread out parallel to the peripheral aspect of the tooth (Type A2) and sometimes at the level of a line of arrested growth, the intrinsic fibres were undermined and cemental flakes were partially or fully detached from the underlying peripheral cementum (Figs 4.32, 4.33, 4.34, 4.35, 4.36, 4.37). Combinations of flake-like and flask-like lesions (Fig 4.38) and a combination of flake-like and ellipsoid type lesions were also observed (Fig 4.33).

A final possible way for oral micro-organisms to penetrate cementum was via the minuscule network of intracemental tubules (Type D) that were possible former cemental vascular sites and micro-organisms were also observed to be present in lacunae (Fig 4.39).

SEM confirmed the destruction of cementum in PC lesions (grade 1.1), both in areas with (Fig 4.40) and without (Fig 4.41) lines of arrested growth. TEM examination of the palatal aspect of a control tooth, both in areas with and an area without discolouration, showed micro-organisms to be present, not only on the peripheral aspect of the cheek tooth but also within cementum, both in lacunae and in what appeared to be former sites of blood vessels (Fig 4.42).

Table 4.1. Description of histological/ultrastructural presentation of different morphological types of cemental peripheral caries lesions in equine cheek teeth.

Type	Shape	Localisation and main direction of spreading
Type A1	Flake-like lesion	Bacteria penetrate between Sharpey's fibres; bacteria spread in a direction perpendicular to peripheral aspect of cheek tooth. Flakes become partially or fully detached from underlying peripheral cementum
Type A2	Flake-like lesion	Bacteria undermine intrinsic fibres; Bacteria spread in a direction parallel to peripheral aspect of cheek tooth at the level of or parallel to lines of arrested growth. Flakes become partially or fully detached from underlying peripheral cementum.
Type B	Flask- like lesion	Bacteria penetrate and then spread in a radiating direction
Type C	Ellipsoid-type lesion	Perpendicular to peripheral aspect of tooth
Type D	Shape defined by the outlines of microtubules/canaliculi and lacunae	Bacteria penetrate into and spread via microtubule/canaliculi und lacunae; multidirectional

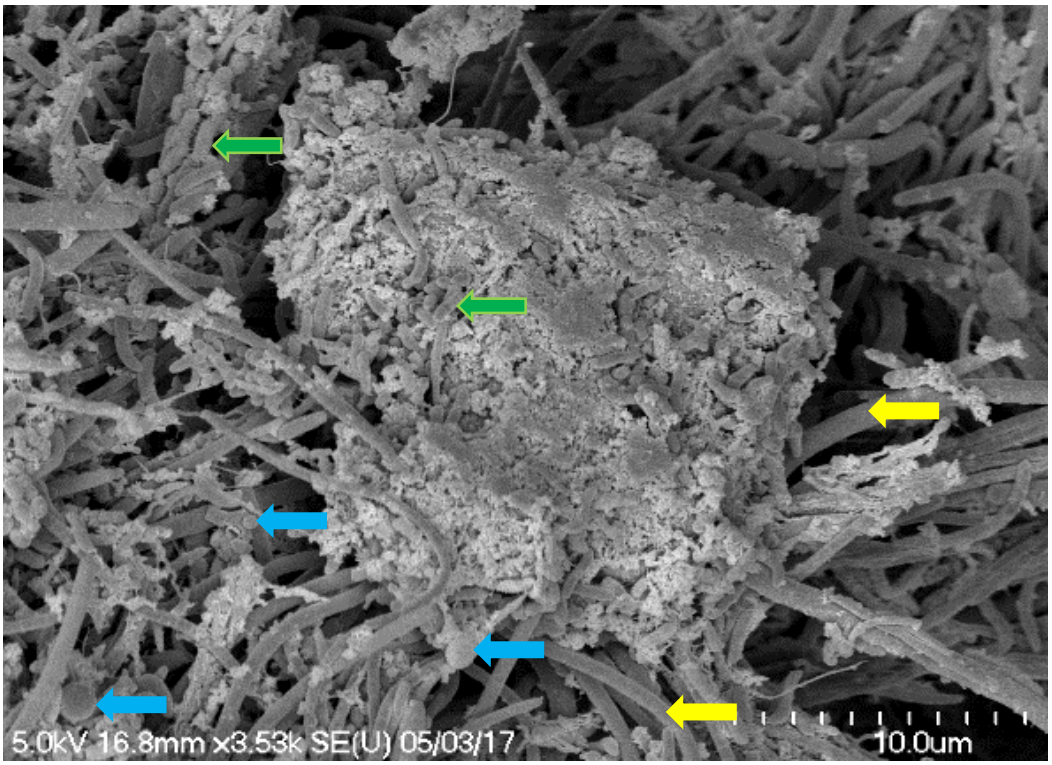


Fig 4.22. SEM image of an undecalcified peripheral section of the palatal aspect of a maxillary cheek tooth (110) with peripheral caries lesions (grade 1.1) in its lines of arrested growth that are covered by a layer of dental plaque. Pictured is the dental plaque with its network of micro-organisms of different shapes and sizes. Yellow arrows = filamentous micro-organisms; blue arrows = large and small cocci; green arrows = bacilli (rods)

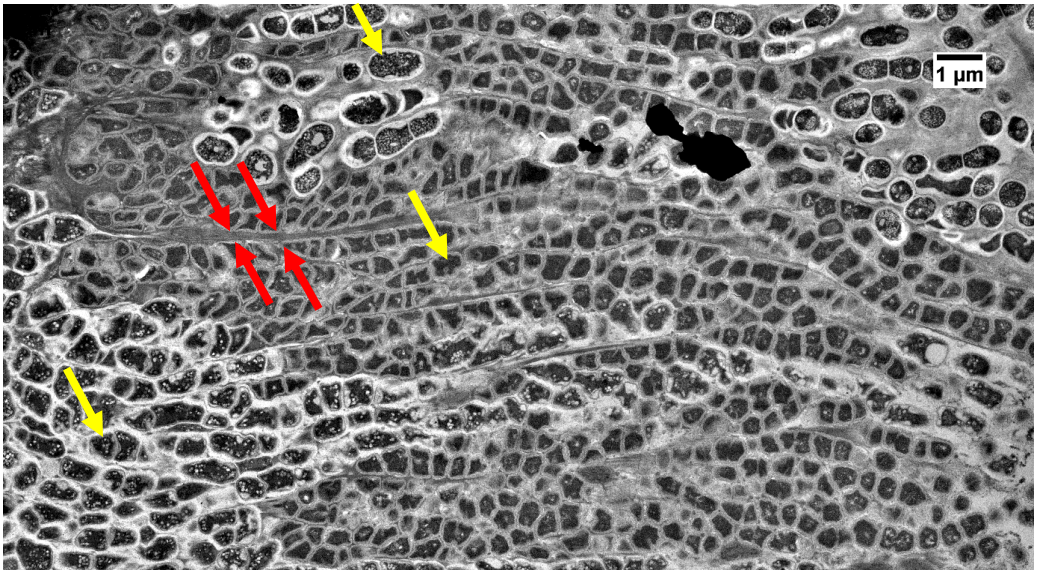


Fig 4.23. SEM of the (lingual) peripheral aspect of a mandibular cheek tooth with grade 1.1 peripheral caries lesion covered with a thick layer of supragingival dental plaque that contains micro-organisms (406). The micro-organisms in the dental plaque are tightly arranged in rows (yellow arrows) with layers of fibrillar material (red arrows) present between them.

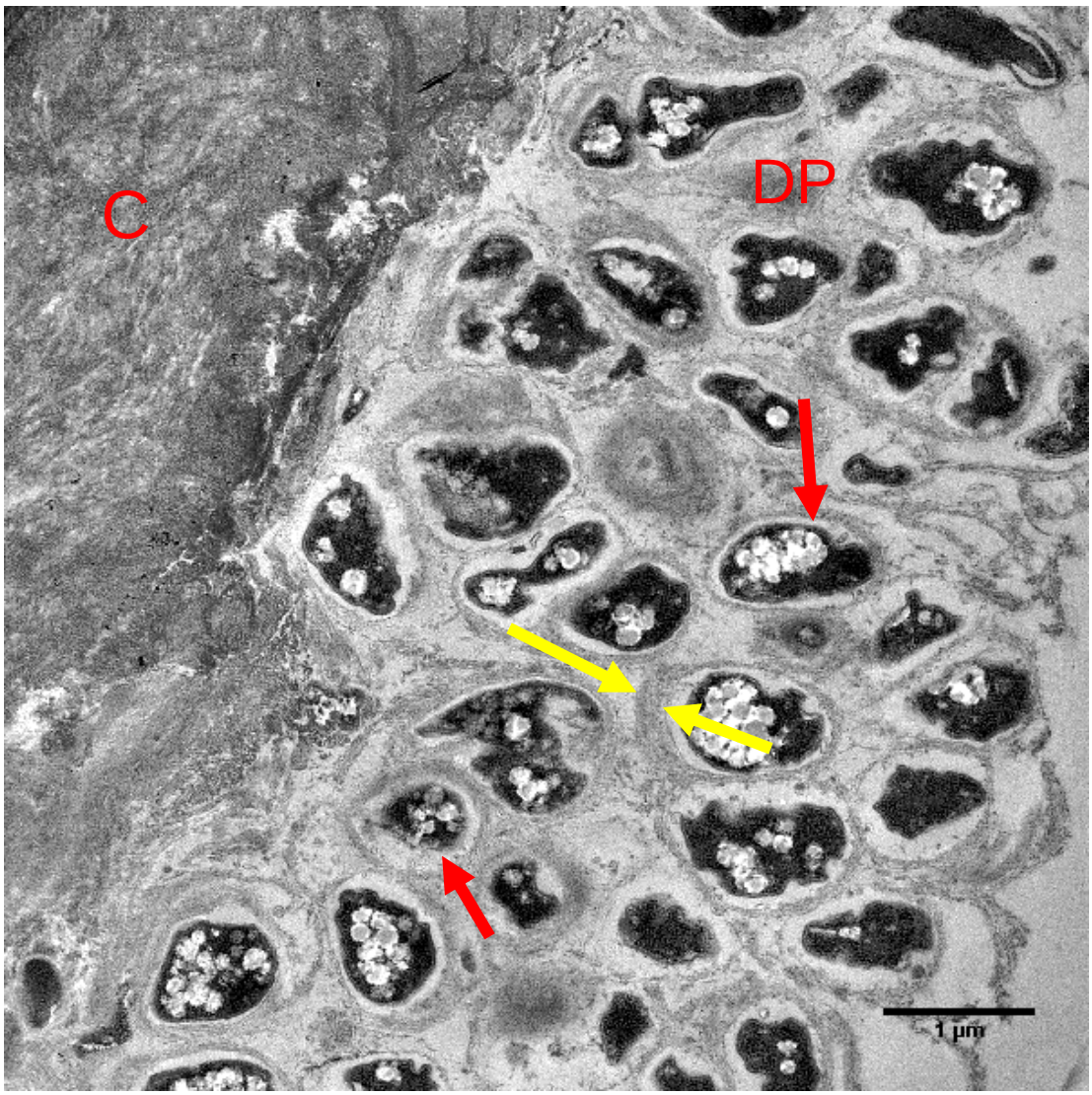


Fig 4.24. TEM image of peripheral cementum (C) with a grade 1.1 peripheral caries lesion at the lingual aspect of a mandibular cheek tooth (406) that is covered by a layer of dental plaque (DP) containing micro-organisms (red arrow) embedded in a honeycomb of fibrillar material (yellow arrows).

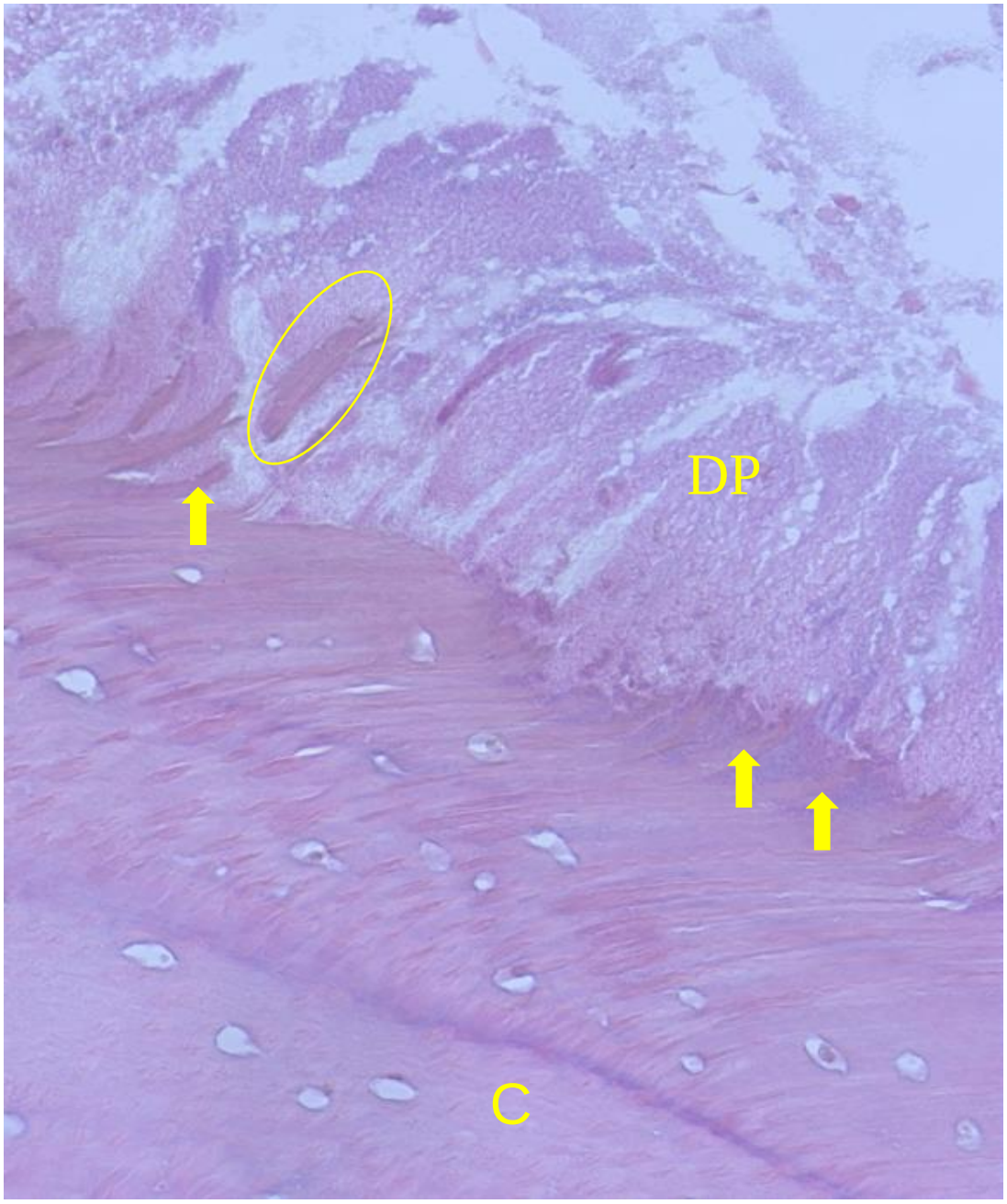


Fig 4.25. Histomicrograph of a decalcified section of the peripheral aspect of a mandibular cheek tooth (310) with grade 1.1 peripheral caries: Flake-like, cemental caries lesions, lying in a thick layer of dental plaque (DP) that covers the tooth periphery are oriented perpendicular to the peripheral aspect of the tooth. Some cemental flakes have become fully detached (circle) from the cementum (C) and dental plaque has penetrated between some Sharpey's fibres (arrows). [Original magnification X 200, H&E]

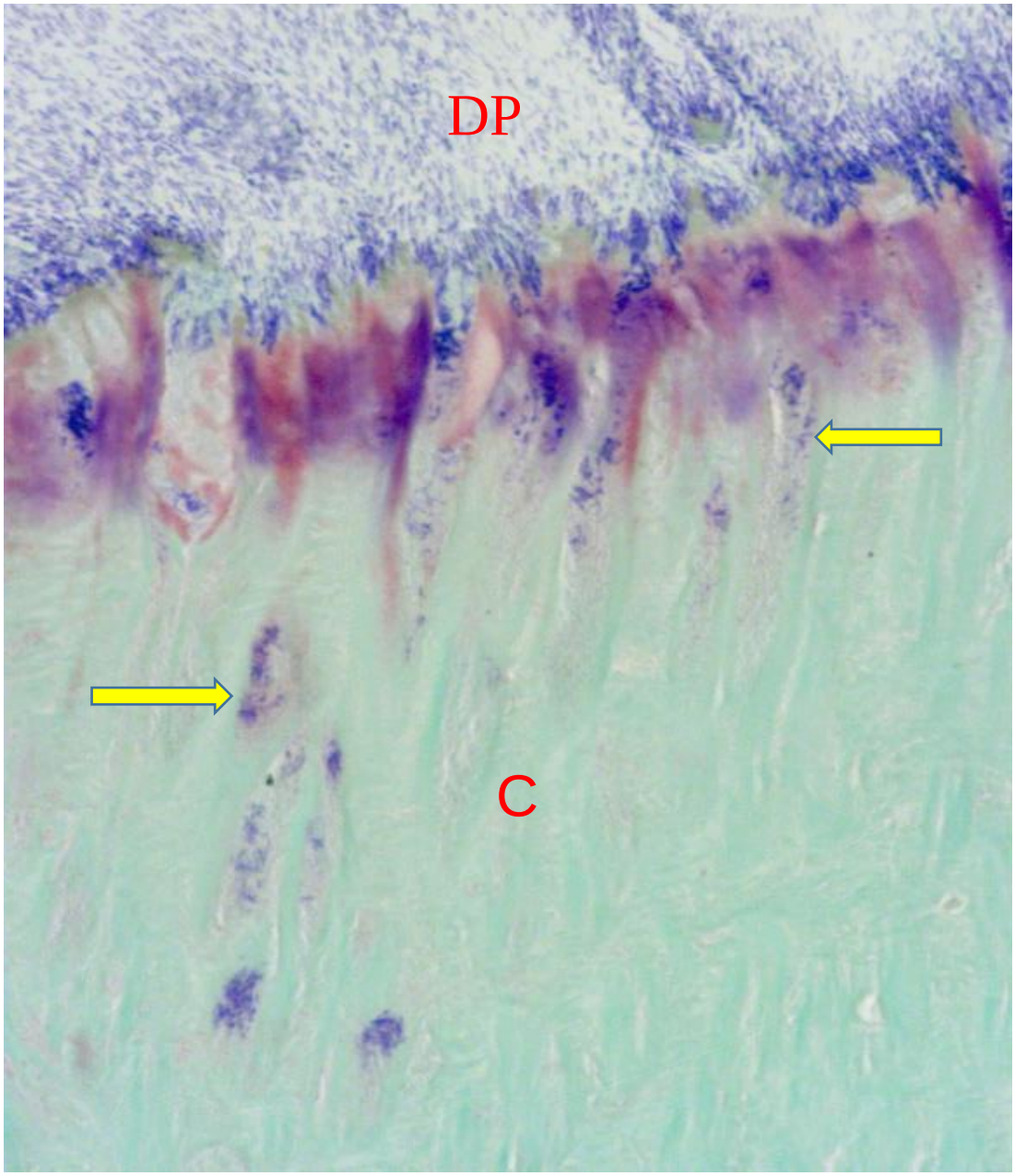


Fig 4.26. Gram stained, decalcified section of the periphery of a maxillary cheek tooth (210) with grade 1.1 peripheral caries. Note the thick layer of overlying dental plaque (DP) containing many Gram-positive bacteria, which have penetrated the cementum, between Sharpey's fibres (arrows) in a direction perpendicular to the periphery of the tooth. [Original magnification X 400]

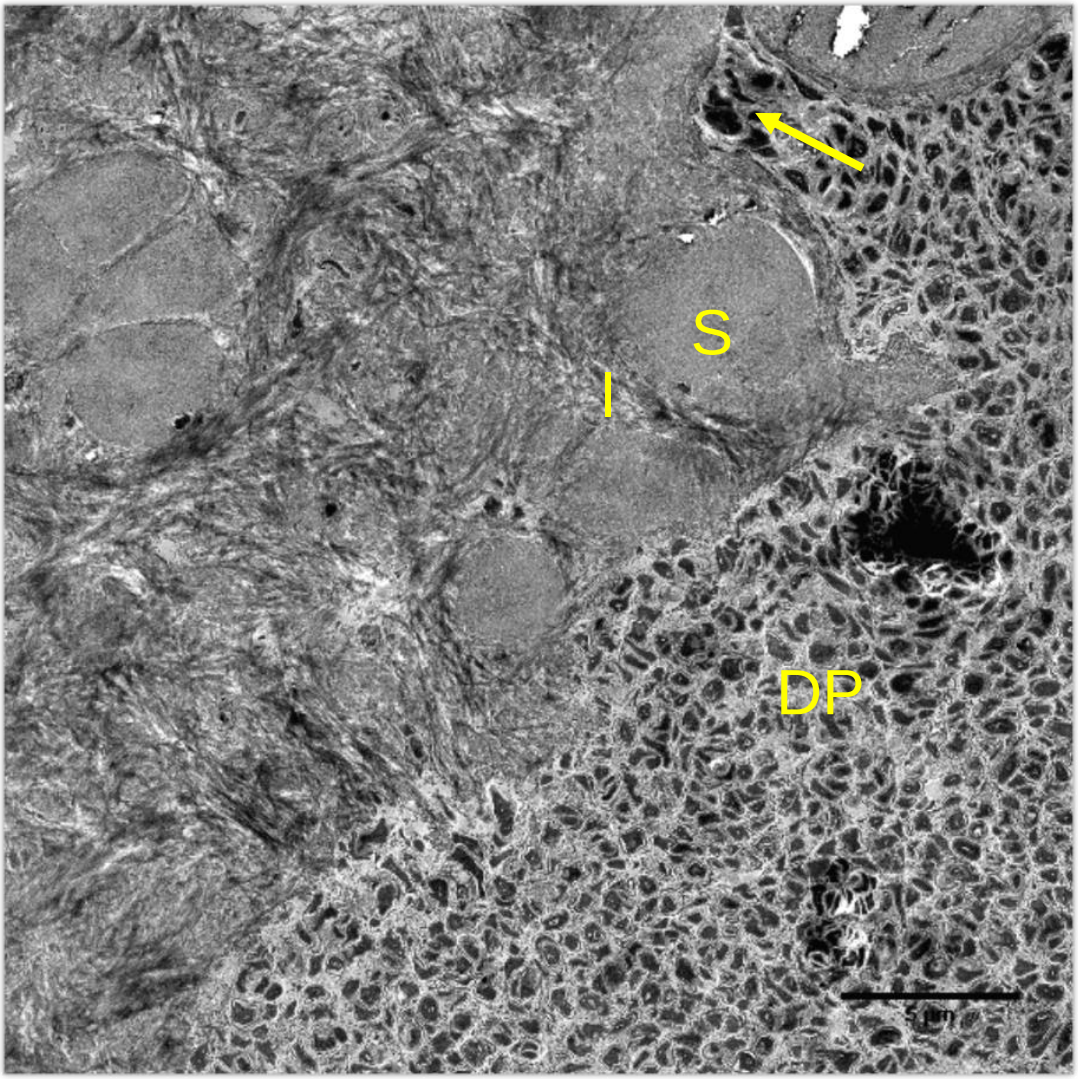


Fig 4.27. TEM image of peripheral cementum with a grade 1.1 peripheral caries lesion at the lingual aspect of a mandibular cheek tooth (406) that is covered by a thick layer of dental plaque (DP). The micro-organisms and DP have penetrated between two Sharpey's fibres (arrow). S = Sharpey's fibres ; I = intrinsic fibres

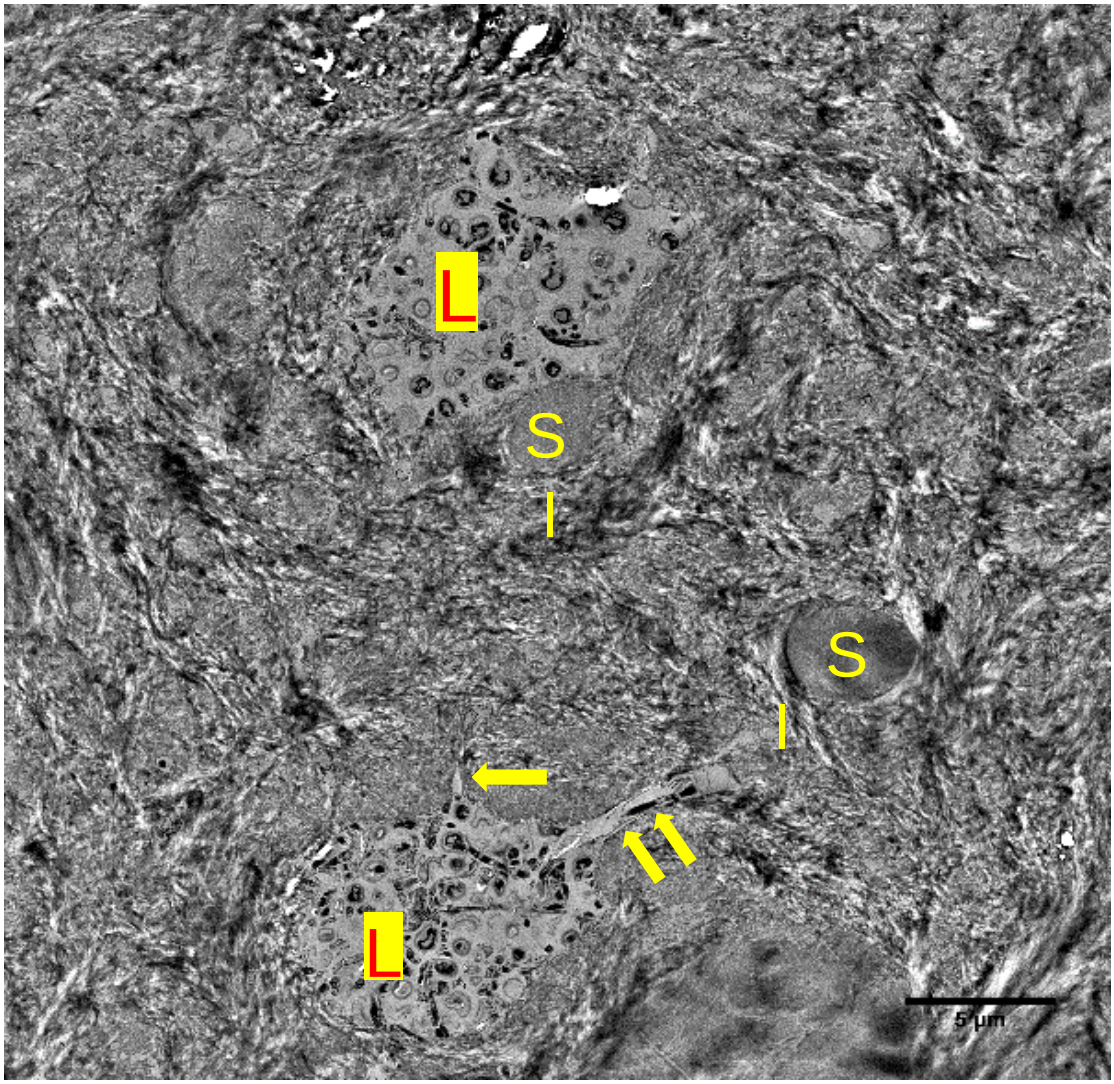


Fig 4.28. TEM image of peripheral cementum with a grade 1.1 peripheral caries lesion at the lingual aspect of a mandibular cheek tooth (406). Dental plaque containing micro-organisms have invaded cemental lacunae (L) and canaliculi (arrows). S = Sharpey's fibres; I = intrinsic fibres.

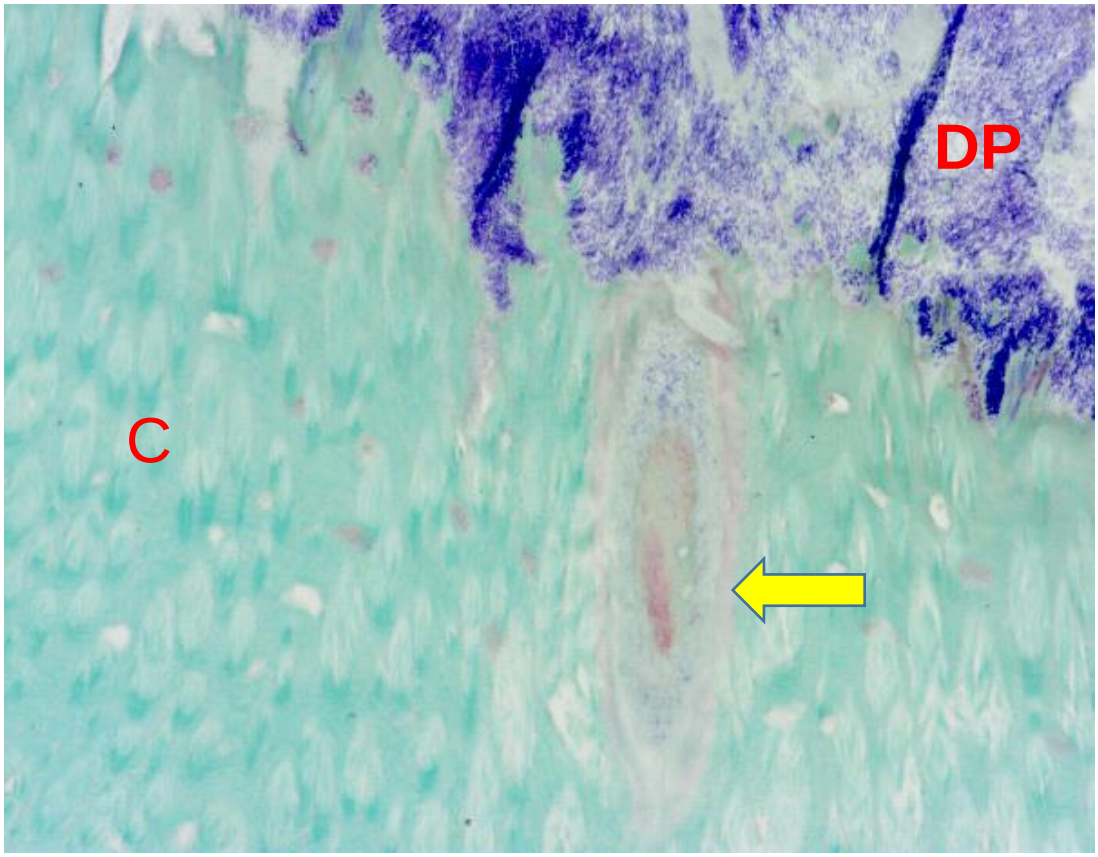


Fig 4.29. Gram stained, decalcified section of a maxillary cheek tooth (210) showing a grade 1.1 peripheral caries lesion. A layer of dental plaque (DP) containing many Gram positive bacteria (blue coloured) covers the peripheral cementum (C). Dental plaque and its associated microorganisms have penetrated the cementum mainly between Sharpey's fibres in a direction perpendicular to the peripheral aspect of tooth. An ellipsoid caries lesion (arrow) contains both Gram positive bacteria (more peripherally) and Gram negative bacteria (more centrally). Gram positive and negative bacteria are also present in some lacunae. [Original magnification X 200]

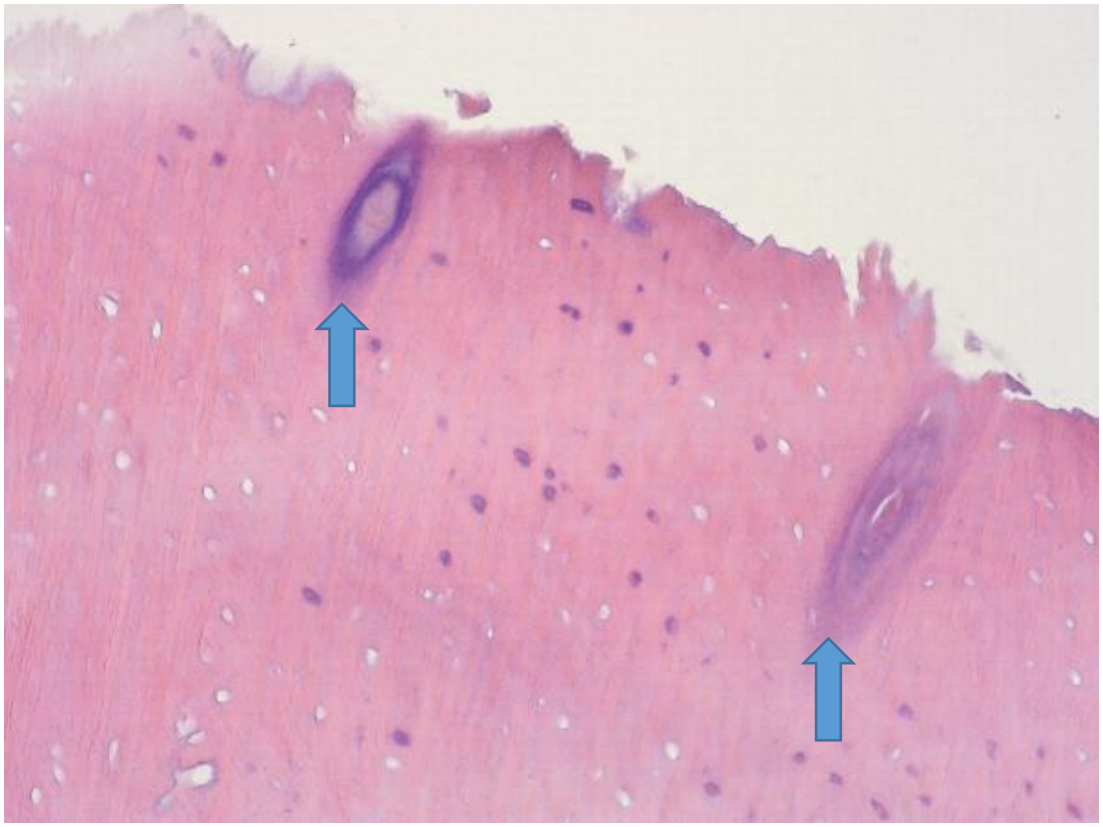


Fig 4.30. Histomicrograph of a decalcified, transverse section of a maxillary cheek tooth (108) at clinical crown with grade 1.1 PC containing two ellipsoid lesions (arrows) oriented perpendicularly to the peripheral aspect of the tooth. Flake-like lesions perpendicular to the peripheral aspect of the tooth are present. Dental plaque is present in some lacunae. [Original magnification X 100, H&E]

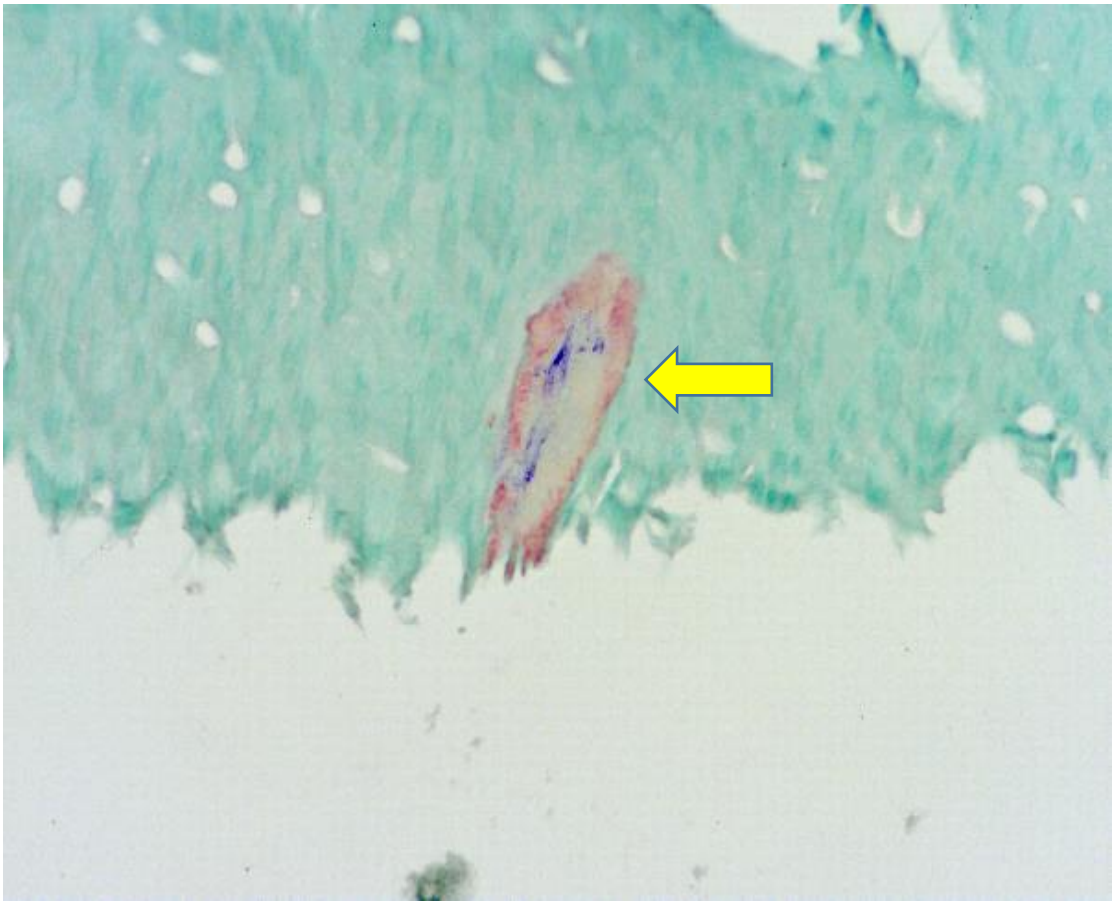


Fig 4.31. Gram stained, decalcified section of a maxillary cheek tooth (210) with a grade 1.1 PC lesion. An ellipsoid type of caries lesion (arrow) is present containing Gram positive and Gram negative bacteria. [Original magnification X 200]

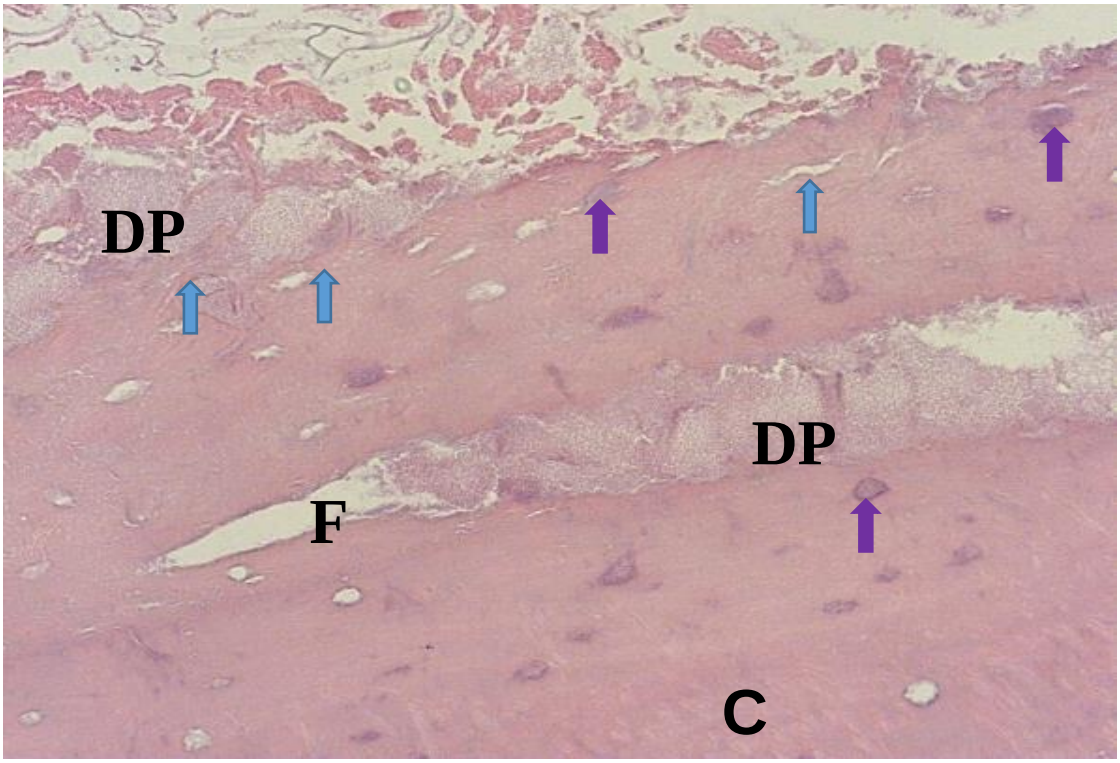


Fig 4.32. Histomicrograph of an undecalcified section of a mandibular cheek tooth (310) with grade 1.1 peripheral caries: flake-type fracture planes have undermined the intrinsic fibres, in a direction parallel to the peripheral aspect of the tooth (blue arrows). Some lacunae are filled with dental plaque (purple arrows). A large fissure fracture (F) containing dental plaque (DP) is also present. C=cementum [Original magnification X 200, H&E]

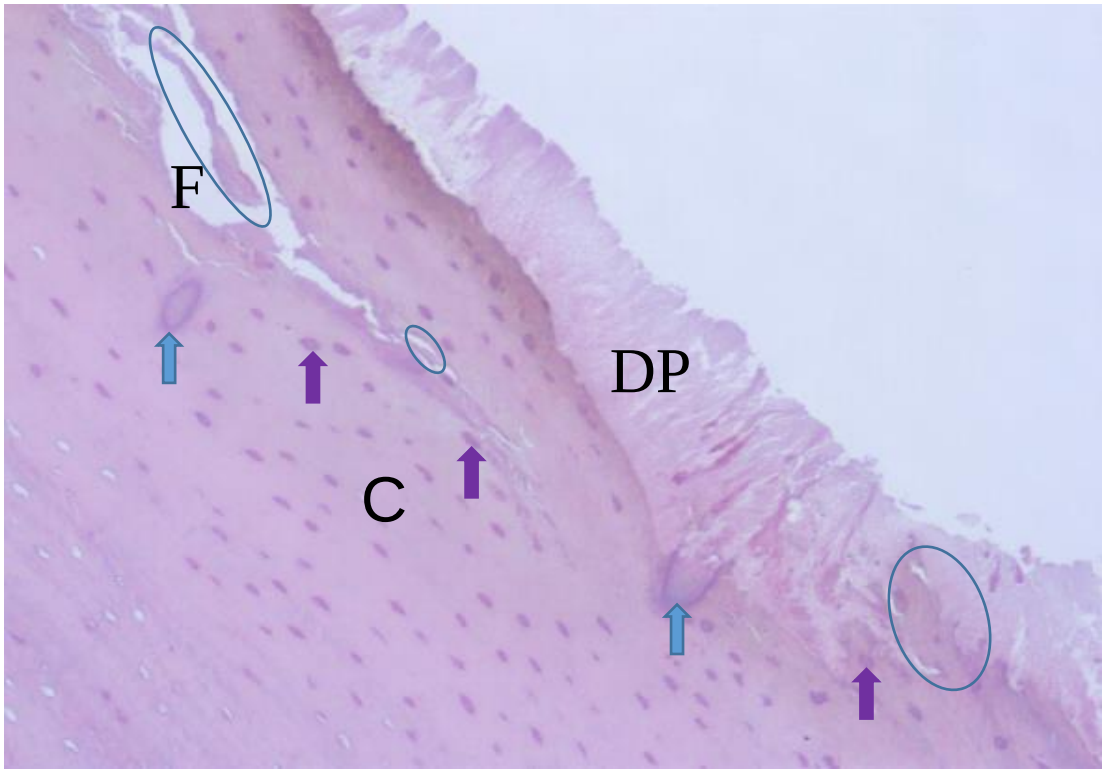


Fig 4.33. Histomicrograph of a decalcified section of a mandibular cheek tooth (310) with grade 1.1 peripheral caries: A thick layer of dental plaque (DP) covers the peripheral aspect of the cementum and is also present within the fissure fracture (F) with undermining of the intrinsic fibres of the cementum (C). Flake-like lesions (ovals) are oriented parallel or tangential to the peripheral aspect of the tooth, along with two ellipsoid-type lesions (blue arrows). Some lacunae are filled with dental plaque (purple arrows). [Original magnification X 40, H&E]

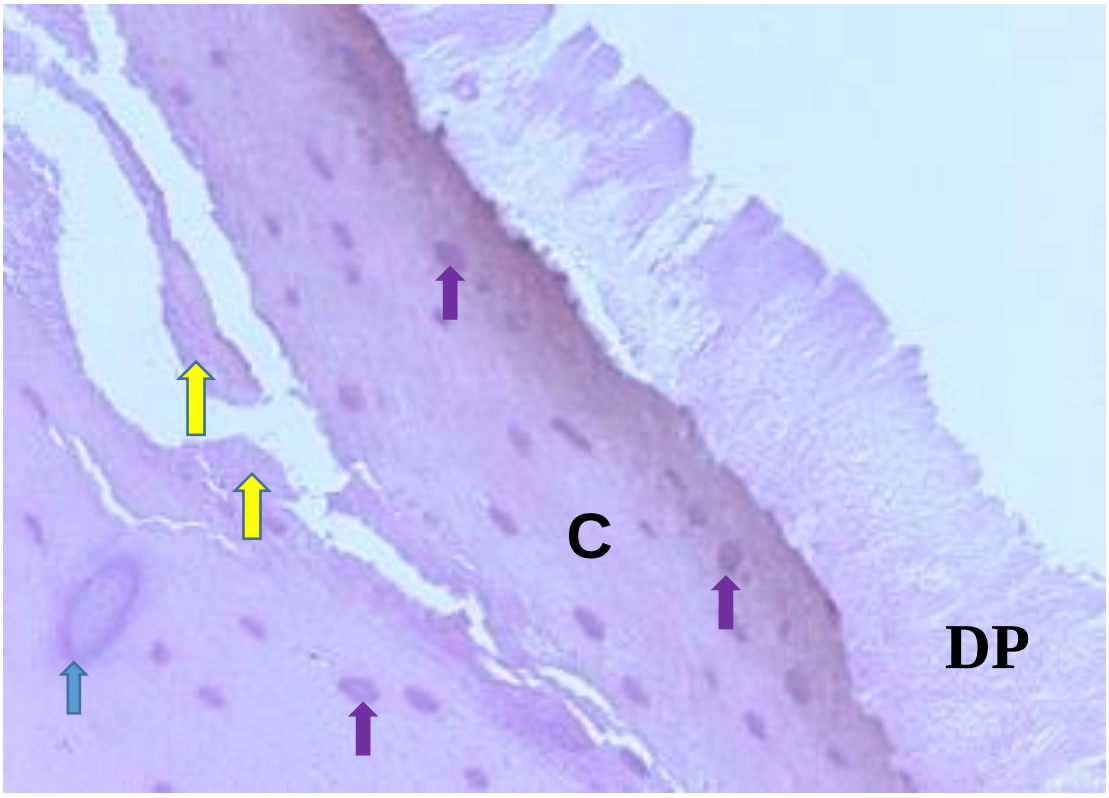


Fig 4.34. Higher magnification of previous image (Fig 4.33), a H&E stained mandibular cheek tooth (310) section with grade 1.1 peripheral caries: A thick layer of dental plaque (DP) covers the peripheral aspect of the cementum and some plaque (yellow arrows) is also present within the fissure fracture (F), undermining the intrinsic fibres of the cementum (C). Flake-like lesions are oriented parallel to the peripheral aspect of the tooth; an ellipsoid-type lesion is also present (blue arrow). Many lacunae are filled with dental plaque and bacteria (purple arrows). [Original magnification X 200]

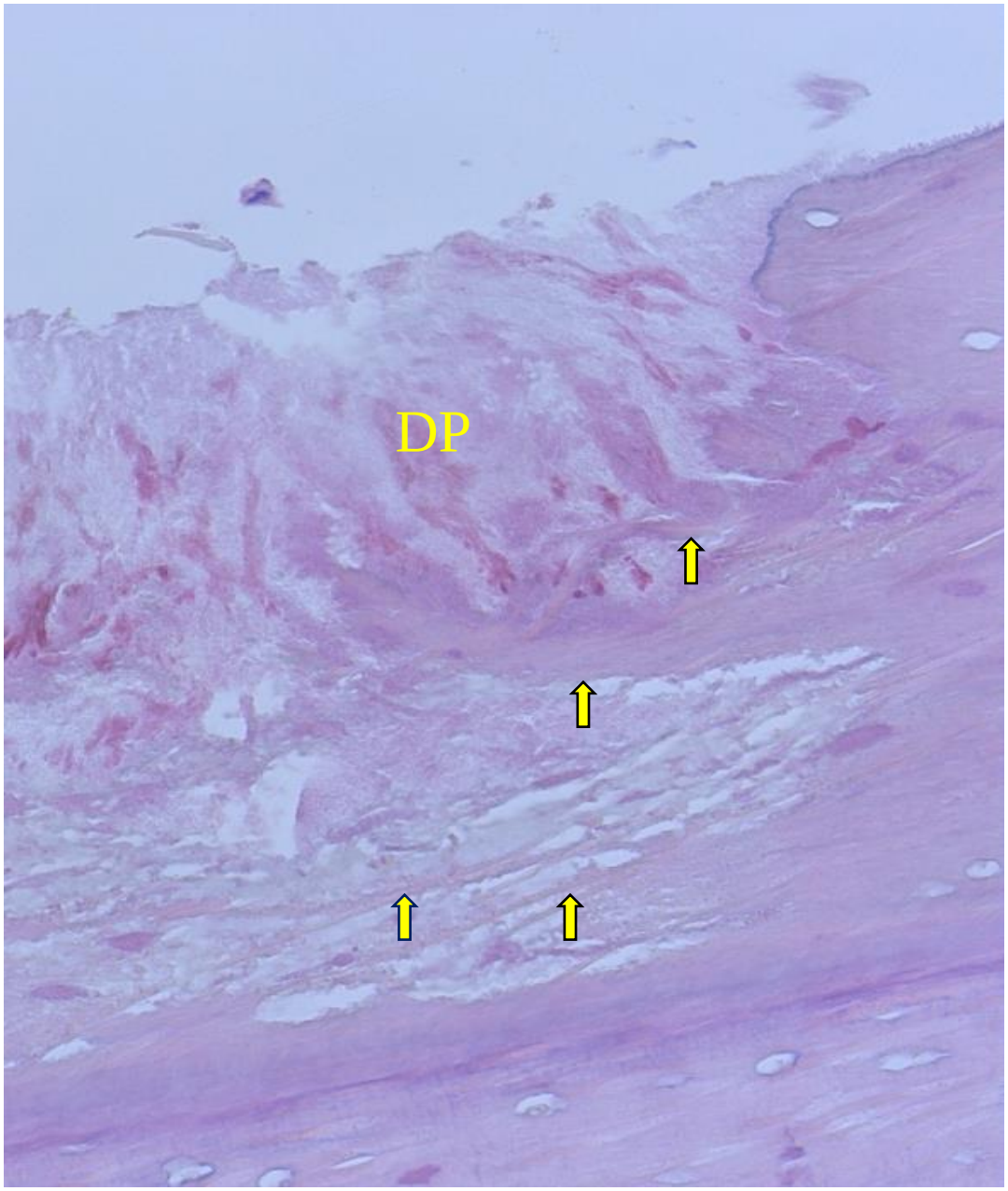


Fig 4.35. Histomicrograph of a decalcified section of a mandibular cheek tooth (310) with grade 1.1 peripheral caries: Extensive flake-like lesions are oriented parallel to the peripheral aspect of the tooth. Disintegration of cementum (arrows) can be observed in these areas, with dental plaque (DP) lying between and on top of the disintegrating cementum that lies in many planes. C=cementum [Original magnification X 200, H&E]

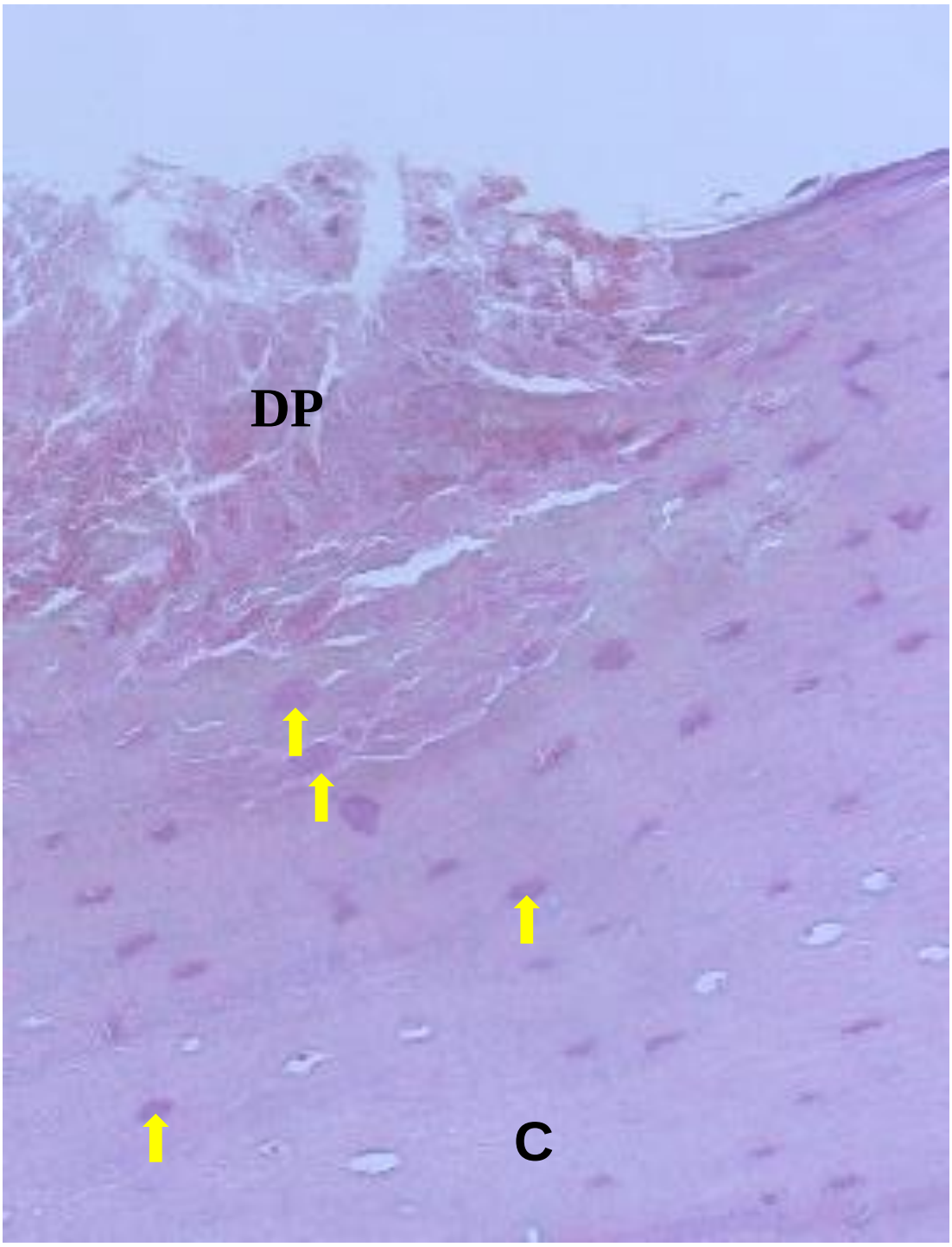


Fig 4.36. Histomicrograph of a decalcified section of a mandibular cheek tooth (310) with grade 1.1 peripheral caries: Deep flake-like lesions have spread in a direction parallel to the peripheral aspect of the tooth, beneath a large accumulation of dental plaque (DP). Most lacunae are filled with dental plaque (arrows). C=cementum. [Original magnification X 200, H&E]

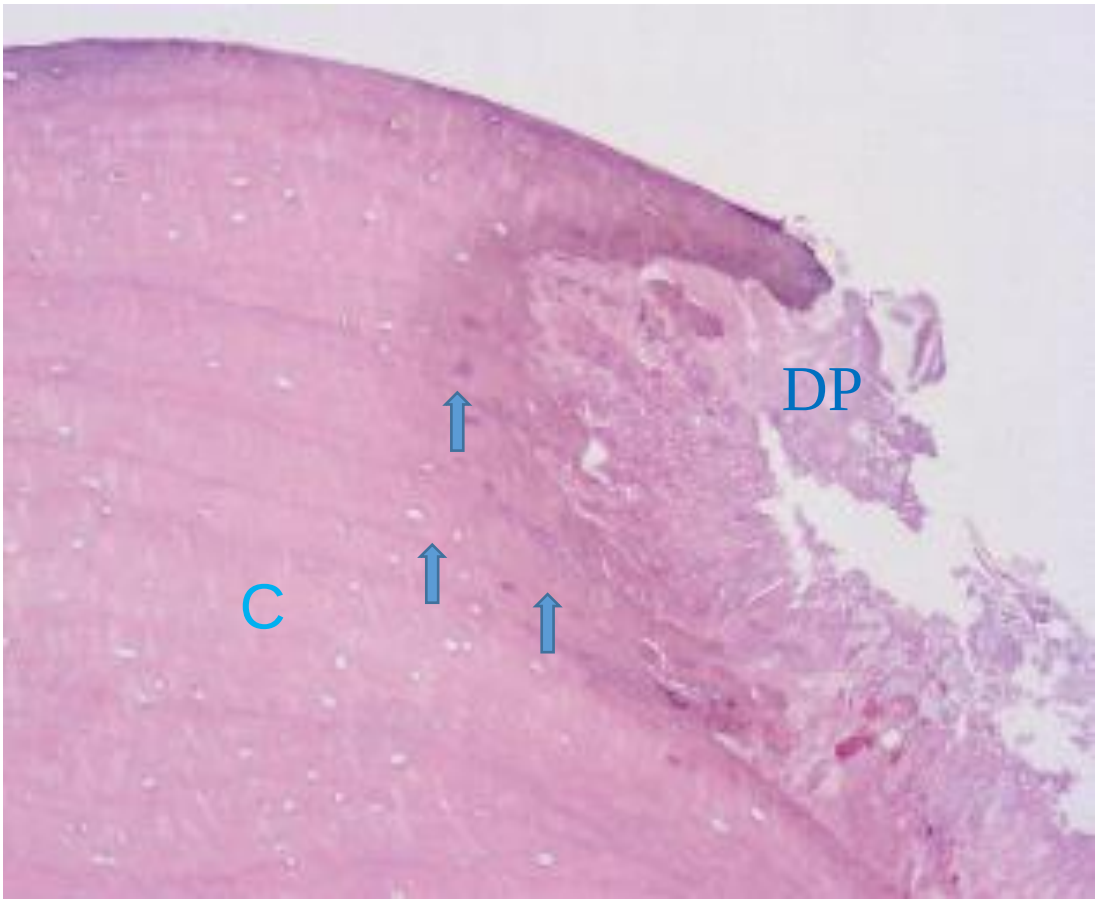


Fig 4.37. Histomicrograph of a decalcified section of a mandibular cheek tooth (310) with grade 1.1 PC covered by a thick layer of dental plaque (DP). Flake-like carious lesions undermine the intrinsic fibres of the cementum (C), in a direction parallel to the peripheral aspect of the tooth, some at the level of lines of arrested growth (arrows). [Original magnification X 100, H&E]

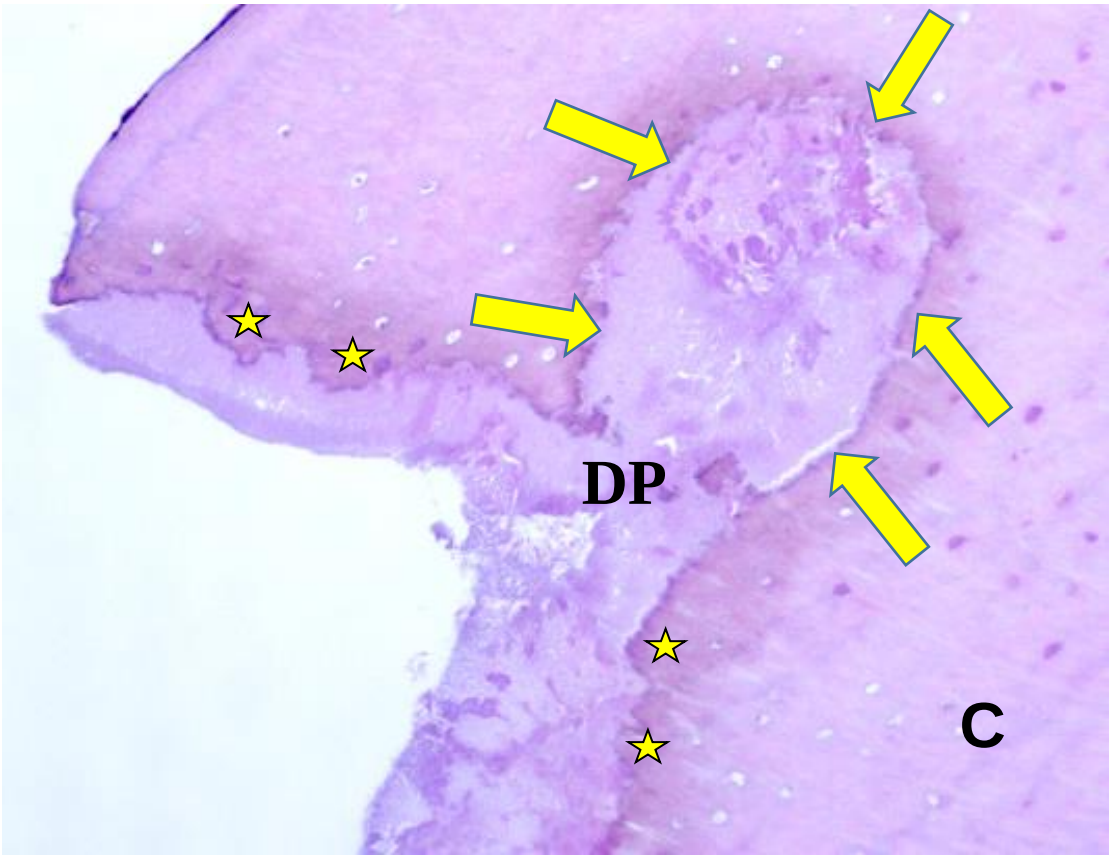


Fig 4.38. Histomicrograph of a decalcified section of a mandibular cheek tooth (409) with grade 1.1 peripheral caries, illustrating different types of cemental caries lesions, including: flake-like lesions (parallel as well as perpendicular to the peripheral aspect of the tooth) (stars), and a large, well defined flask-like lesion (arrows) lying parallel to the peripheral aspect of the tooth. [Original magnification X 100, H&E]

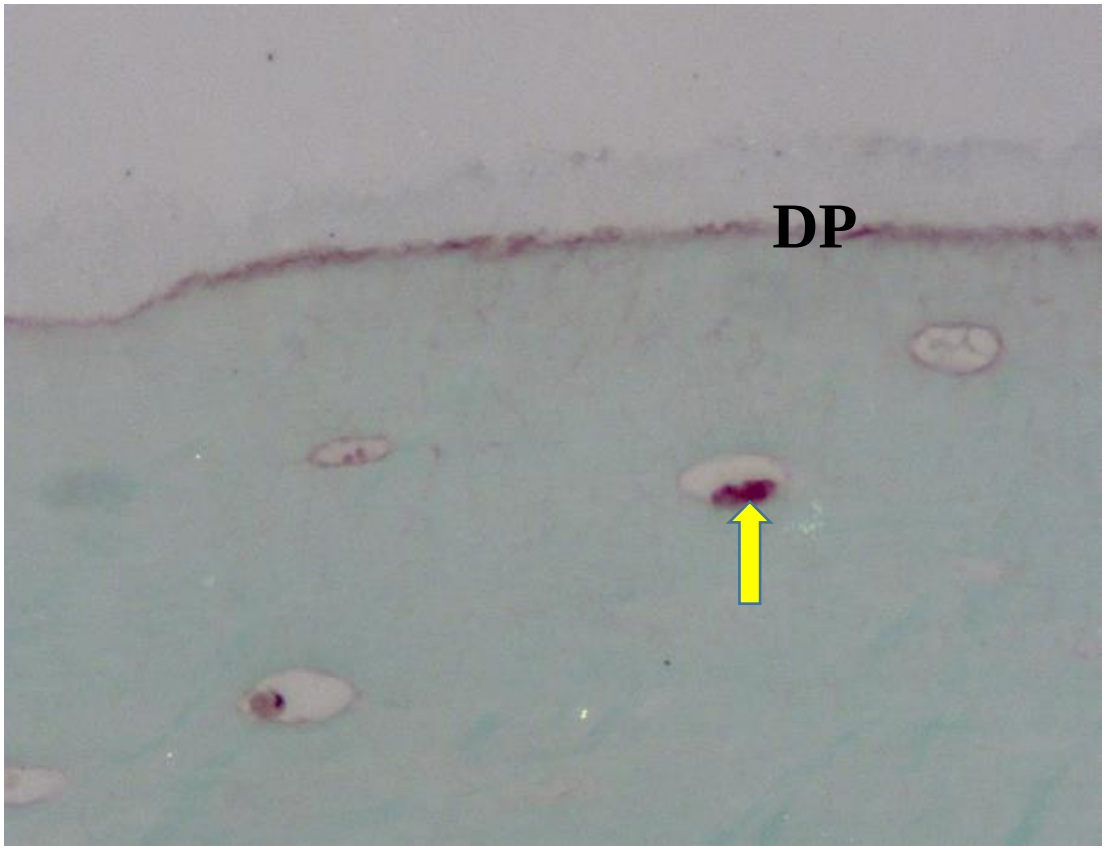


Fig 4.39. Gram stained section of the periphery of a mandibular cheek tooth (310) with an overlying thin layer of dental plaque (DP). Gram negative bacteria that are present in dental plaque have penetrated the cementum via a network of defects or minuscule tubules, possibly including canaliculi, and lie in some lacunae (arrow). [Original magnification X 400]

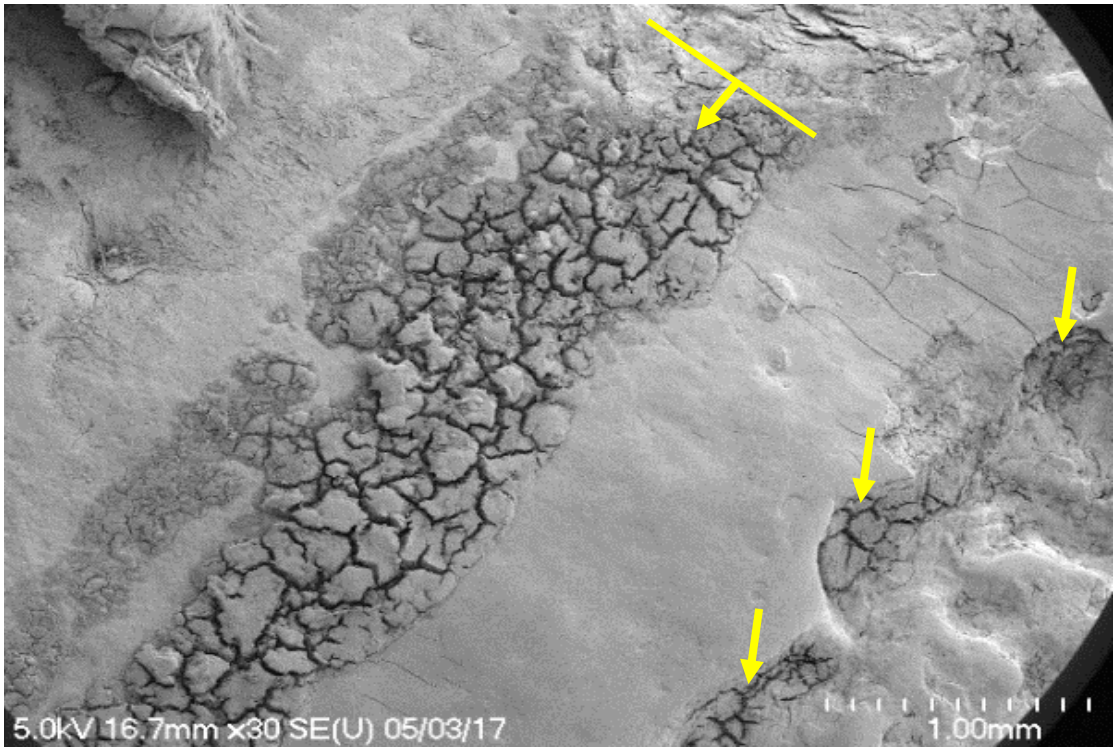


Fig 4.40. SEM image of an undecalcified peripheral section of the palatal aspect of a maxillary cheek tooth (110) showing cementum with grade 1.1 PC lesions (arrows) affecting the surface of areas with lines of arrested growth.

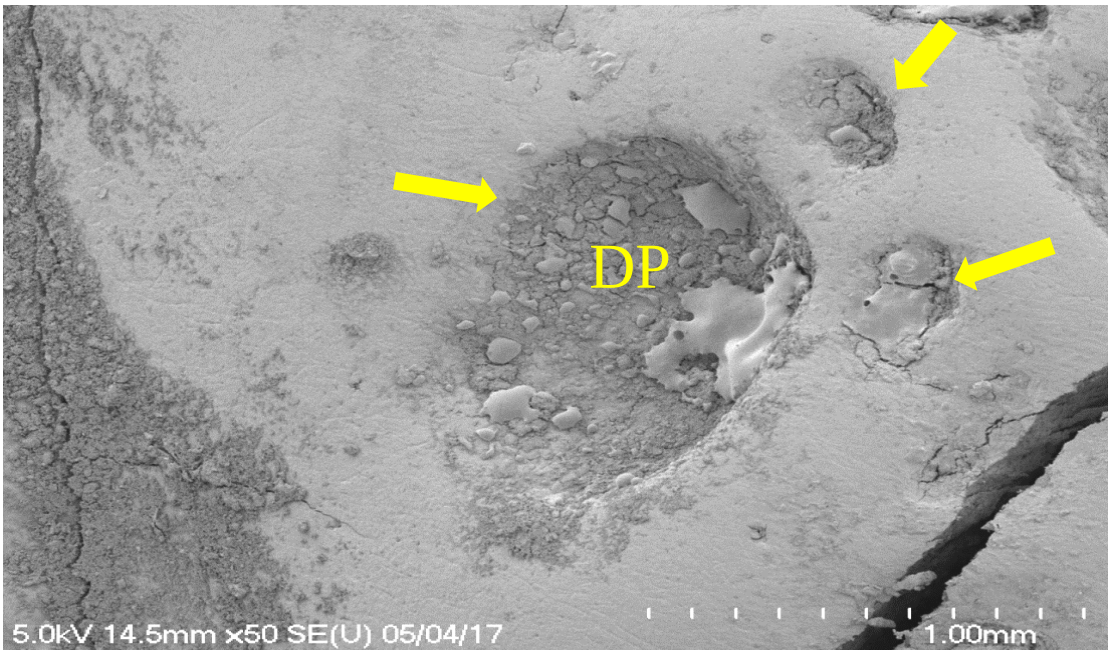


Fig 4.41. SEM image of an undecalcified peripheral section of the buccal aspect of a maxillary cheek tooth (211) with grade 1.1 PC lesions (arrows) covered by dental plaque (DP).

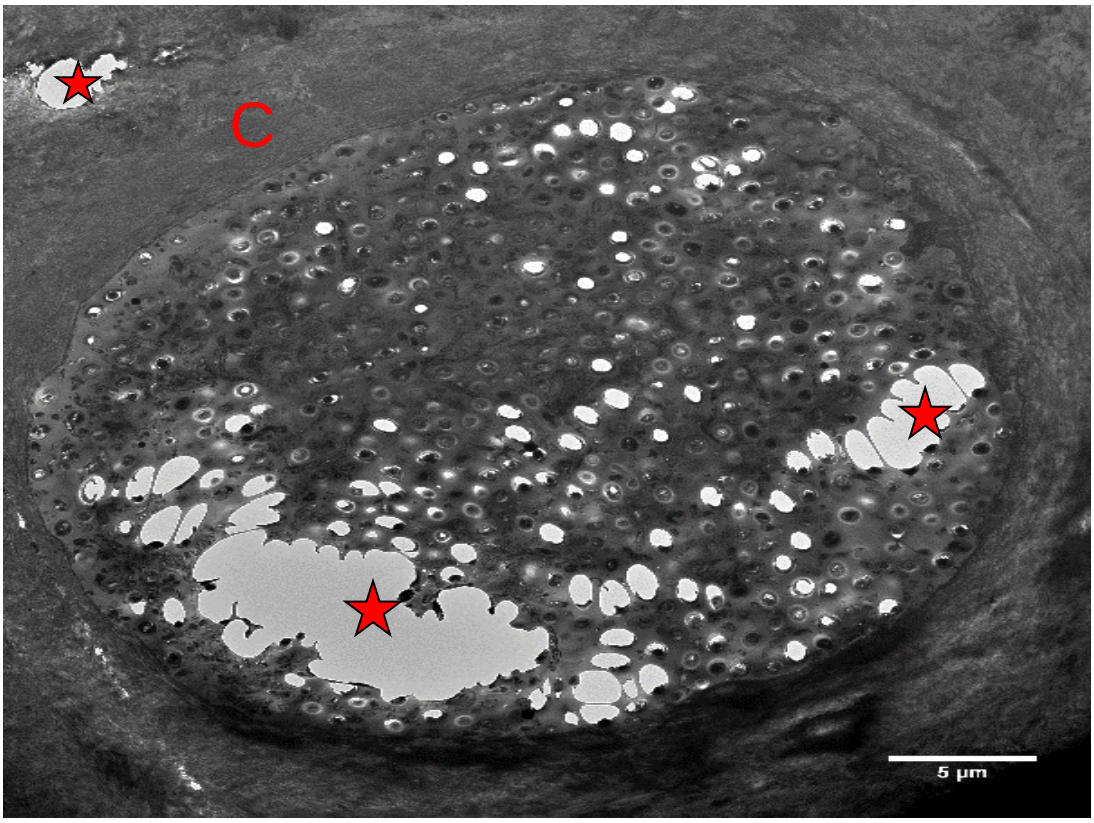


Fig 4.42. TEM of the palatal aspect of a macroscopically normal maxillary cheek tooth (206) which was used as a control. Approximately 40 micron wide, mostly electron dense, discrete circular accumulation of ~1-2 micron wide variably electron dense and electron lucent structures. Peripheral cementum: C; Red stars: artefacts.

4.3.1.3 Histological and Ultrastructural Findings in Equine Cheek Teeth Cementum Affected by Infundibular Caries

Histology showed the destruction of cementum in an IC lesion with (food) debris inside the former central blood vessel and its branches (Figs 4.43, 4.44). Parts of the dentine were also destroyed, possibly by IC (all enamel is removed by decalcification). TEM confirmed the presence of micro-organisms in a cemental IC lesion (Figs 4.45, 4.46). Additionally, SEM confirmed an IC grade 1 lesion with food embedded within the cemental defect (Figs 4.47).

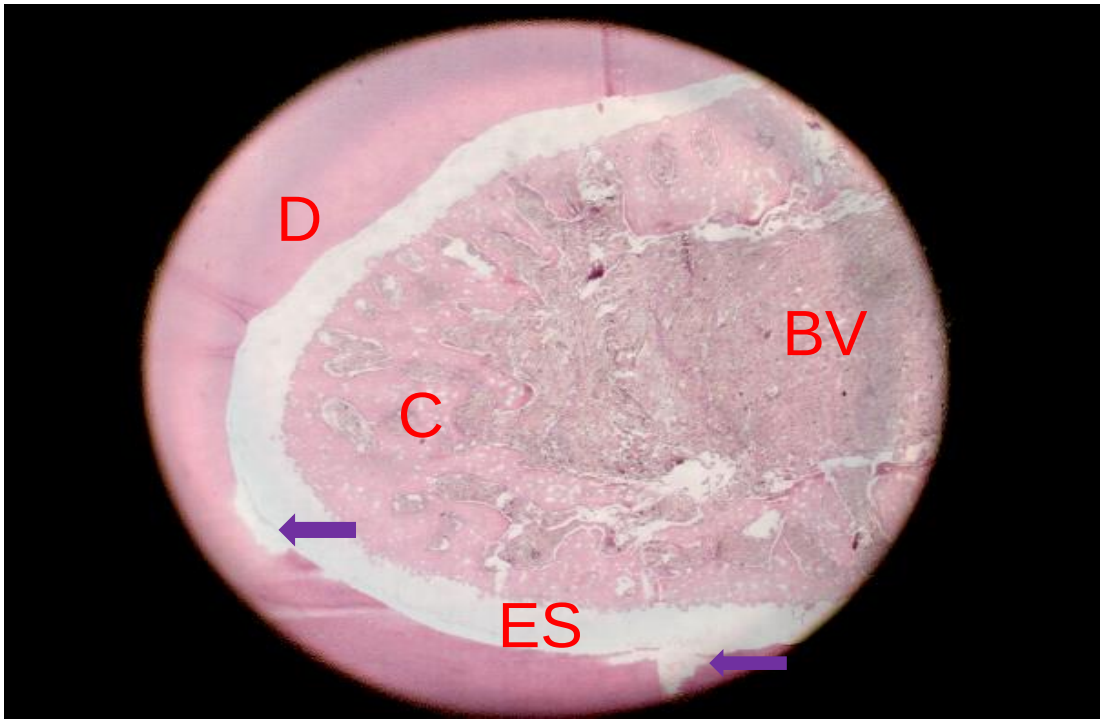


Fig 4.43. Histomicrograph of a decalcified transverse section of maxillary cheek tooth (210) with grade 3 infundibular caries, at the level of the occlusal surface. The central blood vessel and its branches are affected by infundibular caries and are filled with debris. Destruction of the dentine is also appreciable (arrows). D = dentine; ES = enamel space; C = cementum; BV = blood vessel. [Original magnification X 20, H&E]

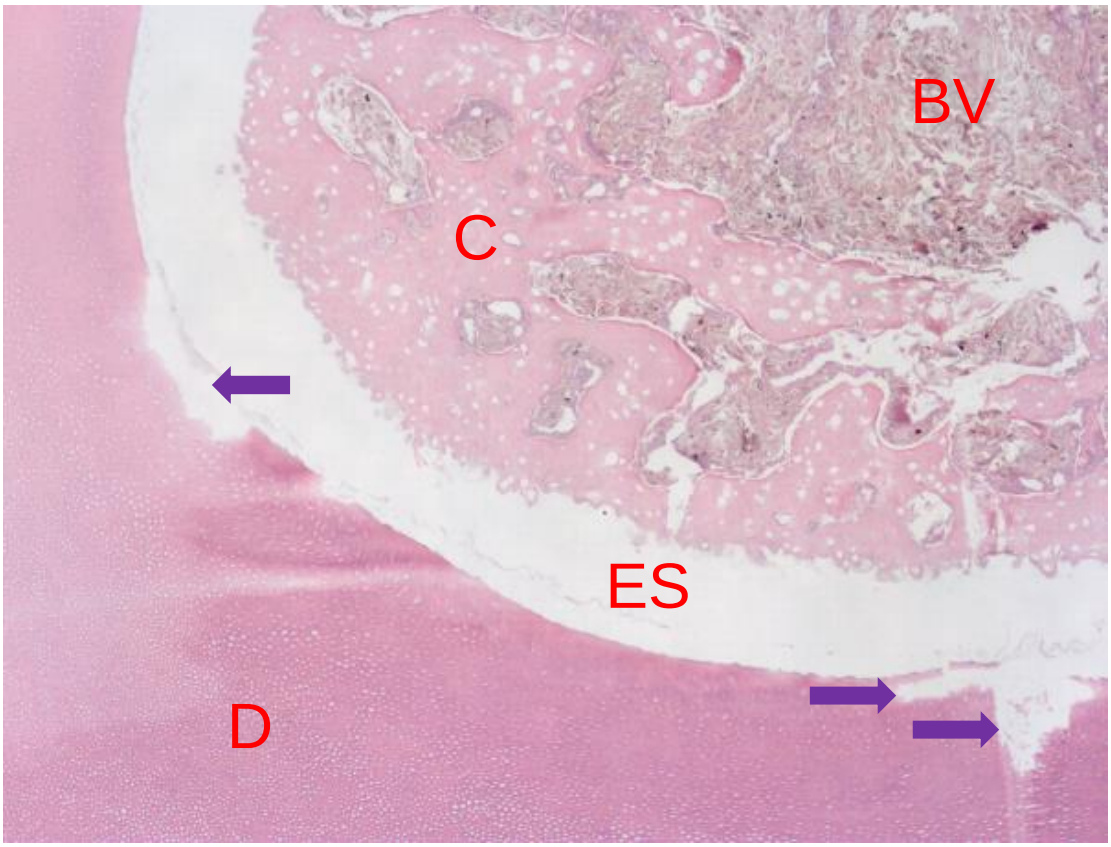


Fig 4.44. Higher magnification of previous image (Fig 4.43), a decalcified transverse section of maxillary cheek tooth (210) with grade 3 infundibular caries at the level of the occlusal surface. The central blood vessel and its branches are affected by IC and are filled with debris. Destruction of the dentine can be observed as well (arrows). D = dentine; ES = enamel space; C = cementum; BV = blood vessel. [Original magnification X 40, H&E]

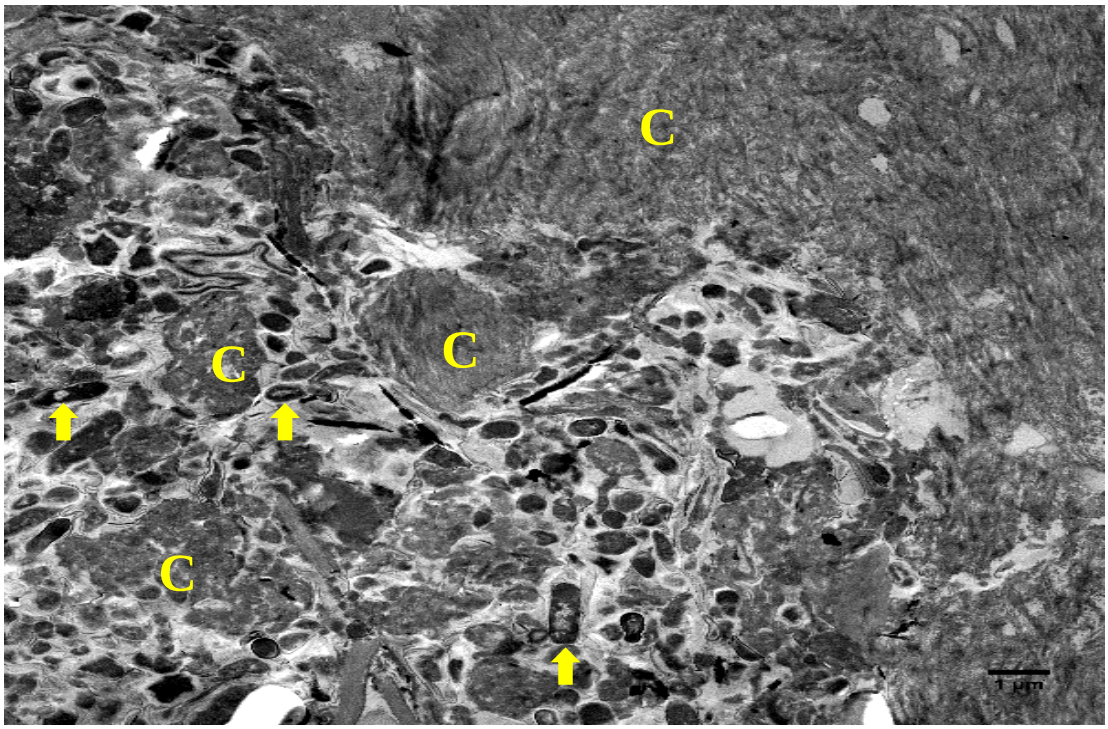


Fig 4.45. TEM of a decalcified transverse section of a maxillary cheek tooth (209) with grade 2 infundibular caries. Micro-organisms (arrows) and degraded cementum (C) are present in an infundibular caries lesion.

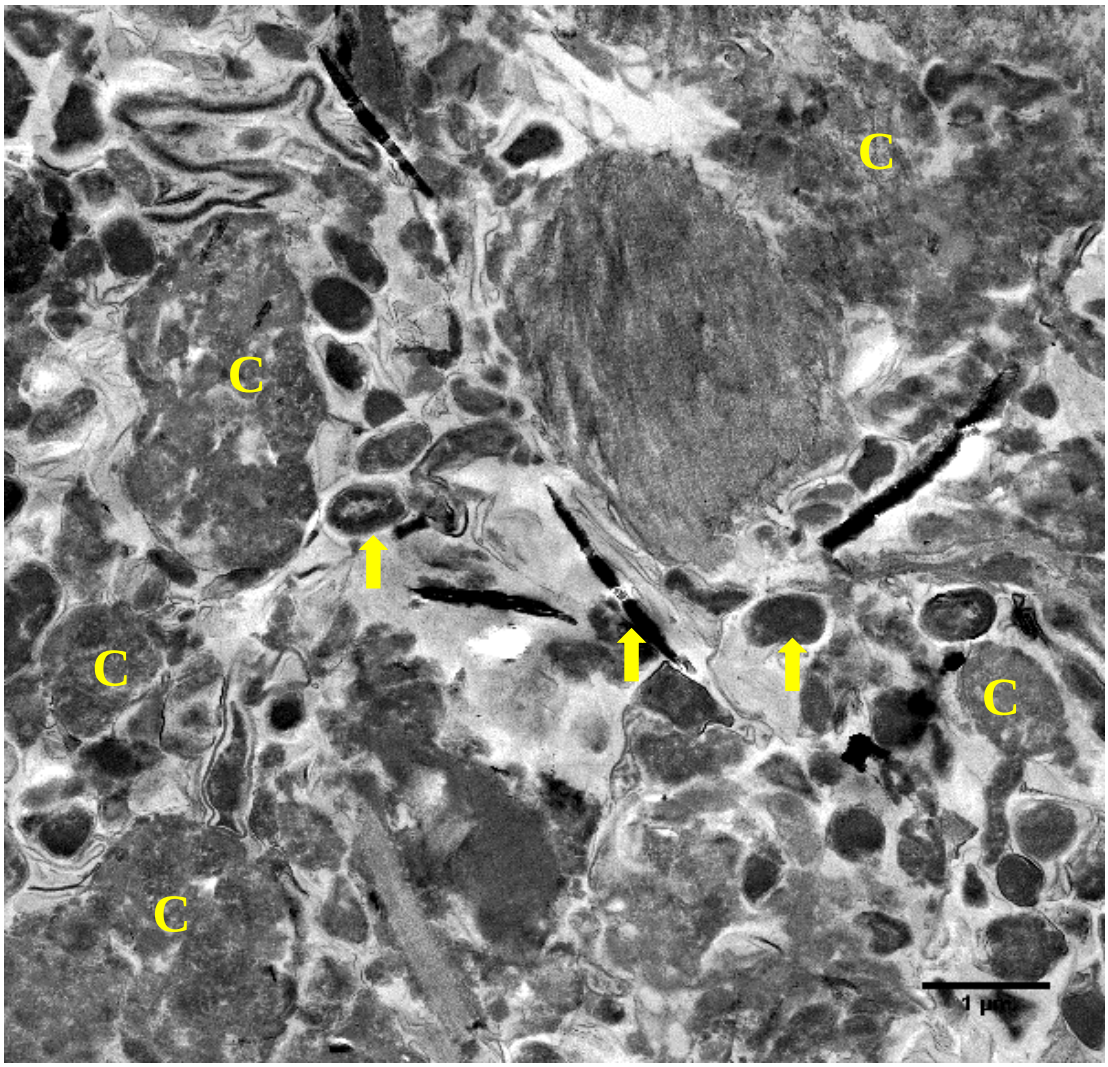


Fig 4.46. Higher magnification of previous TEM image (Fig 4.45) of a decalcified transverse section of a maxillary cheek tooth (209) with grade 2 infundibular caries. Micro-organisms (arrows) and degraded cementum (C) are present in an infundibular caries lesion.

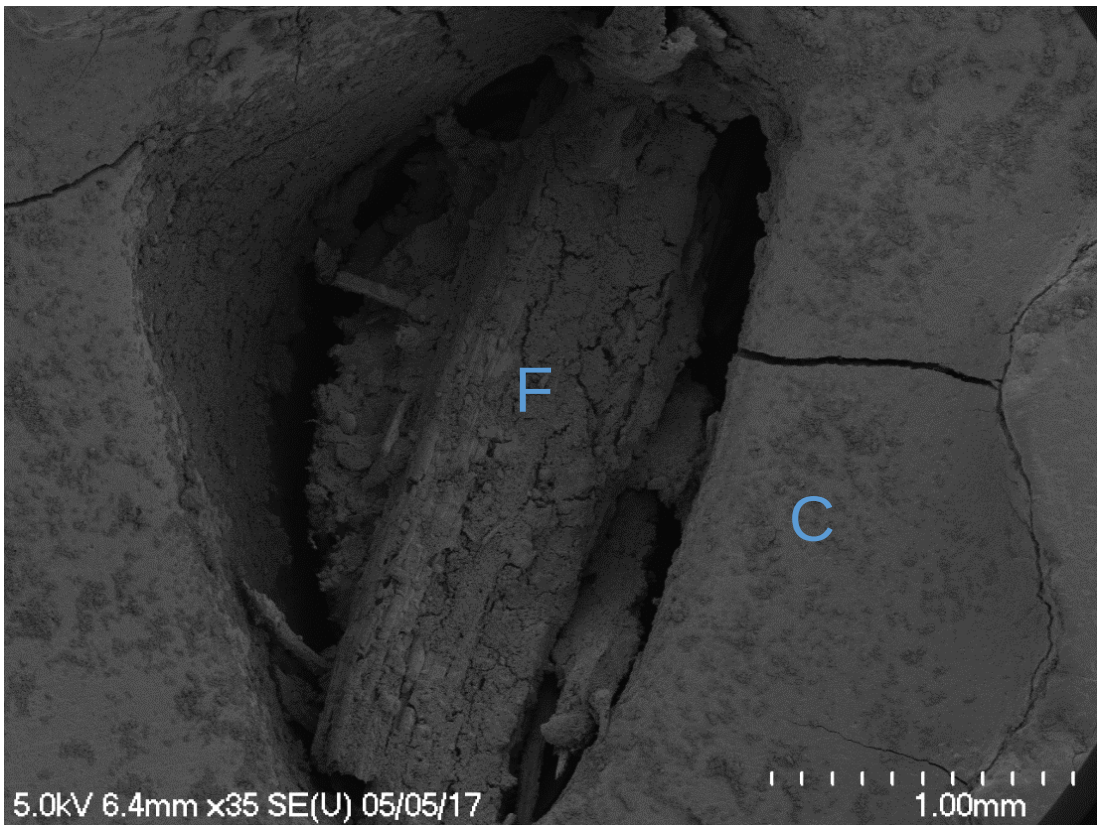


Fig 4.47. SEM image of grade 1 infundibular caries in a maxillary cheek tooth (208), with fibrous food material (F) present in the infundibulum. C = cementum

4.3.2 Enamel

4.3.2.1 General Histological and Ultrastructural Findings in Equine Cheek Teeth Enamel

The cemento-enamel junction was scalloped and the cemental aspect of this region which survived processing (unlike enamel) could be examined in decalcified histological sections (Fig 4.48). In contrast, both cementum and enamel could be observed at the cemento-enamel junction in undecalcified sections of a mandibular and a maxillary cheek tooth (Fig 4.49). Enamel spindles and an enamel lamella were observed in an undecalcified transverse section of equine cheek tooth (Figs 4.50 and 4.51). SEM of enamel at the occlusal surface showed the protruding enamel ridges containing some defects and wear lines. Additionally, some artefactual changes were observed in the enamel and also in the cementum and dentine (Figs 4.52 and 4.53). SEM of peripheral enamel in a control tooth showed a smooth appearance on this normal enamel (Fig 4.54), although in the cementum a small grade 1.1 PC lesion was present. An enamel defect was found in a cheek tooth examined using SEM, which did not appear to be due to PC macroscopically (Fig 4.55). In this defect, fissures were present which did not follow a straight line but instead formed a pattern which may have followed the outlines of several enamel rods (Fig 4.56).

4.3.2.2 Ultrastructural Findings in Equine Cheek Teeth Enamel Affected by Peripheral Caries

Macroscopically, vertical ridges (in an apico-occlusal direction) were identified in the peripheral enamel of cheek teeth with grade 1.2 or 2 PC. On SEM examination, some peripheral aspects of enamel ridges and areas of non-protruding enamel of the exposed peripheral enamel were smooth while, in other areas, small irregularities were present in the enamel (Fig 4.57Figs 4.57, 4.58 and 4.59).

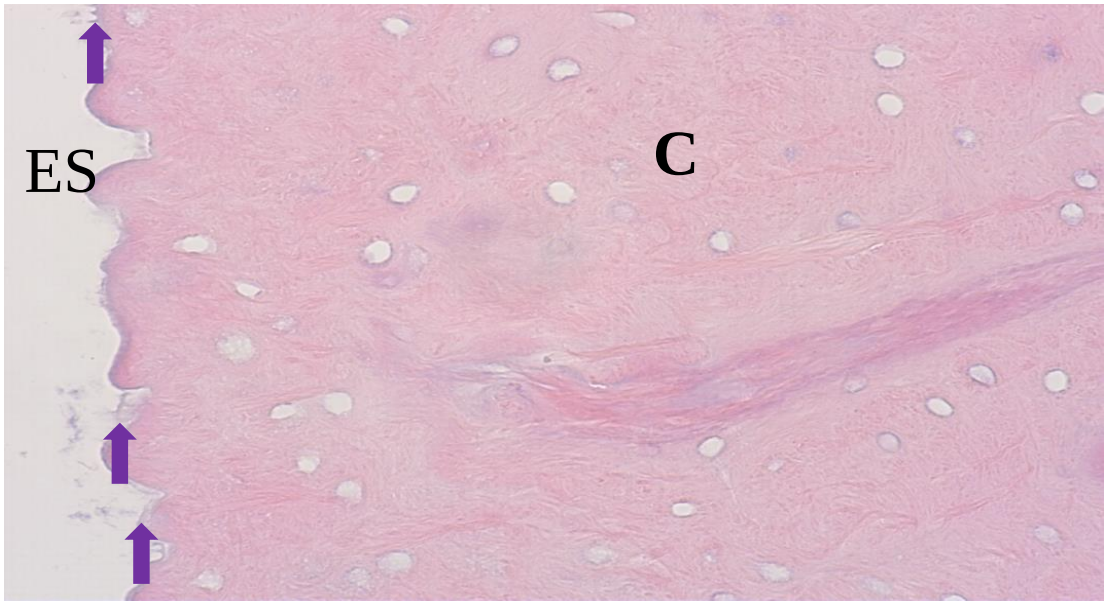


Fig 4.48. Transverse decalcified section of a control mandibular cheek tooth (309) showing the cemento-enamel junction with a slightly slight basophilic layer (arrows) which is the remnant of decalcified enamel. C = cementum; ES = Enamel space. [Original magnification X200, H&E]

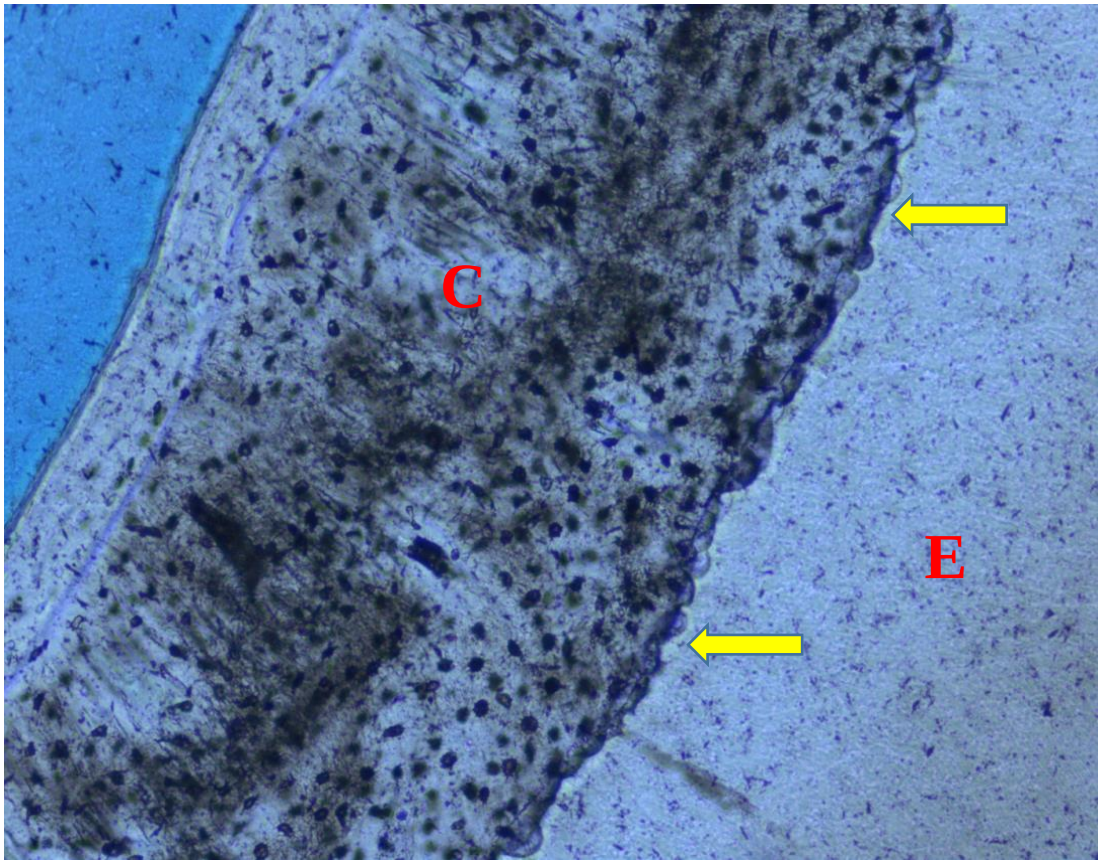


Fig 4.49. Transverse section of an undecalcified maxillary cheek tooth showing the scalloped cemento-enamel junction (arrows). C=cementum; E=enamel [Original magnification X 100]

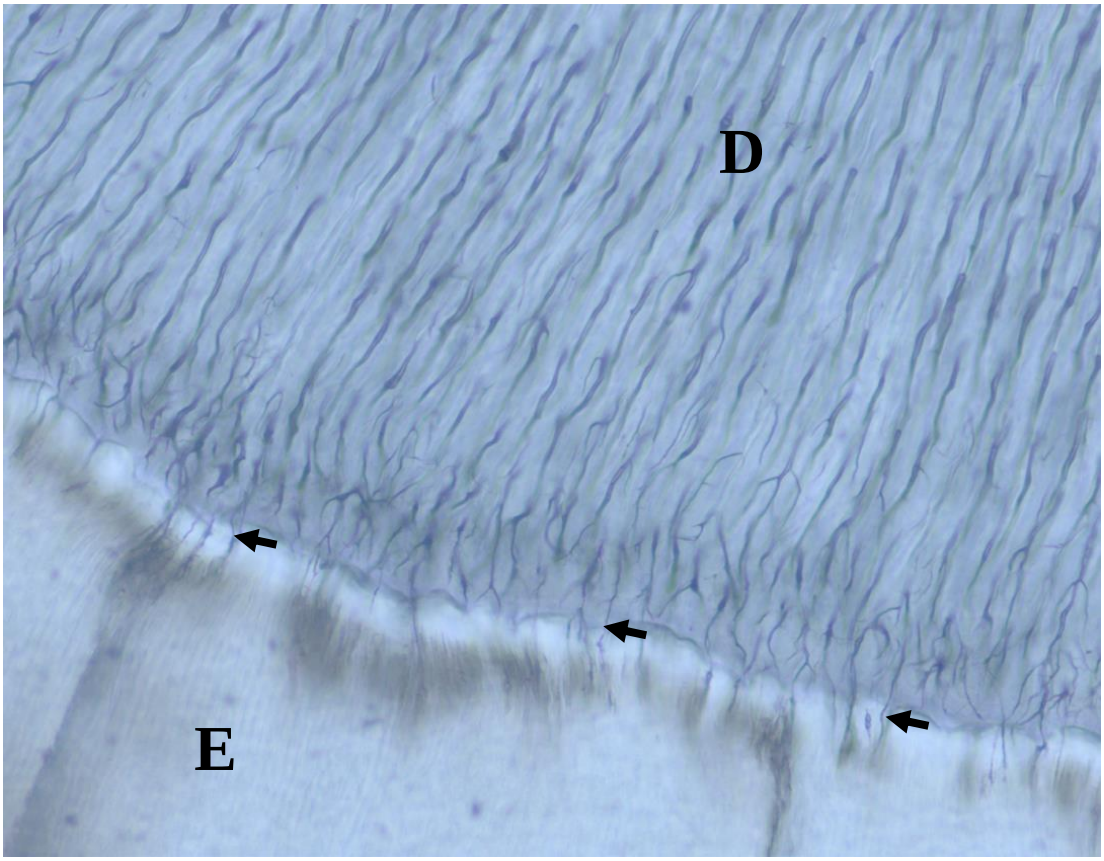


Fig 4.50. Undecalcified section of an equine mandibular cheek tooth showing enamel spindles (arrows) extending into the enamel (E) at the dentino-enamel junction. D=dentine [Original magnification X 200]

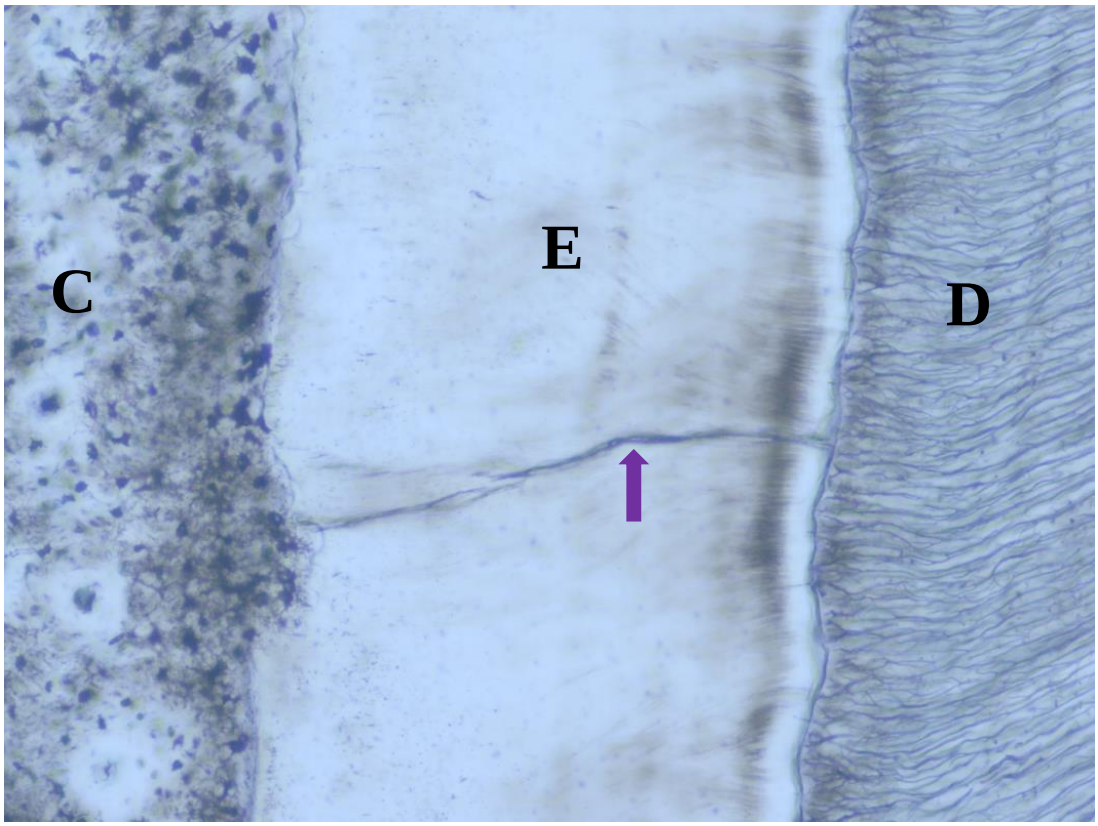


Fig 4.51. Undecalcified section of an equine mandibular cheek tooth showing a fissure-like structure (arrow) filled with organic material extending from the cemento-enamel junction to the dentino-enamel junction. C=cementum; E=enamel; D = dentine [Original magnification X 100]

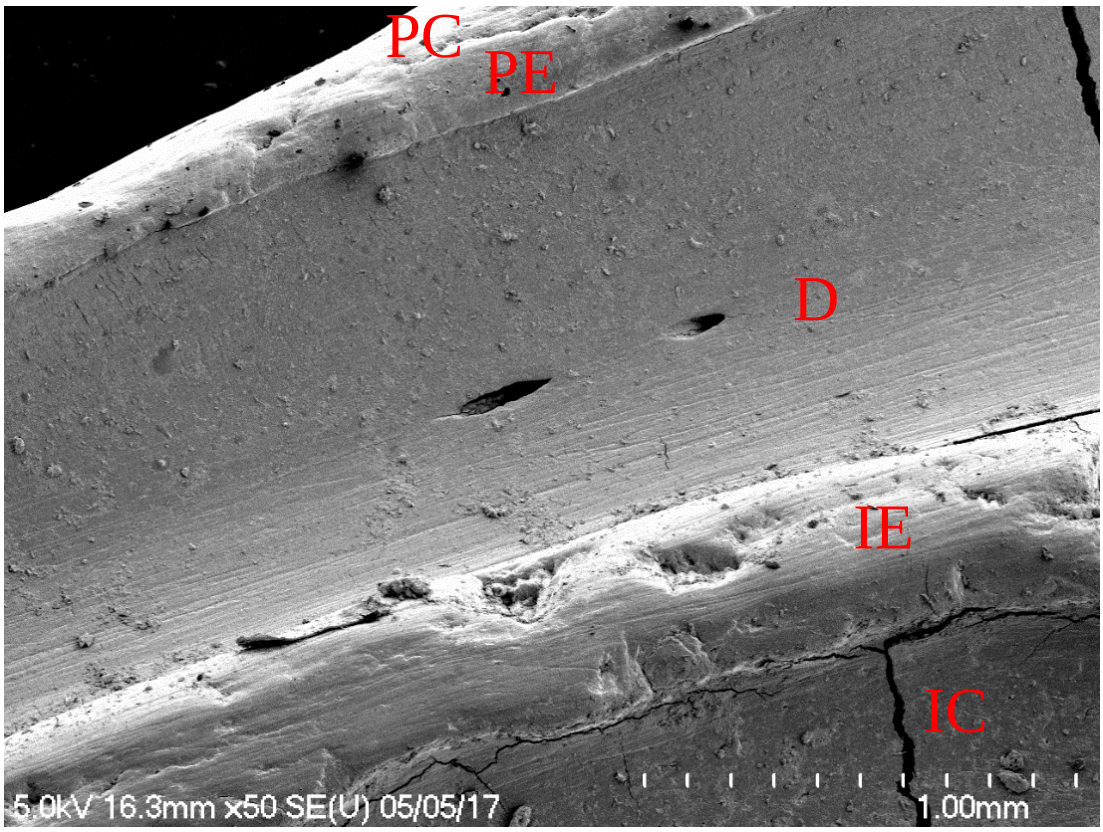


Fig 4.52. SEM of the occlusal surface of a maxillary cheek tooth (208). Enamel is the dental tissue which protrudes most from the occlusal surface. Peripheral cementum (PC) surrounds the peripheral enamel (PE) at the periphery while, in the infundibulae, infundibular enamel (IE) surrounds the infundibular cementum (IC). Dentine (D) is present between peripheral enamel and infundibular enamel. The long striations on the occlusal surface are caused by abrasion of ingested phytoliths during mastication.

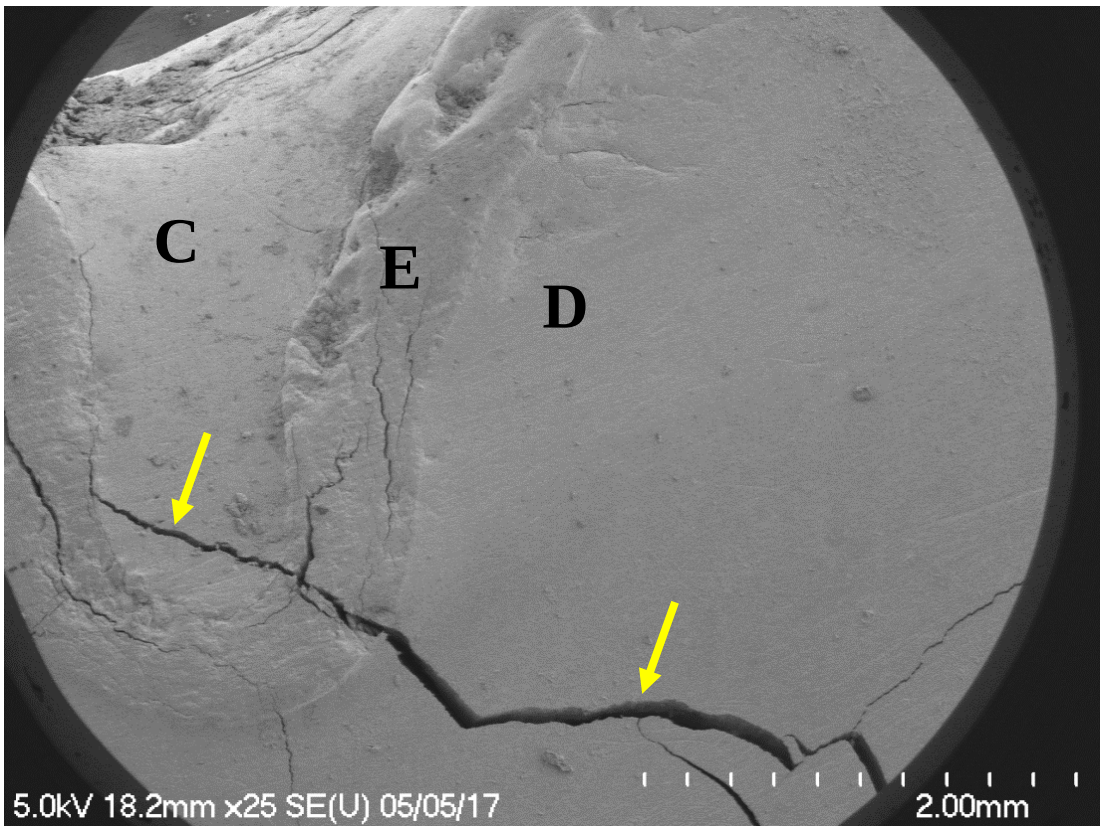


Fig 4.53. SEM of the occlusal surface of a maxillary cheek tooth (208) with cemental infolding. Small defects are present in the cementum (grade 1.1 PC) and enamel (no PC) which were not apparent macroscopically. Yellow arrows = fixation artefact; C=cementum; E=enamel; D = dentine

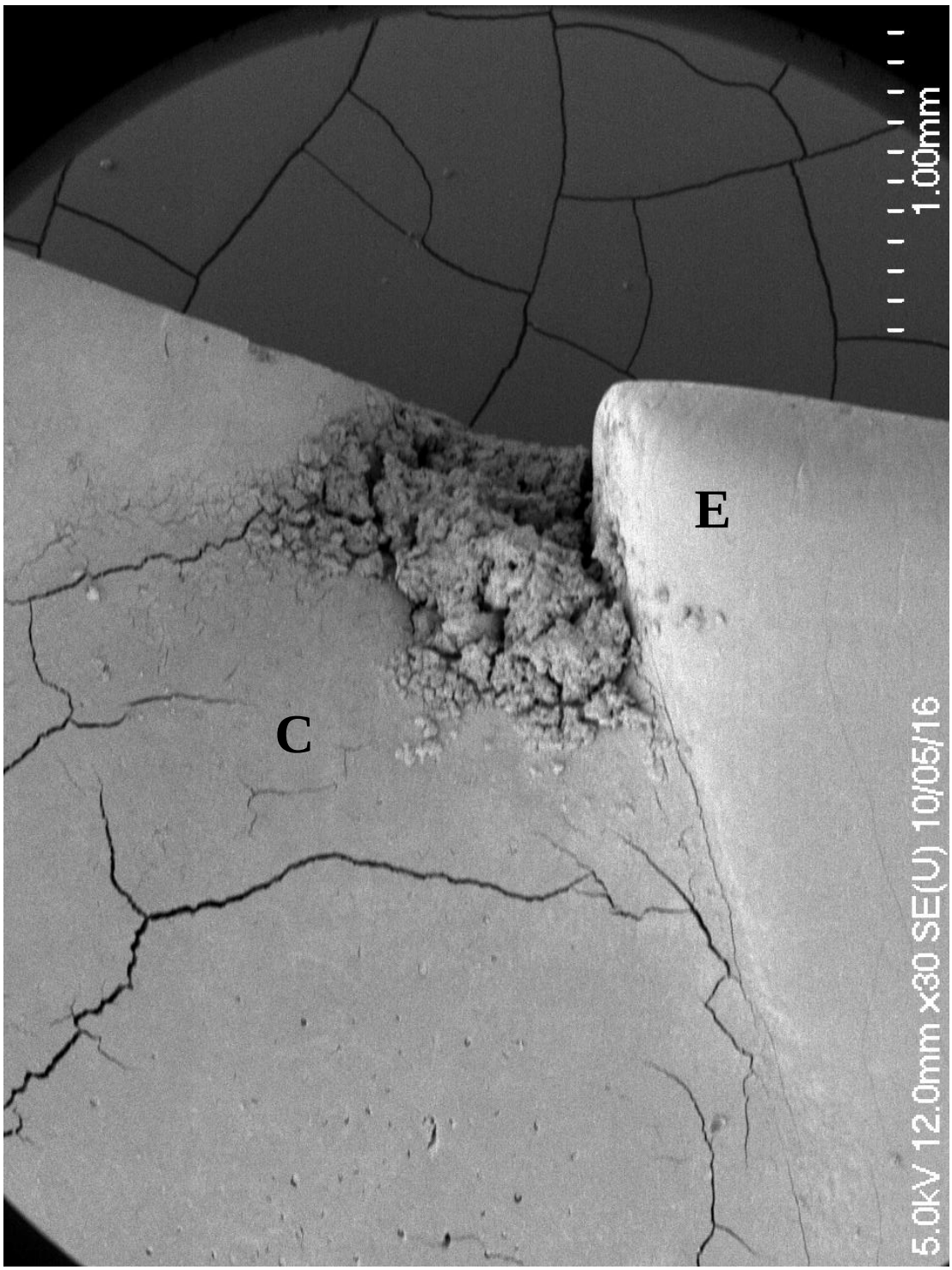


Fig 4.54. SEM of the peripheral aspect of a control mandibular tooth (306) showing an enamel ridge protruding above the cementum (C). Note that this peripheral enamel (E) has a smooth surface. A small cemental defect is present in the cementum (grade 1.1 PC), which had a white appearance macroscopically.

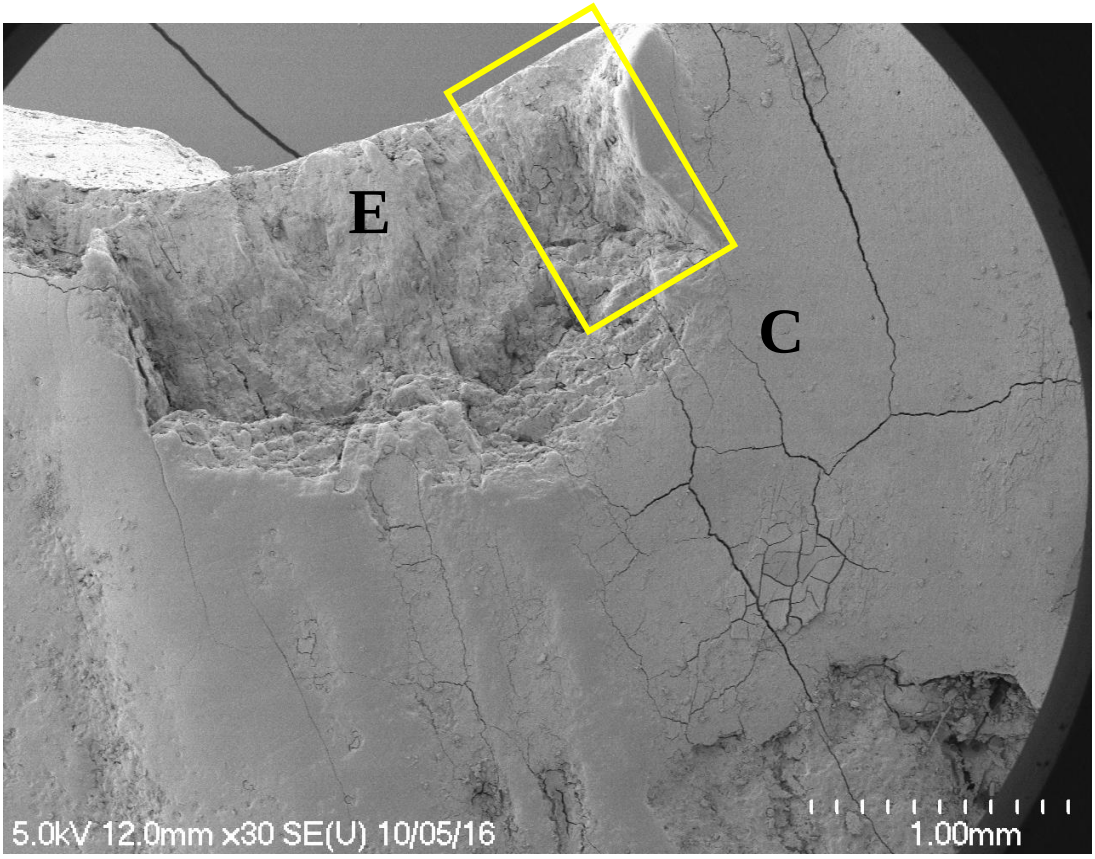


Fig 4.55. SEM image of an undecalcified peripheral section of the palatal aspect of a maxillary cheek tooth (209) showing an enamel defect (yellow box) which may not have been caused by PC, no dark gross discolouration was present. C=cementum; E=enamel.

Fig 4.56. Higher magnification of selected area (yellow square) of SEM image (Fig 4.55) of an undecalcified peripheral section of the palatal aspect of a maxillary cheek tooth (209) showing an enamel defect which does not appear to have been caused by PC because, grossly, no dark discolouration was present. Small cracks are present in the enamel which may follow the outlines of several rods that are near-horizontally oriented to the tooth periphery.

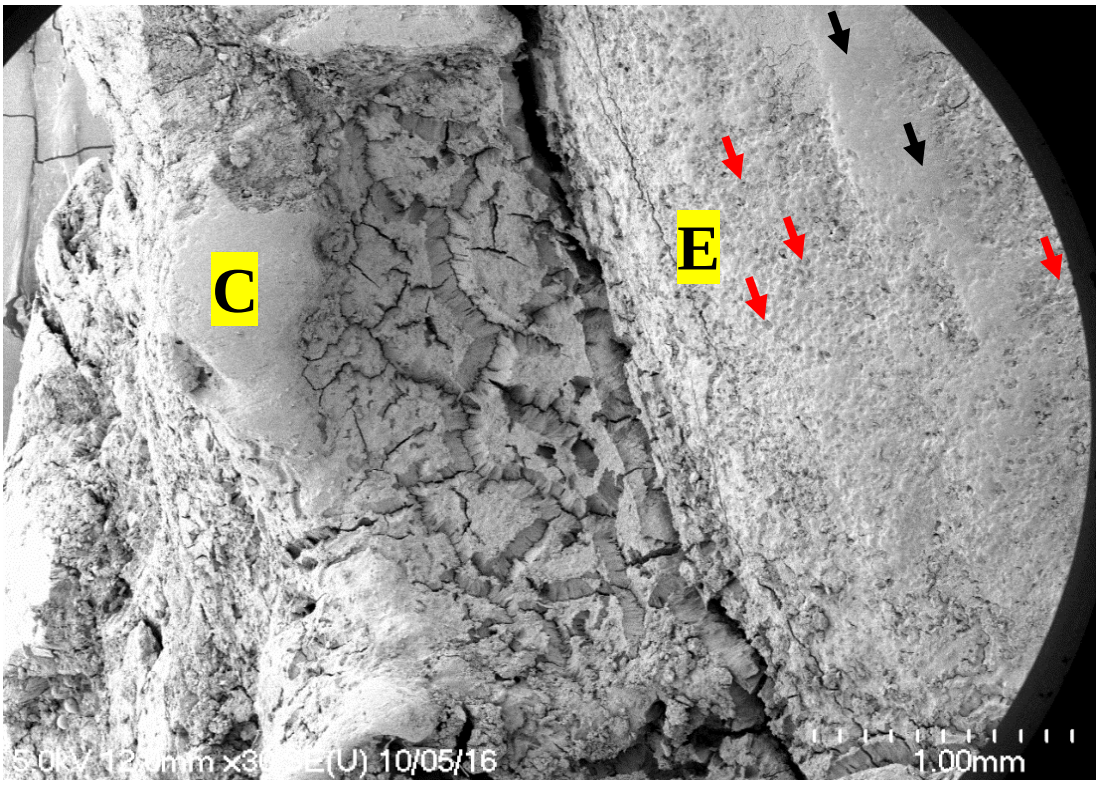


Fig 4.57. SEM image of an undecalcified peripheral section of the palatal aspect of a maxillary cheek tooth (209) with grade 1.2 PC, showing destruction of cementum (left side of image) and exposed enamel (right side of image). The enamel surface has some smooth areas (black arrows) and pitted areas (red arrows). C=cementum; E=enamel.

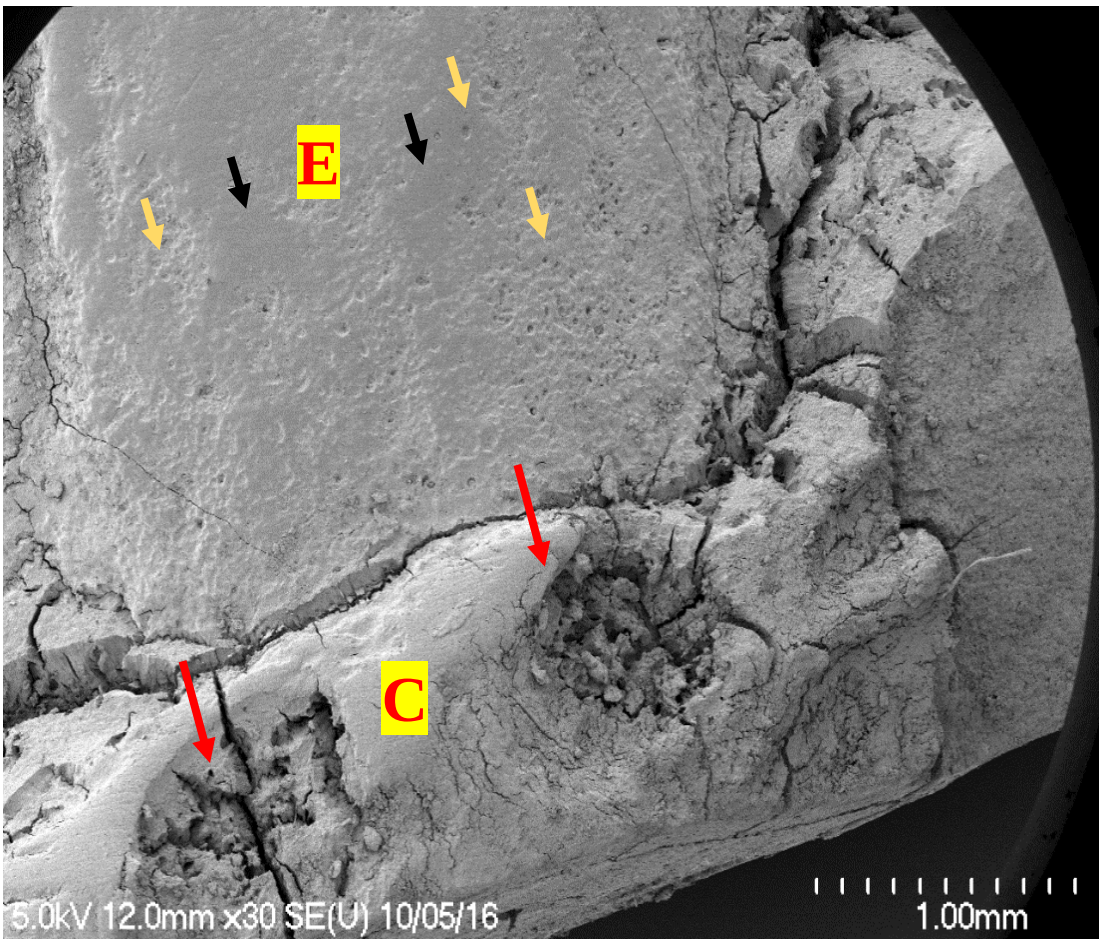


Fig 4.58. SEM image of an undecalcified peripheral section of the palatal aspect of a maxillary cheek tooth (209) with grade 1.2 PC, with most of its enamel (E) exposed, and only a small layer of cementum (C) left (which in turn has grade 1.1 PC in some areas [red arrows]). The enamel has some smooth areas (black arrows) and some areas which are pitted (yellow arrows).

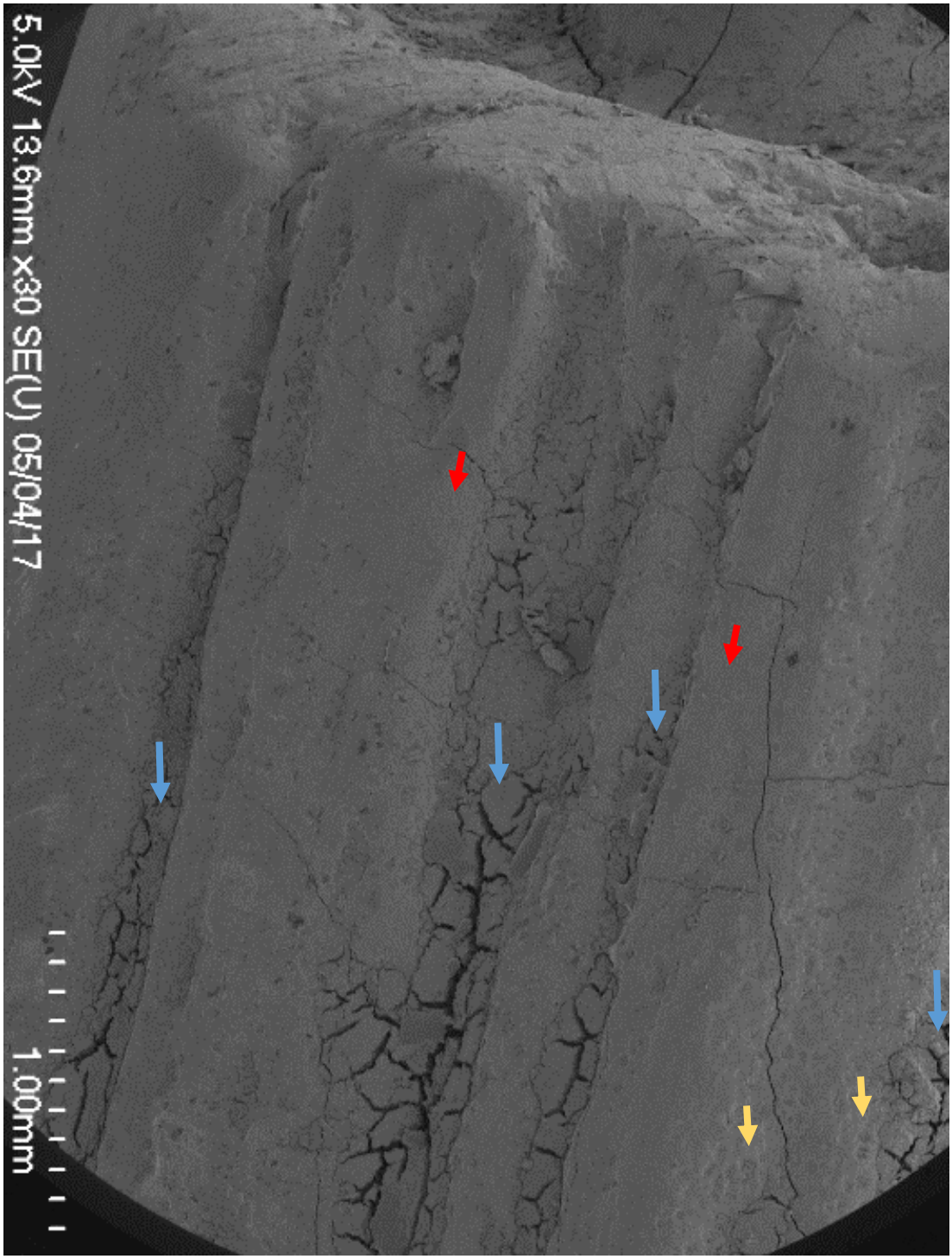


Fig 4.59. SEM image of an undecalcified peripheral section of the buccal aspect of a mandibular cheek tooth (406) with grade 2 PC, showing smooth enamel (red arrows); and enamel irregularities on and between enamel ridges (yellow arrows). The remnants of cementum are present in enamel grooves (blue arrows).

4.3.3 Dentine

4.3.3.1 General Histological and Ultrastructural Findings in Dentine and Pulp of Control Equine Cheek Teeth

Regular secondary dentine surrounded the pulp and was, in turn, surrounded by primary dentine (Figs 4.60 and 4.61). Histologically, pulp contained many blood vessels, and odontoblasts were observed lining the pulp periphery (Fig 4.61). Denticles ("pulp stones") were found in the pulp or secondary dentine (Figs 4.62 and 4.63). Predentine was observed on H&E stained sections as a pale pink region lying axial to the regular secondary dentine surrounding the pulp. In some sections, odontoblast processes were identified in the dentinal tubules (Fig 4.64). In undecalcified sections, odontoblast processes were observed to have multiple branches towards the dentino-enamel junction (Fig 4.65). Using SEM, a granular smear layer (formed from opposing calcified dental tissues wearing off each other) was observed to cover some dentinal tubules containing odontoblast processes on the occlusal surface, while other occlusal dentinal tubules appeared to be empty. In other sections, a smooth calcified layer partly or completely occluded some of the dentinal tubules on the occlusal surface. Odontoblast process were observed at the occlusal surface, but did not protrude above the occlusal surface itself (Fig 4.66). TEM examination of dentine showed the ultrastructure of odontoblast processes, intratubular dentine and intertubular dentine (Figs 4.67 and 4.68).

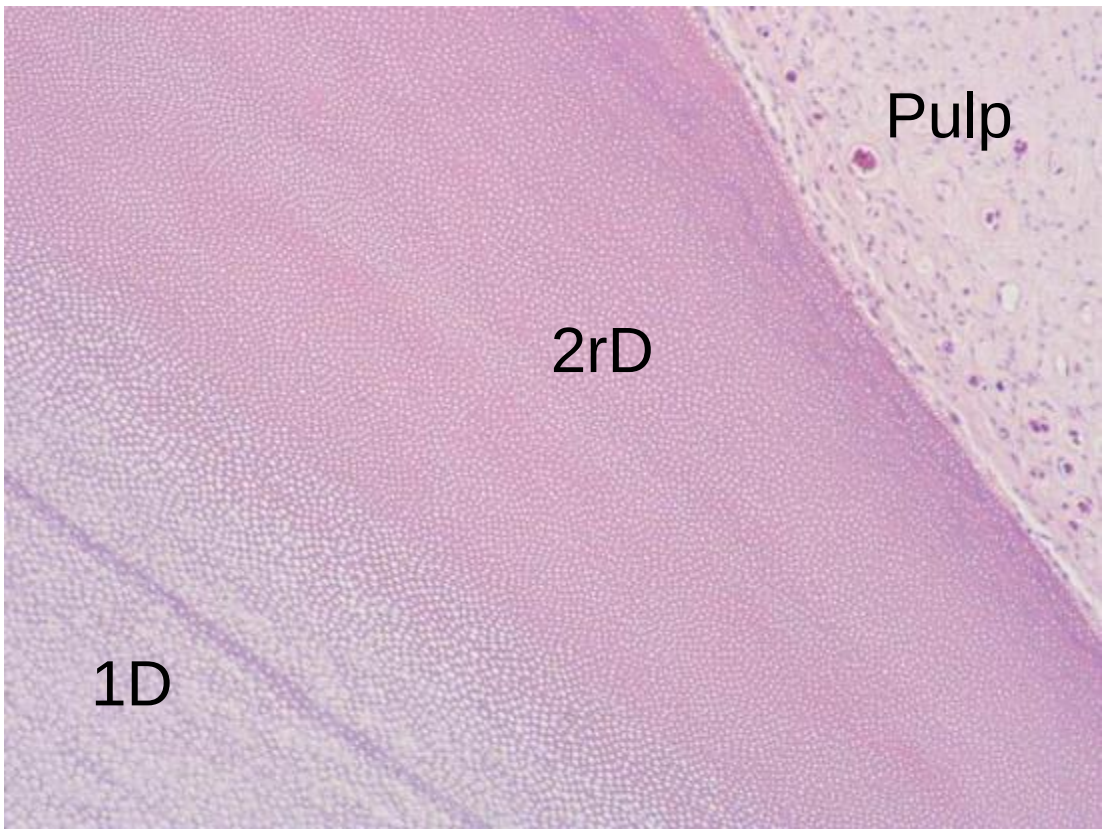


Fig 4.60. Decalcified transverse section of a control maxillary cheek tooth (108) at the level of the gingiva, that includes the dentino-pulp complex. The pulp contains many capillaries and is surrounded by secondary regular dentine (2rD), which in turn is surrounded by primary dentine (1D). [Original magnification X 100, H&E]

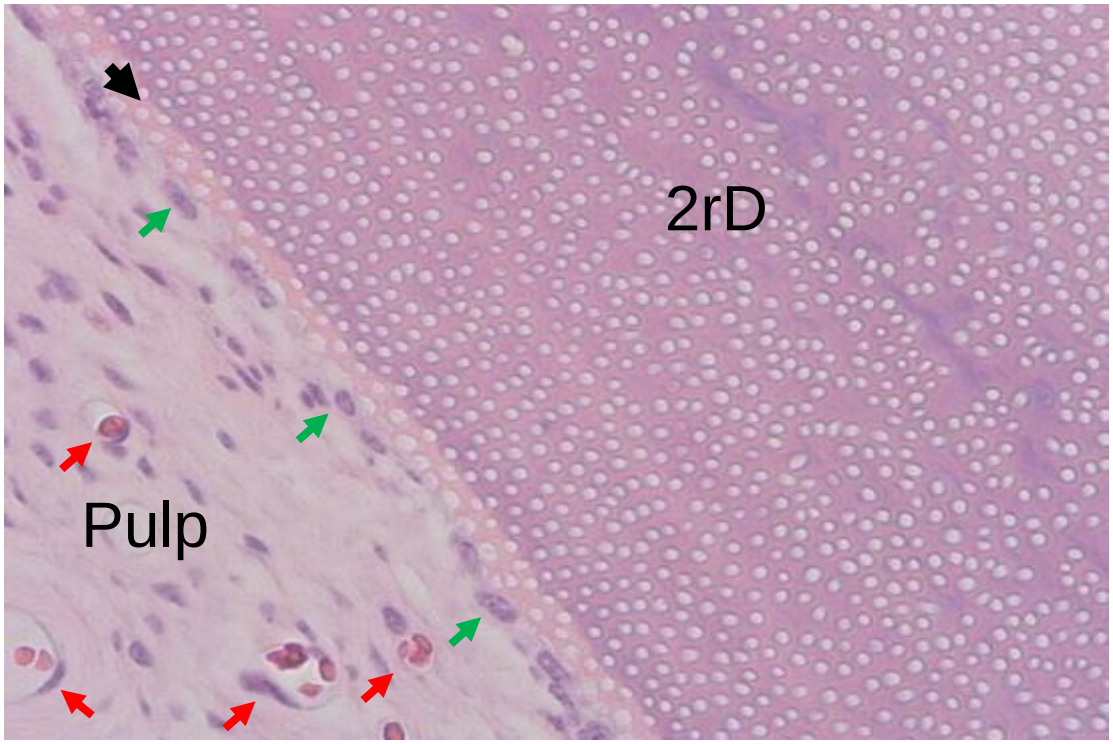


Fig 4.61. Decalcified transverse section of a control maxillary cheek tooth (108) at the level of the gingiva that includes the dentino-pulp complex with odontoblasts (green arrows) and predentine (black arrow - which the odontoblasts produce) lining the pulp; 2rD= secondary regular dentine; red arrows = pulpar blood vessels. [Original magnification X 400, H&E]

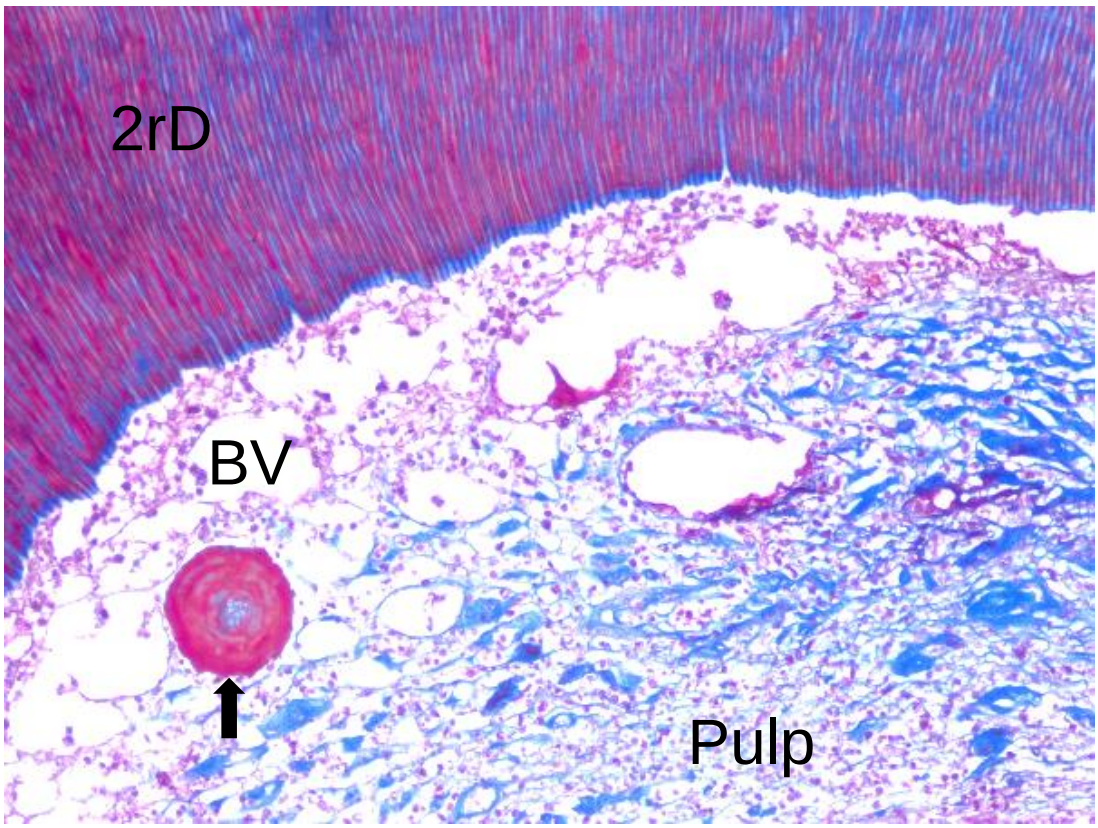


Fig 4.62. Decalcified transverse section of a maxillary cheek tooth (210) including pulp and secondary regular dentine (2rD). The pulp contains a pulp stone (black arrow) and blood vessels (BV). [Original magnification X 100, Masson's trichrome]

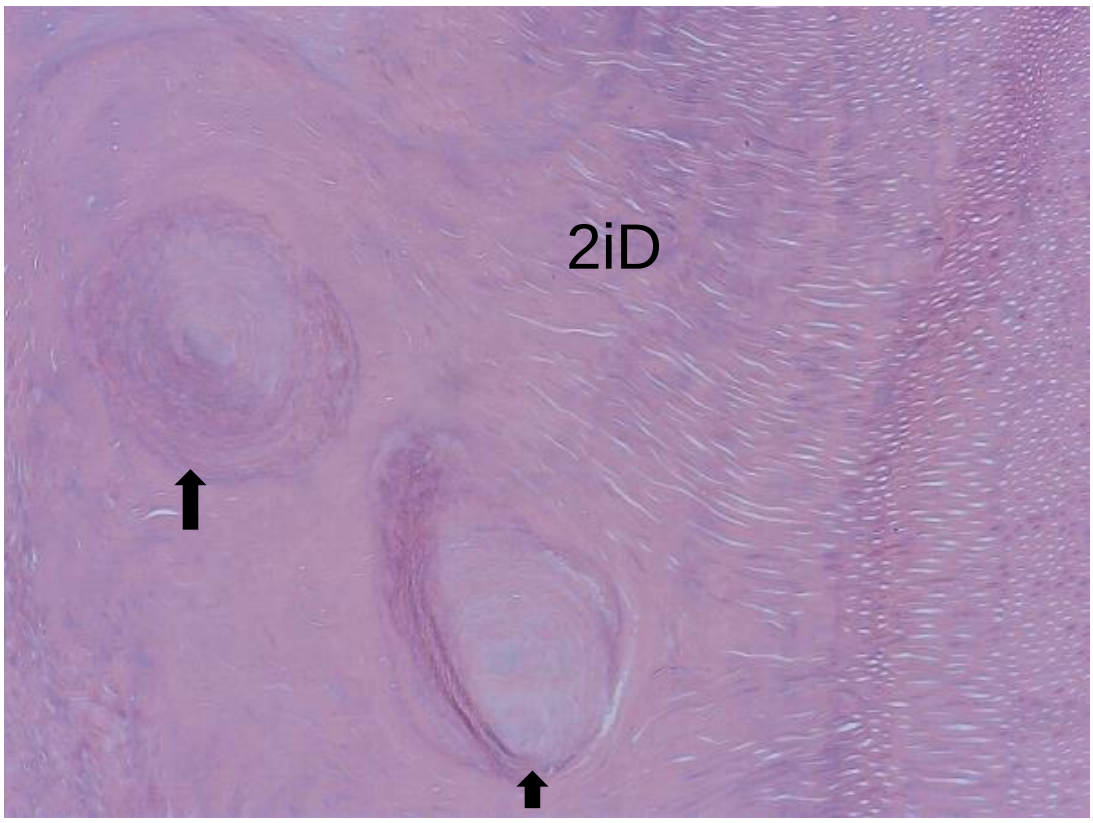


Fig 4.63. Decalcified transverse section of a maxillary cheek tooth (209) with pulp stones (arrows) present in the irregular secondary dentine (2iD). [Original magnification X 100, H&E]

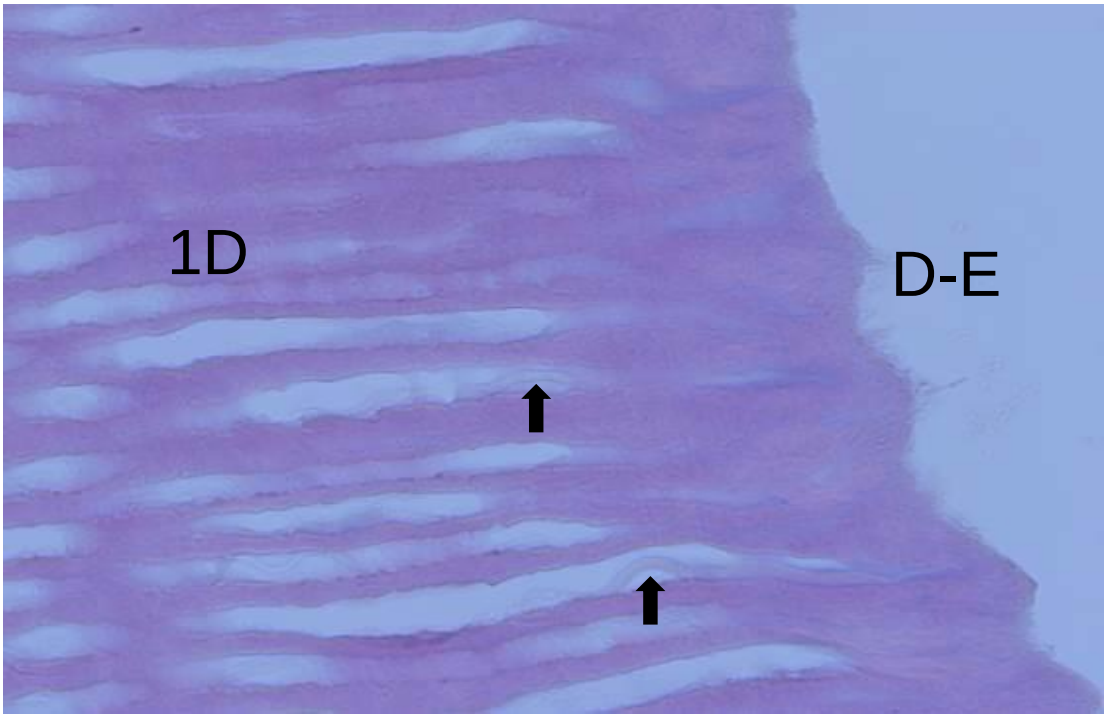


Fig 4.64. Decalcified transverse section of a control mandibular cheek tooth (310) illustrating dentinal tubules of primary dentine (1D), some of which contain odontoblast processes (arrows). D-E=dentino-enamel junction. [Original magnification X 400, H&E]

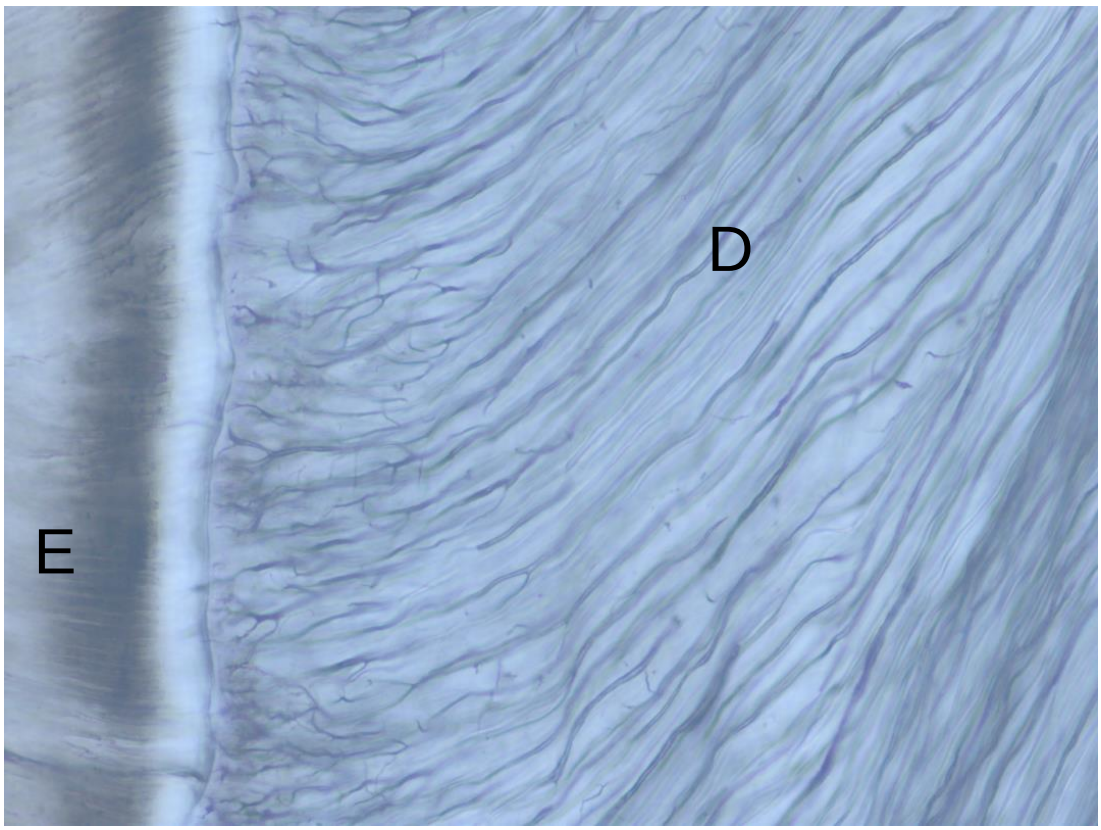


Fig 4.65. Undecalcified transverse section of a control mandibular cheek tooth showing branching of odontoblast processes towards the dentino-enamel junction; E= enamel; D= dentine. [Original magnification X 400]

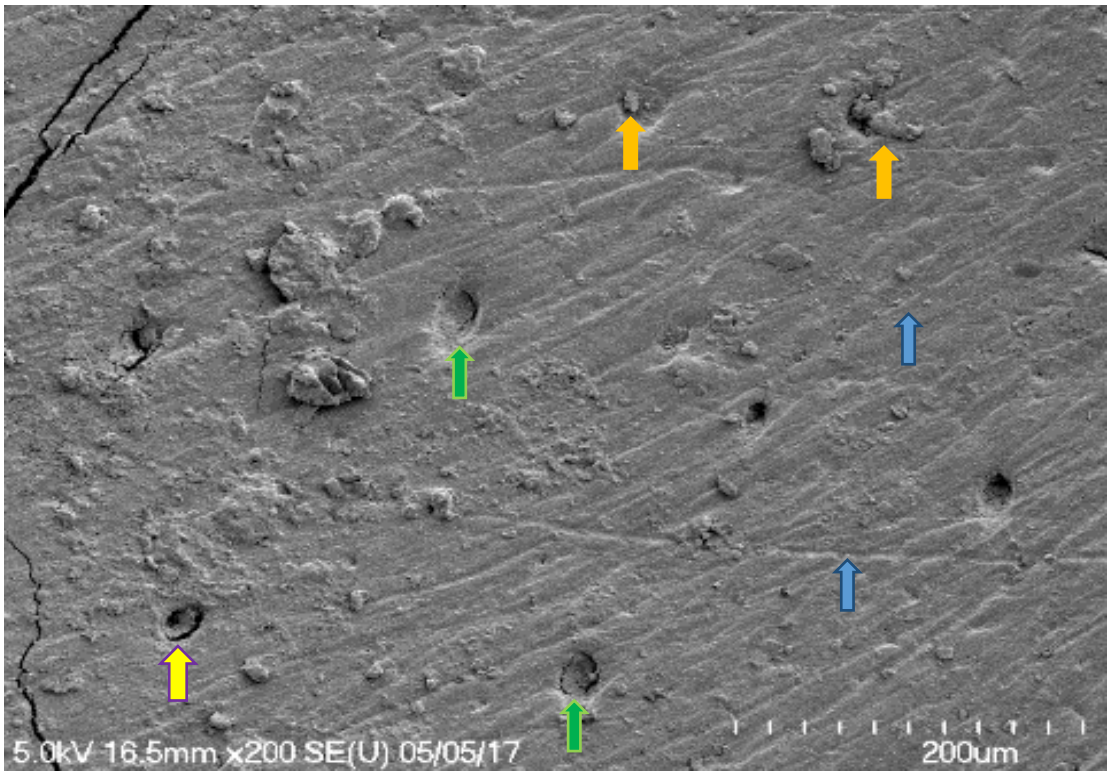


Fig 4.66. SEM of primary dentine on the occlusal surface of an undecalcified control maxillary cheek tooth (209). Some dentinal tubules are covered by a smear layer (orange arrows) consisting of granular dental calcified material; some appear to contain calcified tissue overlying the odontoblastic processes (green arrows). A hollow odontoblastic process can be identified in one dentinal tubule (yellow arrow). Wear lines (blue arrows) caused by mastication are present on the occlusal surface.

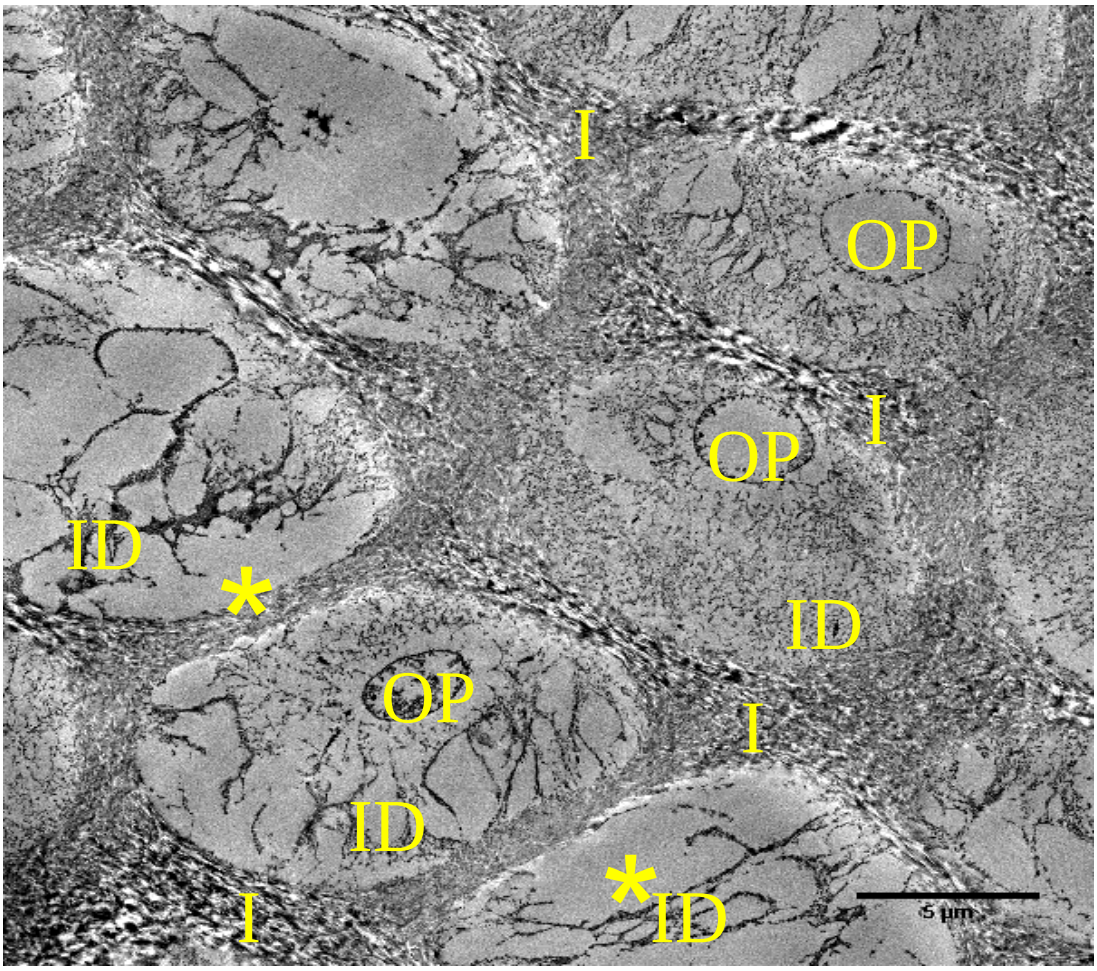


Fig 4.67. TEM of primary dentine on the occlusal surface of a decalcified control maxillary cheek tooth (209). ID= intratubular dentine; OP= odontoblastic process; * = periodontoblastic space; I = intertubular dentine.

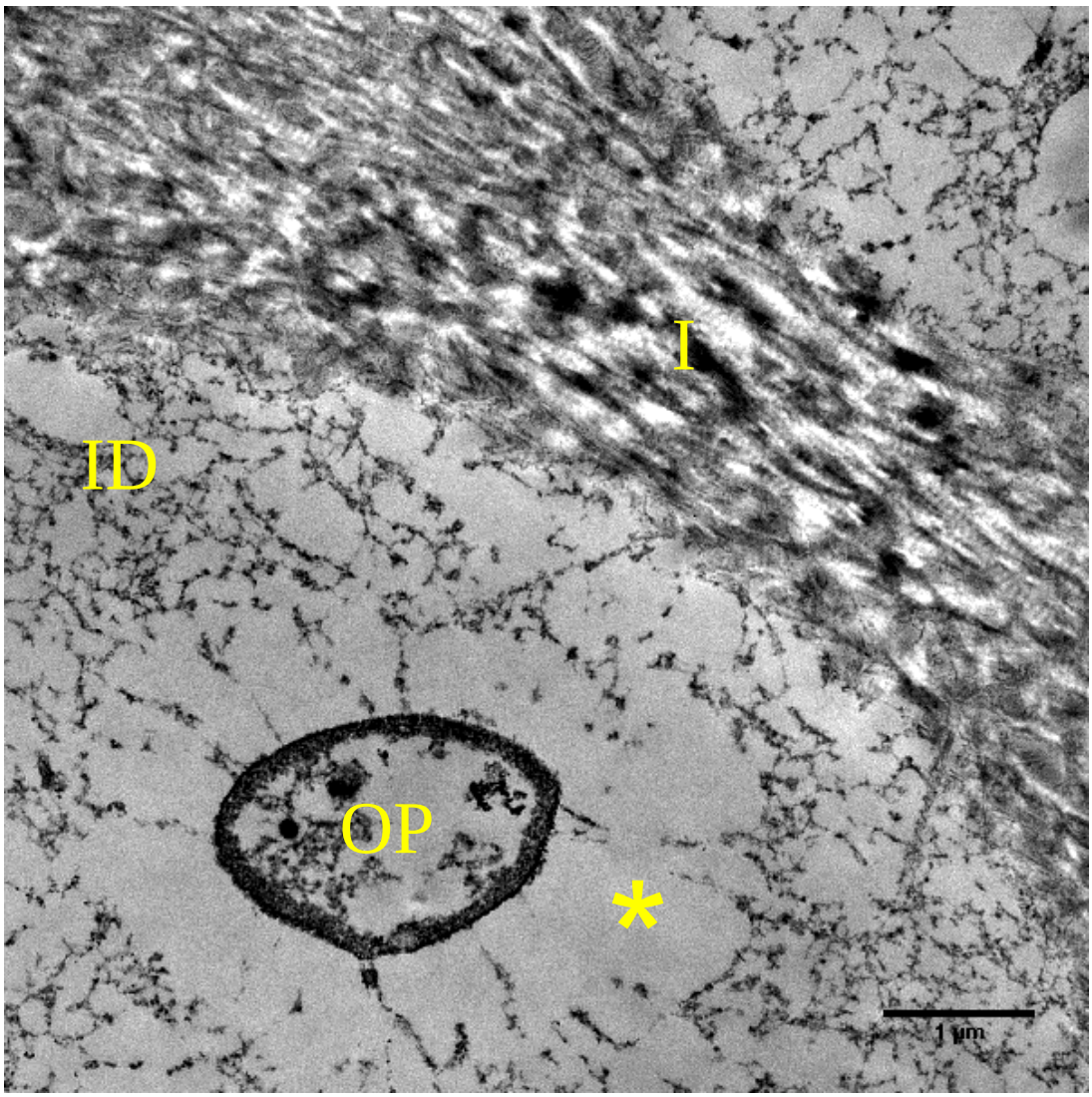


Fig 4.68. Higher magnification of the previous figure (Fig 4.67). TEM of primary dentine on the occlusal surface of a decalcified maxillary cheek tooth (209). ID= intratubular dentine; OP= odontoblastic process; *= periodontoblastic space; I= intertubular dentine.

4.3.3.2 Histological Findings in Equine Cheek Teeth Dentine Affected by Peripheral Caries

Dentinal PC was not investigated using SEM or TEM, but only histologically. In dentinal PC, bacteria were found in primary and/or secondary dentine. These micro-organisms could potentially have invaded from the occlusal surface (Fig 4.69) or from the peripheral aspect of the cheek tooth at the dentino-enamel junction, if the overlying dental tissues (cementum and enamel) were affected by PC (Fig 4.70). Bacteria were found to enter primary dentine at the dentino-enamel junction, at sites where dentine (both intratubular as well as intertubular dentine) was destroyed. Dental plaque that contained bacteria was observed between degraded and immediately adjacent intact dentine (Fig 4.71). Some fissure fractures involving the occlusal surface were identified in PC-affected dentine and these fractures could expose many dentinal tubules to oral contamination, creating an entrance portal for micro-organisms (Fig 4.72).

Bacteria were also observed in pulp, and in irregular and regular secondary dentine (Figs 4.73, 4.74, 4.75, 4.76). It was unclear whether the bacteria found in dentine spread in a peripheral direction from the pulp/irregular secondary dentine into the regular secondary dentine or vice versa, or whether the bacterial invasion followed a vertical route from the occlusal aspect of the tooth. Tertiary dentine was observed protruding into one pulp, adjacent to an area of bacterial invasion of pulp and adjacent regular secondary dentine (Fig 4.73).

In a tooth which was grossly assessed as a grade 2 PC, bacteria were histologically also found in dentine, justifying a histological score of grade 3 PC (Figs 4.75 and 4.76).

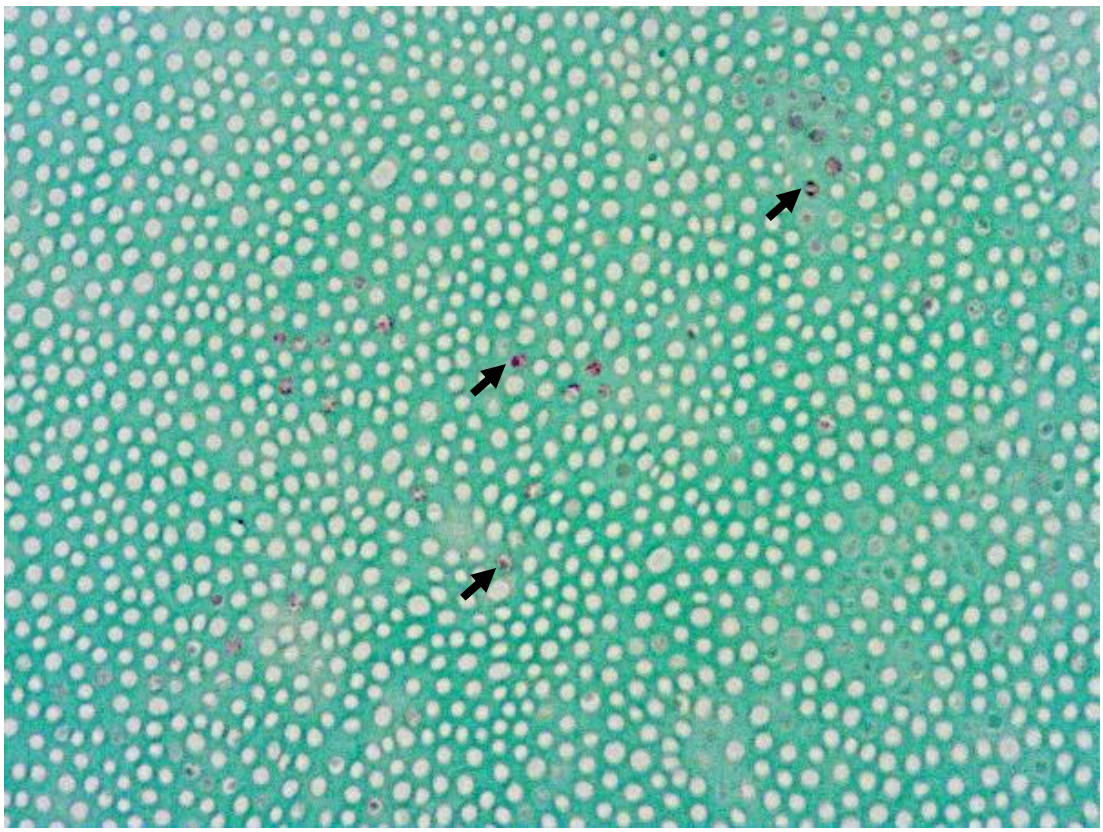


Fig 4.69. Gram stained section of primary dentine from a peripheral caries affected maxillary cheek tooth (210) with grade 3 PC showing bacteria (termed "pioneer organisms" in these dentinal tubules (arrows). [Original magnification X 200]

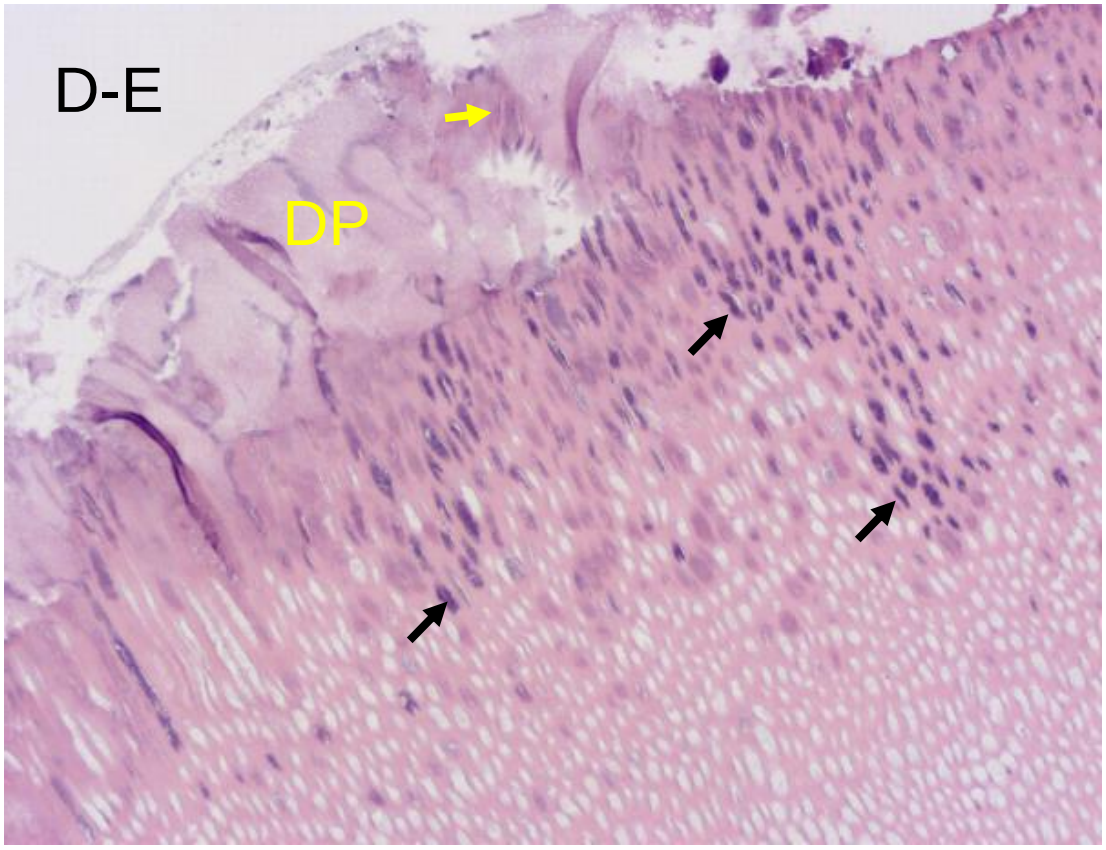


Fig 4.70. Decalcified transverse section of a maxillary cheek tooth (210) with grade 3 PC showing the affected primary dentine. Bacteria (black arrows) originating from the overlying dental plaque (DP) have penetrated into dentinal tubules, which appear to be widened, and have caused disintegration of the peripherally located primary dentine (yellow arrow) near the dentino-enamel junction (D-E). [Original magnification X 100, H&E]

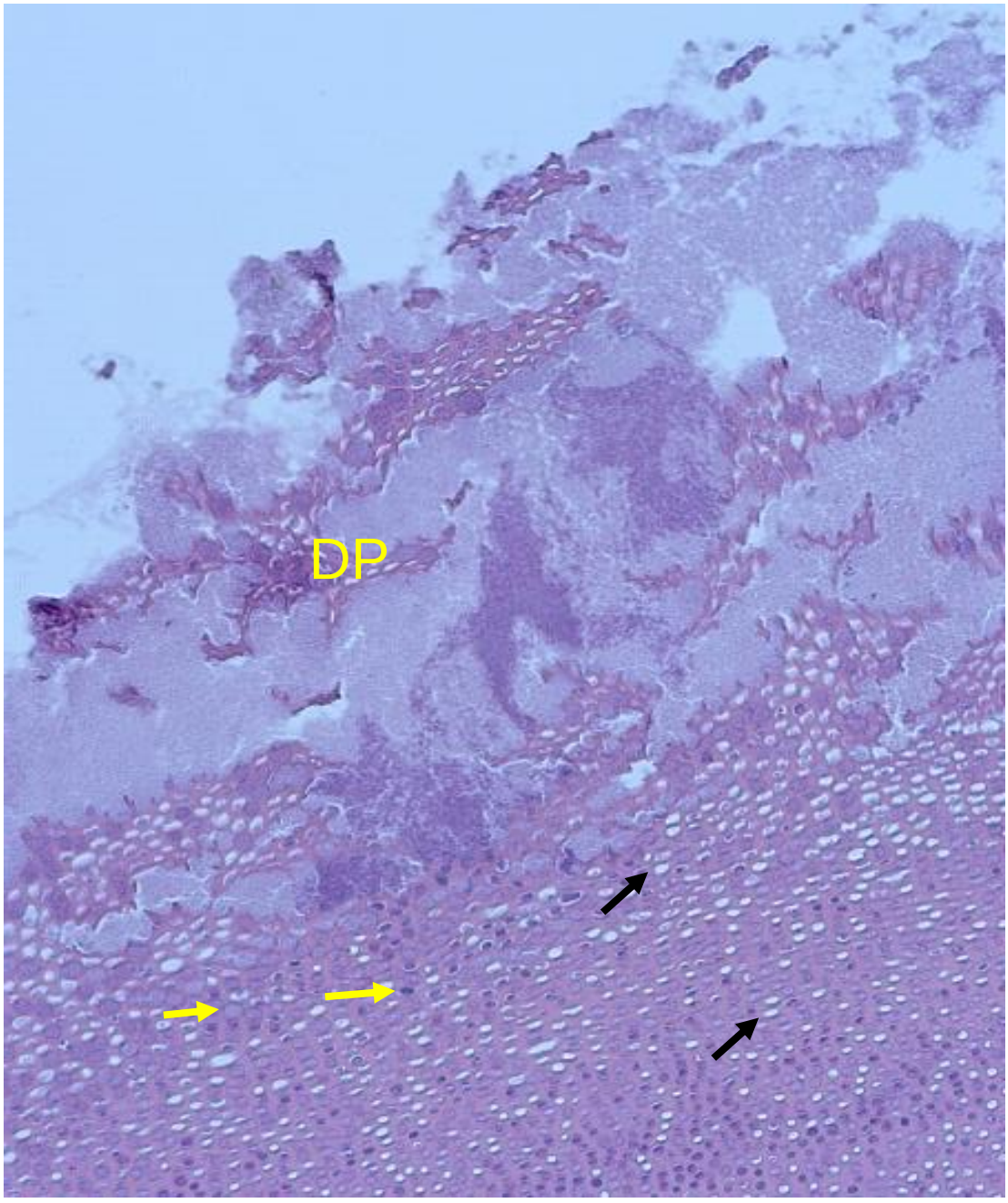


Fig 4.71. Decalcified transverse section of a maxillary cheek tooth (209) with grade 3 PC, showing multiple layers of PC-affected primary dentine that is disintegrating and is undermined by dental plaque on its peripheral aspect. Some tubules appear empty (black arrows), while others are filled by dental plaque (DP) containing bacteria (yellow arrows). [Original magnification X 200, H&E]

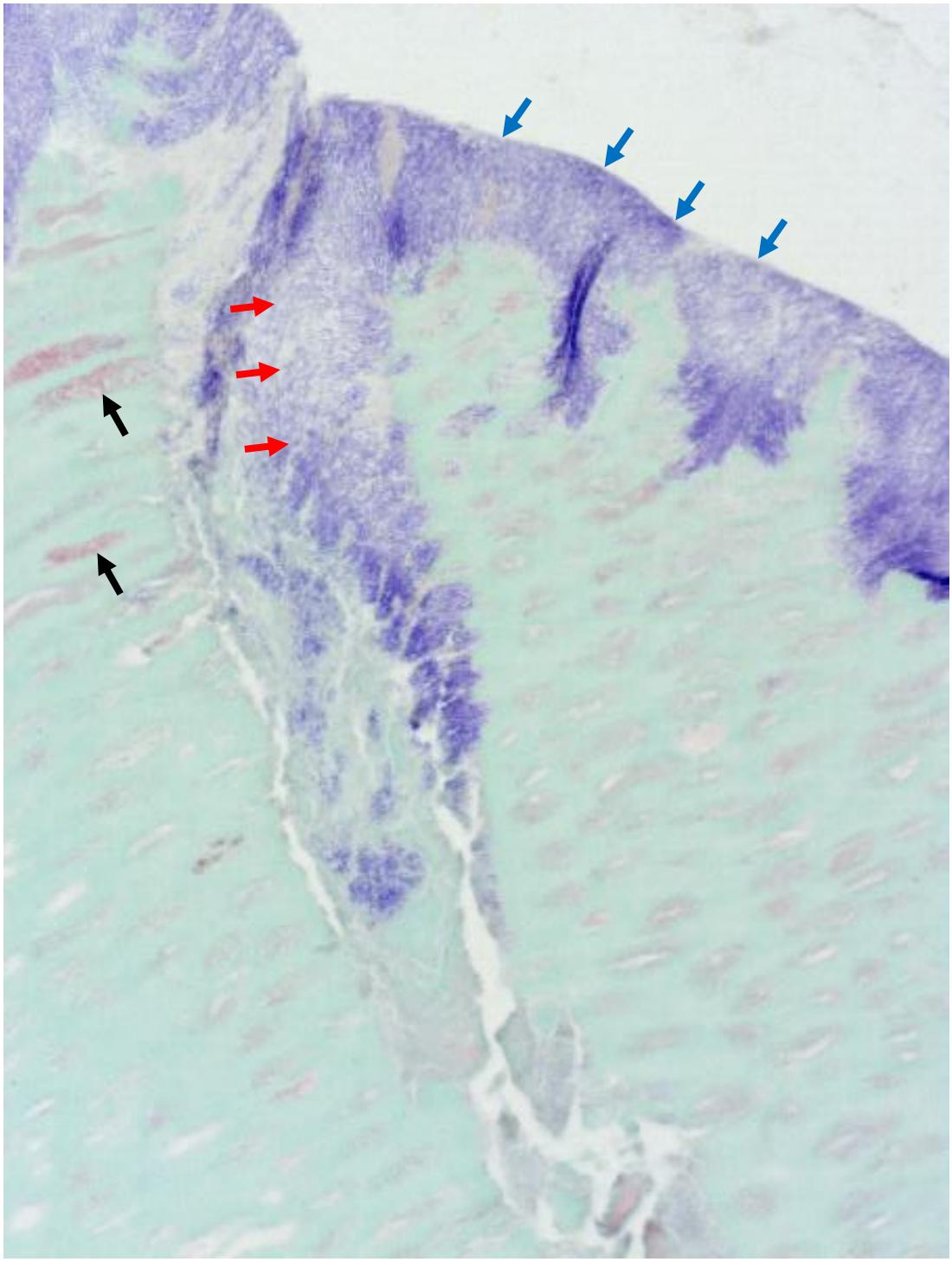


Fig 4.72. Gram stained decalcified tangential section of a maxillary cheek tooth (210) with grade 3 PC. A thick layer of dental plaque with multiple Gram positive bacteria is present at the dentino-enamel junction (blue arrows) and in a fissure fracture (red arrows) in the primary dentine. Gram positive (blue) and what appear to be Gram negative bacteria (red coloured) (black arrows) have penetrated into the dentinal tubules. [Original magnification X 200]

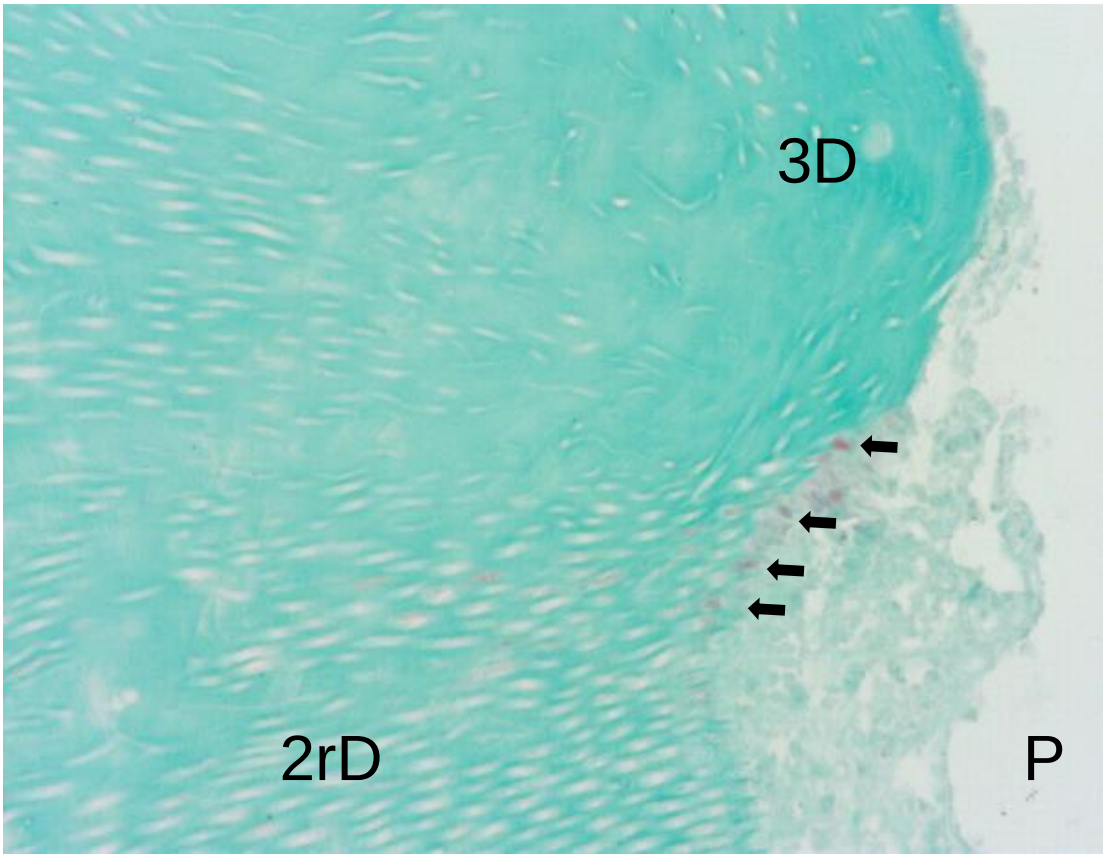


Fig 4.73. Gram stained decalcified transverse section of a maxillary cheek tooth (210) with grade 3 PC, including a caries-affected pulp (P) and secondary regular dentine (2rD). Tertiary dentine (3D) has formed in response to this bacterial invasion and the pulp cavity is now surrounded partly by regular secondary dentine and partly by tertiary dentine. Bacteria (arrows) from the pulp cavity penetrate the secondary dentine. Although individual bacteria are not distinguishable at this magnification, their red-tinged colour is indicative of Gram-negative bacteria. [Original magnification X 200]

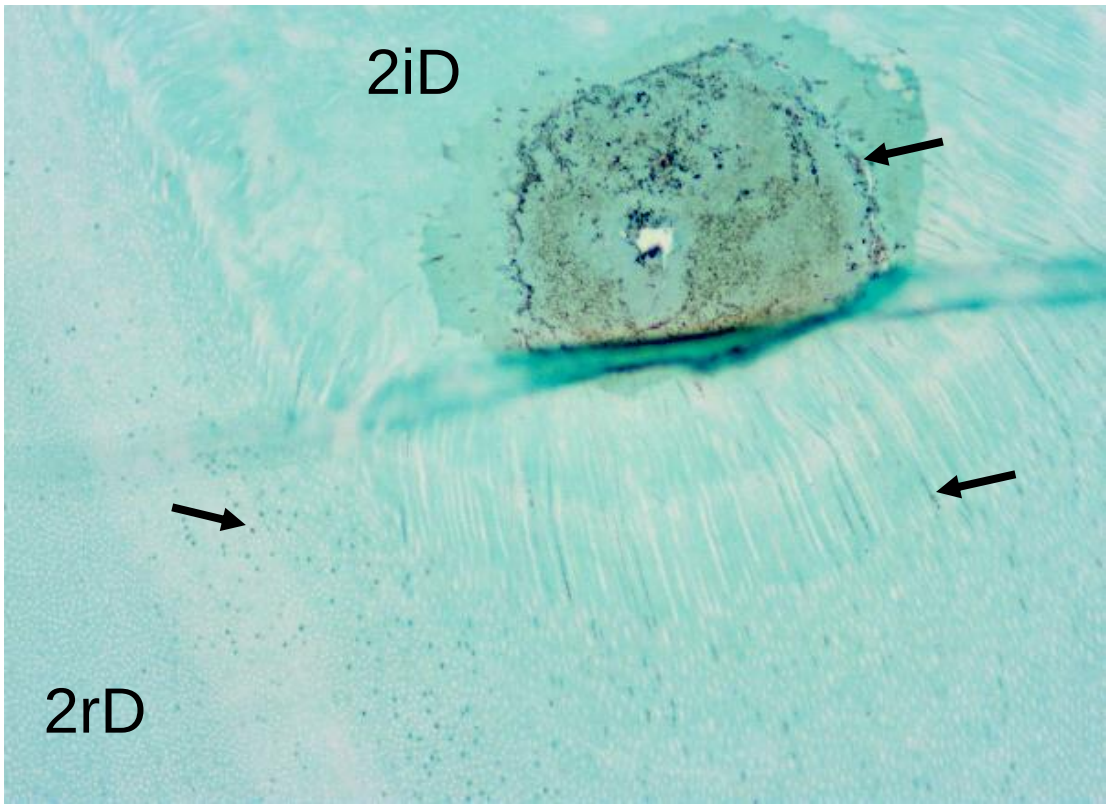


Fig 4.74. Gram stained decalcified transverse section of a maxillary cheek tooth (210) with grade 3 PC showing affected secondary irregular (2iD) and regular (2rD) dentine infiltrated by bacteria (arrows) which appear to be Gram positive (blue stained). [Original magnification X 100]

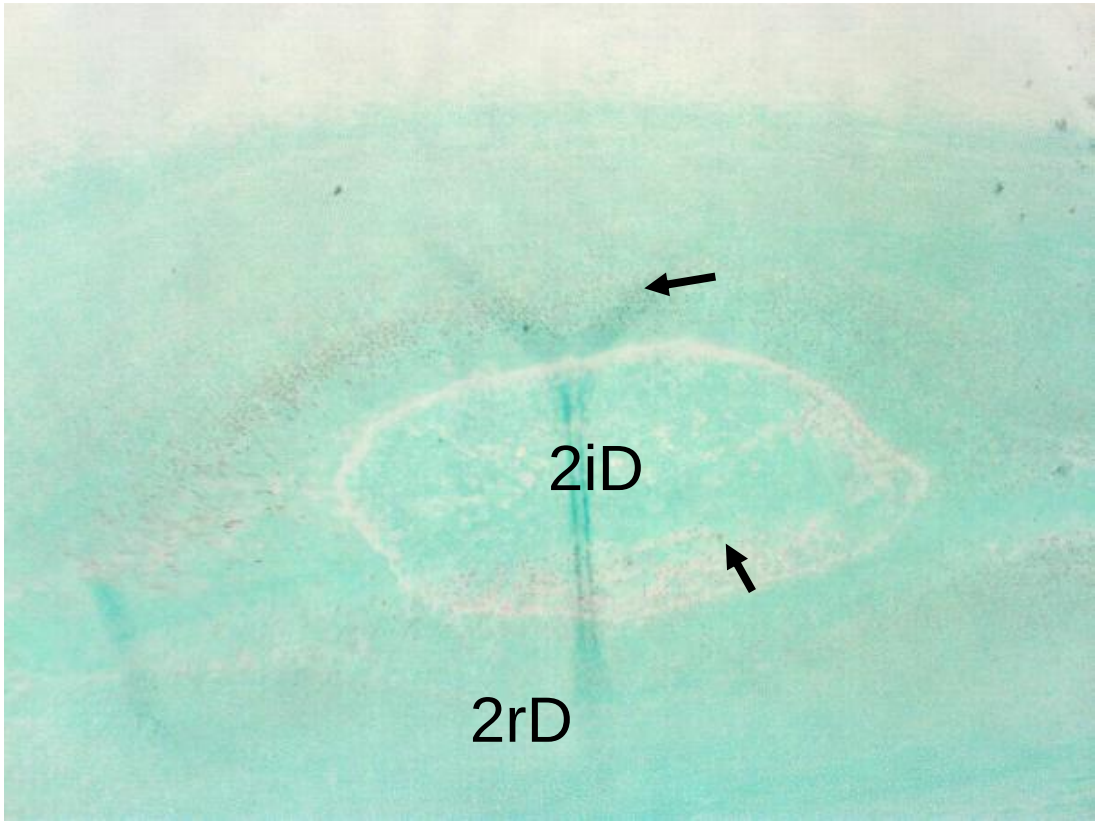


Fig 4.75. Gram stained decalcified transverse section of a maxillary cheek tooth (111) grossly affected with grade 2 PC, which was subsequently proven to be grade 3 PC histologically by the presence of caries-affected dentine. Pink-stained bacteria (arrows) have penetrated the dentinal tubules in both secondary irregular dentine (2iD) and secondary regular dentine (2rD). [Original magnification X 40]

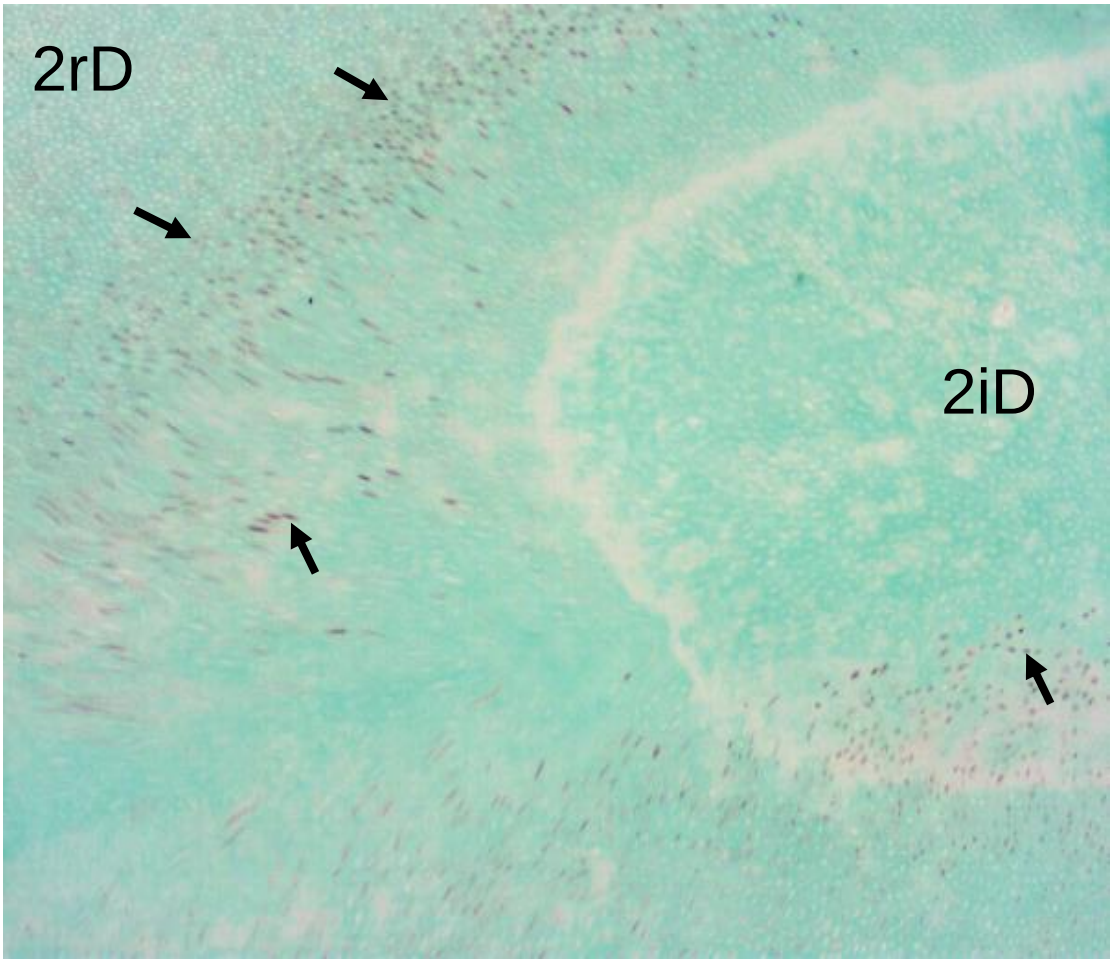


Fig 4.76. Higher magnification of the previous figure (4.75) of a Gram stained transverse section of a maxillary cheek tooth (111) with clinical grade 2 PC, which was shown to be grade 3 PC histologically. Rod-shaped bacteria (arrows) have penetrated the dentinal tubules of both secondary irregular dentine (2iD) and secondary regular dentine (2rD). [Original magnification X 100]

4.4 Discussion

4.4.1 Cementum

The histological and ultrastructural findings of normal cementum were similar to those described in other studies (Kilic et al., 1997c; Mitchell et al., 2003). Histologically, cementum can be classified as primary, secondary or tertiary. In all three types, cementocytes reside in lacunae whose long axes are parallel to the peripheral aspect of the tooth. Although cementocytes are in fact stellate-shaped (Krstic, 1994), the observed cementocytes in this study were usually shrunken and circular or oval- shaped, likely artefactually due to fixing and later histological processing. However, the shape of living cementocytes can also be variable (Zhao et al., 2016) and also depends on the distribution of the surrounding cemental fibres, resulting in oval-shaped cementocytes if there are few surrounding fibres, or disc-to stellate-shaped cementocytes, if adjacent fibres are oriented tangentially (Schroeder, 1986). Moreover, the final histological shape also depends on the plane of sectioning.

In this study, primary cementum, the most axial layer, was recognised by its scalloped cemento-enamel junction, the absence or scarcity of Sharpey's fibres; and the presence of many lines of arrested growth towards its border with secondary cementum. The middle and peripheral cemental layers i.e. the secondary and tertiary cementum, respectively, contained many Sharpey's fibres that were continuous with the extrinsic PDL fibres.

The morphological differences between secondary and tertiary cementum were most obvious in longitudinal sections, where an increase in thickness of tertiary cementum could be observed at the subgingival level, once the tooth had erupted above the rigid confines of the alveolus. Additionally, tertiary cementum had more lines of arrested growth than secondary cementum. This is in line with the findings of Mitchell et al. (2003) who also found tertiary cementum to be present at the gingival level of the reserve crown and in the clinical crown, that it is first deposited at the level of the alveolar crest when the tooth exits the alveolus and that its thickness quickly increases at the gingival aspect of the reserve crown. Although blood vessels were observed in all three cemental layers, secondary cementum generally contained more

blood vessels than primary and tertiary cementum. The cemental infoldings (entoflexid and metaflexid of mandibular cheek teeth and postprotoconal valley and preprotoconal groove of the maxillary cheek teeth) contained many blood vessels, as also described by Mitchell et al. (2003). This vasculature appeared to comprise branches of a larger blood vessel that entered at the tooth periphery and penetrated all cemental layers. In these infoldings, primary cementum contained most blood vessels.

Although infundibulae of younger horses also receive two lateral blood vessels, as previously described (Suske et al., 2016b), these were not identified in older teeth. Branching of the central infundibular blood vessel in infundibular cementum was observed in both undecalcified and calcified maxillary cheek teeth sections. In decalcified, H&E stained sections, some blood vessel lumina appeared empty, while others were occluded, possibly by surrounding, newly formed cementum.

Histologically, the ellipsoid canals observed in peripheral cementum in this study did not appear to be blood vessels, but resembled intra-cemental protrusions of PDL, including Sharpey's fibres, fibroblasts and sometimes capillaries. New cementum appears to have been formed in some inclusions.

Although nerves have been found in peripheral cementum (Mitchell et al., 2003), they were not identified in this study using histology, SEM or TEM.

Cemental resorption can be recognised histologically by the presence of a scalloped, basophilic line called a reversal line. Resorption and repair of cementum in equine teeth has been observed in teeth with Equine Odontoclastic Tooth Resorption and Hypercementosis (EOTHR) (Staszyk et al., 2008; Moore et al., 2016). Although most of the cemental inclusions observed in this study were surrounded by a basophilic line, this line was not scalloped and cementoclasts were not observed within or on the surface of any cemental site. Consequently, the cemental destruction observed in this study is very unlikely to be a resorptive lesion.

Based on morphology, three patterns of PC were histologically observed: flake-like, flask-like and ellipsoid-shaped lesions. Erridge et al. (2012) have previously

described the first two types of lesions in PC. Lesions could progress parallel or perpendicular to the peripheral aspect of the affected tooth. In flake-like lesions, which usually progressed parallel to the peripheral aspect of the tooth (Type A2), at the level of or parallel to a LAG (Newbrun, 1983), the intrinsic fibres were undermined and flakes of cementum became loose and fully detached eventually. The most extensive cemental caries lesions observed in this study were flake-like lesions in this orientation. The presence of cemental LAGs predisposed to plaque accumulation because of their grooves and irregular surface and, in turn, predisposed cementum to develop PC. Equine cemental LAGs run vertically (apico-occlusally) as well as horizontally (in a direction parallel to the periphery of the tooth) (Burke and Castanet, 1995). This could explain why a flake-like lesion at the level of a LAG can be so extensive, despite a LAG being hypermineralised compared to faster growing cemental regions.

When a flake-type lesion occurred in a direction perpendicular to the peripheral aspect of the tooth (Type A1), cemental penetration by dental plaque bacteria usually started between Sharpey's fibres. The affected Sharpey's fibres later became detached from the cementum. Further progression of flake-like lesions was possible in both directions (parallel or perpendicular to the peripheral aspect of the tooth). Bacteria often appeared to use lacunae and their canaliculi to spread further within cementum (Type D). Flask-like lesions (Type B), which had a more circular appearance than ellipsoid lesions, seemed to proceed in a radiating pattern. Ellipsoid type-lesions (Type C) had the same appearance as the cemental protrusions/inclusions and blood vessels, and Gram positive and Gram negative bacteria were present within these structures. A combination of different types of carious lesions was sometimes present within the same tooth.

Owen (1845) found that canaliculi radiating from cemental lacunae intercommunicate with small branches of vascular channels. In some Gram stained sections, it appeared that Gram negative bacteria (or possibly some red-staining inert non-calcified material), extended from the peripheral aspect of the tooth into lacunae via canaliculi.

TEM of a control tooth showed micro-organisms in the dental plaque on its peripheral aspect but also within the cementum, where they were present in lacunae

and in what appeared to be blood vessels (because of their diameter and shape). Overall in this study, PC was histologically more severe than expected based on gross examination. For example, one cheek tooth had macroscopic grade 2 PC which, using Gram stain, was found to be grade 3 PC histologically. Macroscopic underestimation of PC grades compared to histology was also reported by Erridge et al. (2012)

In conclusion, these differences in morphological patterns could reflect differences in pathogenesis. As discussed above, LAGs could predispose to PC because of their rough surface. Because these LAGs are oriented horizontally, in a direction parallel to the periphery of the tooth (and vertically (apico-occlusally)), flake-type lesions are likely to form at these sites. In flake-like lesions intrinsic fibres seemed to get destroyed more readily than Sharpey's fibres which then provide a potential pathway for bacteria to penetrate into the cementum. Ellipsoid-shaped and flask-like lesions could possibly develop from former vascular sites or protrusions/inclusions of PDL including Sharpey's fibres, fibroblasts and sometimes capillaries which predispose to caries, whereas bacteria could also follow microtubules/canaliculi and lacunae to invade the cementum.

In normal infundibulae and in infundibulae affected by IC, food debris was often found histologically and ultrastructurally with SEM, while TEM images more clearly showed destruction of cementum and the presence of micro-organisms in an IC lesion.

4.4.2 Enamel

Macroscopically, vertical ridges (in an apico-occlusal direction) were observed in exposed peripheral enamel, where PC had removed the overlying cementum. In decalcified histological sections, enamel was almost completely or completely lost due to decalcification, which makes it impossible to histologically study enamel caries. Additionally, TEM required decalcification because the tooth needed to be cut into very thin slices. Consequently, enamel could not be studied using TEM also and

could only be effectively examined by histology of undecalcified sections (expensive and difficult to produce) or by SEM.

Using undecalcified sections of a mandibular and a maxillary cheek tooth, the cheek tooth cemento-enamel junction was found to be scalloped, as previously described (Itoh and Saito, 1992). Using decalcified histology, the cemental part of the cemento-enamel junction could be studied and, using undecalcified sections, cemental protrusions were observed filling enamel depressions in the scalloped cemento-enamel junction, (Jones and Boyde, 1974; Kilic et al., 1997c), although some areas of the cemento-enamel junction were flat (Wang et al., 2006). Mitchell et al. (2003) reported that the function of the "peg and pit" design of the cemento-enamel junction is to create a tight junction with increased surface area between the enamel and cementum, helping to prevent fractures, keeping a tight junction and thus preventing penetration of food particles and bacteria between these two layers.

Enamel spindles, which are also reported to be present in brachydont teeth, were found in equine cheek teeth using undecalcified histology (Nanci, 2008), in donkey cheek teeth (du Toit et al., 2008b), but not in equine incisors (Muylle et al., 2000). Additionally, true lamellae or fissure fractures were observed in undecalcified sections of cheek teeth, which might be a pathway for cariogenic oral bacteria to penetrate the tooth (Walker et al., 1998). Fine enamel cracks (fissures) were also observed on SEM examination in the current study. Large continuous cracks were believed to be slide processing artefacts, while more localised cracks on the peripheral surface of exposed enamel appeared to be pre-existing cracks. It is unclear whether these were cracks of superficial enamel or cracks of local residual cementum, although macroscopically cementum seemed to have been completely destroyed by PC in that area, leaving the enamel exposed and affected by PC.

The outer layer of equine enamel is usually a thin layer of Equine Type-3 enamel, which is present as a thin layer at the cemento-enamel junction and consists of rounded rods and large amounts of inter-rod substance (Kilic et al., 1997a). du Toit et al. (2008b) proposed that Equine Type-3 enamel could in fact be a transitional enamel present at enamel junctions. However, Equine Type-3 enamel is not always present, so that sometimes the outer layer of enamel at the cemento-enamel junction

of a cheek tooth may be composed of Equine type-2 enamel, which contains mainly circular, keyhole to horseshoe-shaped rods and little or no inter-rod enamel (Kilic et al., 1997a).

In the current study, no enamel rods or inter-rod enamel were observed. This is because our samples were not acid etched prior to preparation for SEM, in contrast to previous studies (Kilic et al., 1997a; du Toit et al., 2008b). One aim of the current study was to assess the effect of naturally occurring PC on peripheral enamel and acid etching would have interfered with the results. Because no rods or inter-rod enamel were observed at the peripheral aspects of cheek teeth with PC grade 1.2 or 2, even after exposure to cariogenic bacterial acids, this means that the enamel is still heavily mineralised with little dissolution of rod cores and boundary regions or that there is an extra outer layer of the enamel present which covers rods and inter-rod material.

In brachydont teeth, the first layer of enamel (formed at the dentino-enamel junction) and the final layer of enamel do not contain any rods and are therefore termed the rodless (or aprismatic) layers (Nanci, 2008). The rodless layers are continuous with the interprismatic layer and each rodless layer is formed by the proximal portion of Tomes' process (Nanci, 2008). The crystal orientation in the rodless layers is similar to inter-rod enamel (Kierdorf et al., 2013). In human dental enamel, the rodless layer is 5-15µm thick (Goldberg, 2016) and present in all deciduous and permanent, maxillary and mandibular teeth (Gwinnett, 1966, 1967; Fava et al., 1997), although another study suggested it is present in all deciduous teeth but only in 70% of permanent human teeth (Ripa et al., 1966). Hydroxyapatite crystals in the rodless layer are arranged parallel to each other and perpendicular to the enamel surface in human teeth (Gwinnett, 1966; Fava et al., 1997).

These rodless enamel layers are also present in the enamel of tribosphenic molars (three cusped shaped teeth functioning as grinding wedge) in bats, which is interesting because all mammalian molar types appear to have evolved from these tribosphenic molars (Spoutil et al., 2010). If there is an extra layer covering the

peripheral enamel surface with its rod/interrod substance in equine teeth, then it is likely that this is also a rodless layer. Similarly to human teeth, the observed enamel irregularities in equine cheek teeth may be the result of partial dissolution of the rodless layer caused by cariogenic challenge or dietary acids, e.g. acidic silage/haylage additives in horses (Miranda et al., 2005; Eissaa et al., 2013; Briso et al., 2015). Fine cracks which were mainly observed in the vertical grooves of peripheral enamel could either reflect cracks in the rodless layer or cracks in residual layers of cementum within enamel grooves - this is such a thin layer that it cannot be recognised macroscopically as cementum.

In order to study the effect of bacterial acids in equine PC, an *in vitro* study could be performed in which an acid-resistant varnish could be applied to some areas of the tooth to protect against acid damage, and then expose the tooth to acid and assess the acid-etching patterns on the occlusal and peripheral aspects of the teeth using SEM. The aprismatic layer could be removed by wet-grinding with 600-gritt SiC paper before acid etching to improve the etching effects (Shinohara et al., 2006). A similar study could be performed on equine cementum and dentine to improve our knowledge on the morphological effects of acid erosion on equine teeth.

4.4.3 Dentine

4.4.3.1 Normal Dentine

Some dentinal tubules and odontoblast processes on the occlusal surface were covered by a granular smear layer or a smooth calcified layer, while other dentinal tubules appeared to be patent. Muylle et al. (2000) showed that the smear layer on equine incisors consisted of calcified material by etching specimens with phosphoric acid which removed this layer. The smooth calcified layer might be intratubular dentine or a calcified plug.

Using TEM, intratubular dentine was shown to contain a small number of fibres. Kilic et al. (1997b) described these fibres in equine cheek teeth as collagen fibres, but in TEM images in the current study, fibres in intratubular dentine had a different

appearance to the collagen fibres of intertubular dentine. For example, the fibres in intratubular dentine had a more granular aspect and no periodicity was visible, in contrast to the intertubular dentine. Bertassoni et al. (2012) examined permanent human third molars and found the organic matrix of the intratubular dentine to be mainly composed of glycosaminoglycans, which serves as a scaffold to support and facilitate hypermineralisation of the intratubular dentine. They also found that a sheet-like membrane (lamina limitans), which lined the entire dentinal tubule and contained proteoglycans protein cores, was the origin of the organic intratubular dentine network.

4.4.3.2 Dentinal Peripheral Caries

Similarly to the findings of Erridge et al. (2012), the macroscopic grading of equine PC underestimated the severity as assessed histologically, i.e. bacteria were found in dentine where dentinal caries was not observed macroscopically.

In brachydont teeth, intratubular dentine possesses a lower collagen content and a higher content of sulphated proteoglycans and minerals than intertubular dentine, making the former a harder tissue, which is more readily dissolved by acid than intertubular dentine (Cohen and Burns, 1998). Similarly in equine teeth, intratubular dentine may be more readily destroyed by acids produced by bacteria in the dental plaque than intertubular dentine, due to its lower collagen content (Kilic et al., 1997b) or possibly even due to the absence of collagen fibres (Boyde, 1997; Muylle et al., 2001).

In teeth affected by PC, plaque containing bacteria was present in fissure fractures in both cementum and dentine. In dentine, these fissure fractures involved intratubular as well as intertubular dentine; thus the fissure fractures connected multiple dentinal tubules with each other, allowing bacterial spread. Fissure fractures were recognised on histological examination within PC lesions, but were not recognised on gross inspection and thus they could be termed microscopic fissure fractures. More research is needed to investigate the potential pathway of bacteria from a microscopic or macroscopic fissure fracture to the pulp, which in turn could lead to

pulpar and apical infections (Dacre et al., 2008c; Dacre et al., 2008b; Simhofer et al., 2008; van den Enden and Dixon, 2008).

It has been suggested that macroscopic occlusal fissure fractures do not necessarily cause clinically apparent dental disease. For instance, one study found such fractures in 54.3% of all horses examined and were present in teeth that were otherwise healthy with no associated clinical signs (Simhofer et al., 2008). These findings were similar to those described by Ramzan and Palmer (2010) who reported a cheek teeth fissure fracture prevalence of 58.2% in horses examined for dental disease investigation or treatment. However, it would still be helpful to investigate both microscopic and macroscopic fissure fractures grossly, histologically and ultrastructurally to assess any potential role for these fractures in spreading oral bacteria deeper into the tooth.

It is believed that in brachydont teeth (possibly also in hypsodont equine teeth), that acidogenic bacteria initially invade dentinal tubules and demineralise their walls. Proteolytic bacteria follow, destroying the organic matrix and further enlarging the dentinal tubules (Cohen and Burns, 1998). Most bacteria which cause dental caries in human teeth are non-motile and can only invade dentinal tubules by cell division or by the hydrostatic pressure created by masticatory forces (Hargreaves and Berman, 2016). Such bacteria can eventually reach the pulp by intratubular migration in the presence of dentinal caries and also if the pulp is exposed to the oral environment by a dental fracture (i.e. primary pulp exposure). This can cause pulpitis and the resultant pulpar inflammation in brachydont teeth would lead to compression of the fine apical and pulpar vasculature and ischaemic death of the pulp. In equine teeth that have prolonged eruption and secondary dentine deposition, the apical foramina are wider and the pulpar vasculature is larger, especially in young horses. Consequently, pulpitis does not necessarily lead to ischaemic pulpar death (van den Enden and Dixon, 2008). Intra-pulpar bacteria can spread readily through the pulp towards the apical foramina and cause (peri)apical infection, initially involving the periodontium, although this route was not identified as an important pathway of apical infection in mandibular or maxillary cheek teeth in other studies (Dacre et al., 2008b; Dacre et al., 2008c).

In human teeth, bacteria can also reach the pulp via dentinal tubules from deep carious lesions before actual pulpar exposure occurs; if the pulp remains vital, it may be able to clear the infection or seal it off with tertiary dentine. If the pulp is non-vital and defence mechanisms are impaired, then pulpar infection can become established, even if only a few bacteria are present (Hargreaves and Berman, 2016).

The pulp can also be infected if a bacterial periodontitis proceeds to the periapical region and the pulp (periodontal-endodontic lesion) (Crabill and Schumacher, 1998; Dacre et al., 2008b; Dacre et al., 2008c). Anachoresis is another pathway of pulpar infection, in which circulating bacteria (i.e. during bacteraemia) can be attracted to, and become localised within, inflamed tissue (in this case the pulp) (Cohen and Burns, 1998). Anachoresis was the main diagnosed cause (68%) of apical infection in a study by van den Enden and Dixon (2008) of 79 clinically extracted cheek teeth. In the case of anachoresis, the pulp horn is anatomically intact and the bacteria cause an insult to the vital pulp which may result in a local or generalised decrease in, or cessation of, secondary dentine production in the involved pulp (Dacre et al., 2008c; Dacre et al., 2008b; Dacre et al., 2008d; van den Enden and Dixon, 2008). Due to further wear of the tooth, the remaining secondary dentine is gradually worn away and eventually the devitalised or dead pulp will be occlusally exposed (i.e. secondary occlusal pulpar exposure) (van den Enden and Dixon, 2008).

Dacre et al. (2008c) and Suske et al. (2016a) found an association between IC and pulpar and apical infections (16% and 27% of diseased maxillary cheek teeth, respectively). More research is needed to investigate the micro-organisms involved in IC, pulpar and apical infections, and a possible relationship between PC and pulpar and apical infections could be explored using molecular microbiology studies combined with histological and ultrastructural investigation.

CHAPTER 5: GENERAL DISCUSSION

5.1 Epidemiological Study

A UK-wide survey was performed to assess the prevalence and severity of equine PC and IC and to examine for possible risk factors for the development of both of these types of dental caries in horses.

One of the survey findings was a strong association between the presence of PC and cheek teeth diastemata/periodontal disease. When a diastema is present, food can become chronically impacted between the teeth, providing a constant substrate for bacteria in dental plaque. These bacteria can then cause periodontal disease and PC, although the causal bacteria involved in these two diseases appear to differ, as discussed later.

IC was positively associated with age. This could be because areas of incompletely formed cementum (cemental hypoplasia) are mainly found towards the apical area of the tooth. With age the tooth wears down and exposes these defects on the occlusal surface; food can then penetrate into the infundibular defects where it can become entrapped and bacteria can thrive on this substrate and cause IC. Rostral infundibulae were more often affected by IC than the distal infundibulae. This is likely because there is a less complete cementogenesis in rostral infundibulae compared to distal infundibulae (Suske et al., 2016b).

It was of surprise to find that there was only a limited association found between the prevalence of PC and diet (only linked with feeding 2.1-3 kg concentrates/day), no association with feeding haylage (silage) and no association between IC and diet. In a Western-Australian clinical study, Jackson et al. (2017) found a positive association between the presence of PC and feeding of oaten hay. Horses receiving meadow hay and horses with pasture access all year round versus horses with no pasture access were less likely to have PC.

A possible future clinical study in which all horses receive concentrates with a known amount of fermentable carbohydrates and graze on the same pasture could be performed to further assess if there is indeed limited or no association between diet and PC and/or IC. The volume and pH of saliva produced and contents of saliva, including calcium and fluoride could also be analysed in this proposed study.

Observer was included as random effect in this epidemiological study because there can be differences in findings between different observers, even though all passed the same test on ability to grade. In the grading test in this survey, the most important was that all observers could distinguish between a tooth with PC or IC and a control tooth, rather than between grades of these disorders.

Limitations of the survey were that the number of horses investigated was limited, i.e. 706 horses, despite great efforts for over a 6 month period to obtain more records from suitably qualified practitioners. A further study with a bigger horse population could increase the power of the study.

5.2 Bacteriology

Rather than only establishing risk factors for the development of PC and IC, it was also important to examine the actual aetiology of these two disorders. Dental caries is caused by acidogenic micro-organisms, but it was unknown which micro-organisms are important in the development of equine dental caries, consequently we performed a conventional and molecular microbiological study.

One of the research questions of the survey was to assess for a possible association between PC and IC. The survey showed no such significant association, if observer was taken into account. A further research question was to assess for potential differences in the bacteria which cause these two types of dental caries. The microbiological study showed that there was a difference between the bacterial genera most commonly associated with PC and IC, although the comparison was not performed directly due to big difference in sample size, i.e. larger numbers of PC

samples (the main focus of this PhD study) and a small sample size of IC. A future study could assess higher numbers of IC samples to investigate this area further.

The molecular bacteriological findings of PC were compared to the findings of the periodontal disease study of Kennedy et al. (2016) to compare the microbiota of these two diseases. Although the main bacterial genera identified in equine PC (i.e. *Streptococcus*) differed from those identified in equine periodontal disease (i.e. *Prevotella*) (similar to the differences between human dental caries and human periodontal disease), at family level some bacteria were common to both diseases. The families of bacteria associated with PC in our study (PC versus control) and the periodontal disease study by Kennedy et al. (2016) (periodontal disease vs control), when the results of both LefSE analyses (LDA score PC>2 and LDA score PD >3, both $p < 0.05$) at the family or higher level were compared, were Propionibacteriaceae, Bifidobacteriaceae and Coriobacteriaceae. The families of the control groups which corresponded between these two studies were Burkholderiaceae, Neisseriaceae and Pasteurellaceae. A further study is required to investigate the bacterial flora of the gingiva and dental surface in horses which have both periodontal disease and PC, and of horses which have only periodontal disease or PC, versus control samples.

The disadvantages of using a swab to collect dental plaque for microbiological examination of PC is that it does not remove all dental plaque and therefore may only reflects the microbiota of the plaque surface, because the bacteria which directly overly and actually penetrate into the tooth are not sampled by use of such swabs. However, this is a commonly used method which is not invasive, therefore well tolerated by patients, and still can give a good indication of the bacteriological changes in PC lesions compared to control teeth. This has been the only molecular bacteriological study to examine PC or IC versus control teeth, and has given good insight into the bacteria involved in equine dental caries development. Follow-up studies could use a sterile dental burr to collect deeper samples from the tooth surface as well as the base of the dental plaque.

5.3 Pathology

A pathological study was performed to examine the histological and ultrastructural changes present in PC and IC and to directly examine for the presence of micro-organisms within carious dental tissues. Histologically, Gram positive as well as Gram negative bacteria were found in PC and IC lesions using Gram staining, which generally corresponded to the findings of the microbiological study.

Using TEM and histology, 3 different types of PC lesions were described. It was also observed that the PC lesions were more histologically severe than was expected following gross grading of PC lesions. Even one control tooth was shown to contain micro-organisms in cemental lacunae on its peripheral aspect, and also in what appeared to be a blood vessel deeper in the cementum. These bacterial were found in areas with and without macroscopic cemental discoloration.

Histologically, ellipsoid canals were observed in normal peripheral cementum; some were identified as blood vessels, and others as protrusions or inclusions of PDL containing Sharpey's fibres, fibroblast-like cells and small blood vessels. New cementum had apparently formed within some of these inclusions.

Mitchell et al. (2003) suggested that cementocytes lying within mature peripheral cementum remain active and may continue to play a role in the secretion and deposition of calcium hydroxyapatite crystals. They also found these cementocytes to contain active nuclei and other vital cell organelles. Because cementocytes were not observed using TEM in the current study, we cannot confirm these observations. However, it appears that new cementum was produced inside cemental inclusions, possibly by surrounding cementocytes or alternatively by fibroblast-like cells present in the PDL inclusions. In the PDL, fibroblasts prevent mineralisation of the PDL by secreting specific factors (Nanci, 2008), which might also be the case initially with cemental inclusions that contain viable fibroblasts. Later these inclusions could mineralise when these fibroblasts die off or stop producing such anti-mineralisation factors.

An in vitro model could be developed to imitate PC and IC development in equine teeth to allow future, more objective pathological studies without the use of live horses. A possible study on the prevention and treatment of PC and IC would be to investigate the potential effect of fluoride supplementation, such as by giving horses fluoride tablets. The concentration of fluoride in their drinking water and feedstuffs would need to be established beforehand so that the total dietary fluoride levels are known and could then be supplemented with safe levels of fluoride. The use of chlorhexidine mouthwashes is widely used in Australia to treat and prevent equine PC (Kirsten Jackson, personal communication). Anecdotally this treatment is claimed to be very effective and a critical assessment of its potential value in European horses is also needed.

BIBLIOGRAPHY

Aas, J. A., Griffen, A. L., Dardis, S. R., Lee, A. M., Olsen, I., Dewhirst, F. E., Leys, E. J. and Paster, B. J. (2008) Bacteria of Dental Caries in Primary and Permanent Teeth in Children and Young Adults. *J. Clin. Microbiol. 46*(4), 1407-1417.

Afgan, E., Baker, D., van den Beek, M., Blankenberg, D., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Eberhard, C., Grüning, B., Guerler, A., Hillman-Jackson, J., Von Kuster, G., Rasche, E., Soranzo, N., Turaga, N., Taylor, J., Nekrutenko, A. and Goecks, J. (2016) The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res. 44*(W1), W3-W10.

Alam, S., Brailsford, S. R., Adams, S., Allison, C., Sheehy, E., Zoitopoulos, L., Kidd, E. A. and Beighton, D. (2000) Genotypic heterogeneity of Streptococcus oralis and distinct aciduric subpopulations in human dental plaque. *Appl Environ Microbiol 66*(8), 3330-6.

Auschill, T. M., Arweiler, N. B., Netuschil, L., Brecx, M., Reich, E. and Sculean, A. (2001) Spatial distribution of vital and dead micro-organisms in dental biofilms. *Arch Oral Biol 46*, 471-476.

Avery, J. K. (1992) Essentials of Oral Histology and Embryology: A Clinical Approach, St. Louis: Mosby Year Book, pp. 93-131.

Azaz, B., Ulmansky, M., Moskev, R. and J., S. (1974) Correlation between age and thickness of cementum in impacted teeth. *Oral Surg. 38*, 691-694.

Baker, G. J. (1974) Some aspects of equine dental decay. *Equine Vet. J. 6*, 127-130.

Baker, G. J. (1979) *A study of dental disease in the horse*. Unpublished PhD.

Barbieri, D. d. S. A. V., Vicente, V. A., Fraiz, F. C., Lavoranti, O. J. and Svidzinski, T. I. E. (2007) Analysis of the in vitro adherence of Streprococcus mutans and Candida albicans. *Braz J Microbiol 38*, 624-663.

Bardow, A., Pederson, A. M. L. and Nauntofte, B. (2004) Saliva. in Miles, T. S., Nauntofte, B., Svensson, P., (ed.) *Clinical Oral Physiology*,Copenhagen: Quintessence. pp. 17-33.

Benítez-Páez, A., Belda-Ferre, P., Simón-Soro, A. and Mira, A. (2014) Microbiota diversity and gene expression dynamics in human oral biofilms. *BMC Genomics 15*(1), 311.

Bennet, D. (2008) The evolution of the horse. in Evans, J. W., (ed.) *Horse Breeding and Management*,Amsterdam: Elsevier. pp. 1-37.

Berkovitz, B. K. B., Holland, G. R. and Moxham, B. (2009) Oral Anatomy, Histology and embryology, 4th Edn. ed., Edinburgh: Elsevier, pp. 169-179.

Berkovitz, B. K. B. and Moxham, B. (1981) Development of dentition: early stages of tooth development. in *Dental Anatomy and Embryology*,Oxford: Blackwell Scientific, pp. 166-174.

Bertacci, A., Chersoni, S., Davidson, C. L. and Prati, C. (2007) In vivo enamel fluid movement. *Eur J Oral Sci, 115*, 169–173.

Bertassoni, L. E., Stankoska, K. and Swain, M. V. (2012) Insights into the structure and composition of the peritubular dentin organic matrix and the lamina limitans. *Micron, 43*(2), 229-236.

Bhaskar, S. N. (1991) Orban's Oral Histology and Embryology, 11th edn. ed., St. Louis: Mosby Year Book, pp. 28-138.

Blake, R. (1798) De dentium formatione et structura in homine et in varíis animalibus. MD Thesis, University of Edinburgh.

Borggreven, J. M. P. M., Van Dijk, J. W. E. and Driessens, F. C. M. (1977) A quantitative radiochemical study of ionic and molecular transport in bovine dental enamel. *Arch Oral Biol 22*, 467-472.

Borkent, D. and Dixon, P. M. (2015) Equine peripheral and infundibular dental caries: a review and proposals for its investigation. *Equine Vet. Educ. 29* (11), 621-628.

Borkent, D., Reardon, R. J. M., McLachlan, J., Smith, S. and Dixon, P. M. (2016) An epidemiological survey on the prevalence of equine peripheral dental caries

in the United Kingdom and possible risk factors for its development. *Equine Vet J.*49 (4), 480-485.

Boyde, A. (1989) Enamel. in Berkovitz, B. K. B., Boyde, A., Frank, R.M., Höhling, H.J., Moxham, B.J., Nalbandian, J., Tonge C.H., (ed.) *Teeth. Handbook of Microscopic Anatomy*,New York: Springer Verlag. pp. 309-473.

Boyde, A. (1997) Equine dental tissues: a trilogy of enamel, dentine and cementum. *Equine Vet. J. 29* (3), 171-172.

Braden, M., Duckworth, R. and Joyston-Bechal, S. (1971) The uptake of 24Na by human dental enamel. *Arch Oral Biol 16*, 367-374.

Bradford, E. W. (1967) Microanatomy and histochemistry of dentine. in Miles, A. E. W., (ed.) *Structural and Chemical Organisation of Teeth*,New York: Academic Press. pp. 3-34.

Bradley, O. C. (1923) The Topographical Anatomy of the Head and Neck of the Horse, Edinburgh: W Green & Son Ltd, pp. 85-92.

Briso, A. L. F., Gonçalves, R. S., Costa, F., Gallinari, M. O., Cintra, L. T. A. and Santos, P. H. (2015) Demineralization and Hydrogen Peroxide Penetration in Teeth with Incipient Lesions. *Brazilian Dental Journal 26*, 135-140.

Brunelle, J. A. and Carlos, J. P. (1990) Recent trends in dental caries in US children and the effect of water fluoridation. *J. Dent. Res. 69*, 723-727.

Buchalla, W. (2013) Histological and clinical appearance of caries. in Meyer-Lueckel, H., Paris, S., Ekstrand, K.R., (ed.) *Caries Management-Science and Clinical Practice*,Stuttgart: Georg Thieme Verlag KG.

Büdras, K.-D., Sack, W. O. and Röck, S. (2003) Anatomy of the Horse: An Illustrated Text, 4th edn ed., Hannover: Schlütersche, pp. 28-35.

Bühler, M., Fürst, A., Lewis, F. I., Kummer, M. and Ohlert, S. (2014) Computed tomographic features of apical infection of equine maxillary cheek teeth: A retrospective study of 49 horses. *Equine Vet J. 46*, 468-473.

Burke, A. and Castanet, J. (1995) Histological Observations of Cementum Growth in Horse Teeth and their Application to Archaeology. *Journal of Archaeological Science 22*, 479-493.

Butler, P. (1991) Dentition in function. in Osborn, J. W., (ed.) *Dental Anatomy and Embryology*, Oxford: Blackwell Scientific, pp. 345-346.

Byers, M. R. and Yoon Lin, K. J. (2003) Patterns of fluoro-gold entry into rat molar enamel, dentine, and pulp. *J Dent Res 82*, 312-317.

Callaway, T. R., Dowd, S. E., Edrington, T. S., Anderson, R. C., Krueger, N., Bauer, N., Kononoff, P. J. and Nisbet, D. J. (2010) Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. *J Anim Sci 88*(12), 3977-83.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N. and Knight, R. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences 108*(Supplement 1), 4516-4522.

Carlsson, J. (1997) Bacterial metabolism in dental biofilms. *Adv Dent Res, 11*(1), 75-80.

Chandra, S., S., C., M., C. and Chandra, N. (2004) Textbook of Dental and Oral Histology and Embryology and Multiple Choice Questions, New Delhi: Jaypee Brothers Medical Publishers Ltd, pp. 41-132.

Chang, Y.-J., Pukall, R., Saunders, E., Lapidus, A., Copeland, A., Nolan, M., Glavina Del Rio, T., Lucas, S., Chen, F., Tice, H., Cheng, J.-F., Han, C., Detter, J. C., Bruce, D., Goodwin, L., Pitluck, S., Mikhailova, N., Liolios, K., Pati, A., Ivanova, N., Mavromatis, K., Chen, A., Palaniappan, K., Land, M., Hauser, L., Jeffries, C. D., Brettin, T., Rohde, M., Göker, M., Bristow, J., Eisen, J. A., Markowitz, V., Hugenholtz, P., Kyrpides, N. C. and Klenk, H.-P. (2010) Complete genome sequence of Acidaminococcus fermentans type strain (VR4(T)). *Standards in Genomic Sciences 3*(1), 1-14.

Chao, A. (1984) Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics 11*(4), 265-270.

Chávez de Paz, L. E., Hamilton, I. R. and Svensäter, G. (2008) Oral bacteria in biofilms exhibit slow reactivation from nutrient deprivation. *Microbiology 154*, 1927-1938.

Chen, L., Qin, B., Du, M., Zhong, H., Xu, Q., Li, Y., Zhang, P. and Fan, M. (2015) Extensive Description and Comparison of Human Supra-Gingival Microbiome in Root Caries and Health. *PLoS ONE, 10*(2), e0117064.

Chen, T., Shi, Y., Wang, X., Wang, X., Meng, F., Yang, S., Yang, J. and Xin, H. (2017) Highthroughput sequencing analyses of oral microbial diversity in healthy people and patients with dental caries and periodontal disease. *Mol Med Rep 16*(1), 127-132.

Chen, T., Yu, W.-H., Izard, J., Baranova, O. V., Lakshmanan, A. and Dewhirst, F. E. (2010) The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database, 2010*.

Chhour, K. L., Nadkarni, M. A., Byun, R., Martin, F. E., Jacques, N. A. and Hunter, N. (2005) Molecular analysis of microbial diversity in advanced caries. *J Clin Microbiol 43*(2), 843-9.

Cohen, S. and Burns, R. C. (1998) Pathways of the pulp, St. Louis: Mosby, Inc., pp. 386-475.

Colwell, R. K. and Coddington, J. A. (1994) Biodiversity: measurement and estimation - Estimating terrestrial biodiversity through extrapolation. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 345*(1311), 101.

Colyer, J. F. (1906) Variations and diseases of teeth of horses. *Transactions of the Odontological Society of Great Britain 38*, 47–74.

Colyer, J. F. (1906) Variations and diseases of the teeth of horses. *Trans Odontol Soc GB, 38*.

Cook, G. M., Rainey, F. A., Chen, G., Stackebrandt, E. and Russell, J. B. (1994) Emendation of the description of Acidaminococcus fermentans, a trans-aconitate- and citrate-oxidizing bacterium. *Int J Syst Bacteriol 44*(3), 576-8.

Costalonga, M. and Herzberg, M. C. (2014) The oral microbiome and the immunobiology of periodontal disease and caries. *Immunology letters, 162*(2a), 22-38.

Cox, A., Dixon, P. M. and Smith, S. (2012) Histopathological lesions associated with equine periodontal disease. *Vet J 194*, 386-391.

Crabill, M. R. and Schumacher, J. (1998) Pathophysiology of acquired dental diseases of the horse. *Vet. Clin. N. Am.: Equine Pract 14*, 291-307

Culling, C. F. A. (1974) Handbook of Histopathological and Histochemical Techniques, 3rd ed. ed., London: Butterworths, pp. 211-467.

Dacre, I., Kempson, S. A. and Dixon, P. M. (2007) Equine idiopathic cheek teeth fractures. Part 1: Pathological studies on 35 fractured cheek teeth. *Equine Vet. J. 39*, 310-318.

Dacre, I. T. (2005a) Equine dental pathology. in Baker, G. J. and Easley, K. J., (eds.) *Equine Dentistry*,Edinburgh: Elsevier Saunders, pp. 91-110.

Dacre, I. T. (2005b) A pathological study of equine dental disorders, Edinburgh: PhD Thesis. University of Edinburgh.

Dacre, I. T. (2006) Caries of peripheral cementum. in *Focus on Dentistry*,American Association of Equine Practitioners, Indianapolis, USA. pp. 91-92.

Dacre, I. T., Kempson, S. A. and Dixon, P. M. (2008a) Pathological studies of cheek teeth apical infections in the horse: 1. Normal endodontic anatomy and dentinal structure of equine cheek teeth. *Vet J 178*, 311-320.

Dacre, I. T., Kempson, S. A. and Dixon, P. M. (2008b) Pathological studies of cheek teeth apical infections in the horse: 4. Aetiopathological findings in 41 apically infected mandibular cheek teeth. *Vet J 178*(3), 341-351.

Dacre, I. T., Kempson, S. A. and Dixon, P. M. (2008c) Pathological studies of cheek teeth apical infections in the horse: 5. Aeticopathological findings in 57 apically infected maxillary cheek teeth and histological and ultrastructural findings. *Vet J 178*, 352-363.

Dacre, I. T., Shaw, D. J. and Dixon, P. M. (2008d) Pathological studies of cheek teeth apical infections in the horse: 3. Quantitative measurements of dentine in apically infected cheek teeth. *Vet J 178*(3), 333-40.

Dawes, C. (2003) What is the critical pH and why does a tooth dissolve in acid? *J Can Dent Assoc 69*(11), 722-724.

Dean, H. T., Arnold Jr, F. A., Jay, P. and Knutson, J. W., 1950. Studies on mass control of dental caries through fluoridation of the public water supply. Public Health Reports (1896-1970), pp.1403-1408. (1950) Studies on mass control of dental caries through fluoridation of the public water supply. *Public Health Reports (1896-1970)*, 01403-1408

Dellmann, H. D. and Brown, E. (1976) Textbook of Veterinary Histology, Philadelphia: Lea and Febiger, pp. 214–219.

Dent, V. E. (1979) The bacteriology of dental plaque from a variety of zoo-maintained mammalian species. *Archives of Oral Biology 24*, 277-282.

Dewhirst, F. E., Paster, B. J., Tzellas, N., Coleman, B., Downes, J., Spratt, D. A. and Wade, W. G. (2001) Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the family Coriobacteriaceae: description of olsenella gen. nov., reclassification of Lactobacillus uli as Olsenella uli comb. nov. and description of Olsenella profusa sp. nov. *International Journal of Systematic and Evolutionary Microbiology 51*(5), 1797-1804.

Dixon, P. M. (2002) The gross, histological, and ultrastructural anatomy of equine teeth and their relationship to disease, Orlando: Proceedings of the 48th AAEP Annual Convention, pp. 421-437.

Dixon, P. M. and Copeland, A. N. (1993) The radiological appearance of mandibular cheek teeth in ponies of different ages. *Equine Vet Educ 5*, 317-323.

Dixon, P. M. and du Toit, N. (2010) Dental anatomy. in Easley, K. J., Dixon, P. M. and Schumacher, J., (eds.) *Equine Dentistry*,Edinburgh: Elsevier Saunders. pp. 51-77.

Dixon, P. M., du Toit, N. and Dacre, I. T. (2010) Equine dental pathology. in Easley, K. J., Dixon, P. M. and Schumacher, J., (eds.) *Equine Dentistry*,Edinburgh: Elsevier Saunders. pp. 129-147.

Dixon, P. M., Savill, D., Horbyl, A., Reardon, R. J. M. and Liuti, T. (2014) Critical evaluation of ex-vivo restoration of carious equine maxillary cheek teeth infundibulae following high pressure gas and aluminium hydroxide micro-particle abrasion. *Vet. J. 200*, 368-374.

Dixon, P. M., Tremaine, W. H., Pickles, K., Kuhns, L., Hawe, C., McCann, J., McGorum, B. C., Railton, D. I. and Brammer, S. (2000) Equine dental disease Part 3: A long-term study of 400 cases: disorders of wear, traumatic damage and idiopathic fractures, tumours and miscellaneous disorders of the cheek teeth. *Equine Vet J. 32*(1), 9-18.

Dohoo, I., Martin, W. and Stryhn, H. (2003) Veterinary Epidemiologic Research, Charlottetown: AVC Inc.

Douglas, C. W. (1994) Bacterial-protein interactions in the oral cavity. *Advances in dental research 8*(2), 254-262.

du Toit, N., Kempson, S. A. and Dixon, P. M. (2008a) Donkey dental anatomy Part 1: Gross and computed axial tomography examinations. *Vet J 176*, 338-244.

du Toit, N., Kempson, S. A. and Dixon, P. M. (2008b) Donkey dental anatomy Part 2: Histological and scanning electron microscopy examinations. *Vet J 176*, 345-353.

du Toit, N., Kempson, S. A. and Dixon, P. M. (2008c) Pathological investigation of caries and occlusal pulpar exposure in donkey cheek teeth using computerised axial tomography with histological and ultrastructural examination. *Vet J 178*, 387-395.

Dyce, K. M., Sack, W. O. and Wensing, C. J. G. (1987) Textbook of Veterinary Anatomy, Philadelphia: WB Saunders, pp. 473-477.

Eissaa, O. E., El-Shourbagy, E. M. and Ghobashy, S. A. (2013) In vivo effect of a fluoride releasing adhesive on inhibition of enamel demineralization around orthodontic brackets. *Tanta Dental Journal 10*(2), 86-96.

Engelking, L. R. (2002) Review of Veterinary Physiology, Jackson: Teton NewMedia, pp. 321-322.

Erridge, M. E., Cox, C. L. and Dixon, P. M. (2012) A histological study of peripheral dental caries of equine cheek teeth. *J. Vet. Dent. 29*, 150-156.

Esty, W. W. (1986) The Efficiency of Good's Nonparametric Coverage Estimator. *The Annals of Statistics 14*(3), 1257-1260.

Fava, M., Watanabe, I. S., Fava-de-Moraes, F. and Costa, L. R. R. S. (1997) Prismless enamel in human non-erupted deciduous molar teeth: a scanning electron microscopic study. *Revista de Odontologia da Universidade de Sao Paulo, 11*.

Ferguson, M. (1990) The dentition throughout life. in Elderton, R. J., (ed.) *The Dentition and Dental Care*,Oxford: Heinemann. pp. 1-18.

FitzGerald, C. M. (1998) Do enamel microstructures have regular time dependency? Conclusions from the literature and a large-scale study. *J Hum Evol 35*, 371-386.

Fitzgibbon, C. M., du Toit, N. and Dixon, P. M. (2010) Anatomical studies of maxillary cheek teeth infundibula in clinically normal horses. *Equine Vet. J. 42*, 37-43.

Frank, R. M. (1990) Structural events in the caries process in enamel, cementum and dentin. *Acta Zoologica Fennica 180*, 1-76.

Fuller, R. (1966) Some Morphological and Physiological Characteristics of Gram Negative Anaerobic Bacteria isolated from the Alimentary Tract of the Pig. *Journal of Applied Bacteriology 29*(2), 375-379.

Galan, J. C., Reig, M., Navas, A., Baquero, F. and Blazquez, J. (2000) ACI-1 from Acidaminococcus fermentans: characterization of the first beta-lactamase in Anaerobic cocci. *Antimicrob Agents Chemother 44*(11), 3144-9.

Gao, W., Chan, Y., You, M., Lacap-Bugler, D. C., Leung, W. K. and Watt, R. M. (2016) In-depth snapshot of the equine subgingival microbiome. *Microb Pathog 94*, 76-89.

Genco, R. J., Yecies, L. and Karush, F. (1969) The immunoglobulins of equine colostrum and parotid fluid. *The Journal of Immunology 103*(3), 437-444.

Gere, I. and Dixon, P. M. (2010) Post mortem survey of peripheral dental caries in 510 Swedish horses. *Equine Vet. J. 42*, 310-315.

Gingerich, P. D. (1981) Variation, sexual dimorphism, and social structure in the early Eocene horse Hyracotherium (Mammalia, Perissodactyla). *Paleobiology 7*, 443-455.

Goldberg, M. (2016) Understanding Dental Caries. From Pathogenesis to Prevention and Therapy., Basel: Springer International Publishing, pp. 29-40.

Gorrel, C. (2013) Veterinary Dentistry for the General Practitioner, 2nd edn ed., London: Elsevier Saunders, pp. 191.

Gotelli, N. J. and Colwell, R. K. (2011) Estimating species richness. in Magurran, A. E. and McGill, B. J., (eds.) *Frontiers in Measuring Biodiversity*,New York: Oxford University Press. pp. 39-54.

Gross, E. L., Beall, C. J., Kutsch, S. R., Firestone, N. D., Leys, E. J. and Griffen, A. L. (2012) Beyond Streptococcus mutans: Dental Caries Onset Linked to Multiple Species by 16S rRNA Community Analysis. *PLoS ONE, 7*(10), e47722.

Gwinnett, A. J. (1966) The ultrastructure of the "prismless" enamel of deciduous teeth. *Archives of Oral Biology 11*(11), 1109-IN16.

Gwinnett, A. J. (1967) The ultrastructure of the "prismless" enamel of permanent human teeth. *Archives of Oral Biology 12*(3), 381-IN30.

Hannig, C., Hannig, M., Rehmer, O., Braun, G., Hellwig, E. and Al-Ahmad, A. (2007) Fluorescence microscopic visualization and quantification of initial bacterial colonization on enamel in situ. *Archives of Oral Biology 52*(11), 1048-1056.

Hannig, M. and Joiner, A. (2006) The structure, function and properties of the acquired pellicle. in Duckworth, R., (ed.) *The teeth and their environment*,Basel: Monogr Oral Sci, Karger. pp. 29-64.

Hargreaves, K. M. and Berman, L. H. (2016) Cohen's Pathways of the Pulp, 11th edn. ed., St. Louis: Elsevier Inc., pp. 532-660.

Havers, C. (1691) Osteologia nova, or, Some new observations of the bones, and the parts belonging to them, with the manner of their accretion, and nutrition, communicated to the Royal Society in several discourses to which is added a fifth discourse of the cartilages, London: Samuel Smith, pp. 78-88.

Henry, R. J. (2008) Plant Genotyping II: SNP Technology, Wallingford: CAB International, pp. 227-237.

Hillson, S. (2005a) Dental Tissues. in *Teeth*,Cambridge: Cambridge University Press. pp. 147-171.

Hillson, S. (2005b) Teeth, Cambridge: Cambridge University Press, pp. 194-198.

Hoffman, R. M. (2009) Carbohydrate metabolism and metabolic disorders in horses. *Revista Brasileira de Zootecnia 38*, 270-276.

Holland, G. R. (1994) Morphological features of dentin and pulp related to dentin sensitivity. *Archives of Oral Biology 39*, supplement 3-11.

Honma, K., Yamakawa, M., Yamauchi, S. and Hosoya, S. (1962) Statistical study on the occurrence of dental caries of domestic animal: I. Horse. *Jpn. J. Vet. Res. 10*, 31-36.

Hosmer, D. W., Lemeshow, S. and Sturdivant, R. X. (2013) Applied logistic regression, Third edition ed., New York: Wiley.

Huidekoper, R. S. (1981) Age of Domestic Animals, London: F.A. Davis, pp. 33-35.

Itoh, I. and Saito, H. (1992) Coronal cementum in the horse molar teeth. *Jpn. J. Oral Biol. 34*, 274-279.

Jackson, K., Kelty, E. and Tennant, M. (2017) Equine peripheral dental caries: An epidemiological survey assessing prevalence and possible risk factors in Western Australian horses. *Equine Veterinary Journal*, Epub ahead of printed version DOI: 10.1111/evj.12718.

Jardine, P. E., Janis, C. M., Sahney, S. and Benton, M. J. (2012) Grit not grass: Concordant patterns of early origin hypsodonty in Great Plains ungulates and Glires. *Palaeography, Palaeoclimatology, Palaeoecology 365-366*, 1-10.

Joest, E. (1915) Odontologische Notizen. *Berl. Tierärztl. Wschr., 31*, 61-66.

Joest, E. (1926) Handbuch der Speziellen Pathologischen Anatomie der Haustiere, 2nd edn ed., pp. 115-597.

Joest, E., Chorin, A., Finger, H. and Westman, O. (1922) Studien über das Backzahngebiss des Pferdes: mit besonderer Berücksichtigung seiner postembtyonalen Entwicklung und seines Einflusses auf den Gesichsschädel und die Kieferhöhle, Berlin: Schoetz, pp. 1-133.

Jones, S. J. (1981) Human tissue: cement. in Osborn, J. W., (ed.) *Dental Anatomy and Embryology*,Oxford: Blackwell Scientific. pp. 193-209.

Jones, S. J. and Boyde, A. (1974) Coronal cementogenesis in the horse. *Archs oral Biol. 19*, 605-614.

Jones, S. J. and Boyde, A. (1994) Questions of quality and quantity – a morphological view of bone biology. *Actu Anatoniica Nipponicu 69*, 229-243.

Joyston-Bechal, S., Duckworth, R. and Braden, M. (1971) Diffusion of radioactive ions into human dental enamel. *Arch Oral Biol 16*, 375–384.

Jubb, K. V. F., Kennedy, P. C. and Palmer, N. (1985) Pathology of Domestic Animals, 3rd edn ed., London: Acacemic Press Inc., pp. 9-10.

Jumas-Bilak, E., Carlier, J. P., Jean-Pierre, H., Mory, F., Teyssier, C., Gay, B., Campos, J. and Marchandin, H. (2007) Acidaminococcus intestini sp. nov., isolated from human clinical samples. *Int J Syst Evol Microbiol 57*(Pt 10), 2314-9.

Kang, Y. J., Cheng, J., Mei, L. J., Hu, J., Piao, Z. and Yin, S. X. (2010) Multiple copies of 16s rRNA gene affect the restriction patterns and DGGE profile as revealed by analysis of genome database. *Mikrobiologiia 79*(5), 664-71.

Karpiński, T. M. and Szkaradkiewicz, A. K. (2013) Microbiology of dental caries. *3*(1), 4.

Kennedy, R., Lappin, D. F., Dixon, P. M., Buijs, M. J., Zaura, E., Crielaard, W., O'Donnell, L., Bennett, D., Brandt, B. W. and Riggio, M. P. (2016) The microbiome associated with equine periodontitis and oral health. *Veterinary Research 47*(1), 49.

Keyes, P. H. (1960) Infectious and Transmissable Nature of Experimental Dental Caries. *Archive of Oral Biology 1*(4), 304-320.

Kianoush, N., Adler, C. J., Nguyen, K. T., Browne, G. V., Simonian, M. and N., H. (2014) Bacterial profile of dentine caries and the impact of pH on bacterial population diversity. *PLoS ONE, 9*(3).

Kidd, E. A. M. (2005) Essentials of Dental Caries: The Disease and Its Management, 3rd edn ed., Oxford: Oxford University Press, pp. 4-135.

Kierdorf, H., Kierdorf, U., Frölich, K. and Witzel, C. (2013) Lines of Evidence–Incremental Markings in Molar Enamel of Soay Sheep as Revealed by a Fluorochrome Labeling and Backscattered Electron Imaging Study. *PLoS ONE, 8*(9), e74597.

Kilic, S., Dixon, P. M. and Kempson, S. A. (1997a) A light microscopic and ultrastructural examination of calcified dental tissues of horses: 2. Ultrastructural enamel findings. *Equine Vet. J. 29*(3), 198-205.

Kilic, S., Dixon, P. M. and Kempson, S. A. (1997b) A light microscopic and ultrastructural examination of calcified dental tissues of horses: 3. Dentine. *Equine Vet. J. 29*(3), 206-212.

Kilic, S., Dixon, P. M. and Kempson, S. A. (1997c) A light microscopic and ultrastructural examination of calcified dental tissues on horses: 4. Cement and the amelocemental junction. *Equine Vet J. 29*(3), 213-219.

Kleinberg, I. (2002) A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to Streptococcus mutans and the specific-plaque hypothesis. *Crit Rev Oral Biol Med 13*, 108-125.

Kleter, G. A. (1998) Discoloration of dental carious lesions (a review). *Archives of Oral Biology 43*, 629-632.

Klugh, D. O. (2010) Principles of Equine Dentistry, London: Manson Publishing Ltd, pp. 27-188.

Koch, R. (1890) in Xth International Congress of Medicine, Berlin.

Kollar, E. J. and Lumsden, A. G. S. (1979) Tooth morphology: the role of innervation during induction and pattern formation. *J Biol Buccale 7*, 49-60.

König, H. E. and Liebich, H. G. (2007) Veterinary Anatomy of Domestic Mammals, 3rd edn ed., Stuttgart: Schattauer GmbH, pp. 301-368.

Kopke, S., Angrisani, N. and Staszyk, C. (2012) The dental cavities of equine cheek teeth: three-dimensional reconstructions based on high resolution micro-computed tomography *Veterinary Research 8*, 173-189.

Kottke-Marchant, K. and Davis, B. (2012) Laboratory Hematology Practice,

, Chichester, UK: Blackwell Publishing Ltd., pp. 148-150.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. and Schloss, P. D. (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. . *Applied and Environmental Microbiology 79*(17), 5112-20.

Kraatz, M., Wallace, R. J. and Svensson, L. (2011) Olsenella umbonata sp. nov., a microaerotolerant anaerobic lactic acid bacterium from the sheep rumen and pig jejunum, and emended descriptions of Olsenella, Olsenella uli and Olsenella profusa. *International Journal of Systematic and Evolutionary Microbiology 61*(4), 795-803.

Krstic, R. V. (1994) Human Microscopic Anatomy: An atlas for Students of Medicine and Biology, Springer-Verlag Berlin Heidelberg GmbH, pp. 168-169.

Kumar, G. S. (2014) Orban's Oral Histology & Embryology, Elsevier Health Sciences, pp. 50-87.

Latshaw, W. K. (1987) Veterinary Developmental Anatomy: A Clinically Oriented Approach, Toronto: B.C. Decker, pp. 87-112.

Lee, L., Reardon, R. J. M. and Dixon, P. M. (2017) A post-mortem study on the prevalence of peripheral dental caries in Scottish horses. *Equine Veterinary Education*, Epub ahead of printed version DOI: 10.1111/eve.12783.

Levallois, B., Terrer, E., Panayotov, Y., Salehi, H., Tassery, H., Tramini, P. and Cuisinier, F. (2012) Molecular structural analysis of carious lesions using micro-Raman spectroscopy. *Eur J Oral Sci*, 1-8.

Lindén, L. A. (1968) Microscopic observations of fluid flowing through enamel in vitro. *Odontol Rev 19*, 349–365.

Linkous, M. B. (2006) Dental conditions affecting the juvenile performance horse (2-5 years). AAEP Focus Meeting, Indianapolis.

Liuti, T., Reardon, R. and Dixon, P. M. (2017) Computed tomographic assessment of equine maxillary cheek teeth anatomical relationships, and paranasal sinus volumes. *Veterinary Record*.

Loesche, W. J., Rowan, J., Straffon, L. H. and Loos, P. J. (1975) Association of Streptococcus mutans with Human Dental Decay. *Infection and Immunity, 11*(6), 1252-1260.

Lundström, T. and Pettersson, H. (1988) Den Svenska hästens munhålestatus. *Svensk Veterinartidning, 40*, 247-252.

Lundström, T. and Pettersson, H. (1990) Den svenska hästens munhålestatus II. *Svensk Veterinartidning 42*, 559-563.

Lundström, T. S., Dahlen, G. G. and Wattle, O. S. (2007) Caries in the infundibulum of the second upper premolar tooth in the horse. *Acta Vet Scand 49*, 10.

MacFadden, B. J. (1992) Fossil horses: systematics, paleobiology, and evolution of the family Equidae., New York: Cambridge University Press.

MacFadden, B. J. (2010) Equine Dental Evolution: Perspective from the Fossil Record. in Easley, J., Dixon, P. M. and Schumacher, J., (eds.) *Equine Dentistry*.

Maripandi, A., Arun Kumar, T. and Al Salamah, A. A. (2011) Prevalence of dental caries bacterial pathogens and evaluation of inhibitory concentration effect on different tooth pastes against Streptococcus spp. *African Journal of Microbiology Research 5*(14), 1778-1783.

Marsh, P. D. (2005) Dental plaque: biological significance of a biofilm and community life-style. *J. Clin. Periodontol. 32*(suppl. 6), 7-15.

Marsh, P. D., Martin, M. V., Lewis, M. A. O. and Williams, D. W. (2009) Oral Microbiology, Edinburgh: Elsevier, pp. 1-213.

Marshall, R., Shaw, D. J. and Dixon, P. M. (2012) A study of sub-occlusal secondary dentine thickness in overgrown equine cheek teeth. *Vet. J. 193*(1), 53-57.

Martin, M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal 17*(1), 10-12.

McGeady, T. A., Quinn, P. J., FitzPatrick, E. S. and Ryan, M. T. (2006) Veterinary Embryology, Oxford: Blackwell Publishing Ltd, pp. 268-285.

McLean, J. S., Fansler, S. J., Majors, P. D., McAteer, K., Allen, L. Z., Shirtliff, M. E., Lux, R. and Shi, W. (2012) Identifying Low pH Active and Lactate-Utilizing Taxa within Oral Microbiome Communities from Healthy Children Using Stable Isotope Probing Techniques. *PLoS ONE, 7*(3), e32219.

Metwalli, K. H., Khan, S. A., Krom, B. P. and Jabra-Rizk, M. A. (2013) Streptococcus mutans, Candida albicans, and the human mouth: a sticky situation. *PLoS Pathog 9*(10).

Miles, A. E. W. and Grigson, C. (1990) Colyer's Variations and Diseases of the Teeth of Animals (Revised Edition).

Miller, W. D. (1889) Die Mikroorganismen der Mundhohle, Leipzig: Georg Thieme.

Miranda, C. B., Pagani, C., Benetti, A. R. and Matuda, F. S. (2005) Evaluation of the bleached human enamel by Scanning Electron Microscopy. *Journal of Applied Oral Science 13*, 204-211.

Mitchell, S. R., Kempson, S. A. and Dixon, P. M. (2003) Structure of peripheral cementum of normal equine cheek teeth. *J Vet Dent 20*, 199-208.

Moore, N. T., Schroeder, W. and Staszyk, C. (2016) Equine odontoclastic tooth resorption and hypercementosis affecting all cheek teeth in two horses: Clinical and histopathological findings. *Equine Veterinary Education 28*(3), 123-130.

Morrison, J., Laurie, C. C., Marazita, M. L., Sanders, A. E., Offenbacher, S., Salazar, C. R., Conomos, M. P., Thornton, T., Jain, D., Laurie, C. A., Kerr, K. F., Papanicolaou, G., Taylor, K., Kaste, L. M., Beck, J. D. and Shaffer, J. R. (2016) Genome-wide association study of dental caries in the Hispanic Communities Health Study/Study of Latinos (HCHS/SOL). *Hum Mol Genet 25*(4), 807-16.

Muylle, S., Simoens, P. and Lauwer, H. (2001) The distribution of intratubular dentin in equine incisors: a scanning electron microscopic study. *Equine Vet. J. 33*(1), 65-69.

Muylle, S., Simoens, P. and Lauwers, H. (1999) Age-related morphometry of equine incisors. *J. Vet. Med. A 46*, 633-643.

Muylle, S., Simoens, P. and Lauwers, H. (2000) The dentinal structure of equine incisors: a light and scanning electron-microscopic study. *Cells Tissues Organs 167*(4), 273-84.

Nanci, A. (2008) Ten Cate's Oral Histology: Development, Structure and Function. in,Missouri: Mosby Elsevier. pp. 79-268.

Nascimento, M. M., Zaura, E., Mira, A., Takahashi, N. and Ten Cate, J. M. (2017) Second Era of OMICS in Caries Research: Moving Past the Phase of Disillusionment. *J Dent Res 96*(7), 733-740.

Newbrun, E. (1983) Cariology, 2nd Edn. ed., Baltimore: Williams & Wilkins, pp. 161-255.

Obata, J., Takeshita, T., Shibata, Y., Yamanaka, W., Unemori, M., Akamine, A. and Yamashita, Y. (2014) Identification of the Microbiota in Carious Dentin Lesions Using 16S rRNA Gene Sequencing. *PLoS ONE 9*(8), e103712.

Oliver, J. D. (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev. 34*, 415-425.

Olsen, I. (2006) New principles in ecological regulation - features from the oral cavity. *Microbial Ecology in Health and Disease 18*, 26-31.

Owen, R. (1845) Odontography: A Treatise On The Comparative Anatomy Of The Teeth., London: Hippolyte Bailliere, pp. 572-582.

Perkins, J. (2001) Quantitative and qualitative anatomy of the equine maxillary sinuses with particular reference to the nasomaxillary aperture, Edinburgh: MScThesis, University of Edinburgh

Peterson, S. N., Meissner, T., Su, A. I., Snesrud, E., Ong, A. C., Schork, N. J. and Bretz, W. A. (2014) Functional expression of dental plaque microbiota. *Frontiers in Cellular and Infection Microbiology 4*, 108.

Peterson, S. N., Snesrud, E., Schork, N. J. and Bretz, W. A. (2011) Dental caries pathogenicity: a genomic and metagenomic perspective. *International dental journal, 61*(0 1), 11-22.

Poole, D. F., Tailby, P. W. and Berry, D. C. (1963) The movement of water and other molecules through human enamel. *Arch Oral Biol 38*, 771–772.

R Core Team (2014) R: A language and environment for statistical computing. Vienna, Austria. URL http://www.R-project.org/.

Ramzan, P. H. L. and Palmer, L. (2010) Occlusal fissures of the equine cheek tooth: Prevalence, location and association with disease in 91 horses referred for dental investigation. *Equine Vet J. 42*(2), 124-128.

Ramzan, P. H. L. and Palmer, L. (2011) The incidence and distribution of peripheral caries in the cheek teeth of horses and its association with diastemata and gingival recession. *Vet. J. 190*, 90-93.

Richardson, J. D., Lane, J. G. and Waldron, K. R. (1994) Is dentition an accurate indication of the age of a horse? *Veterinary Rec 137*, 88-90.

Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N. N., Anderson, I. J., Cheng, J.-F., Darling, A., Malfatti, S., Swan, B. K., Gies, E. A., Dodsworth, J. A., Hedlund, B. P., Tsiamis, G., Sievert, S. M., Liu, W.-T., Eisen, J. A., Hallam, S. J., Kyrpides, N. C., Stepanauskas, R., Rubin, E. M., Hugenholtz, P. and Woyke, T. (2013) Insights into the phylogeny and coding potential of microbial dark matter. *Nature 499*(7459), 431-437.

Ripa, L. W., Gwinnett, A. J. and Buonocore, M. G. (1966) The "prismless" outer layer of deciduous and permanent enamel. *Arch Oral Biol 11*(1), 41-8.

Rodrigues, J. B., Dixon, P. M., Bastos, E., San Roman, F. and Viegas, C. (2013) A clinical survey on the prevalence and types of cheek teeth disorders present in 400 Zamorano-Leonés and 400 Mirandês donkeys (Equus asinus). *Veterinary Rec 173*, 581.

Rogosa, M. (1969) Acidaminococcus gen. n., Acidaminococcus fermentans sp. n., anaerobic gram-negative diplococci using amino acids as the sole energy source for growth. *J Bacteriol 98*(2), 756-66.

Sahara, N. (2014) Development of coronal cementum in hypsodont horse cheek teeth. *The Anatomical Record, 297*, 716-730.

Samaranayake, L. (2012) Essential Microbiology for Dentistry, 4th edition ed., London: Elsevier, pp. 139-286.

Schloss, P. D. and Westcott, S. L. (2011) Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. *Appl Environ Microbiol 77*(10), 3219-26.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. and Weber, C. F. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol 75*(23), 7537-41.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods 9*, 671.

Schroeder, H. E. (1986) The Periodontium. Handbook of Microscopic Anatomy, Berlin: Springer, pp. 23-129.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. and Huttenhower, C. (2011) Metagenomic biomarker discovery and explanation. *Genome Biol, 12*.

Shaffer, J. R., Feingold, E., Wang, X., Lee, M., Tcuenco, K., Weeks, D. E., Weyant, R. J., Crout, R., McNeil, D. W. and Marazita, M. L. (2013) GWAS of Dental Caries Patterns in the Permanent Dentition. *Journal of Dental Research 92*(1), 38-44.

Sharpton, T. J. (2014) An introduction to the analysis of shotgun metagenomic data. *Frontiers in Plant Science 5*, 209.

Shellis, R. P. (2000) Transport processes in enamel and dentine. in Addy, M., Embery, G., Edgar, W. M. and Orchardson, R., (eds.) *Tooth wear and sensivity*,London: Martin Dunitz Publishers. pp. 19-24.

Shinohara, M. S., de Oliveira, M. T., Hipólito, V. D., Giannin, M. and de Goes, M. F. (2006) SEM ANALYSIS OF THE ACID-ETCHED ENAMEL PATTERNS PROMOTED BY ACIDIC MONOMERS AND PHOSPHORIC ACIDS. *Journal of Applied Oral Science 14*(6), 427-435.

Simhofer, H., Griss, R. and Zetner, K. (2008) The use of oral endoscopy for detection of cheek teeth abnormalities in 300 horses. *Vet J 178*(3), 396-404.

Singer, D. L. and Kleinberg, I. (1983) The free amino acids in human dental plaque. *Arch Oral Biol, 28*(9), 873-8.

Siquera, W. L., Custodio, W. and McDonald, E. E. (2012) New insights into the composition and functions of the acquired enamel pellicle. *J Dent Res 91*(12), 1110-1118.

Soames, J. V. and Southam, J. C. (2005) Dental caries. in Soames, J. V. and Southam, J. C., (eds.) *Oral Pathology*,Oxford: Oxford University Press. pp. 401-420.

Spoutil, F., Vlcek, V. and Horacek, I. (2010) Enamel microarchitecture of a tribosphenic molar. *J Morphol 271*(10), 1204-18.

St Clair, L. E. (1975) Teeth. in Getty, R., (ed.) *Sisson and Grossman's the Anatomy of the Domestic Animals*, Philadelphia: WB Saunders. pp. 460-470.

Stanley, H. R., White, C. L. and McCray, L. (1966) The rate of tertiary reparative dentine formation in the human tooth. *Oral Surgery, Oral Medicine and Oral Pathology 21*, 180-189.

Staszyk, C., Bienert, A., Kreutzer, R., Wohlsein, P. and Simhofer, H. (2008) Equine odontoclastic tooth resorption and hypercementosis. *Vet J 178*(3), 372-9.

Stebbins, G. L. (1981) Coevolution of grasses and herbivores. *Ann. Missouri Bot. Gard. 68*, 75-86.

Stoker, H. S. (2016) General, Organic and Biological Chemistry, 7th edn. ed., Boston: Cengage Learning, pp. 580-640.

Suske, A., Pöschke, A., Müller, P., Wöber, S. and Staszyk, C. (2016a) Infundibula of equine maxillary cheek teeth Part 2: Morphological variations and pathological changes. *Vet. J. 209*, 66-73.

Suske, A., Pöschke, A., Schrock , P., Kirschner, S., Brockmann, M. and Staszyk, C. (2016b) Infundibula of equine maxillary cheek teeth. Part 1: Development, blood supply and infundibular cementogenesis. *Vet. J. 209*, 57-65.

Svensäter, G., Sjögreen, B. and Hamilton, I. R. (2000) Multiple stress responses in Streptococcus mutans and the induction of general and stress-specific proteins. *Microbiology 146*, 107-117.

Syed, S. A. and Loesche, W. J. (1972) Survival of Human Dental Plaque Flora in Various Transport Media. *Applied Microbiology 24*(4), 638-644.

Takahashi, N. and Nyvad, B. (2011) The role of bacteria in the caries process: ecological perspectives. *J Dent Res 90*.

Takahashi, N. and Nyvad, B. (2016) Ecological Hypothesis of Dentin and Root Caries. *Caries Res 50*(4), 422-431.

Takuma, S., Katagiri, S. and Ozasa, S. (1966) Electron-probe microanalysis of horse dentine. *J. Electron Microsc. 15*, 86-89.

Tanner, A. C., Mathney, J. M., Kent, R. L., Chalmers, N. I., Hughes, C. V., Loo, C. Y., Pradhan, N., Kanasi, E., Hwang, J., Dahlan, M. A., Papadopolou, E. and Dewhirst, F. E. (2011) Cultivable anaerobic microbiota of severe early childhood caries. *J Clin Microbiol 49*(4), 1464-74.

Tanzer, J. M. (1992) Microbiology of dental caries. in M., S. J. a. T., (ed.) *Contemporary Oral Microbiology and Immunology*, St. Louis: Mosby Year Book. pp. 342-372.

Tarbet, W. J. and Forsick, L. S. (1971) Permeability of human dental enamelto acriflavine and potassium fluoride. *Arch Oral Biol 16*, 951–961.

Towner, P. (1991) Purification of DNA. in Brown, T. A., (ed.) *Essential Molecular Biology. A Practical Approach*,Oxford: Oxford University Press. pp. 47–68.

Tremaine, W. H. and Dixon, P. M. (2001) Equine sinonasal disorders: a long term study of 277 cases Part I. Historical clinical and ancillary diagnostic findings. *Equine Veterinary Journal 33*, 274-282.

van den Enden, M. S. D. and Dixon, P. M. (2008) Prevalence of occlusal pulpar exposure in 110 equine cheek teeth with apical infections and idiopathic fractures. *The Veterinary Journal 178*(3), 364-371.

van Loveren, C., Broukal, Z. and Oganessian, E. (2012) Functional foods/ingredients and dental caries. *Eur. J. Nutr. 51*(suppl 2), S15-S25.

Vanuspong, W., Eisenburger, M. and Addy, M. (2002) Cervical tooth wear and sensitivity: erosion, softening and rehardening of dentine; effects of pH, time and ultrasonication. *Clin Periodontol 29*, 351-357.

Veraa, S., Voorhout, G. and Klein, W. R. (2009) Computed tomography of the upper cheek teeth in horses with infundibular changes and apical infection. *Equine Vet J. 41*(9), 872-876.

Verstraete, F. J. M. (1999) Veterinary Dentistry, London: Manson Publishing Ltd, pp. 44.

Větrovský, T. and Baldrian, P. (2013) The Variability of the 16S rRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses. *PLoS ONE 8*(2), e57923.

Wafa, N. S. Y. (1988) *A Study of Dental Disease in the Horse*. Unpublished MVM Thesis, National University College Dublin, Ireland.

Walker, B. N., Makinson, O. F. and Peters, M. C. (1998) Enamel cracks. The role of enamel lamellae in caries initiation. *Aust Dent J 43*(2), 110-6.

Walmsley, J. P. (1993) Some observations on the value of ageing 5-7 year-old horses by examination of their incisor teeth. *Equine Vet Educ 5*, 195-298.

Wang, R., Hu, Y. and Ng, C. (2006) Microstructure and interfacial fracture at the cementum-enamel junctions in equine and bovine teeth. *J Mater Res 21*(8), 2146-2155.

White, C. and Dixon, P. M. (2012) A study of the thickness of cheek teeth subocclusal secondary dentine in horses of different ages. *Equine Vet. J. 42*(2), 119-123.

Wiggs, R. B. and Lobprise, H. B. (1997) Veterinary Dentistry: Principles and Practice, Philadelphia: Lippincott-Raven, pp. 57.

Windley, Z., Weller, R., Tremaine, W. H. and Perkins, J. D. (2009) Two- and three-dimensional computed tomographic anatomy of the enamel, infundibulae and pulp of 126 equine cheek teeth. Part 2: findings in teeth with macroscopic occlusal or computed tomographic lesions. . *Equine Vet. J. 41*, 441-447.

Yue, J. C. and Clayton, M. K. (2005) A Similarity Measure Based on Species Proportions. *Communications in Statistics - Theory and Methods 34*(11), 2123-2131.

Zander, H. A. and Hurzeler, B. (1958) Continuous cementum apposition. *J. Dent. Res. 37*, 1035-1044.

Zhao, N., Foster, B. L. and Bonewald, L. F. (2016) The Cementocyte—An Osteocyte Relative? *Journal of Dental Research 95*(7), 734-741.

APPENDICES

Supplementary Item 1. Guidelines for grading caries

GUIDELINES FOR PARTICIPANTS IN THE EQUINE DENTAL CARIES SURVEY

1. Please use consecutive dental examination cases whenever possible, because we are trying to establish the true prevalence of peripheral caries around the UK. If horses are specifically selected for this survey (e.g. known cases of peripheral caries) this will affect the results.

2. Likewise, please fill out the dental chart and questionnaire (2 pages in total) for all horses, even if they **do not have caries**.

3. Each tooth should be graded separately

4. If no caries is present – write 0 or alternatively do not fill anything in – all blank lines or boxes will be assumed to show that the adjacent teeth have no caries (Grade 0)

5. If there are areas with different grades of peripheral caries on the **same** tooth, use the grade of the **worst affected area** for grading that tooth

6. Please use the lines provided for grading **peripheral** caries: i.e. use the palatal aspects of the maxillary cheek teeth and the buccal aspects of the mandibular cheek teeth

7. The reason we grade the palatal aspects of the maxillary cheek teeth and the buccal aspects of the mandibular cheek teeth is because these sites are worst affected by peripheral caries

8. If you have difficulty in grading the buccal aspects of the mandibular cheek teeth (e.g. in a difficult, unsedated horse), you can instead grade the lingual aspects of these teeth and record grade on the chart at the corresponding **lingual** side of the mandible.

9. The boxes in the maxillary teeth are for recording the grade of caries in each infundibulum.

10. The chart will be different from the one you are used to, please pay attention to the order of the arcades. In particular, the position of the 3rd and 4th arcades can differ from other charts.

11. Sometimes incisors and/or canines can be affected by peripheral caries. Please examine
these teeth also and record grade of caries (if any) beside any affected tooth.

12. Please record if diastemata are present between the cheek teeth and grade severity as **mild** (e.g. shallow/absent periodontal pockets); **moderate** (some periodontal pockets with food trapping) and **severe** (deep periodontal pockets with food trapping)

13. Likewise grade dental overgrowths on a similar scale; e.g. **mild** (buccal/lingual enamel points); **moderate** (e.g. larger overgrowths such as moderate upper 06/lower 11 overgrowths) and **severe** (e.g. wavemouth, stepmouth or shearmouth)

14. Record all cheek teeth fractures – maxillary midline fractures ae also recorded in infundibular caries grading

15. If anything is unclear, please do not hesitate to contact Paddy Dixon or Dewi Borkent at these email addresses: p.m.dixon@ed.ac.uk or dewiborkent@gmail.com

If you have difficulty with any aspect of this survey, other dental practitioners may have the same difficulties. Therefore, it would be really helpful to everyone if you could let us know at an early stage about any problems you have. We will hopefully find a solution and send out an email for clarification to all survey participants. Thank you very much for taking part!

Paddy Dixon, Dewi Borkent and Richard Reardon

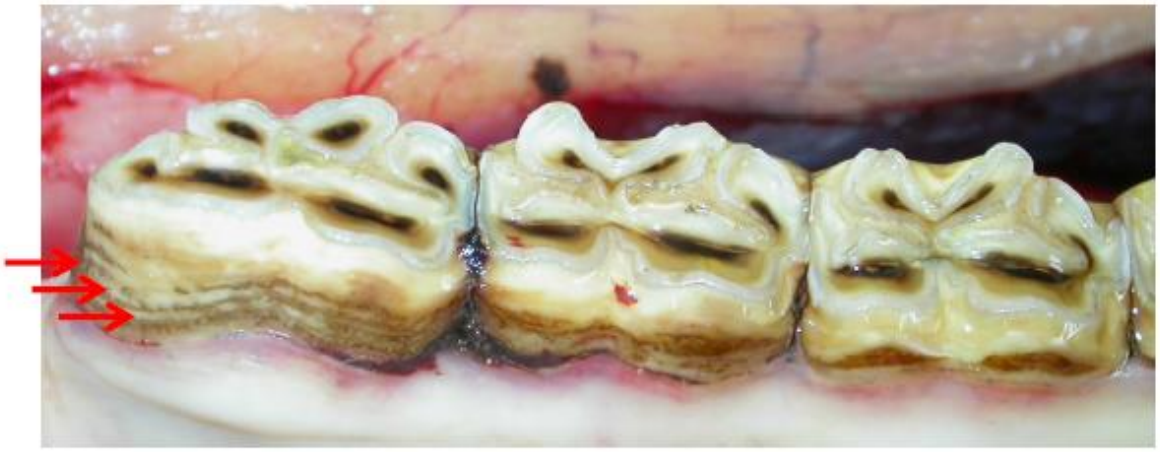
Supplementary Item 2. Grading system peripheral caries: guidelines with images

Peripheral Caries Grading System

The system used for grading peripheral caries is discussed first and is the modification of the Honma system described by Dacre (2005) (Dacre, I.T. (2005). Equine dental pathology. *In: Equine dentistry* (2nd Edition) Eds. Baker G.J., Easley K.J., Elsevier Saunders, Edinburgh, pp. 91-110).

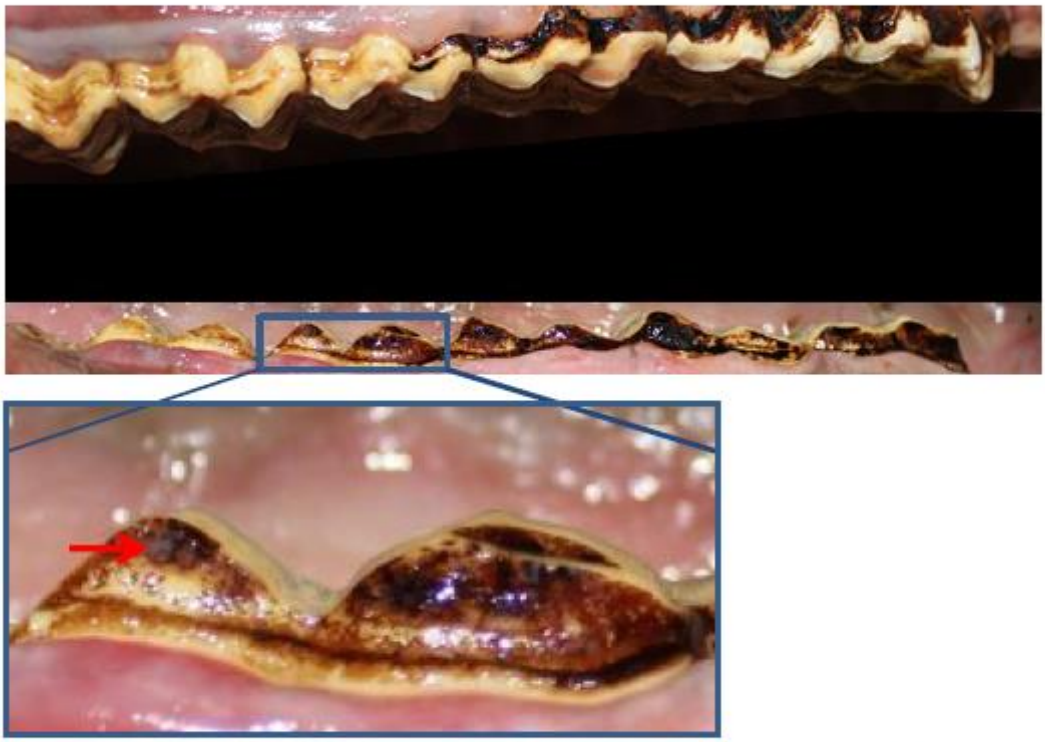
- **Grade 0**: normal tooth i.e. no macroscopic peripheral caries visible
- **Grade 1**: only cementum is affected
  - Grade 1, Class 1 (Grade 1.1): lesions appear as superficial erosions or pitting lesions or even as an extensive erosions of the cementum (cement) surface, although there is still some underlying cementum left.
  - Grade 1, Class 2 (Grade 1.2): more severe peripheral caries where the cementum is completely lost in some areas of the tooth, exposing the underlying (but unaffected) enamel.
- **Grade 2**: cementum and the underlying **enamel** are affected
- **Grade 3**: cementum, **enamel** and **dentine** are affected
- **Grade 4**: tooth integrity is affected (i.e. secondary dental fractures present)

GRADE 0 (Normal Teeth)

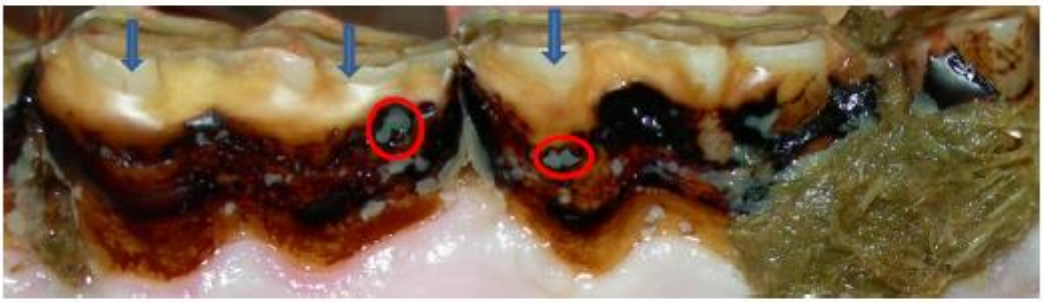


Normal peripheral cementum with slight areas of discolouration. The horizontal ridges (red arrows) in the peripheral cement are a common normal feature ("growth lines") and may be diet-related.

GRADE 1.1 (Grade 1, Class 1) - Superficial Erosions of Cementum

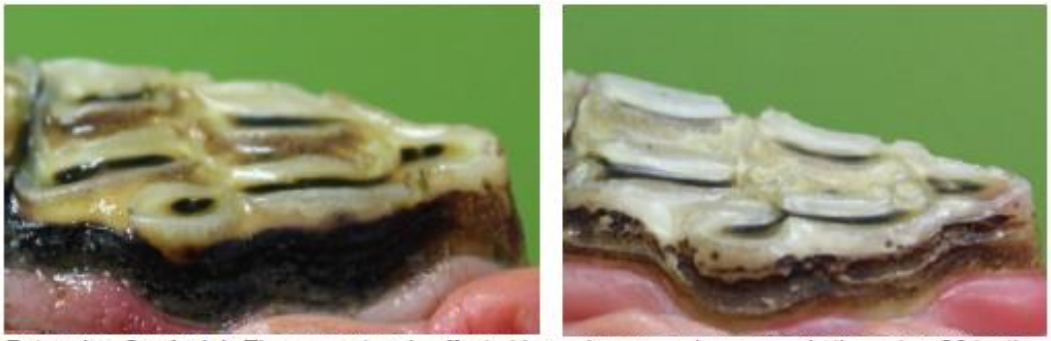


Grade 1.1 - If these teeth just had food staining – all cheek teeth would likely be affected and the surface would be just discoloured and not pitted – as is present here (arrow). This case is unusual in that the rostral 3 cheek teeth are affected with Grade 1 Class 1 (Grade 1.1) peripheral caries and the caudal 3 cheek teeth are unaffected – usually the opposite situation occurs.



Grade 1.1- Notice the plaque (red circles) lying within pitting peripheral caries lesions and also the food overlying the teeth at a diastema. The small area of total peripheral cementum loss near the occlusal surface (blue arrows) is a feature in normal horses where the cementum is worn away by abrasion with food. This is grade 1.1. peripheral caries.

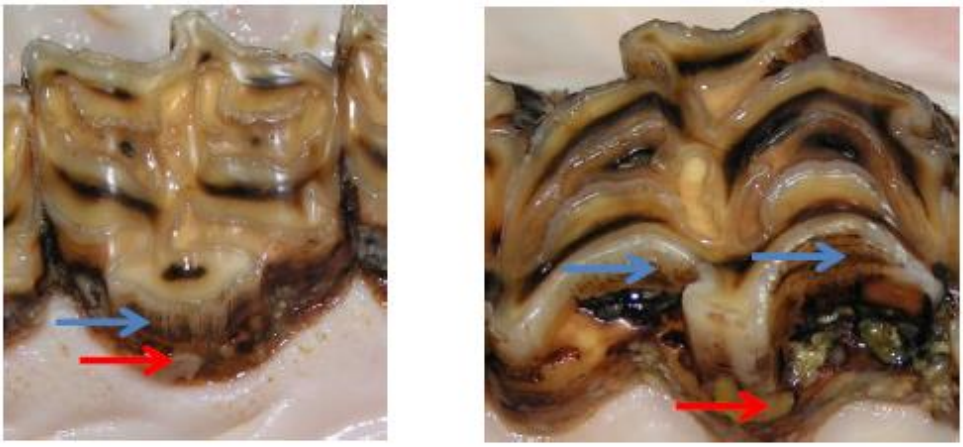
Grade 1.1 – The peripheral cementum is discoloured and pitted but not completely lost over any tooth (i.e. is therefore Grade 1.1). The loss of cementum (and underlying dentine) on the cingulae (vertical ridges) (arrows) is due to floating – and horizontal float blade marks are visible here.



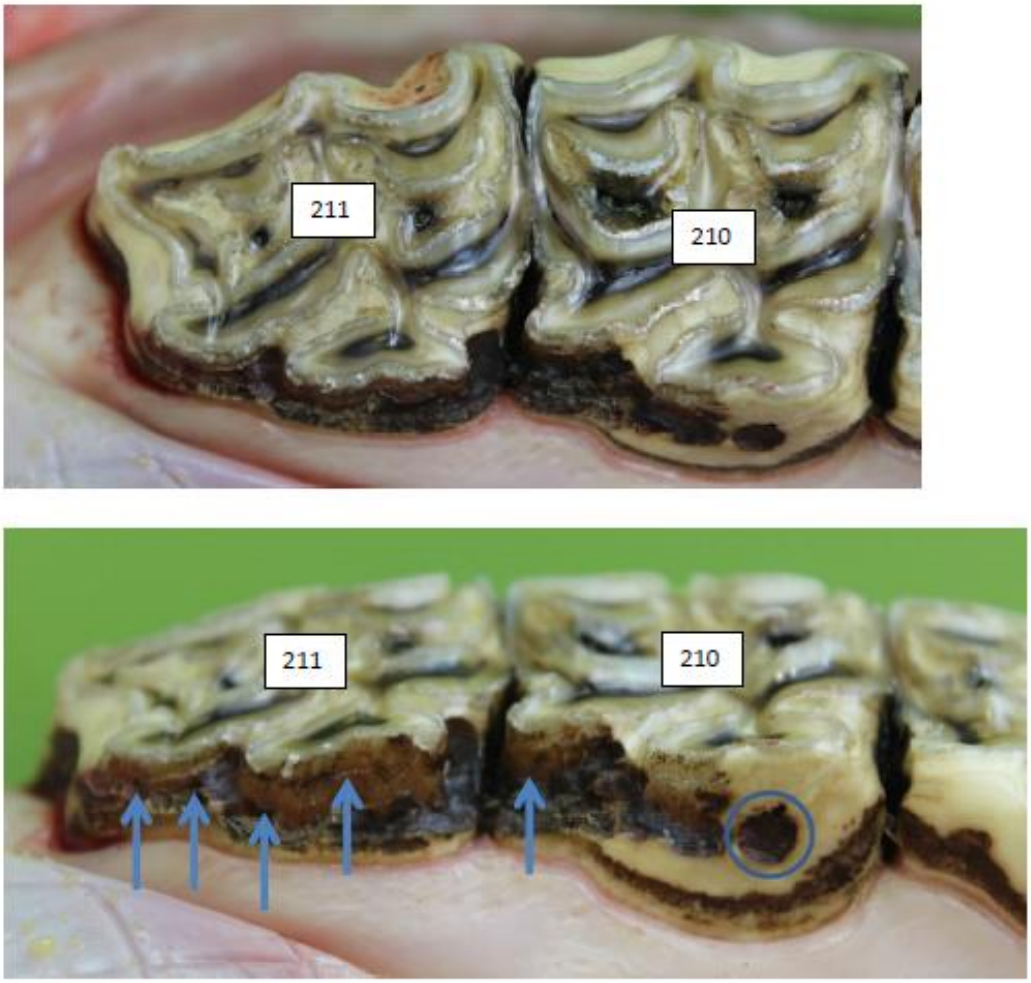
Extensive Grade 1.1- The cementum is affected by caries over a large area in these two 06 teeth, but still there is no total loss of peripheral cementum and so they are grade 1.1.

Extensive grade 1.1- Peripheral cementum is widely affected in all of these teeth but there is no total loss of cementum –therefore grade 1.1. (Image courtesy of I. Gere)

GRADE 1.2 (Grade 1, Class 2) - Total Loss of Cement over some areas exposing the underlying enamel.



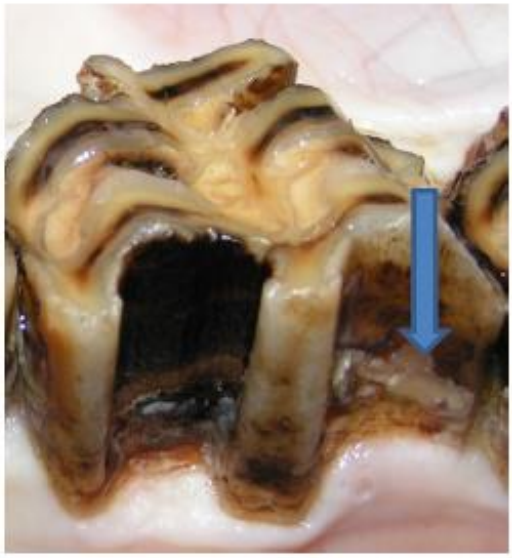
Grade 1.2: **Left** and **Right**: There is total loss of peripheral cementum in areas in both teeth (Grade 1.2). The underlying enamel is discoloured (blue arrow) but does not appear to have caries – as it still has a transparent appearance. Note the plaque attached to the tooth above the gingival margin (red arrow).



Grade 1.2 – Occlusal (**top**) and palatal (**bottom**) views of these same teeth show total loss of peripheral cementum in some areas (arrows) over the 211 and on the caudal aspect on the 210 (grade 1.2) with superficial cementum loss rostrally on the 210 – blue circle - (Grade 1.1). Tooth 210 should be graded according to the worst affected areas i.e. as Grade 1, Class 2 (Grade 1.2). The underlying enamel is discoloured but does not appear to have caries. In particular – the exposed peripheral enamel ridges appear translucent and not black as would occur in enamel caries (Grade 2 caries).

Grade 1.2- In normal equine incisors the more occlusal peripheral cementum is usually worn away by food abrasion (blue arrows) on the labial (rostral) aspects and less commonly on the lingual aspects, as is present in this case. Additionally, incisors can less commonly be affected by peripheral caries (red arrows). This picture shows areas with total loss of cementum but the underlying enamel still seems to be intact - therefore the peripheral caries present in these incisors is graded as Grade 1.2.

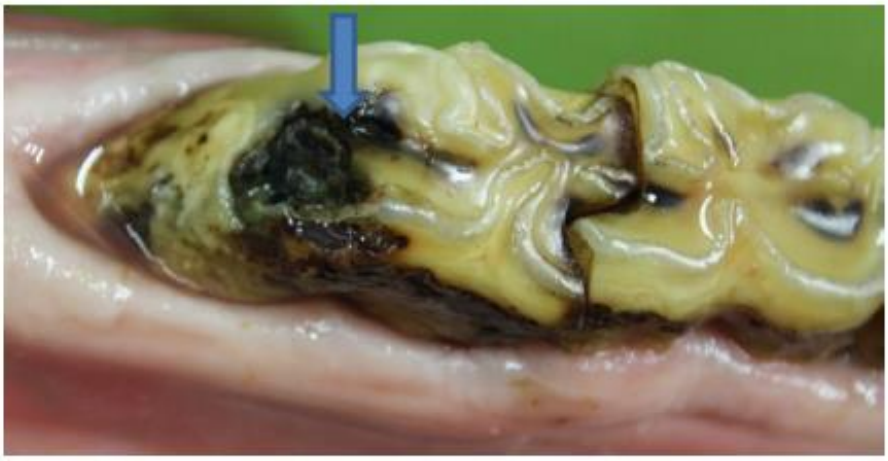
GRADE 2 - Involvement of cement and ENAMEL



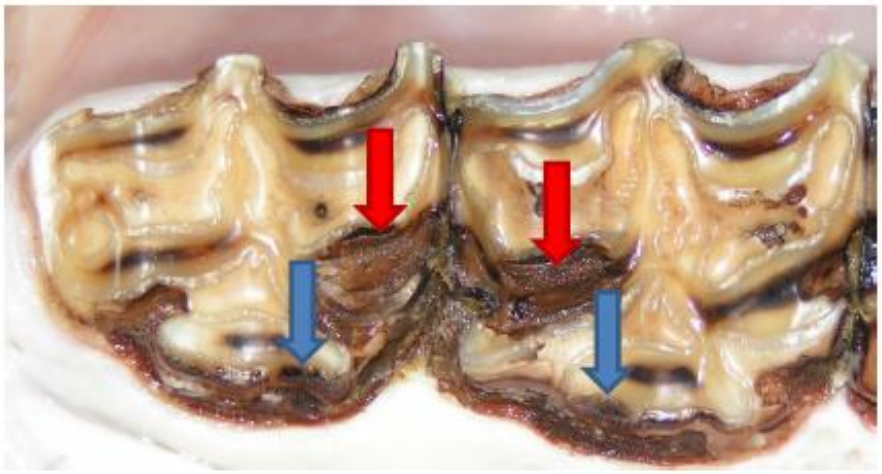
Grade 2- The peripheral cementum and also the underlying enamel are affected by caries in these two cheek teeth. Notice the dark-coloured enamel (**left** and **right**)) and so these can be classified as Grade 2 caries.

There is no peripheral cementum left over this tooth and all of the surface of the peripheral enamel is darkly discoloured. The enamel itself is also eroded and has a greyish colour insome areas (arrow) indicating it has caries (Grade 2).

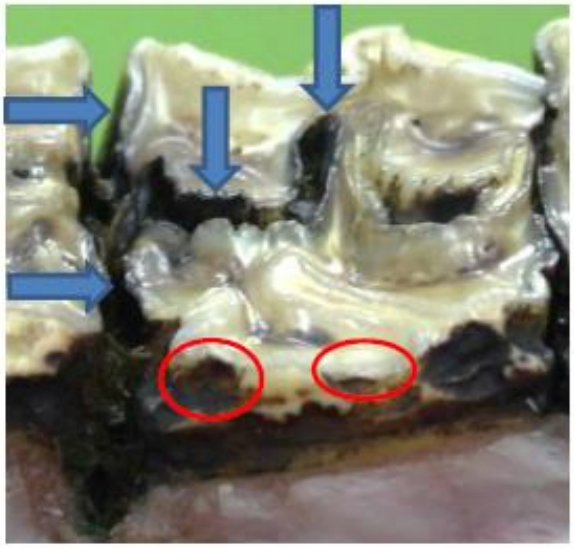
Grade 3 – Involvement of cementum, enamel and DENTINE



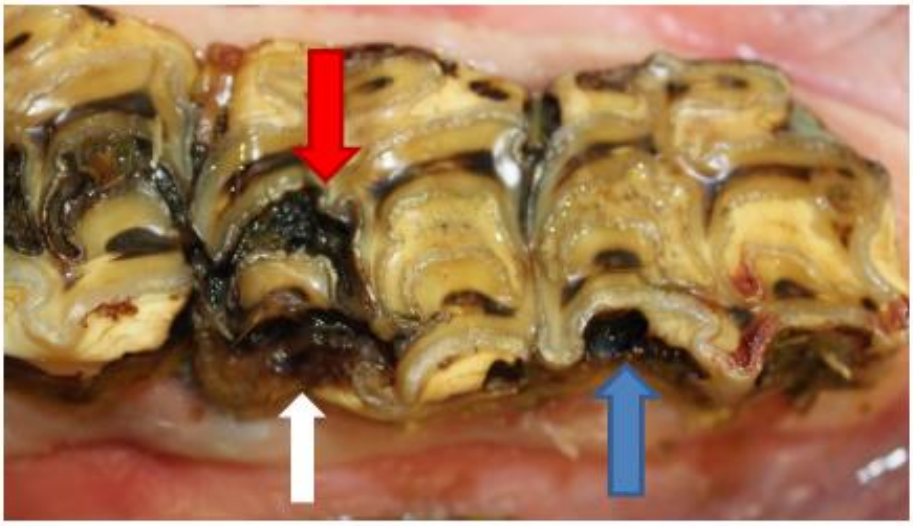
Grade 3. The caries in this tooth involves peripheral cementum, enamel and dentine (blue arrow)



Grade 3- The caries in this tooth involves peripheral cementum, enamel and also dentine (blue arrows). The dentine overlying pulp chambers number 3 and 4 also has caries (red arrows).



Grade 3- This 109 tooth has marked cemental caries (Grade 1.2 – red circles) and the peripheral (and infundibular) enamel is carious and dark (Grade 2 – blue arrows). Additionally there is carious loss of dental tissue with a connection now between the peripheral and (rostral) infundibular caries – this defect must involve the adjacent dentine – therefore there is Grade 3 caries in this tooth.

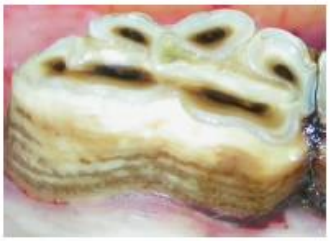


Grade 3- Grade 2 Infundibular (red arrow) and grade 1.2 peripheral caries (blue arrow) is present in this tooth. Additionally the first pulp horn of the 110 has a slab fracture (white arrow) – but with our current knowledge, this cannot be related to peripheral caries

Supplementary Item 3. Field guide for grading peripheral caries

Grading Equine Peripheral Caries

Grade 0



Grade 1.1

Grade 1.2

Grade 2

Grade 3

Grading System Equine Peripheral Caries

Grade	Description
0	normal tooth i.e. no macroscopic peripheral caries visible
1.1	only cementum affected: lesions appear as superficial erosions or pitting lesions or even as extensive erosions of the cementum (cement) surface, although there is still some underlying cementum left.
1.2	only cementum affected: more severe peripheral caries where the cementum is completely lost in some areas of the tooth, exposing the underlying (but unaffected) enamel.
2	cementum and underlying enamel are affected
3	cementum, enamel and dentine are affected
4	tooth integrity is affected (i.e. secondary dental fracture present)

Supplementary Item 4. Results of univariable logistic regression analysis of risk factors for peripheral caries in horses in the UK, showing some forms of the variables evaluated. Best fit of each variable, according to lowest AIC-value, in grey boxes.

Variable	Odds Ratio and 95% Confidence Intervals	P-value	Total (n=706)	Prevalence PC (%)	Number with PC
IC		**<0.001**			
No	1 (ref)		385	42.6	164
Yes	2.26 (1.67, 3.06)	*<0.001*	321	62.6	201
Concurrent dental disorder other than IC		**0.005**			
No	1 (ref)		211	43.6	92
Any concurrent	1.58 (1.14, 2.19)	*0.006*	482	55.2	266
Diastema		**<0.001**			
No	1 (ref)		540	45.9	248
Yes	3.11 (2.1, 4.61)	*<0.001*	153	72.5	111
Periodontal disease not associated with diastema		**0.176**			
No	1 (ref)		682	51.5	351
Yes	2.51 (0.66, 9, 56)	*0.176*	11	72.7	8
Dental overgrowths		**0.651**			
No	1 (ref)		334	52.7	176
Yes	0.93 (0.69, 1.26)	*0.651*	359	51.0	183
Dental fracture		**0.009**			
No	1 (ref)		654	50.6	331
Yes	2.48 (1.22, 5.07)	*0.013*	39	71.8	28
Other dental disorder		**0.006**			
No	1 (ref)		546	49.1	268
Yes	1.69 (1.16, 2.45)	*0.006*	147	61.9	91
Concurrent dental disorder other than IC		**< 0.001**			

No	1 (ref)		211	43.6	92
Dental fracture	9.05 (1.09,74.9)	*0.041*	8	87.5	7
Diastema/PD	4.89 (2.23,10.7)	*< 0.001*	43	79.1	34
Multiple	2.68 (1.76,4.06)	*< 0.001*	175	67.4	118
Other	1.12 (0.57,2.19)	*0.747*	41	46.3	19
Overgrowths	0.91 (0.62,1.34)	*0.645*	215	41.4	89
Breed		**0.155**			
Thoroughbred	1 (ref)		111	58.6	65
Pony	0.57 (0.36,0.93)	*0.023*	183	44.8	82
Warmblood	0.82 (0.54,1.27)	*0.38*	366	53.8	197
Arab	0.94 (0.31,2.9)	*0.919*	14	57.1	8
Coldblood	0.57 (0.14,2.22)	*0.415*	9	44.4	4
Saddlebred	0 (0,Inf)	*0.979*	1	0.0	0
Breed		**0.155**			
Pony	1 (ref)		183	44.8	82
Thoroughbred	1.74 (1.08,2.8)	*0.023*	111	58.6	65
Warmblood	1.44 (1.01,2.05)	*0.047*	366	53.8	197
Arab	1.64 (0.55,4.92)	*0.376*	14	57.1	8
Coldblood	0.99 (0.26,3.79)	*0.983*	9	44.4	4
Saddlebred	0 (0,Inf)	*0.98*	1	0.0	0
Breed		**0.022**			
Pony	1 (ref)		183	44.8	82
Other	1.49 (1.06,2.09)	*0.022*	501	54.7	274
Sex		**0.303**			
Stallion	1 (ref)		14	64.3	9
Gelding	0.63 (0.21,1.92)	*0.417*	436	53.2	232
Mare	0.52 (0.17,1.6)	*0.254*	244	48.4	118
Sex		**0.191**			
Female	1(ref)		244	48.4	118
Male	1.23 (0.9, 1.68)	*0.191*	450	53.6	241
Age (years)	0.9975 (0.9724,1.0232)	**0.847**			
Age (years)		**0.025**			
0 to 5	1 (ref)		86	38.4	33
6 to 10	2 (1.21,3.32)	*0.007*	236	55.5	131
11 to 15	1.95 (1.1,3.44)	*0.015*	180	54.4	98
16 to 20	1.95 (1.1,3.44)	*0.022*	115	54.8	63

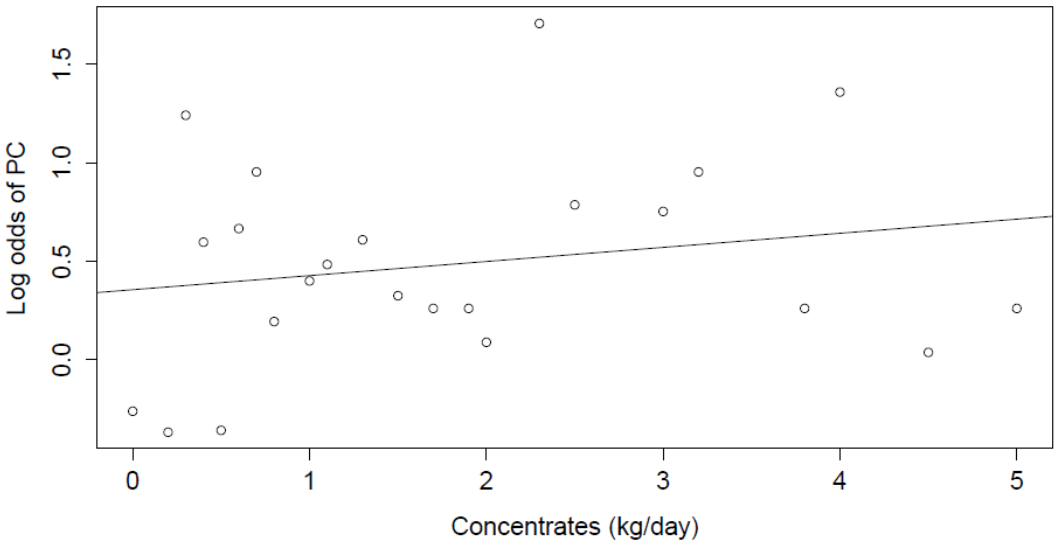
20+	1.15 (0.6,2.21)	*0.668*	67	41.8	28
Age (years)		**0.004**			
0 to 5	1 (ref)		86	38.4	33
6 to 20	1.96 (1.23,3.13)	*0.005*	531	55.0	292
20+	1.15 (0.6,2.21)	*0.668*	67	41.8	28
Work type		**0.208**			
Recreational	1 (ref)		416	49.5	206
Sports	1.32 (0.96, 1.82)	*0.088*	241	56.4	136
Racing	1.36 (0.56, 3.29)	*0.497*	21	57.1	12
Region		**< 0.001**			
Scotland	1 (ref)		173	48.6	84
Midlands	0.6 (0.28,1.26)	*0.176*	36	36.1	13
North England	1.01 (0.7,1.47)	*0.941*	319	48.9	156
South East England	4.84 (2.03,11.57)	*< 0.001*	39	82.1	32
South West England	1.41 (0.85,2.36)	*0.185*	91	57.1	52
Wales	1.23 (0.62,2.43)	*0.557*	41	53.7	22
Fluoridation of water	1 (ref)	**0.757**			
No			627	51.2	321
Yes	0.89 (0.41, 1.91)		27	48.1	13
Type of Concentrates		**0.243**			
None	1 (ref)		124	44.4	55
Nuts	1.35 (0.89, 2.07)	*0.16*	289	51.9	150
Grain/Mix	1.22 (0.76, 1.96)	*0.401*	160	49.4	79
Both	1.95 (0.98, 3.89)	*0.058*	46	60.9	28
Concentrates (kg/day)	1.16 (1.02,1.31)	**0.02**			
Concentrates (kg/day)		**0.009**			
0	1 (ref)		131	43.5	57
0.1 to 0.5	0.96 (0.56,1.66)	*0.886*	87	42.5	37
0.6 to 1	1.41 (0.86,2.32)	*0.175*	121	52.1	63
1 to 1.5	1.47 (0.9,2.38)	*0.123*	132	53.0	70
1.6 to 2	1.14 (0.58,2.24)	*0.713*	45	46.7	21
2.1 to 2.5	3.05 (1.57,5.94)	*<0.001*	57	70.2	40
2.6 to 3	2.3 (1.23,4.31)	*0.009*	62	64.5	40

3+	1.23 (0.58,2.59)	*0.593*	35	49%	17
Concentrates (kg/day)		**0.001**			
0			131	43.5	57
0.1 to 2	1.28 (0.86,1.9)	*0.228*	385	49.6	191
2.1 to 3	2.63 (1.57,4.41)	*<0.001*	118	66.9	79
3+	1.23 (0.58,2.59)	*0.593*	35	48.6	17
Concentrates		**0.043**			
No	1 (ref)		131	43.5	57
Yes	1.48 (1.01, 2.18)	*0.044*	538	53.3	287
Hay		**0.878**			
No	1 (ref)		280	51.4	144
Yes	1.02(0.75, 1.39)	*0.878*	396	52.3	207
Haylage	1.09 (0.81, 1.48)	**0.57**			
No	1 (ref)		348	50.9	177
Yes	1.09 (0.81, 1.48)	*0.57*	238	73.1	174
Chaff		**0.132**			
No	1 (ref)		537	53.6	288
Yes	0.75 (0.52, 1.09)	*0.132*	144	45.8	66
Forage		**0.867**			
Hay	1 (ref)		260	53.1	138
Haylage	0.99 (0.69, 1.4)	*0.941*	237	52.7	125
Chaff	0.66 (0.15, 3.02)	*0.595*	7	42.9	3
Pasture (hours/day)	0.98 (0.96,1)	**0.074**			
Pasture (groups)		**0.526**			
0	1 (ref)		14	57.1	8
1 to 4	1.11 (0.36,3.49)	*0.853*	87	59.8	52
5 to 8	0.9 (0.3,2.72)	*0.855*	163	54.6	89
9 to 12	0.77 (0.25,2.33)	*0.644*	148	50.7	75
13 to 16	0.59 (0.18,2.01)	*0.401*	43	44.2	19
17 to 20	0.71 (0.23,2.24)	*0.563*	80	48.8	39
21 to 24	0.69 (0.23,2.09)	*0.513*	146	47.9	70
Fed Treats		**0.805**			
No	1 (ref)		510	51.8	264
Yes	1.04 (0.74, 1,47)	*0.805*	176	52.8	93
Fed		**0.232**			

supplements					
No	1 (ref)		497	53.5	266
Yes	0.81 (0.58, 1.14)	*0.232*	188	48.4	91

Key: PC=Peripheral caries; IC = infundibular caries; PD= periodontal disease;vs= versus; TB= Thoroughbred type; P-values in bold are from the likelihood ratio test, while those in italics are from the wald test.

Supplementary Item 5. Log odds of peripheral caries (PC) categorised by concentrates (kg/day).



Supplementary Item 6. Results of univariable logistic regression analysis of risk factors for infundibular caries in horses in the UK, showing some forms of the variables evaluated. Best fit of each variable, according to lowest AIC-value, in grey boxes.

Variable (within horse)	Odds Ratio and 95% Confidence Intervals	P-value	Total (n=706)	Prevalence IC (%)	Number with IC
PC		**<0.001**			
No	1 (referent)		321	37.4	120
Yes	2.26 (1.67,3.06)	*<0.001*	365	55.1	201
Concurrent dental disorder other than PC		**0.181**			
No	1 (referent)		212	41.5	88
Any concurrent	1.25 (0.9,1.73)	*0.182*	481	47	226
Diastema		**< 0.001**			
No	1 (referent)		540	39.4	213
Yes	2.98 (2.05,4.34)	*< 0.001*	153	66	101
Periodontal disease not associated with diastema		**0.012**			
No	1 (referent)		682	44.7	305
Yes	5.56 (1.19,25.93)	*0.029*	11	81.8	9
Dental overgrowths		**0.576**			
No	1 (referent)		334	46.4	155
Yes	0.92 (0.68,1.24)	*0.576*	359	44.3	159
Dental fracture		**< 0.001**			
No	1 (referent)		654	43.7	286
Yes	3.28 (1.6,6.69)	*0.001*	39	71.8	28
Other dental disorder		**0.013**			
No	1 (referent)		546	42.9	234
Yes	1.59 (1.1,2.3)	*0.013*	147	54.4	80
Concurrent dental disorder other than PC		**< 0.001**			
No	1 (referent)		215	42.8	92
Dental fracture	4.01 (0.79,20.33)	*0.093*	8	75	6
Diastema/PD	2.04 (1.05,3.99)	*0.036*	43	60.5	26

Multiple	2.1 (1.4,3.16)	*< 0.001*	175	61.1	107
Other	0.95 (0.48,1.86)	*0.875*	41	41.5	17
Overgrowths	0.65 (0.44,0.96)	*0.029*	215	32.6	70
Breed		**0.531**			
Thoroughbred	1 (referent)		111	43.2	48
Pony	1.14 (0.71,1.83)	*0.593*	183	46.4	85
Warmblood	1.08 (0.7,1.65)	*0.733*	366	45.1	165
Arab	1.75 (0.57,5.38)	*0.329*	14	57.1	8
Coldblood	2.63 (0.62,11.03)	*0.188*	9	66.7	6
Saddlebred	0 (0,Inf)	*0.98*	1	0	0
Breed		**0.56**			
Pony	1 (referent)		183	46.4	85
Thoroughbred	0.88 (0.55, 1.41)	*0.593*	111	43.2	48
Warmblood	0.96 (0.66, 1.35)	*0.762*	366	45.1	165
Arab	1.54 (0.51, 4,61)	*0.443*	14	57.1	8
Coldblood	2.31 (0.56, 9.5)	*0.248*	9	66.7	6
Saddlebred	0 (0, Inf)	*0.98*	1	0	0
Sex		**0.347**			
Stallion	1 (referent)		14	35.7	5
Gelding	1.43 (0.47,4.34)	*0.528*	436	44.3	193
Mare	1.74 (0.57,5.35)	*0.332*	244	49.2	120
Sex		**0.191**			
Female	1 (referent)		244	49.2	120
Male	0.81 (0.59, 1.11)	*0.191*	450	44	198
Age (years)	1.09 (1.06, 1.12)	**<0.001**			
Age (years)		**<0.001**			
0 to 5	1 (referent)		86	23.3	20
6 to 10	3.02 (1.69,5.39)	*<0.001*	236	37.3	88
11 to 15	4.77 (2.56,8.9)	*<0.001*	180	47.8	86
16 to 20	7.23 (3.52,14.83)	*<0.001*	115	59.1	68
20+	1.96 (1.11,3.45)	*0.02*	67	68.7	46
Work type		**0.261**			
Recreational	1 (referent)		416	49.3	205
Sports	0.79 (0.58,1.09)	*0.158*	241	43.6	105
Racing	0.63 (0.26,1.56)	*0.321*	21	38.1	8
Region		**< 0.001**			
Scotland	1 (referent)		173	61.3	106

Midlands	1.44 (0.66,3.11)	*0.358*	36	69.4	25
North England	0.39 (0.26,0.57)	*< 0.001*	319	37.9	121
South East England	0.91 (0.45,1.84)	*0.791*	39	59	23
South West England	0.12 (0.07,0.23)	*< 0.001*	91	16.5	15
Wales	1.1 (0.54,2.22)	*0.8*	41	63.4	26
Region		**<0.001**			
South West England	1 (referent)	*<0.001*	91	16.5	15
Scotland	8.02 (4.26, 15.09)	*<0.001*	173	61.3	106
Midlands	11.52 (4.68, 28.32)	*<0.001*	36	69.4	25
North England	3.1 (1.7, 5.63)	*<0.001*	319	37.9	121
South East England	7.28 (3.13, 16.95)	*<0.001*	39	59	23
Wales	8.78 (3.78, 20.4)	*<0.001*	41	63.4	26
Region		**<0.001**			
South West England	1 (referent)		91	16.5	15
North England	3.1 (1.7, 5.63)	*<0.001*	319	37.9	121
Other regions	8.37 (4.58, 15.29)	*<0.001*	289	62.3	180
Fluoridation of water		**0.096**			
No	1 (referent)		607	47.1	286
Yes	0.5 (0.22, 1.16)	*0.108*	27	29.6	8
Type of Concentrates		**0.732**			
None	1 (referent)		124	40.3	50
Nuts	0.74 (0.37, 1.46)	*0.38*	289	44.6	129
Grain/Mix	0.88 (0.47, 1.64)	*0.686*	160	46.3	74
Both	0.94 (0.49, 1.81)	*0.85*	46	47.8	22
Concentrates (kg/day)	0.97 (0.86, 1.09)	**0.592**			
Concentrates (kg/day)		**0.348**			
0	1 (referent)				
0.1 to 0.5	1.16 (0.67, 2)	*0.598*	131	41.2	54
0.6 to 1	1.4 (0.85, 2.31)	*0.183*	87	44.8	39
1 to 1.5	1.52 (0.93, 2.47)	*0.095*	121	49.6	60

1.6 to 2	1.25 (0.63, 2.47)	*0.524*	132	51.5	68
2.1 to 2.5	1.2 (0.64, 2.24)	*0.576*	45	46.7	21
2.6 to 3	0.7 (0.37, 1.32)	*0.265*	57	45.6	26
3+	1.2 (0.57, 2.54)	*0.633*	35	45.7	16
Concentrates (kg/day)		**0.152**			
0	1 (referent)		131	41.2	54
0.1 to 1.5	1.38 (0.92, 2.07)	*0.125*	340	49.1	167
1.6 to 2.5	1.22 (0.72, 2.05)	*0.458*	102	46.1	47
2.5+	0.86 (0.5, 1.47)	*0.571*	96	37.5	36
Concentrates		**0.278**			
No	1 (referent)		131	41.2	54
Yes	1.24 (0.84, 1.82)	*0.28*	538	46.5	250
Hay		**0.212**			
No	1 (referent)		280	42.9	120
Yes	1.22 (0.89,1.66)	*0.212*	394	47.7	188
Haylage		**0.108**			
No	1 (referent)		348	48.9	170
Yes	0.78 (0.58,1.06)	*0.108*	328	42.7	140
Chaff		**0.91**			
No	1 (referent)		537	45.6	245
Yes	1.02 (0.71,1.48)	*0.91*	143	46.2	66
Forage		**0.286**			
Hay	1 (referent)		260	49.2	128
Haylage	0.75 (0.53,1.07)	*0.116*	237	42.2	100
Chaff	0.77 (0.17,3.52)	*0.74*	7	42.9	3
Pasture (hours/day)	1.0057 (0.9866,1.0253)	**0.56**			
Pasture (hours/day)		**0.526**			
0	1 (referent)		14	42.9	6
1 to 4	1.11 (0.36,3.49)	*0.853*	87	52.9	46
5 to 8	0.9 (0.3,2.72)	*0.855*	163	41.7	68
9 to 12	0.77 (0.25,2.33)	*0.644*	148	39.9	59
13 to 16	0.59 (0.18,2.01)	*0.401*	43	44.2	19
17 to 20	0.71 (0.23,2.24)	*0.563*	80	45.0	36
21 to 24	0.69 (0.23,2.09)	*0.513*	146	52.1	76
Fed Treats		**0.534**			

No	1 (referent)		510	45.9	234
Yes	0.9 (0.63,1.27)	*0.535*	176	43.2	76
Fed Supplements		**0.159**			
No	1 (referent)		497	43.5	216
Yes	1.27 (0.91,1.78)	*0.159*	188	49.5	93
Reason		**0.858**			
12 months	1 (referent)		412	43.2	178
3 months	0 (0,Inf)	0.98	1	0	0
6 months	1.09 (0.73,1.62)	0.687	126	45.2	57
9 months	1.1 (0.33,3.65)	0.882	11	45.5	5
10 months	0 (0,Inf)	0.98	1	0	0
18 months	0.88 (0.14,5.3)	0.886	5	40	2
24 months	1.97 (0.33,11.93)	0.46	5	60	3
24+ months	1.05 (0.28, 3.97)	0.941	9	44.4	4
Dental/Bitting problem	1.84 (1, 3.37)	0.049	48	58.3	28
Other reason	1.31 (0.78,2.23)	0.309	64	50	32
Reason		**0.108**			
Routine	1 (referent)		570	43.7	249
Dental/Bitting	1.8 (0.99, 3.28)	*0.053*	48	58.3	28
Other	1.29 (0.77, 2.16)	*0.336*	64	50	32

Key: PC = peripheral caries; PD= periodontal disease; P-values in bold are from the likelihood ratio test, while those in italics are from the wald test. Grey highlight= lowest AIC-value

Supplementary Item 7. Results of linear discriminant analysis effect size (LEfSe) at OTU level of peripheral caries of rostral cheek teeth (T06-09; Rcaries) and peripheral caries of caudal cheek teeth (T09-11; Ccaries) versus control group (healthy).

OTU_ID and taxon	Log of the highest class average	Class	LDA effect size	P-value
Otu000130_k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;unclassified;unclassified;	2.700517	Ccaries	2.636896	0.0400535
Otu000202_k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__Leptotrichia;unclassified;	3.144214	Ccaries	2.778493	0.0076349
Otu000108_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;s__delbrueckii;	2.570183	Ccaries	2.793939	0.0321491
Otu000046_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;unclassified;	2.667093	Ccaries	2.845875	0.032262
Otu000030_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;unclassified;	3.237636	Ccaries	2.894639	0.0002333
Otu000138_k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__Leptotrichia;unclassified;	3.333611	Ccaries	2.974535	0.0001448
Otu000411_k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Selenomonas;unclassified;	2.731551	Ccaries	3.079874	0.0346409
Otu000041_k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;unclassified;unclassified;	3.49704	Ccaries	3.097591	0.000074

Otu000047_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;unclassified;	2.047304	Ccaries	3.444549	0.0162132
Otu000072_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;unclassified;	3.913592	Ccaries	3.496163	0.0292276
Otu000639_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;unclassified;	1.968123	Ccaries	3.636941	0.0033016
Otu000008_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;unclassified;	4.995718	Ccaries	4.538301	0.0000523
Otu000001_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;unclassified;	5.656792	Ccaries	4.959444	0.0015269
Otu000277_k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;unclassified;unclassified;	2.901176	Rcaries	2.710439	0.0016193
Otu000027_k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium;unclassified;	3.094301	Rcaries	2.758493	0.0377289
Otu000037_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;s__sobrinus;	2.804266	Rcaries	2.762923	0.0000667
Otu000434_k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Atopobium;unclassified;	2.707356	Rcaries	2.795381	0.0382955
Otu001010_k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;unclassified;	2.348334	Rcaries	2.80824	0.040681
Otu000122_k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;unclassified;	2.348334	Rcaries	2.853946	0.037218

Otu000160_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;unclassified;	1.980358	Rcaries	3.184487	0.0112474
Otu000442_k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Olsenella;unclassified;	2.105296	Rcaries	3.364117	0.0112678
Otu001468_k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;unclassified;unclassified;unclassified;	1.804266	Rcaries	3.43051	0.0112474
Otu001359_k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__CF231;unclassified;	1.804266	Rcaries	3.543861	0.0112474
Otu000003_k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella;s__parvula;	4.348334	Rcaries	3.701624	0.0052476
Otu000011_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;unclassified;	4.585303	Rcaries	4.207325	0.0154793
Otu000121_k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Alysiella;s__crassa;	3.094886	healthy	2.581415	0.0206003
Otu000240_k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];unclassified;unclassified;	2.69283	healthy	2.662482	0.0312015
Otu000395_k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__Leptotrichia;unclassified;	2.682365	healthy	2.729287	0.0195457
Otu000248_k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__Mogibacterium;unclassified;	2.3918	healthy	2.742673	0.0487584

Otu000803_k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;unclassified;unclassified;unclassified;	2.625882	healthy	2.84481	0.0180717
Otu000172_k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium;unclassified;	3.082418	healthy	2.878042	0.0032607
Otu000640_k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;unclassified;unclassified;	2.500944	healthy	2.895802	0.0098936
Otu000191_k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;unclassified;unclassified;	3.114904	healthy	2.972618	0.0009408
Otu001972_k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__Leptotrichia;unclassified;	2.449792	healthy	2.990448	0.0027252
Otu000073_k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;unclassified;unclassified;	3.215709	healthy	3.031987	0.00134
Otu000360_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;unclassified;	1.972671	healthy	3.190091	0.022533
Otu000081_k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Moraxella;unclassified;	3.577436	healthy	3.272886	0.0081667
Otu000023_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;unclassified;	3.944642	healthy	3.539123	0.0027864
Otu000056_k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Lautropia;unclassified;	3.976992	healthy	3.610522	0.0002123

Otu000009_k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;unclassified;unclassified;	3.957198	healthy	3.667913	0.0000389
Otu000058_k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Eikenella;unclassified;	4.186852	healthy	3.818389	0.0003219
Otu000020_k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;unclassified;unclassified;	4.469773	healthy	4.072474	0.0001716
Otu000057_k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Actinobacillus;s__porcinus;	4.581598	healthy	4.166981	0.0065088
Otu000004_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;s__minor;	4.904701	healthy	4.435321	0.0099689
Otu000018_k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;unclassified;	4.76075	healthy	4.437053	0.0011666

Supplementary Item 8. Relative abundance of anaerobically and aerobically cultured bacteria (genus level) present in the peripheral caries group versus control group.

Genus	Relative Abundance (%) Anaerobic Cultures of Peripheral Caries Group	Relative Abundance (%) Anaerobic Cultures of Control Group	Relative Abundance (%) Aerobic Cultures of Peripheral Caries Group	Relative Abundance (%) Aerobic Cultures of Control Group
[Eubacterium]	0.007757043	0.006607856	0	0.001651963
[Prevotella]	0.054299309	0.075990304	0	0.001651963
02d06	0	0	0	0
Acholeplasma	0	0	0	0
Achromobacter	0	0	0.02133187	0
Acidaminococcus	1.5455918	0.885452381	0.012605196	0.302309522
Acidovorax	0	0	0.004848152	0
Acinetobacter	0	0	0.208470833	0.085902
Actinobacillus	0.004848154	0.003303926	0.023271135	0.018171596
Actinobaculum	0.00096963	0	0	0
Actinomyces	0.666136639	0.528628641	0.229802461	0.300657541
Actinotelluria	0	0	0	0
Adlercreutzia	0.002908891	0.001651963	0	0
Aerococcus	0	0	0	0
Aeromicrobium	0	0	0	0
Aeromonas	0	0	0.002908891	0
Agrobacterium	0	0	0	0
Agrococcus	0	0	0	0
Akkermansia	0	0	0	0
Alcaligenes	0	0	0	0.110681481
Alkanindiges	0.00096963	0	0.004848152	0
Alloiococcus	0	0	0	0
Alloscardovia	0.00096963	0.006607852	0.00096963	0
Alysiella	0.00096963	0	0	0
Amaricoccus	0	0	0	0
Aminobacter	0	0	0	0
Ammoniphilus	0	0	0	0
Anaerosinus	0	0	0	0
Anaerovibrio	0	0	0	0
Anaerovorax	0	0	0	0
Arcanobacterium	0	0	0	0
Arcobacter	0	0	0	0
Arthrobacter	0.006787415	0	0.015514087	0.004955889
Asticcacaulis	0	0	0	0

Atopobium	0.010665939	0.029735344	0.00096963	0
Atopostipes	0	0	0	0
Aurantimonas	0	0	0	0
B-42	0	0	0	0
Bacillus	0.004848152	0.001651963	0.018422978	1.478506148
Bacteroides	0.121203983	0.469157633	0.00096963	0
Balneimonas	0	0	0	0
Bifidobacterium	0.00096963	0.001651963	0	0
Bilophila	0	0	0	0
Blastococcus	0	0	0	0
Blautia	0	0	0	0
Bosea	0	0	0	0
Brachybacterium	0	0	0.026180043	0
Bradyrhizobium	0	0	0.009696304	0
Brevibacillus	0	0	0.108598761	0
Brevibacterium	0	0	0.030058591	0
Brevundimonas	0.001939261	0	0	0.997787778
Brochothrix	0	0	0	0
Brooklawnia	0	0	0	0
Bulleidia	0.035876339	0.066078563	0	0
Butyrivibrio	0	0	0	0
Campylobacter	0.016483722	0.031387304	0	0
Candidatus	0	0	0	0
Capnocytophaga	0.55365968	0.028083378	0.002908891	0
Cardiobacterium	0.015514089	0	0	0
Carnobacterium	0	0	0	0
Catonella	0	0	0	0
Cellulomonas	0	0	0	0
Cellvibrio	0	0	0	0
CF231	0	0	0	0
Chryseobacterium	0	0	0.001939263	0
Chthoniobacter	0	0	0	0
Citricoccus	0	0	0	0
Clostridium	0.00096963	0.001651963	0	0
Comamonas	0	0	0.181321087	0
Coprococcus	0.00096963	0.001651963	0	0
Corynebacterium	0.00096963	0	0.223015109	0.109029563
Cupriavidus	0	0	0.00096963	0
DA101	0	0	0	0
Dehalobacterium	0	0	0	0
Deinococcus	0	0	0	0
Delftia	0	0	0.00096963	0
Dermacoccus	0.004848154	0	0.518752985	0.127201148

Desulfobulbus	0	0	0	0
Desulfomicrobium	0.00096963	0	0	0
Desulfovibrio	0	0	0	0
Devosia	0	0	0	0
Dialister	0.105689748	0.16684833	0	0.004955889
Dietzia	0	0	0.002908891	0
Dokdonella	0	0	0	0
Dorea	0	0	0	0
Dyadobacter	0	0	0	0
Dysgonomonas	0	0.001651963	0	0
Eikenella	0.386882157	0.008259815	4.083118135	3.518680633
Elizabethkingia	0	0	0.095023761	0
Enhydrobacter	0.006787413	0	0.528449676	0
Enterococcus	0.058177807	0	0.012605198	0
Escherichia	0.471240652	0.006607852	0.836791435	0.052862704
Euzebya	0	0	0	0
Exiguobacterium	0	0	0	0.014867667
Facklamia	0	0	0	0
Fibrobacter	0	0	0	0
Filifactor	0.008726676	0.004955893	0	0
Flavobacterium	0.00096963	0	0	0
Fluviicola	0	0	0	0
Frigoribacterium	0	0	0	0
Fusobacterium	5.856573537	11.14910555	0.006787413	0.009911781
Gemella	0.0126052	0.011563741	0.036845963	0.023127489
Gordonia	0	0	0	0
GW-34	0	0	0	0
Haemophilus	0.00096963	0.001651963	0.004848152	0.004955893
Halomonas	0.004848152	0.008259815	0.007757048	0.003303926
Helcococcus	0	0	0	0
Hymenobacter	0	0	0	0
Jan-68	0.008726676	0.00330393	0	0
Janthinobacterium	0	0	0	0
Johnsonella	0.002908893	0.004955889	0	0
Kineococcus	0	0	0	0
Kingella	0.068843848	0.001651963	0.964782374	1.797338889
Kocuria	0.567235065	0	0.152232133	0
L7A	0	0	0	0
Laceyella	0	0	0	0
Lachnoanaerobaculum	0.003878524	0.003303926	0	0
Lactobacillus	0.744676672	0.619486315	0.152232074	0.029735352
Lactococcus	0.06981337	0.006607852	0.015514109	0
Lautropia	0.00096963	0.001651963	0.118294961	0.132157304

Legionella	0	0	0	0
Leptotrichia	0.003878522	0.044603004	0	0.001651963
Leucobacter	0	0	0	0
Leuconostoc	0	0	0	0
Luteibacter	0	0	0	0
Magnetospirillum	0	0	0	0
Malus	0	0	0	0
Mannheimia	0.00096963	0	0.21137963	0.004955889
Marmoricola	0	0	0	0
Massilia	0	0	0.046542391	0
Megasphaera	1.181010361	0.698780867	0.009696304	0.115637374
Methylobacterium	0	0	0	0
Methylosinus	0	0	0	0
Methyloversatilis	0	0	0	0
Microbacterium	0	0	0.030058543	0
Micrococcus	0	0	0.034906696	0.01651963
Micromonospora	0	0	0	0
Mitsuokella	0.038785241	0.013215707	0	0
Mogibacterium	0.128960878	0.221363311	0.00096963	0
Moraxella	0.002908893	0	1.165497093	0.644266263
Moryella	0.006787413	0.009911778	0.00096963	0
Mycobacterium	0	0	0	0
Mycoplana	0	0	0	0
Mycoplasma	0	0	0	0
Neisseria	3.271534785	0.460899074	9.379241346	14.83959106
Nelumbo	0	0	0	0
Nesterenkonia	0	0	0	0
Nitrobacteria	0	0	0	0.001651963
Nocardioides	0	0	0	0
Novosphingobium	0	0	0	0
Odoribacter	0	0	0	0
Olsenella	0.100841576	0.112333485	0.00096963	0.006607852
Oribacterium	0.080479352	0.132157122	0.001939261	0
Oscillospira	0	0	0	0
p-75-a5	0.00096963	0	0	0
Paenibacillus	0	0	0.002908891	0.001651963
Paludibacter	0	0	0	0
Pantoea	0.00096963	0	0.07854013	0.464201963
Parvimonas	0.10375048	0.155284719	0	0
Pasteurella	0	0	0.015514091	0.004955889
Paucibacter	0	0	0	0
Pediococcus	0	0	0	0
Pedobacter	0	0	0	0

Pelotomaculum	0	0	0	0
Peptococcus	0.003878524	0.006607859	0	0
Peptostreptococcus	2.064344546	2.968581522	0.012605196	0.001651963
Peredibacter	0	0	0	0
ph2	0.011635567	0.016519637	0	0
Phascolarctobacterium	0	0	0	0
Phenylobacterium	0	0	0.004848152	0
Phycicoccus	0	0	0	0
Phyllobacterium	0	0	0	0
Planifilum	0	0	0	0
Planomicrobium	0	0	0	0
Polaromonas	0	0	0	0
Porphyromonas	0.345188748	0.193279926	0	0.001651963
Prevotella	2.992280989	3.137079411	0.015514091	0.127201296
Propionibacterium	0	0	0	0
Propionicimonas	0	0	0	0
Propionimicrobium	0.00096963	0	0	0
Propionivibrio	0	0	0	0
Proteiniclasticum	0.002908893	0.006607852	0	0
Proteocatella	0	0	0	0
Pseudobutyrivibrio	0.00096963	0	0	0
Pseudochrobactrum	0	0	0.00096963	0
Pseudomonas	0.003878524	0.011563741	0.569173263	0.158588593
Pseudonocardia	0	0	0	0
Pseudoramibacter	0.001939261	0.016519633	0	0.001651963
Pseudoxanthomonas	0	0	0.005817783	0
Psychrobacter	0	0	0.004848152	0.001651963
Pyramidobacter	0.045572717	0.07929427	0	0
Ralstonia	0	0	0.001939263	0
Rathayibacter	0	0	0	0
Rhizobium	0	0	0	0
Rhodobacter	0	0.001651963	0.00096963	0
Rhodococcus	0	0	0	0
Rhodoplanes	0	0	0	0
Riemerella	0	0	0.020362239	0
Roseburia	0	0	0	0
Roseococcus	0	0	0	0
Rothia	0.565295635	0.013215704	14.58519557	12.20636491
Rudanella	0	0	0	0
Ruminococcus	0	0.001651963	0	0
Salinibacterium	0	0	0	0
Sanguibacter	0	0	0	0
Sarcina	0	0	0	0

Scardovia	0	0	0	0
Selenomonas	0.032967435	0.04790693	0	0
Sharpea	0.01745335	0.033039263	0	0.018171593
SHD-231	0	0	0	0
Shewanella	0.001939261	0.001651963	0	0
Shigella	0	0	0.002908891	0
Shuttleworthia	0.001939263	0	0	0
Skermanella	0	0	0	0
Slackia	0.003878524	0.004955889	0	0
SMB53	0	0	0	0
Sphingobacterium	0	0	0	0
Sphingobium	0	0	0	0
Sphingomonas	0	0	0.007757043	0
Stenotrophomonas	0	0.004955889	0.32870472	0.337000852
Streptococcus	36.39025574	36.29530564	6.481983435	3.9316777
Streptomyces	0	0	0.180351435	0.01651963
Succiniclasticum	0.022301526	0.016519641	0.00096963	0
Sutterella	0.00096963	0	0	0
Suttonella	0	0	0.017453348	0.080946152
Syntrophomonas	0	0	0	0
T78	0	0	0	0
Tannerella	0.002908893	0	0	0
Tessaracoccus	0	0	0	0.00330393
TG5	0	0.004955889	0	0
Thermoactinomyces	0	0	0	0
Thermoanaerobacterium	0	0	0	0
Treponema	0	0	0	0
Trichococcus	0	0	0	0
unclassified	23.34193202	14.12594605	56.22698032	57.78571687
Veillonella	17.77721899	27.02117841	0.088236378	0.087554115
Wautersiella	0	0	1.767639261	0.004955893
Weissella	0.002908891	0.003303926	0.018423	0.003303926
Williamsia	0	0	0	0
Wolinella	0	0	0	0
Yaniella	0	0	0	0
Yersinia	0	0	0.009696304	0
YRC22	0	0	0	0