Microbiological analysis of molars with advanced furcation involvement before and after non-surgical or surgical periodontal therapy

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Microbiological analysis of molars with advanced furcation involvement before and after non-surgical or surgical periodontal therapy

Thesis submitted for the Degree of

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By

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Abstract

Background: Very little is known about the effects of microbial composition of furcation defects. Limited results about the furcation microbiome reported higher levels of both overall microbiological counts and anaerobic counts for furcation areas compared with non-furcation after the debridement. Interestingly, it was also stated that the microbiological environment of furcation defects appeared different from non-furcation defects, with few periodontopathogenic species such as *Porphyromonas* and *Treponema* (including *P. gingivalis* and *T. denticola*), in particular advanced furcation involvements (FIs) seemed to experience progressive disease regardless of the presence or absence of well-known periodontal pathogens.

Aim: To describe the furcation microbiological environment of subgingival dental plaque, comparing the level of aerobic, anaerobic, and facultative genera in furcation sites untreated, and treated with a surgical or non-surgical approach.

Materials and methods: A cross-sectional study with 57 participants, a single-centre randomized controlled clinical study with 20 participants and a multicentre randomised controlled clinical trial with 135 participants were designed to answer the research question. In the cross-sectional study, each participant contributed with a furcation defect, a matched non-furcation periodontal defect (NF) of similar probing pocket depth and a periodontally healthy site (HS). For the two RCTs, surgical and non-surgical periodontal treatment (NSPT) was provided on molars with advanced FI and a matched periodontally healthy molar was used as control. For all three projects, demographic, clinical parameters, and subgingival plaque samples were collected and analysed. Gingival crevicular fluid (GCF) was also collected in the cross-sectional study. Subgingival microbial composition was analysed sequencing the V3–V4 region of the 16S rRNA gene, and a multiplex bead immunoassay was carried out to estimate the level of 18 GCF biomarkers, associated with inflammation, connective tissue degradation and repair.

Results: For the cross-sectional study, 171 subgingival plaque and 84 GCF samples were included, while for each RCTs, 160 samples were analysed including test, control and healthy sites at the baseline and 4/6 months follow-up visit. Differences in richness, diversity, oxygen metabolism, microbial composition, GCF volume and biomarkers were observed when comparing different periodontal defects (FI and NF) with HS ($p<0.05$). Lower aerobic levels ($p< 0.05$) were detected in sites with untreated FI compared to NF and furcation sites treated with NSPT compared to surgically treated sites. IL-6, MMP-3, MMP-8, BMP-2, SOST, EGF, and TIMP-1 levels were increased in untreated furcation defects compared with non-furcation periodontal sites, despite no difference in probing pocket depths (PPD). Specific bacteria as *S. mutans*, *S. oralis*, and *S. gordonii* showed significant increased levels in surgically treated sites compared to NSPT. A significant lower level of facultative ($p<0.05$), and a barely significant lower level of aerobic bacteria ($p=0.05$) was detected between Treatment A and Treatment B, 4 months after treatment, in the multicentre study. Interestingly 4 months after treatment, higher level of *Cardiobacterium valvarum* were detected

in treatment B, and higher level of *Fusobacterium periodonticum* were detected in treatment A. No significant difference was detected in level of *Streptococcus* genera between Treatment B and HS after treatment.

Conclusion: The anatomical complexity of furcation defects with untreated periodontitis is characterized by a subgingival microbial composition with a reduced level of aerobic bacteria and an increase of several inflammatory, connective tissue degradation and repair markers compared with matched-periodontal defects in the same individual. Periodontal therapy using a non-surgical approached resulted in a less aerobic environment, while surgical treatments may interact with local anatomical factors selecting a unique microbiological profile characterised by higher level of aerobic bacteria, including streptococci species associated with root caries.

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Santamaria P, Sari A, Nibali L. Molecular profiling of gingival crevicular fluid fails to distinguish between infrabony and suprabony periodontal defects. J Clin Periodontol. 2023 Oct;50(10):1315-1325. doi: 10.1111/jcpe.13849. Epub 2023 Jul 12. PMID: 37438680.

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Troiano G, Nibali L, Petsos H, Eickholz P, Saleh MHA, Santamaria P, Jian J, Shi S, Meng H, Zhurakivska K, Wang HL, Ravidà A. Development and international validation of logistic regression and machine-learning models for the prediction of 10-year molar loss. J Clin Periodontol. 2023 Mar;50(3):348-357. doi:10.1111/jcpe.13739. Epub 2022 Dec 11. PMID: 36305042.

Rexhepi I, Paolantonio M, Romano L, Serroni M, Santamaria P, Secondi L, Paolantonio G, Sinjari B, De Ninis P, Femminella B. Efficacy of inorganic bovine bone combined with leukocyte and platelet-rich fibrin or collagen membranes for treating unfavorable periodontal infrabony defects: Randomized non-

inferiority trial. J Periodontol. 2021 Nov;92(11):1576-1587. doi: 10.1002/JPER.20-0305. Epub 2021 Feb 25. PMID: 33547808.

Role of the candidate in the thesis.

Pasquale Santamaria (PS) was responsible for the ethics approval, coordination, recruitment, randomisation and examiners calibration of the cross-sectional study presented in chapter 3, and the single-centre study presented in chapter 4. PS was primary involved in the ethics approval, coordination, randomisation and calibration of the multicentre study reported in chapter 5. PS was responsible of the collection of subgingival plaque samples for chapter 3 and 4 projects. PS proceeded with the storage and DNA extraction process of all subgingival plaque samples analysed. PS conducted the statistical analysis of clinical parameters and explorative analysis of microbiological data.

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List of Abbreviations

°C: Centigrade

ASVs: Amplicon Sequence Variants

BMI: Body Mass Index

BMP-2: Bone Morphogenic Protein-2

Bp: Base Pair

BOP: Bleeding On Probing

CAL: Clinical Attachment Loss

CBCT: Cone Beam Computed Tomography

CEJ: Cement-Enamel Junction

CEP: Cervical Enamel Projection

CI: Confidence Interval

CS. Control Sites

EGF: Epidermal Growth Factor

Fapα: Fibroblast Activation Protein Alpha

FI: Furcation Involvement

FMBS: Full Mouth Bleeding Score

FMPS: Full Mouth Plaque Score

FRA: Furcation Roof Area

GCF: Gingival Crevicular Fluid

HOMD: Human Oral Microbiome Database

HS: Healthy Sites

IL-10: Interleukin-10

IL-17: Interleukin-17

IL-1α: Interleukin-1α

IL-1β: Interleukin1-B

IL-6: Interleukin-6

IFN- γ: Interferon-Gamma

KT: Keratinized Tissue

MMP-3: Matrix Metalloproteinase-3

MMP-8: Matrix Metalloproteinase-8

MTA: Material Transfer Agreement

MWF: Modified Widman's Flap (MWF)

NF: Non-Furcation Defects

NGS: Next-Generation Sequencing

NSPT: Non-Surgical Periodontal Therapy

OFD: Open Flap Debridement

OHIP: Oral Health Impact Profile

OPN: Osteopontin

OR: Odds Ratio

PCoA: Principal Coordinates Analysis

PCR: Polymerase Chain Reaction

PDGF-AA: Platelet-Derived Growth Factor-AA

PDGF-BB: Derived Growth Factor-BB

Pg: Picogram

PGE: Prostaglandins

PMPR: Professional Mechanical Plaque Removal

PPD: Probing Pocket Depth

PPE: Personal Protective Equipment

PROMS: Patient-Reported Outcomes

RANKL: Receptor Activator of Nuclear Factor Kappa-beta

RCT: Randomised Controlled Trial

REC: Gingival Recession

RTL: Root Trunk Length

SD: Standard Deviation

SEM: Scanning Electron Microscopy (SEM)

SOP: Standard Operating Procedure

SOST: Sclerostin

SPC: Supportive Periodontal Care

TIMP-1: Tissue Inhibitor Metalloproteinase-1

TS: Test Sites

UDIs: Unique Dual Indexes

VEGF: Vascular Endothelial Growth Factor

µl: Microliter

List of Contents

1 Chapter: Furcation Defect: Anatomy, Diagnosis and Prognosis.

1.1 Introduction

Management of moderate to advanced furcation involvement represents one of the greatest challenges for the success of periodontal therapy (Nibali, Krajewski, et al., 2017). High tooth mortality, poor prognosis, and reduced efficacy of periodontal treatment in molars with furcation involvement have been reported in several retrospective studies analysing tooth loss (Costa et al., 2022). Several reasons have been suggested to support these findings including the difficult accessibility for root instrumentation due to the complex anatomy of the furcation area and, consequently, the persistence of a dysbiotic microbiome.(Loos et al., 1988; Parashis et al., 1993; Svärdström & Wennström, 1996) Reduced responsiveness to therapy, unlike what happens in single-rooted teeth, can be further explained by the greater root surface susceptible to biofilm formation and calculus deposits. Substantial removal of plaque, calculus and bacterial products from the subgingival environment appears to play a key role in the success of periodontal therapy.(Abusleme et al., 2013; Caruso et al., 1982)

1.2 Classification of furcation involvement

A furcation is defined as "the anatomical area of a multi-rooted tooth where the roots separate" and furcation involvement corresponds to the "pathological resorption of alveolar bone within the furcation" (American Academy of Periodontology, 2001).

The horizontal degree of furcation involvement is a significant factor for assigning tooth prognosis and the decision-making process for setting up the periodontal treatment of a molar with furcation involvement (Nibali et al., 2018). Assigning the horizontal grade of a furcation defect offers the classification of the amount of horizontal clinical attachment loss in millimeters (also known as horizontal probing/clinical attachment)(Hamp et al., 1975). To determine the severity of furcation involvement is required the use of a rigid, curved probe, to measure the furcation site in question horizontally to determine the severity of involvement. One of the first classifications was developed by Glickman (Glickman, 1950), taking into account radiographic data, which is known to have

poor reliability, and offers somewhat ambiguous criteria to differentiate between classes of furcation involvement.

1.2.1 Horizontal classification systems

Several classification systems have been developed to assess the depth of the horizontal and vertical bone loss in the furcation area:

Goldman (H. M. Goldman, 1958)

- Grade I: incipient;
- Grade II: cul de sac.
- Grade III: Through-and-through lesion.

-Hamp (Hamp et al., 1975):

- Grade I: Horizontal loss of supporting periodontal tissue < 3 mm.
- Grade II: Horizontal attachment loss >3 mm, but not including the full width of the furcation.

- Grade III: loss of horizontal attachment with probe passing through the furcation zone.

Ramfjord & Ash (Ramfjord SP, Ash MM., 1979)

- Grade I: Initial involvement. Loss of horizontal attachment inside the furcation <2 mm.
- Grade II : Horizontal attachment loss >2 mm, but not through.
- Grade III: Through-and-through destruction.

Furcation involvement must be routinely checked for during periodontal examination at the sites where furcation entrances are typically expected. Essential components of a periodontal examination are the detection of and scoring of furcation involvement (De Beule et al., 1998). Furcation entrances do not lie open, especially in patients with untreated periodontal disease since gingiva covers them in most cases. Furcation diagnosis requires the use of specific curved furcation probes due to the peculiar anatomy of furcation defects

(Schroeder, 1991), and the fact that the furcation entrances of maxillary premolars and molars open into interproximal spaces. The location of the expected furcation entrance is marked by the placement of the probe coronally to the gingival margin on the tooth surface (e.g. the mid-lingual aspect of the mandibular molar). After that, the probe is pushed apically, gradually moving the gingiva in zigzag patterns until it reaches the bottom of the sulcus or pocket (Graetz et al., 2014). Furcation involvement has typically been found if the probe falls into a pit horizontally. Rigorous, straight periodontal probes (e.g. PCP UNC 15) do not follow the curved course of most furcation defects, making them unsuitable for furcation diagnosis. There is a significant chance that their application will underestimate the degree of furcation involvement (Eickholz & Kim, 1998).

1.2.2 Degree II and degree III in complex cases

The clinician may find difficult to easily insert the periodontal probe completely through the furcation area due to the interference of dentinal ridges or

buccal/lingual bone margins (Nibali et al., 2018). However, a grade III may also be suspected if the sum of the buccal and lingual measurements is equal to or greater than the buccal/ lingual dimension of the tooth at the furcation entrance (Nibali et al., 2018). Furcation involvement degree III refers to the possibility of both soft and hard tissue detaching from the furcation fornix (Abdallah et al., 1987).

1.2.3 Vertical classification of furcation defects

Vertical attachment and bone loss in the furcation area contributes in addition to horizontal attachment and bone loss. It was shown that initial vertical bone loss as well as initial horizontal furcation involvement determine the survival of molars following furcation therapy (Dannewitz et al., 2006; Park et al., 2009).

A subclassification (Tarnow & Fletcher, 1984), refers to the loss of vertical clinical attachment loss, which proposes 3 subclasses to be associated independently with one of the 3 horizontal grades listed above:

A) Vertical attachment loss of 1-3 mm;

B) Vertical attachment loss of 4-6 mm;

C) Vertical attachment loss greater than 7 mm.

According to the authors (Tarnow & Fletcher, 1984), furcation sites are categorised as IA, IB, IC, IIA, IIB, IIC, and IIIA, IIIB, IIIC. The long-term prognosis of molars with furcation involvement depends on a mutual correlation between the horizontal and the vertical component (Tonetti et al., 2017).

1.3 Anatomy of Molars

Only teeth with multiple roots exhibit furcation involvement. Maxillary and mandibular molars, as well as first maxillary premolars, frequently have multiple roots. Several anatomical features related to the anatomy of furcation defects have been investigated as potential key factors in the decision-making process of the periodontal care (Abitbol et al., 1997; Paolantonio et al., 1992). Furcation entrance area, (bi)furcation ridges, root surface area, root divergence, and root trunk length are thought to play an important role in survival of molars with furcation involvement due to the higher mortality and compromised diagnoses

of furcation-involved molars, as well as the reduced efficacy of periodontal therapy in multi-rooted teeth (Al-Shammari et al., 2001; Chace & Low, 1993; Leknes et al., 1997). Orban and Mueller (Orban & Mueller, 1929) focused their early studies on the anatomy of multirooted teeth before and after development of furcation sites offering interesting graphic visualisation of the aforementioned anatomical features. Their three-dimensional representations enable a thorough impression of the root area similar to those reported by other authors (Svärdström & Wennström, 1988). Subsequently, a growing interest has been developed on researching on micro-anatomical and histological characteristics of molars with furcation involvement in diagnoses and prognosis of periodontal patients (Bower, 1979; G. L. Hou et al., 1994; G.-L. Hou et al., 2005; H. K. Lu, 1992)

1.3.1 Anatomical features of multi-rooted teeth

The root complex can be divided into 3 parts: the root trunk, (2) the root cone, and the root fornix (Figure 1.1).

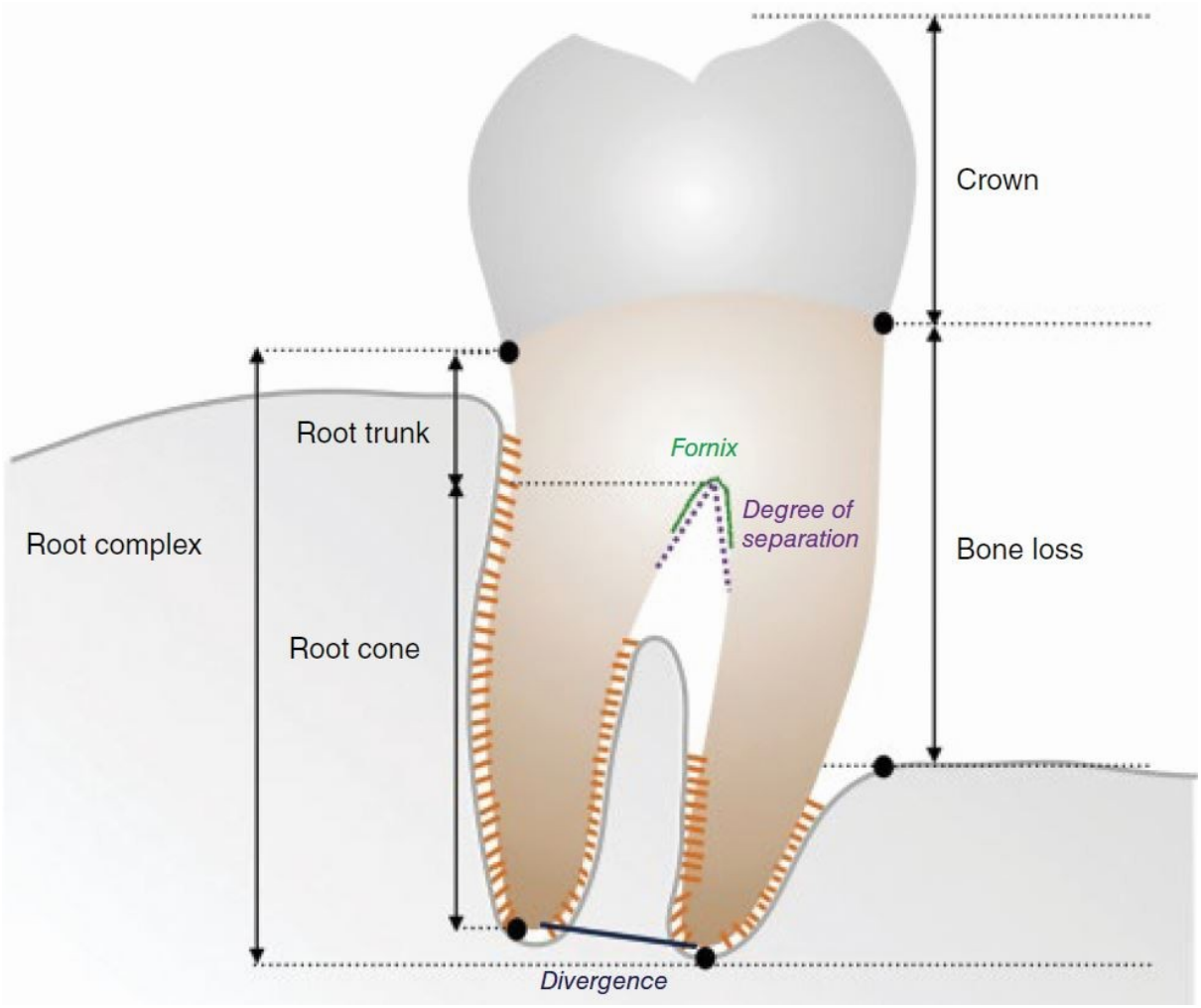


Figure 1.1 Illustration of a mandibular molar with FI

It is highlighted the primary anatomical characteristics such as the root cones and trunk. Additionally indicated are the root divergence and the degree of separation. Reproduced from Nibali (Nibali, 2018).

Several factors including the length of the root trunk, the area of entry to the furcation, the divergence of the roots, and the root surface can influence the diagnosis and, consequently, the choice of therapeutic approach in molars with furcation involvement (Nishihara et al., 1983).

In addition, several morphological features in teeth with furcation involvement may contribute to a poor prognostic picture including the presence of enamel beads, furcation ridges, root cavities, and cervical enamel projections.(Blanchard et al., 2012; Chrcanovic et al., 2010; G. L. Hou & Tsai, 1997)

Bower et al. (Bower, 1979) reported that the mesio-distal width at the CEJ level of the maxillary and mandibular first molars showed a negative correlation with the diameter of the area of entry to the furcation. Similarly, it has been found that, in the lower molars, the width of the entrance area of the buccal furcation is smaller than that of the lingual furcation (Bower, 1979; Paolantonio et al., 1992). Moreover, in maxillary molars, buccal furcation defect had an area of extension smaller than both the mesio- and disto-palatal ones. Bower (Bower, 1979) found that in 81% of cases the diameter of the furcation area was below1mm, and that

in 58% of the samples it was below 0.75mm. Considering that the thickness of the blade of a traditional curette is between 0.75 and 1.10 mm, the author concluded that exclusive use of curettes was not sufficient for adequate achieve a complete debridement of the furcation area (Bower, 1979).

A subsequent study used sections of molar teeth to analyse the anatomical complexity of the root surface in the furcation area (Paolantonio et al., 1992). These studies found concavities on almost all the roots of the mandibular molars, with more pronounced depressions detected more frequently on the mesial roots. In maxillary molars, concavities were detected on the surface of 94% of mesio-buccal roots, 31% of disto-buccal roots and 17% of palatal roots (Paolantonio et al., 1992).

1.3.2 Furcation ridges

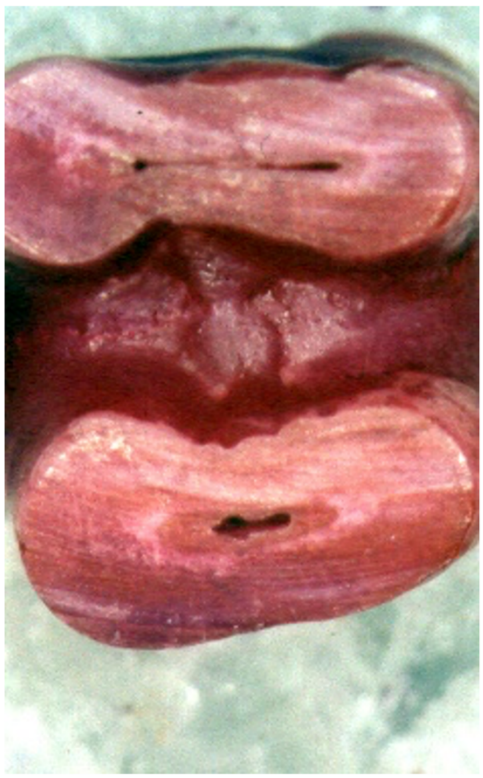
In a morphometric analysis conducted by Paolantonio and co-workers (Paolantonio et al., 1998), 414 molars (207 maxillary molars and 207 mandibular molars) extracted for advanced periodontitis, caries, endodontic infections and

orthodontic reasons were evaluated. It was found that in both types of molars the width of the interradicular angle (IRA) decreased from the first to the second molars and that this decrease was more evident in the maxillary molars. As a result, the amplitude of the furcation roof area FRA was greater in the first molars in both arches. In addition, first molars had longer roots and a shorter root trunk than second molars. The mesio-distal diameter and buccolingual diameter were greater in the first molars, also a positive correlation between FRA and IRA in both maxillary and mandibular molars and an inverse correlation between IRA and root trunk length (RTL) was found (Paolantonio et al., 1998).

The use stereo-microscopic equipment, optical transmission microscopy and scanning electron microscopy (SEM) was also used to evaluate the anatomy of the furcation area from a morphological point of view. Stereo-microscopic examination showed the extreme complexity of the roof surface of the furcation in observed teeth (Paolantonio et al., 1998). In 40% of mandibular molars, the so-called furcation ridge was observed in the middle third of the furcation roof (Figure 1.2), which, on the other hand, was less frequently found in maxillary

molars. In 20% of maxillary molars, the crest of the furcation tended to converge towards the centre of the roof from the inner surface of the buccal roots. On both sides of the ridge of the furcation it was possible to observe concavities, within which the presence of small narrow and irregular depressions could be detected. These concavities were present on the surface of the furcation roof even in the absence of the ridge and on the mesial and distal root surfaces facing the furcation area (Paolantonio et al., 1998).

a)



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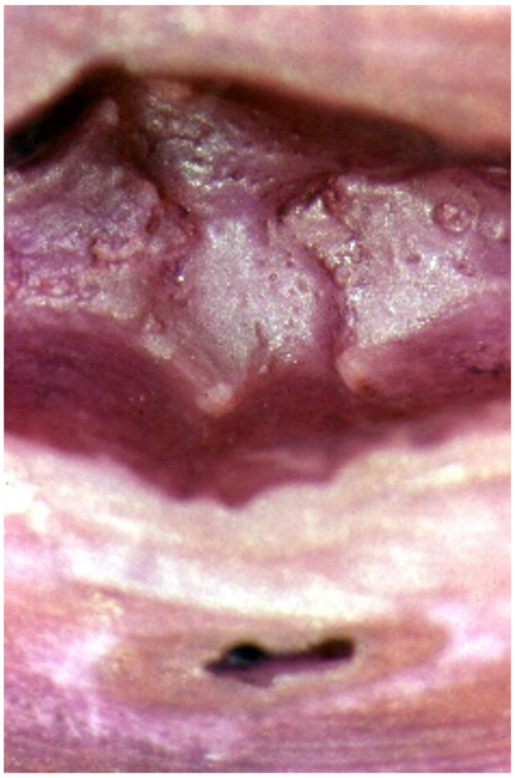


Figure 1.2 Stereo-microscopic examination of the ridge of furcation

a) First mandibula molar with ridge of the furcation evident on the left photograph at 15x magnification; b) on the right side: 45x magnification.

Reproduced with permission from Paolantonio M. (Paolantonio et al., 1998).

Previous studies have described two types of furcation ridges: intermediate ridges and buccal/lingual ridges (Al-Shammari et al., 2001; G. L. Hou & Tsai, 1997). Intermediate ridges connect mesial roots with distal ones and are composed mainly of cementum, while buccal and lingual ridges are primarily characterised by dentinal tissue covered by a thin layer of cementum. Everett (Everett et al., 1958) was the first to report an incidence of 73% of intermediate ridges and 63% of buccal and lingual ridges on first mandibular molars, while (Burch et al., 1992) reported an incidence of 76.3%.

Several analyses confirmed the hypothesis that residual presence of calculus within the concavities adjacent to the crest of the furcation was evident even

following mechanical plaque removal with manual and sonic instrumentation under local anaesthesia (Al-Shammari et al., 2001; G. L. Hou & Tsai, 1997).

1.3.3 Roof of the furcation

Irregularities in the roof of the furcation can be clearly detected in the buccal-lingual sagittal sections in figure 1.3, which reveals the presence of a wide variety of depressions, cavities and pits that promote plaque and calculus retention (Paolantonio et al., 1998). A further factor that could lead to incomplete plaque and calculus removal is the greater proximity (sometimes extreme) of the roots, especially in the second molars of both mandibular and maxillary arches.

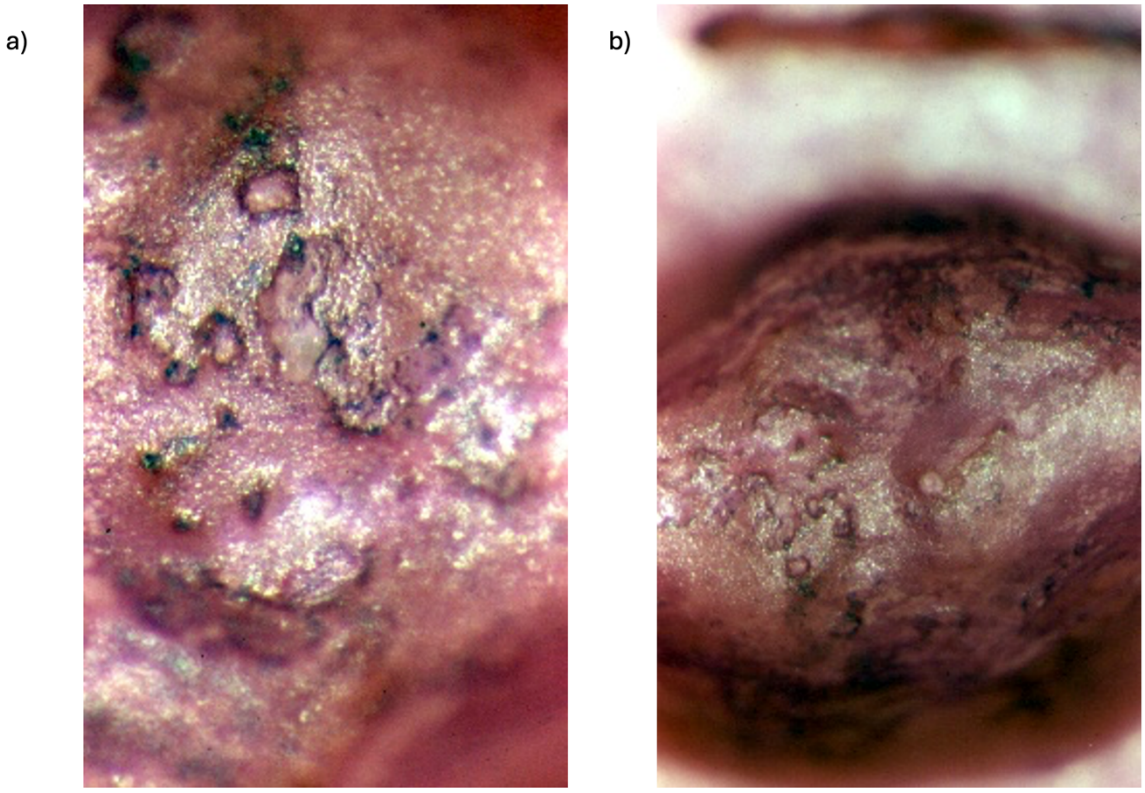


Figure 1.3 Irregularities of roof of furcation area

a) magnification 100x molar with deposit of calculus (black) in the irregular surface of the root of the furcation area. b) magnification 60x, irregularities involving the majority of the roof surface. Reproduced with permission from Paolantonio M. (Paolantonio et al., 1998)

Scanning electron microscopy confirmed the findings described above and showed the presence of a conspicuous number of concavities irregularly

distributed over the entire surface of the furcation roof, representing the external orifices of the accessory canals that connect the pulp system with the interradicular periodontal tissues (Paolantonio et al., 1998). The diameter of these channels varies between 6 and 650 μm. Very frequently it is possible to observe how, especially in the maxillary molars, the pulp canals converge in large depressions that contribute to the largely irregular anatomy of the roof of the furcation (Figure 1.4). The continuous deposition of cementum over the course of life leads to a reduction in the depth of these concavities (Paolantonio et al., 1998).

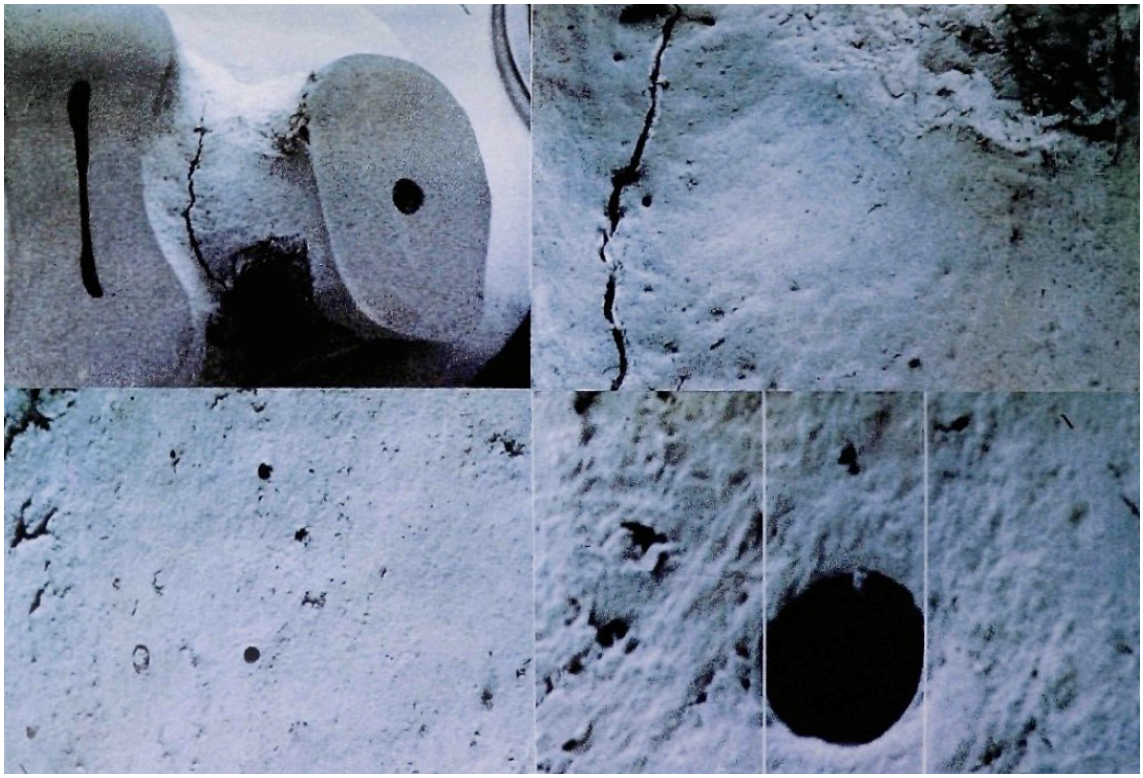


Figure 1.4 Electron microscopy scan of the roof of the furcation area

Top left: mandibular first molar, analysed with electron microscopy scan shows a large number of orifices on the roof of the furcation (10x magnification). Top right: same section at 50x magnification. Bottom left: the same section at 500x magnification highlights several accessory canal orifices with different diameters. Bottom right: small orifice (6-18 μm in diameter) of a furcation channel (5000x magnification). Reproduced with permission from Paolantonio M. (Paolantonio et al., 1998).

1.3.4 Unfavourable anatomical factors

Additional factors that can compromise the outcomes of periodontal therapy in furcation defects are cervical enamel projections (CEPs) and enamel pearls, which can prevent the formation of a new connective tissue attachment (Carranza & Jolkovsky, 1991). These factors could therefore play a role in the development of a furcation defect (Al-Shammari et al., 2001b). Also, it was noted that the presence of CEPs is associated with noticeably higher plaque and gingivitis index values (Carnevale et al., 1995). Masters et al. (Masters & Hoskins, 1964) found an incidence of enamel projections of 28.6% for mandibular molars and 17% for maxillary molars and classified the projections into 3 grades:

Grade I: clear alteration of the profile of the amelo-cemental junction, with a projection of the enamel towards the furcation (<1/3 of the root trunk).

Grade II: Enamel projection near the furcation, but not in contact with it (>1/3).

Grade III: Projection extending into the furcation.

In a study of 1138 molars, Bissada & Abdelmalek (Bissada & Abdelmalek, 1973) reported an incidence of CEPs of 8.6%. The association between CEPs and furcation area involvement was 50%. In addition, they found that the incidence was highest in the second mandibular molars (14.8%), followed by the second maxillary molars (9.1%), the first mandibular molars (7.8%) and finally the first maxillary molars (3.3%). In a more recent morphometric analysis of 134 mandibular first and second molars conducted by Mandelaris and co-workers (Mandelaris et al., 1998), there was an incidence of 56.4% of CEPs, mainly located on the buccal side (61.9%) when compared with the lingual side (50.8%).

The prevalence of enamel pearls is lower than that of CEPs. Moskow & Canut (Moskow & Canut, 1990) reported an incidence of 2.6% (ranging from 1.1% to 9.7%). Mostly composed of enamel with a dentine core, these ectopic globules are evident on the tooth root surface. They are especially common in furcation areas of molar teeth, particularly maxillary third and second molars. Similar to CEPs, enamel projections also prevent the attachment of connective tissue (Nibali, 2018). It is reported that diagnosing enamel pearls at an early stage is

crucial in order to provide an adequate prognosis of molar retention and potentially modify the therapeutic approach (Borg, 1984; Chrcanovic et al., 2010).

1.4 Epidemiology of furcation lesions

Studies on the prevalence of periodontitis have led to believe that severe periodontitis fluctuates between 5% and 20% of prevalence in the various populations analysed. Similarly, longitudinal studies aimed at describing the progression of untreated periodontitis have shown that most severe and rapid rate of periodontal bone loss can be detected in a small subset of the general population (Aimetti et al., 2015).

Although the efficacy of periodontal therapy in successfully arresting the disease has been well documented over the years (Jeffcoat et al., 2014), tooth loss does occur in both treated and untreated teeth, albeit with significantly contrasting annual tooth loss rates, in terms of relative risk, of 0.05-0.1% and 0.14-0.38%, respectively (Bäumer et al., 2011; Carvalho et al., 2021; Costa et al., 2022).

Molars are the least responsive to periodontal therapy and the most susceptible to extraction compared to single-rooted teeth (Checchi et al., 2002; Matuliene et al., 2008; McFall, 1982; Nibali, Krajewski, et al., 2017b). This trend, in part, can be associated with furcation involvement. The destruction of the supporting bone tissue in this area, the peculiar anatomical configuration, the distal position in the maxillary and mandibular arches can accelerate the rate of periodontal tissues destruction, making it extremely difficult for the patient and the dentist to control the disease (Kocher et al., 1998; Matia et al., 1986; Parashis et al., 1993a). It is commonly accepted the existence of a close association between clinical and radiographic involvement of the furcation and risk of tooth loss (Costa et al., 2022; Dannewitz et al., 2006; Nibali et al., 2018).

1.4.1 Cross-sectional studies

Few epidemiological studies have analysed the prevalence of molar furcation involvement in the general population. Most of the available data are derived from observations made on dry skulls (Bissada & Abdelmalek, 1973; Zee &

Bratthall, 2003)[7]. These studies found that maxillary first and second molars have a higher level of furcation involvement than mandibular molars. Moreover, first molars were more frequently affected than second molars. However, these results should be interpreted with caution, since the small sample number and the marked social and ethnic characterizations of the anatomical specimens under analysis could prevent their extrapolation to other populations (Bissada & Abdelmalek, 1973; Zee & Bratthall, 2003).

In the study by Volchansky & Cleaton-Jones (Volchansky & Cleaton-Jones, 1978)[8] conducted on 43 mandibles of the South African Bantu population, furcation involvement was found in 30.9% of molars. Tal (H. Tal, 1982) after examining 100 mandibles taken from skulls of South African populations, detected bone resorption in the furcation area in 85.4% of the mandibular molars surveyed. The same author also showed an increase in the depth of the bone defect proportional to advancing age. Tal & Lemmer (H. Tal & Lemmer, 1982)[9] in subsequent studies, were able to confirm the previous results and highlight a greater involvement of the first molars than the second.

1.4.2 Longitudinal studies

Björn & Hjort (Björn & Hjort, 1982) in a longitudinal study, evaluated the radiographic prevalence, degree and progression of bone destruction in the mandibular molars of 221 workers observed over a period of 13 years. The results showed that the prevalence of lesions increased from 18% to 32% from the beginning to the end of the observation period, and that the second and third molars had a more advanced degree of destruction than the first molars (Björn & Hjort, 1982).

Finally, additional data were obtained from longitudinal studies conducted on subjects undergoing periodontal therapy (Gill et al., 2022; Johansson et al., 2013; Svärdström & Wennström, 1996b). Maxillary molars were more frequently affected than mandibular molars with a furcation involvement ranging from 25 to 52% and from 16 to 35%, respectively. Svärdström & Wennström (Svärdström & Wennström, 1996b) studied the prevalence of furcation lesions in 222 patients receiving periodontal therapy. After the age of 30, about 50% of the maxillary

molars had an advanced furcation defect, while a similar prevalence was observed after the age of 40 for mandibular molars. In the upper maxilla, the most frequently affected sites were the distal furcation sites of first and second molars (53% and 35%, respectively), while in the lower mandible, lingual and buccal furcation sites were affected with equal frequency. Nevins in his book (Nevins et al., 1998) cited an unpublished thesis by Purisi written in 1980,who conducted a study on 83 cadavers, reported a furcation involvement incidence of 26% in subjects aged between 29 and 35 years, and an incidence of 70% in individuals over 35 years of age.

The effects of smoking were analysed by Mullaly (Mullally & Linden, 1994) and found furcation involvement in 36% of non-smokers and in 72% of smokers using more than 10 cigarettes/day.

Interestingly, Wang (Wang et al., 1993) showed in 1993 that molars with prosthetic crowns or interproximal restorations had a significantly higher prevalence of furcation involvement than molars without restorations (prosthetic crowns: 52-63% of FI, and interproximal restoration: 39% of FI).

1.5 Diagnosis

Furcation diagnosis plays a key role in the management of patient with periodontitis, and it also contributes as prognostic factor of tooth loss. It is evident that an advanced furcation defect increases the risk of tooth loss in treated periodontal patients (Dannewitz et al., 2016; Graetz et al., 2014; Hirschfeld & Wasserman, 1978; McGuire & Nunn, 1996; Nibali et al., 2016; Salvi et al., 2014) and in absence of supportive periodontal care (SPC) (Nibali, Krajewski, et al., 2017)

1.5.1 Effectiveness of clinical diagnosis of furcation defects

In a recent survey (Nibali et al., 2021), the role of 'general dental practitioners' (GDPs) in diagnosing periodontal furcation involvement (FI) was explored. An online survey was given to 400 participants asking questions in relation to detection, classification and management of periodontal FI in seven different countries. Approximately 20% of GDPs answered they did not record the

periodontal chart regularly; more than 60% of participants were able to use a curved furcation probe and most of them were able to identify a FI on clinical pictures and radiographs. Although almost half of GDPs showed high level of expertise in classifying a furcation defect, only a tenth were confident on treating them. This study concluded that general dentist should be involved in the treatment of molar with FI after increasing their confidence in approaching these defects, since this can have a good impact on public health.

Moriarty and co-workers (Moriarty et al., 1988) conducted a study on 102 molars with untreated periodontitis, showing II-III horizontal degree of furcation involvement reporting, how the inter-examiner reproducibility of the buccal and lingual furcation defects that were probed decreased with an increase in probing pocket depth and an increased degree of root separation, as this increases the penetrability of the probe and makes more difficult to sound the root surface (Moriarty et al., 1988). Furthermore, the reproducibility of horizontal measurements of furcation defects was lower that the reproducibility of vertical

measurements with only 24 of the 102 sites examined showed overlapping measurements across operators (Moriarty et al., 1988).

The reproducibility of measurements obtained probing the furcation area is still controversial. Some authors argued that a reliable correlation in the measurement of the furcation involvement between non-surgical and open flap measurement (Alsakr et al., 2022; Graetz et al., 2014). Zappa and co-workers compared horizontal measurements obtained from twelve patients assessed according to the Hamp classification (Hamp et al., 1975) with measurements after surgical exposure. The results showed significant discrepancies, with both overestimation and underestimation errors, regardless of the use of a calibrated and non-calibrated Nabers probe (Zappa et al., 1993).

In a histological study, Moriarty and co-workers (Moriarty et al., 1989) assessed the position of the periodontal probe tip when probing the deepest interradicular pocket depth in buccal with II and III degree of furcation involvement on 12 buccal furcation defects, found that probing the deepest interradicular site does not detect the true pocket depth or the true level of attachment in the furcation

area (Moriarty et al., 1989). The tip of the probe was placed on average 0.4 mm apically to the interradicular bone septum, in the context of the inflamed connective tissue of the site under examination. This investigation demonstrated that probing a furcation area does not measure the clinical attachment level of the interradicular root surfaces, but rather records the depth of probe penetration into the inflamed furcation connective tissue (Moriarty et al., 1989).

On the other hand, Eickholz & Staehle and Eickholz (Eickholz, 1995; Eickholz & Staehle, 1994) were able to find satisfactory reliability in terms of furcation involvement in the comparison of clinical and intraoperative measurements, except for disto-palatal sites where only a moderate association could be found.

1.5.2 Bone sounding

Bone and transgingival sounding under local anaesthesia have been thought to assist in the diagnosis of furcation lesions and in the determination of underlying bone contours. Greenberg and co-workers (Greenberg et al., 1976) reported that bone probing can give reliable results when compared with those of

intraoperative measurements. An accurate diagnosis, therefore, requires a combination of radiographic data and Nabers probe and bone sounding (Kalkwarf & Reinhardt, 1988).

1.5.3 Radiographic diagnosis of furcation area

In general, radiographs provide information about the translucency of various tissues to X-rays. When a tooth is affected by a furcation defect, radiographs can offer a partial insight into the bone loss related to the furcation involvement but are not able to highlight changes in the soft tissue profile (Figure 1.5) (Balusubramanya et al., 2012). This limitation is particularly evident after regenerative treatments, where new connective tissue attachment may form in a furcation without the development of new bone (Nibali, 2018). Therefore, it is not possible to reliably diagnose furcation involvement using two-dimensional radiographic techniques (projection radiography: periapical and panoramic radiographs) (Topoll et al., 1988).

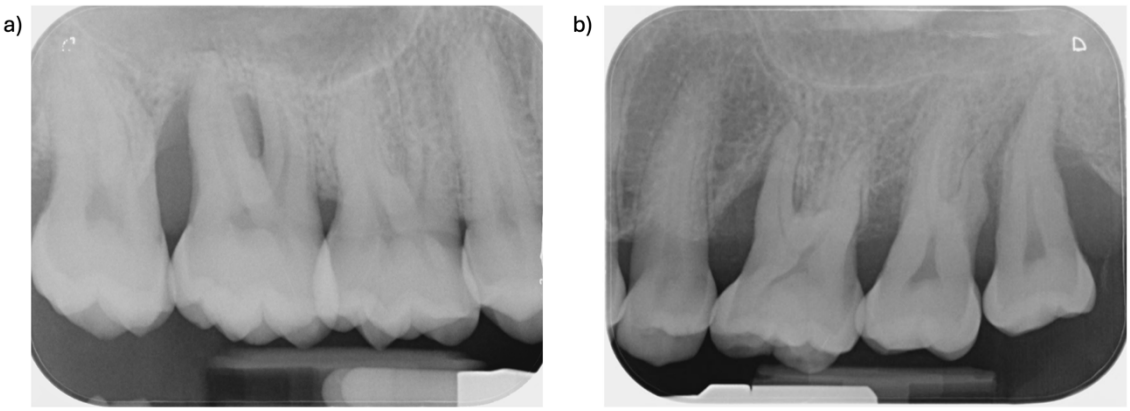


Figure 1.5 Radiological analyses of furcation defects.

a) Second upper right molar with degree III furcation involvement of disto-palatal and buccal furcation in association with intrabony defect, reduced separation for the buccal roots and long root trunk. b) First upper left molar with potential degree III furcation involvement on the mesial-palatal aspect.

Radiographic examination can be of help in the diagnosis of furcation defects, but it shows less accuracy if used as the only diagnostic tool, especially in mild and moderate defects. Ross & Thompson (Ross & Thompson, 1978) found that radiographic examination was able to detect a potential furcation defect in 22%

of cases in the maxillary molars and in 8% of cases in the mandibular molars. This discrepancy has been attributed to the different bone density of the maxillary and mandibular arches. The two authors also underlined the importance of combining the data of the clinical parameters with radiographic findings in order to obtain greater diagnostic accuracy. In relation to the radiological examinations of furcation sites, radiographs may or may not identify the furcation involvement in particular of maxillary molars due to the radiographic overlap of the roots (Ross & Thompson, 1980). In some views, however, the presence of furcation 'arrows' indicates a putative furcation involvement. Hardekopf and co-workers (Hardekopf et al., 1987) found a significant association between the presence of the so-called "furcation arrow" and grade II and III defects of the disto- and mesio-palatal furcation sites of the maxillary molars. An association between radiological evidence of the 'furcation arrow' and clinical evidence of the furcation defect was found in grade I furcation defects on the mesial aspect (19%), grade II furcation defects (44%) and 55% in grade III furcation sites. Distal furcation sites showed an incidence of 'furcation arrow' of 19% for I grades, 30%

for II and 52% for III (Hardekopf et al., 1987). Although the presence of radiological arrow sign is significantly associated to the clinical evidence of a degree II and III furcation involvement of the mesio- and disto-palatal furcation defects in maxillary molars, if not radiologically detected does not necessarily represent the absence of furcation involvement (Figure 1.6).

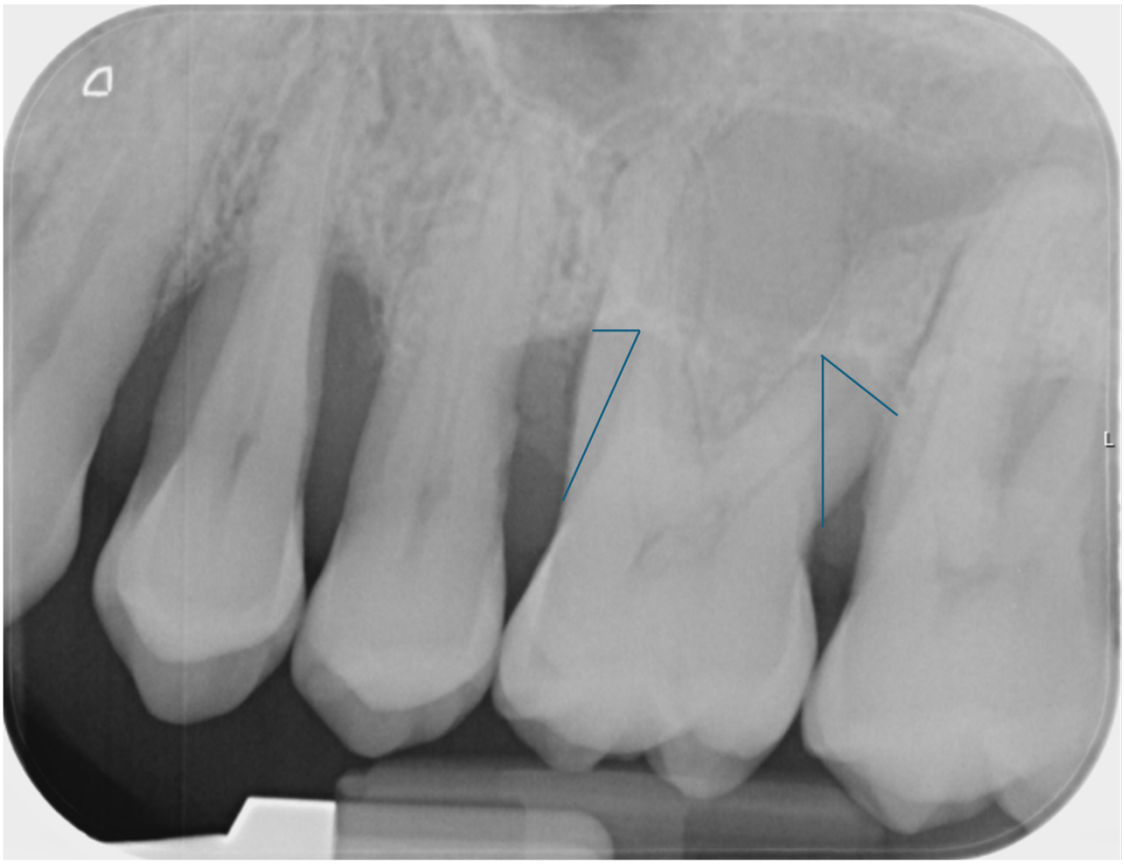


Figure 1.6 The Furcation arrow in upper molars

The external profile of the palatal root, the interdental crestal bone profile and the external profile of the buccal roots outline the 'furcation arrow' (blue lines on both mesial and distal aspect of first maxillary left molar) reflecting a significant association of a grade II-III FI for maxillary molars (Hardekopf et al., 1987).

Interestingly, the presence of a radiolucent area in the furcation site is not always an indication of a periodontal involvement, as endodontic pathologies associated with the presence of accessory channels communicating with the interradicular region can reproduce bone resorption comparable to that of periodontal origin (de Miranda et al., 2013; Paolantonio et al., 1998).

1.5.4 Three-dimensional radiography

Given the potential drawbacks of conventional two-dimensional radiographic imaging, it may be beneficial to examine specific clinical scenarios using a suitable three-dimensional diagnostic approach and the right amount of

radiation exposure, especially in the case of maxillary molar teeth (Laky et al., 2013; Walter et al., 2010). Cone Beam Computed Tomography (CBCT) has been proven to be an effective in vivo method for evaluating maxillary molars involved in furcation sites (Walter et al., 2010). CBCT data were reported to accurately classify the degree of furcation involvement in maxillary molars and assess the amount of periodontal tissue loss (Walter et al., 2009, 2010). Furthermore, multiple findings were revealed by the three-dimensional images, including the bony support surrounding each maxillary molar root, the proximity or fusion of roots, periapical lesions, root perforations, and/or missing bony walls (Walter et al., 2009, 2010b). Maintaining a radiation dose as low as reasonably achievable (ALARA) is the primary objective of diagnostic radiology and should also be a requirement for the use of CBCT in dentistry (Nibali, 2018).

1.6 Management of furcation Involvement in patient with periodontitis

Microbiological plaque has been shown to be the aetiological factor of human gingivitis in an experimental model (Loe et al., 1965). The idea that gingivitis is a precondition for the onset of periodontitis is supported by a 26-year longitudinal study on well-maintained Norwegian males, which revealed that sites with persistent plaque-induced gingival inflammation had 70% more clinical attachment loss (than sites that remained healthy) (Schätzle et al., 2003). Eliminating microbial plaque and its retentive factors from root surfaces and gingival sulcus is the main goal of periodontitis management in order to stop disease onset and progression. The main method for achieving this is through expert supra- and subgingival mechanical debridement, which aims to remove the microbial biofilm that is developing on the root surface (Tonetti et al., 2017).

1.6.1 Guidelines for periodontal therapy

According to British Society of Periodontology and Implant Dentistry (BSP) implementation of European S3 - level evidence-based treatment guidelines for

stage I-III periodontitis in UK clinical practice (West et al., 2021), patients with periodontitis should follow a predetermined, step-by-step therapy regimen that includes various interventions, each of which should be incremental in nature based on the disease stage. Informing the patient about the diagnosis, the condition's causes, risk factors, available treatments, anticipated risks and benefits, and the option to forego treatment is a crucial prerequisite to therapy. After this discussion, a customized care plan should be decided upon (West et al., 2021). Throughout the course of treatment, the plan may need to be adjusted in response to patient preferences, clinical findings, and changes in general health.

The first step in therapy aims to direct behaviour change by guiding the patient on how to successfully remove supragingival dental biofilm and control risk factors:

1. Supragingival dental biofilm control

2. Professional Mechanical Plaque Removal (PMPR), which includes the professional interventions aimed at removing supragingival plaque and calculus,

3. Risk factors control which refers to all health-related behavioural change initiatives that eliminate or reduce the known risk factors for the development and progression of periodontitis. (smoking cessation, improved metabolic control of diabetes, and perhaps physical exercise, dietary counselling and weight loss).

The second step of therapy (cause-related therapy) is to remove the calculus and subgingival biofilm with subgingival instrumentation. Furthermore, in some specific case, the use of additional physical or chemical agents as subgingival locally delivered antimicrobials, and the use of additional systemic antimicrobials was also suggested (West et al., 2021).

Clinicians should proceed with the third stage of therapy if deep periodontal pockets measuring more than 5 mm are still evident after step 2, while patients with no periodontal pockets that means probing depth less than 4mm or equal to 4 mm with no bleeding should be maintained in long-term as part of the supportive periodontal care (West et al., 2021). The third step of therapy aims to manage complex anatomical factor as infrabony and/or furcation defects.

1.6.2 Treatment of furcation involvement: literature evidence.

Furcation lesions are particularly difficult to treat with both non-surgical and surgical periodontal therapy (Gill et al., 2022). The anatomical configuration of the affected area, in particular the access to the furcation area, the presence of root concavities, the irregularities of the roof of the furcation, and peculiar anatomical abnormalities make it difficult to guarantee adequate mechanical debridement of the interradicular region even when resorting to a surgical approach (Gill et al., 2022b; Matia et al., 1986; Parashis & Mitsis, 1993).

Brayer and Fleischer (Brayer et al., 1989; Fleischer et al., 1989) evaluated the amount of residual calculus in molars with furcation involvement, after a single session of scaling and root planing performed as open flap procedure by clinicians with two different degrees of experience. Among experienced operators, open-flap instrumentation was associated with lower amounts of residual calculus compared to procedures performed without opening a flap (open flap: 32% of residual calculus, non-open flap: 56% of residual calculus),

however with no significant difference among the two comparison groups (Brayer et al., 1989; Fleischer et al., 1989). When comparing the low-experienced operators, it was possible to find a significant difference between surgical and non-surgical procedures (open flap procedure: 43% of residual calculus versus non-open flap procedure: 81% of residual calculus). Therefore, it was shown that the level of experience seems to play an important role in the cleansing of the furcation area, and that experienced clinicians, regardless of the type of approach, are able to obtain better results in terms of root surfaces debridement compared to less-experienced practitioners (Brayer et al., 1989; Fleischer et al., 1989).

Buchanan & Robertson (Buchanan & Robertson, 1987) also found similar results between open-flap surgical procedures and non-surgical approach in high-experienced practitioners only. In addition, it was also found that the surgical approaches were less effective on multi-rooted molars when compared with single rooted teeth due to several factors, including difficult surgical access, instrumentation.

In 1986, Matia and co-workers (Matia et al., 1986) compared the percentages of residual calculus in the furcation area after instrumentation carried out by experienced operators in 4 groups: non-surgical approach with manual curettes, non-surgical approach with ultrasonics, surgical approach with manual curettes and surgical approach with ultrasonics. It was concluded that the open flap debridement resulted in lower amounts of residual calculus than the non-surgical techniques and that ultrasonic instrumentation was more efficient compared to manual curettes.

Parashis and co-workers (Parashis et al., 1993) in 1993 evaluated the efficacy of root planing in the furcation area of horizontal degree grade II and III, with open flap debridement and non-surgical techniques comparing the use of manual curettes and diamonds burs. The results showed how only 12.5% of the root surfaces treated with manual-nonsurgical approach and 25% of surfaces treated with manual-surgical approaches were completely free of calculus residues. On the other hand, a significant improvement in the quantity of plaque and calculus removal was achieved by using diamond burs during an open flap debridement,

with a completely calculus free area in 38% of cases, especially in furcation areas with a fornix entrance less than 2.4 mm. However, the presence of a periodontal pockets with a depth equal of more than 7 mm was reported as negative factor with increased amount of root surface still covered by plaque and calculus both after surgical and non-surgical procedures. In general, none of the techniques analysed was able to guarantee a 100% removal of plaque and calculus deposits.

Finally, Loos and co-workers (Loos et al., 1988) in a longitudinal study compared the microbiological effects of subgingival scaling in 24 mono-rooted teeth and 31 molars with grade II horizontal furcation defects. At the end of the 52nd week, both groups reported a significant reduction in bacterial load, but multi-rooted teeth had a significantly higher count of periodontal pathogenic bacteria (*Spirochetes* spp and *Porphyromonas* spp) than the mono-rooted teeth.

Therefore, it can be concluded that experienced operators were able to guarantee a more effective decontamination of the furcation area compared to clinicians with lower level of experience; and that for an expert operator, both

open-flap procedure and a non-surgical approach where able to offer similar advantages, especially when combining the use of curettes with ultrasonic and diamond-coated tips.

However, other investigations focused their attention on the evaluation of clinical parameters after different treatment modalities of molars showing furcation involvement (Kalkwarf et al., 1988; Schroer et al., 1991; Wang et al., 1994).

In particular, Kalkwarf and co-workers (Kalkwarf et al., 1988) evaluated the efficacy of four therapeutic approaches in furcation lesions: supragingival scaling, root planing, modified Widman's flap (MWF) and open flap debridement (OFD) with osteoplasty. The OFD with osteoplasty was found to be the most effective therapy for reducing pocket depth, with a decrease of 1.65 mm, however, this procedure was associated with the highest vertical attachment loss (0.36 mm) following the 2 years of observation. Supragingival scaling, root planing, and WMF showed a clinical attachment level gain of 0.32 mm, 0.44 mm, and 0.40 mm, respectively. In addition, all procedures, except for root planing, resulted in a worst horizontal attachment level in the furcation area (0.51 mm for

OFD osteoplasty surgery, 0.13 mm for supragingival scaling, and 0.14 mm for MWF). It is interesting to note that molars treated with bone surgery were associated with a greater number of tooth loss (n=55). This is probably related to the greater risk of developing caries and tooth fracture when the furcation area is exposed to the oral cavity environment.

Schroer and co-workers (Schroer et al., 1991) evaluated changes in attachment level and pocket depth in grade II lesions of buccal furcation defects, treated with open flap debridement and non-surgical periodontal therapy. At 16 months, both procedures resulted in a reduction in pocket depth of 1.2-1.5 mm. While non-surgical procedures produced minimal gain of new clinical attachment (0.60 mm), surgical procedure techniques were associated with a slight loss of clinical attachment (0.46 mm). However, these differences were not statistically significant.

Similarly, Wang and co-workers (Wang et al., 1994) found no statistically significant differences in terms of pocket depth reduction when comparing surgical and non-surgical procedures in molars with furcation involvement.

The greater gain of attachment related to the non-surgical approach was also confirmed by subsequent studies, that aimed to evaluate the densitometric alterations of the alveolar bone after scaling and root planing and open flap debridement (Payot et al., 1987). Payot and co-workers (Payot et al., 1987) treated grade I and II furcation lesions using non-surgical procedures, open flap debridement alone or in association with osteoplasty. All three procedures induced a resorption of the superficial portion of the interradicular bone septum in the following two months after the treatment. However, the initial bone loss stage was followed by a marked increase in the bone density in the 12 months after the non-surgical therapy. Similar results were also reported from the study by Brägger (Brägger, 1991).

In conclusion, although open flap debridement allows for better visibility of the root surface and ideally better removal of calculus deposits, clinical evaluations do not reveal a consistent difference between surgical and non-surgical procedures, regardless of the degree of furcation involvement. Furthermore, non-surgical procedures, while determining a lower reduction in pocket depth, prove

to be more effective in preserving the level of clinical attachment level and guarantee a faster remineralization of the bone tissue. However, the effectiveness of each method is heavily influenced by operator experience, patient compliance, individual risk factors or, more precisely, a combination of these parameters.

1.7 Tooth loss and furcation involvement

Supportive periodontal care (SPC) aims at maintaining periodontal stability in all treated periodontitis patients combining preventive and therapeutic interventions defined in the first and second steps of therapy, depending on the gingival and periodontal status of the patient's dentition (West et al., 2021). Regular SPC, which includes periodontal charting, supra- and subgingival debridement, and reinforcement and motivation for good oral hygiene, is linked to a lower risk of tooth loss (Nibali et al., 2018).

1.7.1 Tooth loss and non-surgical periodontal therapy

Several studies examined the survival of molars with furcation involvement that were treated exclusively by scaling and root planing. In particular, Hamp and co-workers (Hamp et al., 1975), taking into account molars with grade I furcation involvement, found a 5-year survival of 100%. In the study by Dannewitz and co-workers (Dannewitz et al., 2016), after an observation period of between 62 and 145 months (5-12 years), a survival rate of 90.7% was observed, only 5 molars were extracted for unspecified causes: 3 showed a III-degree furcation involvement, 1 degree II and 1 degree I furcation.

1.7.2 Tooth loss and surgical procedures

Several studies evaluated the survival of molars with furcation involvement after being treated with gingivectomy, apical repositioning flap or modified Widman's flap (with or without bone resection) and introduced into a maintenance periodontal program accordingly (Ariga, 1987; Nibali, Krajewski, et al., 2017; Ross & Thompson, 1978). Ross & Thompson (Ross & Thompson, 1978), in a

retrospective study including 100 patients with chronic periodontitis, investigated the survival of 387 maxillary molars with radiographically appreciable furcation involvement, after an observation period of 5-24 years. All participants were treated with oral hygiene instructions and scaling/root planing, occlusal adjustments, open flap surgery. Three hundred-five of the 387 molars (84%) were assigned as having poor prognosis at the start of the study with at least 50% bone loss of the root length. At the end of the study, 341 molars (88%) remained in the mouth in functional condition, whereas 15 (33%) of the 46 molars extracted were lost after a period of 11-18 years and 10 (22%) after the first 6 years. Few retrospective studies (H. M. Goldman, 1958; Hirschfeld & Wasserman, 1978; McFall, 1982; Wood et al., 1989) were carried out on the basis of long-term observations, taking into account different forms of periodontitis. The degree of treatment response of each individual sample was assessed on the basis of the number of teeth extracted during the observation period. In this way, it was possible to identify 3 categories of patients: well-maintained patients (0-3 lost

teeth), patients moderately compromised (4-9 lost teeth) and patients severely compromised (10-23 lost teeth) (Cattabriga et al., 2000).

1.7.3 Tooth loss and supportive therapy

Hirschfeld & Wasserman (Hirschfeld & Wasserman, 1978), in a retrospective study, evaluated 600 patients undergoing periodontal therapy in private practices after a period of 15 to 53 years (average 22 years). At baseline, 76.5% of the recruited patients had advanced periodontitis, 16.5% had moderate destruction and only 7% showed signs of mild periodontitis. All patients underwent non-surgical periodontal treatment, flap surgery, root amputation (17 teeth) or hemisection, and, finally, placed in a maintenance program. At the end of the study, 499 (83.2%) were in the group of well-maintained patients, 76 (12.6%) patients belonged to the moderately compromised group and the remaining 25 (4.2%) were severely compromised. Although most of the patients recruited had severe forms of periodontitis, most of them reported an excellent response to treatment and only a small number showed an inexorable progression of

periodontal tissue destruction. During the maintenance phase, 7.1% of molars were lost for periodontal reasons and 460 of the remaining 1455 treated teeth (31.2%) were extracted for reasons not further specified. Therefore, it can be argued that molars with furcation involvement are at higher risk to be loss than molars that do not show furcation involvement. Similarly, Mc Fall and co-workers (McFall, 1982), over an observation period of 15-29 years, evaluated 100 patients who had been previously treated and subsequently underwent supportive periodontal therapy. On the basis of the criteria proposed by Hirschfeld & Wasserman (Hirschfeld & Wasserman, 1978), 3 groups of patients could also be identified: 36 patients with advanced periodontitis, 53 with moderate periodontitis and 11 with initial signs of periodontitis (McFall, 1982). In the preliminary phase, all participants were treated with scaling and root planing, occlusal adjustment, and oral hygiene instructions. Five molars were subsequently treated with root amputation, or gingivectomy-gingivoplasty and some defects with mild infraosseous defects were treated with ostectomy and osteoplasty. Finally, all patients were included in a rigorous maintenance program and recalled at

variable intervals of 3, 4 or 6 months. On the basis of the lost teeth, during the study, it was possible to identify 3 groups of patients: a well-maintained group with 77 samples, a moderately compromised group with 15 samples, and an extremely compromised group with 8 individuals (McFall, 1982). Overall, 259 teeth (9.8%) were lost due to periodontal reasons; 56.7% of teeth with furcation involvement were extracted during the observation period, and only 27% of these extracted teeth belonged to the well-maintained group. On the other hand, of the 600 molars without furcation involvement, only 46 (7.6%) were lost. Interestingly, the majority of molars with FI that were extracted remained in function for a period of 14 years in the well-maintained group, 10.5 years in the moderately compromised group, and 9 years in the extremely compromised group before being extracted. Goldman and co-workers (M. J. Goldman et al., 1986) analyzed 211 patients treated and maintained for a period of 15 to 34 years. In the preliminary phase, all participants underwent scaling and root planing, oral hygiene instructions, and occlusal correction. Secondly, 5 cases were treated with root amputation, some with gingivectomy-gingivoplasty, others with apical

flap repositioning or open flap debridement and, subsequently received SPC. Based on response to treatment, patients were distributed as follows: 131 (62%) in the well-maintained group, 59 (28%) in the moderately compromised group, and 21 (10%) in the extremely compromised group. Out of all 630 molars with FI, 270 (43.5%) were extracted and 56 (16.7%) belong to the well-maintained group. On the other hand, among molars without FI, 190 out of 1112 (17.0%) were lost during the course of the study. Wood and co-workers (Wood et al., 1989) evaluated 63 patients, treated with scaling and root planing and oral hygiene instructions and followed for a mean period of 13.6 years, included in a maintenance protocol with sessions at regular intervals between 6 and 9 months. On the basis of response to therapy, it was possible to distinguish: a group of 54 patients (85.7%) well maintained, a group of 7 patients (11.1%) moderately compromised and a group of 2 patients (3.2%) extremely compromised. During the maintenance phase, 5% of the teeth were extracted due to periodontal causes. In the well-maintained group, 21 out of 126 teeth (16.6%) with FI were lost; while out of the 261 molars free from furcation involvement, 36 were

extracted (13.8%). In a study of 24 patients undergoing surgical and non-surgical treatment, Wang and co-workers (Wang et al., 1994) showed that, during supportive periodontal therapy, molars with furcation area involvement are 2.54 times more likely to be extracted than molars without furcation involvement. While it is fairly clear that molars with FI are more likely to be extracted than molars without FI, very few studies have thoroughly evaluated this risk of tooth loss for molar with FI, particularly in populations that did not receive a regular periodontal treatment. Nibali and co-workers (Nibali, Krajewski, et al., 2017) collected data on 3267 molars overall from approximately two thousand subjects participating at the baseline and 11-year follow-up of the Study of Health in Pomerania (SHIP). At baseline, each participant underwent a half-mouth periodontal examination, which included FI measurements using a standard probe in one upper and one lower molar. Just 28% of the subjects said they had received an unidentified "gum treatment" at some point during the observational period. During the follow-up period, 375 subjects (19.8%) lost all of their molars (5.6% no FI, 12.7% degree I FI, 34% degree II FI, and 55.6% degree III FI). In the

11-year follow-up, molar loss was linked to the baseline PPD, CAL ($p < 0.001$). Initial degree I FI was associated with a 1.73 IRR (incidence rate ratio) (95% CI=1.34-2.23, $p < .001$) of tooth loss while degree II-III was associated with a 3.88 IRR (95% CI=2.94-5.11, $p < .001$) of tooth loss compared to molars without FI at baseline. This study concluded that an increased risk of tooth loss for molar with FI is evident in a population of patients with 'untreated' periodontitis. Another interesting retrospective study investigated the effect of horizontal and vertical furcation involvement on molar survival on patients with chronic periodontitis undergoing supportive periodontal therapy in a private practice environment (Nibali, Sun, et al., 2017). 633 molars were analysed in 100 participants undergoing SPC. All molars with furcation involvement were treated with several surgical approaches in accordance with the clinical need. Forty-six molars were extracted, 23 before starting SPC and 23 during SPC. It was reported that horizontal and vertical furcation involvement were related with increased risk of tooth loss during supportive maintenance (OR 5.26, 95% CI: 1.46-19.03, $p = .012$ and OR 9.83, 95% CI: 1.83-50.11, $p = .006$). Therefore, horizontal and vertical FI in

molars is related to increased risk of tooth loss (Nibali, Sun, et al., 2017). An interesting systematic review analysed the tooth loss in molars with and without furcation involvement based on the diagnosis carried out at the baseline with a minimum of 3 years follow-up including clinical parameters of FI and data on tooth loss (Nibali et al., 2016). The results included twenty-one studies and the relative risk of tooth loss gradually increased for molars with a grade II and III of FI. In particular, the relative risk of tooth loss for FI was 1.46 (95% CI = 0.99-2.15, p = 0.06) for studies up to 10 years and 2.21 (95% CI = 1.79-2.74, p < 0.0001) for studies with a follow-up of 10-15 years. The authors concluded that the detection of a furcation defect doubles the risk of molar loss in patient under SPC (Nibali et al., 2016). Molars with III degree of FI offered a favourable outcome during the SPC, strongly recommending to preserve these teeth in function, preferring a conservative approach (Nibali et al., 2016). The longitudinal studies mentioned above clearly indicate a greater susceptibility to extraction of molars with furcation involvement compared to molars without involvement of the furcation area. However, the number of teeth with furcation involvement lost during the

studies could be even less, if we take into account that some molars, mainly third molars, have been extracted for strategic reasons. On the other hand, some molars extracted in the initial stages of treatment were not included in longitudinal studies. Furthermore, the number of molars with furcation involvement lost ranges from 11.8% to 56.7%. However, patients in the well-maintained groups, which account for the majority of enrolled patients (62-85.7%), have significantly lower tooth mortality rates (16.7%-27.3%) of molars with furcation involvement than those in the remaining groups (Nibali et al., 2016). This finding is supported by the hypothesis that some patients, due to lower susceptibility to the disease and better plaque control, responded effectively to periodontal treatment. In general, not clear data were provided regarding the threshold for extraction in most studies accounting for the great variability observed in term of tooth loss. In addition, patient factors which may have some bearing for tooth loss are often ignored.

1.7.4 Summary evidence on tooth loos and furcation

Molars with furcation involvement do not necessarily have to be assigned with a hopeless prognosis, since, as demonstrated, most of them remain in good function for many years in well-maintained patient groups (Sanz et al., 2020). However, the estimated high survival rate should not exempt us from considering the involvement of furcation as a real risk factor, which, as demonstrated in long-term studies, increases the odds ratio for the extraction of the affected molars compared to molars free from lesions of the furcation area.

1.8 Future direction and rationale of this project

In conclusion, this chapter sheds light on how:

- The anatomical configuration of teeth with multiple roots encourages the buildup of dental plaque, which in turn causes periodontal disease within the root separation region

- Removing plaque from inside the furcation area can be a challenging task for patients as well as a daunting one for clinicians
- Thus, it makes sense to believe that teeth with furcation involvement (FI), which are more exposed to the microbial challenge, will experience a faster rate of periodontal progression and a higher risk of tooth loss.

To what extent the microbiological dysbiosis interacts with the anatomical features of the furcation area, leading to an increased risk of tooth loss will be discussed in Chapter 2.

2 Chapter: Periodontal dysbiosis and furcation defects: literature overview, aims and methodology.

In the previous chapter, the complex anatomical factors of molars with furcation involvement were positively associated to a less effective periodontal treatment, with a consequent increased risk of tooth loss. To what extent these anatomical features may affect the subgingival microbiome is still controversial.

2.1 The ecosystem of the oral cavity

The oral cavity is considered a complex ecosystem, hosting a dynamic microbiome with unique characteristics due to the presence of soft tissues and hard tissues (Aas et al., 2005). Different types of mucosae can be recognized in the oral cavity, such as the lining mucosa (oral floor, buccal region, labial region, soft palate), masticatory mucosa (gingival region and hard palate) and specialized mucosa (dorsum of the tongue) (Arweiler & Netuschil, 2016).

The oral mucosa is composed by a layer of squamous epithelium; however, each structure shows unique features according to their function in the oral cavity. Furthermore, the oral cavity is characterised by two different types of

biological fluids, such as saliva and gingival-crevicular fluid (GCF) (Arweiler & Netuschil, 2016).

2.1.1 Oral microbiota homeostasis

The oral microbiota plays a fundamental role in preserving a healthy oral system. Each healthy individual hosts a self-tailored microbiota that with healthy eating habits and good hygiene sustains this oral balance. A dysbiotic process can start when various factors (such as climatic conditions, eating habits, tobacco and alcohol consumption, stress, hormonal imbalance, puberty, poor oral hygiene, diabetes, and gum inflammation) contributes to undermine the healthy balance in the composition and structure of the microbiota, leading to negative effects (Darveau, 2010).

2.1.2 Dysbiosis and microbial diversity

Dysbiosis is illustrated as the decrease of beneficial microorganisms, the increase of pathogenic microorganism, with an overall reduction of microbial

diversity. Several processes underlie the transition from eubiosis to dysbiosis (Hajishengallis & Lamont, 2012). Two factors mutually interact in triggering the dysbiosis, the microbial metabolism and the host's immune response. These factors can cause changes in the local environment that facilitate the abnormal growth of microorganisms associated with a dysbiosis. The microbiota associated with a healthy state is therefore considered more diverse, while the microbiota associated with disease is influenced by a specific pattern of microorganisms that possess metabolic functions and a high virulence potential largely absent in the health state (Hajishengallis & Lamont, 2012). After a community enters a dysbiotic state, the long-term persistence of the condition is facilitated by the structural stability of the specialised microbial components. Oral diseases, such as periodontitis and dental caries, are often chronic and progress slowly (although the acute onset of both diseases can be triggered under host compromised conditions)(Bascones-Martínez et al., 2009; Loesche, 1996).

2.2 Oral microbiota and periodontitis

The most common diseases of the periodontium are gingivitis (inflammation of the gingival tissues) and periodontitis (inflammation and destruction of the periodontium) (Listgarten, 1986). Gingivitis is characterized by inflammation of the gingival tissue without loss of attachment to the tooth (Loe et al., 1965). Periodontitis is defined as a chronic inflammatory disease induced by bacteria that affects the entire periodontium (Listgarten, 1986).

2.2.1 Periodontitis aetiopathogenesis

The development and consolidation of periodontitis are influenced by both extrinsic factors (such as patient behaviour, drug use or environmental factors), and intrinsic factors (such as genetic and epigenetic factors) (Jeffcoat et al., 2014; Katagiri & Izumi, 2012; Klinger, 2004). In addition to these risk factors, there are also "site-specific features" (e.g. anatomical factors) that can locally promote the development of this chronic condition. The phenotype of periodontitis is characterized by disproportionate, poorly effective and non-resolving inflammation that leads to tissue destruction (destructive inflammation), rather

than specifically targeted, effective, and a self-resolving inflammatory process. The initial phase of this disease can manifest itself as inflammation of the gingival tissue (gingivitis). A variable-depth periodontal pocket forms as the disease worsens and the inflammation extends to deeper tissues, eventually destroying the alveolar bone. If left untreated, the bone destruction can continue until tooth loss (Listgarten, 1986).

2.2.2 Periodontitis and polymicrobial synergy

According to World workshop of Periodontology (Tonetti et al., 2018), periodontitis is now classified in four stages based on the severity of this disease: mild periodontitis (stage I), moderate periodontitis (stage II), severe periodontitis (stage III), very severe periodontitis requiring multidisciplinary approaches (stage IV). Over time, several hypotheses have been put forward to try to elucidate the mechanism of the onset and progression of periodontitis. Today, the most likely theory seems to be the one that predicts a model of pathogenesis called

polymicrobial synergy and dysbiosis. In this model, both the unbalanced interaction between microbiota and host, and the impact of different microbial species in the dysbiosis are evaluated (Darveau, 2010). Healthy gingival sites are characterised by a complex biofilm that grows at the gingival margin, inducing a subclinical inflammatory state well controlled by the host (physiological inflammation) (Darveau, 2010).

2.2.3 Periodontitis and host response

In periodontal disease, an increase in microbial diversity is observed, a consequence of an increased availability of nutrients derived from tissue damage and the increase in physical space as the gingival pocket deepens. The resulting inflammatory process is characterized by the failure to eliminate the microbial biofilm leading to host-mediated destructive events (Bascones-Martínez et al., 2009). Innate immunity is the first line of defense against pathogenic microorganisms, which initiates inflammatory reactions using

immune cells as neutrophils, macrophages, and dendritic cells (Figure 2.1). Also, innate immune molecules, such as cytokines, lysozyme, and antimicrobial peptides, inhibit or kill bacteria and initiate and participate in the innate immune response. Neutrophils, the largest group of white blood cells in the blood, play a dual role in periodontal inflammation. They are necessary for the stability of the internal environment of periodontal tissue and can phagocytize bacteria when plaque microorganisms invade the host. Neutrophils in peripheral blood and gingival crevicular fluid can synthesise and secrete inflammatory cytokines, which can promote the degradation of connective tissue matrix and stimulate bone resorption, leading to the destruction of periodontal tissue. Innate immune cells play a crucial role in protecting periodontal tissues, but they cannot effectively remove pathogenic bacteria that constantly colonize and invade the periodontium. Innate immune cells can be used as antigen-presenting cells to induce cellular and humoral immune responses by presenting antigens to initial T cells and B cells, respectively, enhancing the immune response against microorganisms. Humoral immunity involves B cells synthesizing and secreting

antibodies, while cellular immunity involves T cells that specifically recognize antigens and differentiate into effector T cells. B cells, derived from the bone marrow, differentiate into plasma cells and produce antibodies, which can bind to pathogens to prevent infection and activate complement. They also participate in immunoregulation and the inflammatory response by contacting with other cells and producing cytokines. T cells, derived from bone marrow, differentiate into helper T (Th) lymphocytes, regulatory T cells (Tregs), and cytotoxic T lymphocytes. Th1 cells can synthesise proinflammatory cytokines, while Th2 cells can produce anti-inflammatory factors to inhibit macrophage activation. Both innate immune and adaptive immune response therefore plays a key role in destruction of the periodontium. Although an excessive immune response can eliminate periodontal pathogenic bacteria, irreversible destruction of periodontal tissues is also reported.

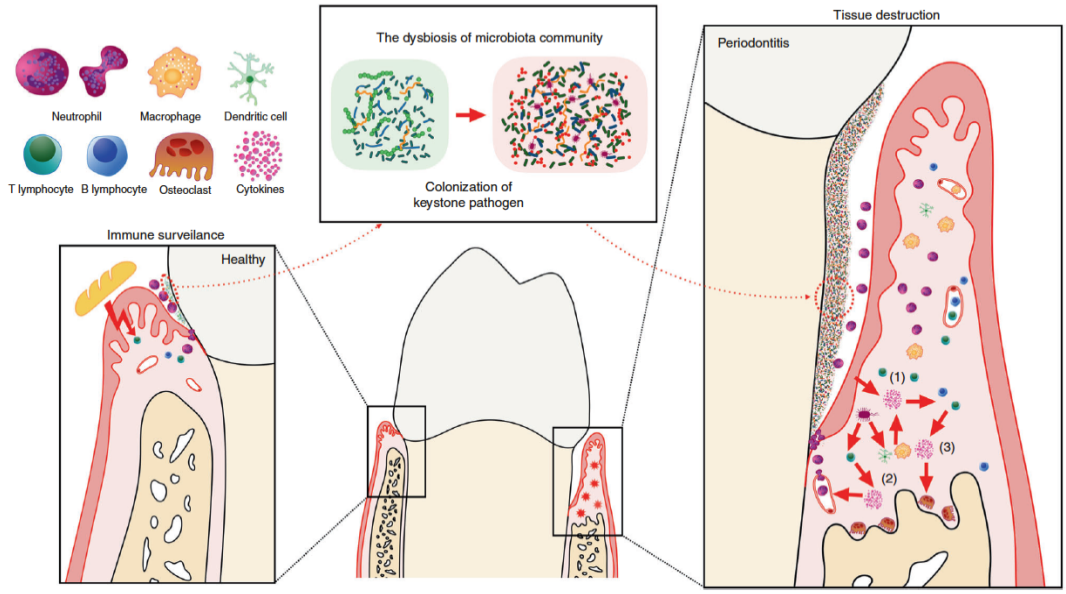


Figure 2.1 Role of host immune response in the pathogenesis of periodontitis. During periodontitis, the interaction between the microbiota and all host cells leads to a pro-inflammatory cytokine cascade and activation and differentiation of specific immune cells. Each of these cell subsets secretes a certain pattern of cytokines, which might act as the positive-feedback factor or direct effector eventually leading to tissue destruction. Figure reproduced from Pan W. et al, 2019.

2.2.4 The role of the biofilm in periodontal dysbiosis

The pathogenetic process in periodontal disease involves an active and reciprocal modification of the response of the organism and biofilm during disease progression (Offenbacher et al., 2007). The biofilm expands, evolves, becomes more complex, and pathological changes in gingival tissues affect commensal microbial species that become pathogenic. Subsequently, the dysbiotic community actively induces the inflammatory state in order to sustain itself (Mosaddad et al., 2019). The onset of periodontitis therefore reflects changes in abundance of species normally present in low levels. The inflammatory process, in fact, causes an increase in gingival crevicular fluid which, in addition to cellular immune factors into the site, represents an essential source of factors especially for anaerobic microorganisms. In addition, the local inflammatory process disrupts the microcirculation, disturbing the blood and oxygen supply (Page & Schroeder, 1976).

2.2.5 Oxygen levels and periodontal communities

The periodontium may undergo a shift from the normal oxygen level phase to low oxygen level as inflammation begins and progresses. The anaerobic environment thus provides a niche for colonisation by gram-negative anaerobic bacteria, whose growth, accompanied by the production of various metabolites, contributes to further lowering the oxygen levels at deeper sites. In the periodontal pocket, therefore, an "invasive" community is established (Socransky & Haffajee, 1991). In fact, this dysbiotic community acts as an inflammatory stimulus capable of activating various types of cells (epithelial cells, periodontal ligament fibroblasts, leukocytes, osteoblasts) to release pro-inflammatory factors, e.g. IL-1 alpha, IL-6, TNF-alpha, matrix metalloproteinase, prostaglandins (PGE), and so on. When the inflammation is not resolved, it can induce not only dysregulation of T or B cells, but also DNA damage, cellular senescence, and oxidative stress, potentially affecting the immune system and causing infections (Barros et al., 2016; Page, 1986). The final event of periodontitis is the loss of the tooth element due to resorption of the alveolar bone. Bone resorption is due to

several factors of both the host and the microorganisms that together contribute to altering the normal balance between osteoblasts (cells that deposit neo-bone) and osteoclasts (cells responsible for bone resorption) (Page & Schroeder, 1976).

2.2.6 Clusters of periodontal microbiomes

Several gram-negative anaerobic and microaerophilic bacterial species, late colonisers of dental plaque, such as *P. gingivalis, T. denticola* and *T. forsythia* are among the most relevant pathogenic microorganisms in periodontitis (Socransky et al., 1998). In 1998, Socransky (Socransky et al., 1998) et al attempted to identify the bacterial complex in subgingival plaque. The purpose was to define such communities using data from large numbers of plaque samples and different clustering and ordination techniques. Subgingival plaque samples collected from in 185 subjects with and without periodontitis were analysed using genomic DNA probes and checkerboard DNA-DNA hybridization. Five major complexes were consistently observed using any of the

analytical methods. *P. gingivalis, T. denticola* and *T. forsythia* were defined as part of the 'red complex', considered to have highest pathogenic potential. The 'orange complex' (*C. gracilis, C, rectus, F. nucleatum, P. micra, P. intermedia* and *P. nigrescens*) may play the role of coaggregation bridge pathogens that facilitates aerobic and obligate anaerobes aggregation (Socransky et al., 1998). The role of *F. nucleatum*, as "bridge species" has been particularly investigated for the onset and progression of periodontitis because it facilitates the colonization of other periodontal pathogenic species such as *P. gingivalis*. Other key microorganisms are those belonging to the 'green complex' (*E. corrodens, C. gracilis, C. ochracea, C. sputigena,* and *A. actinomycetemcomitans*) which, together with those of the 'yellow' (*S. mitis* and *Streptococcus* spp) and 'purple' (*V. parvula, A. odontolyticus*) complexes represent the first colonizers of dental plaque (Figure 2.2)

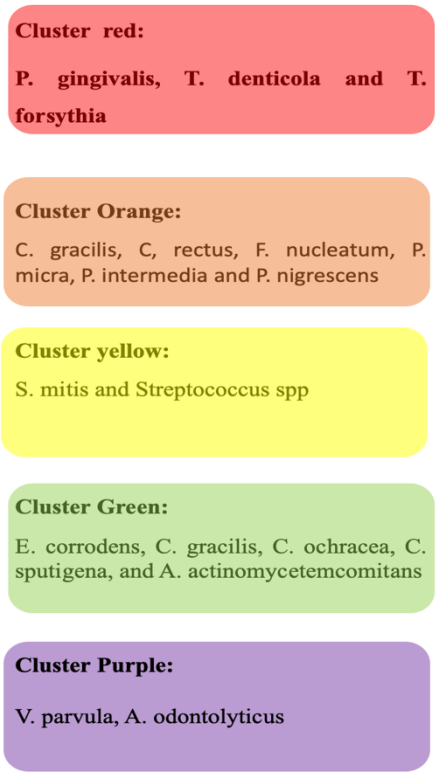


Figure 2.2 Schematic representation of microbial complexes (Socransky et al.,1998).

The presence of 40 subgingival tax was evaluated using the whole genomic DNA probes and checkerboard DNA-DNA hybridization. Green, purple and yellow represent the first colonizers, orange and red include late colonizers species.

In order to trigger an immune response against the invasive bacteria, these Gram-negative bacteria release molecules like lipopolysaccharide and extracellular proteolytic enzymes that interact with the innate host inflammatory surveillance system (Darveau, 2010). Therefore, the inflammatory response causes the connective tissue attachment and supporting bone to break down, resulting in established periodontitis lesions.

2.3 Microbial dysbiosis in molars with furcation involvement

The anatomy of furcation molars may influence the oral microbiome and potentially select for more pathogenic microorganisms due to several factors. First, the intricate anatomy of furcation areas makes them less accessible to routine oral hygiene practices such as brushing and flossing. This limited access can result in plaque accumulation and increased bacterial colonization. Furthermore, furcation areas can provide an anaerobic environment (low oxygen levels), which is conducive to the growth of anaerobic bacteria (Figure 2.3)

(Fleischer et al., 1989; Kocher et al., 1998; Matia et al., 1986; Parashis et al., 1993; Svärdström & Wennström, 1988b).

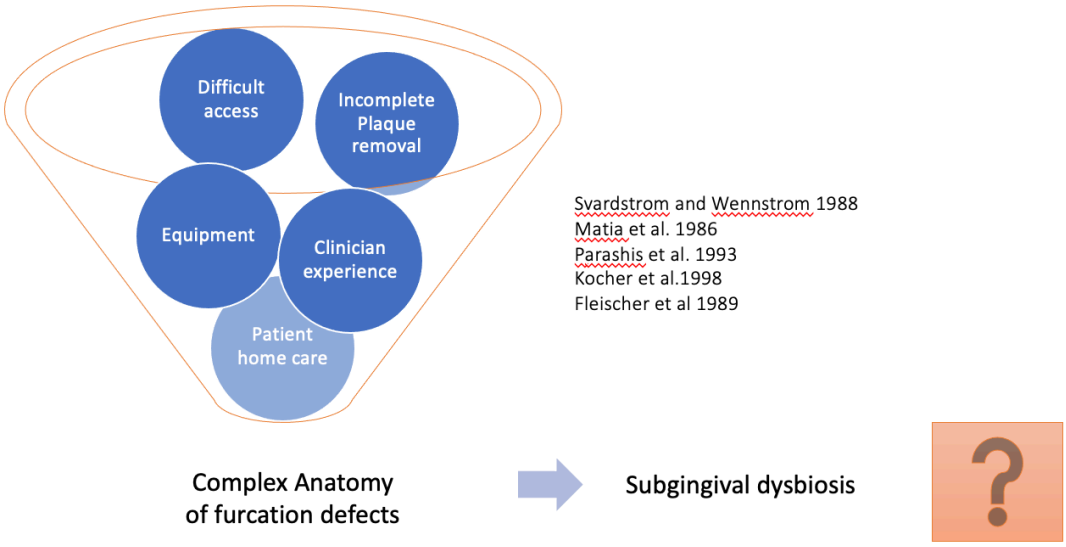


Figure 2.3 Correlation between clinical factors and anatomical features

Previous literature suggests that several factors may be related to complex anatomy of furcation defects, however a clear link between this factor and the subgingival dysbiosis in furcation area was not identified (Fleischer et al., 1989;

Kocher et al., 1998; Matia et al., 1986; Parashis et al., 1993a; Svärdström & Wennström, 1988).

2.3.1 Furcation microbiome and previous studies

To our knowledge, no previous studies were able to comprehensively analyse the furcation microbiome in patients with untreated periodontitis. Only few studies analysed the subgingival microbiome after advanced periodontal procedures (as surgical approaches) on molars with furcation involvement (Loos et al., 1988; Queiroz et al., 2017). Two investigations indicated that compared to interproximal sites, the furcation microbiome is significantly more diverse (Loos et al., 1988; Queiroz et al., 2017). This may suggest that the intrinsic furcation shape influences microbial diversity and leads to distinct environmental features. Queiroz reported that 39 furcation lesions contained about 422 species, with an average of 119±35 bacterial species in each defect (Queiroz et al., 2017). Interestingly, previous studies have found similarities between the furcation

microbiome and that found in cases of severe periodontitis identified using similar sequencing and microbiological bioinformatics techniques (Abusleme et al., 2013; Dabdoub et al., 2016). Moreover, the microbiome associated to a furcation defect seems to differ more from interproximal lesions in terms of both total microbial counts and core microbiome (Queiroz et al., 2017). The Human Microbiome Project coined the term "core microbiome" to refer to bacterial aggregation that are found more frequently in study populations, suggesting that these species are best suited to that specific microenvironment (The Integrative HMP (iHMP) Research Network Consortium, 2019). According to Queiroz et al., the core microbiome detected in the furcation sites represented the 75% of the overall relative abundance. An additional intriguing discovery revealed that although well-known periodontopathogen *Porphyromonas* and *Treponema* species (such as *P. gingivalis* and *T. denticola*) were detected in the furcation defects, they did not form a part of the core microbiome of the furcation sites (Queiroz et al., 2017). The influence of microbiome in furcation defects and clinical parameters as probing pocket depth (PPD), and clinical attachment level

(CAL) following root debridement between non-furcation and furcation sites has only been compared in one investigation (Loose et al., 1988), suggesting that even following non-surgical periodontal therapy (NSPT), there is still a clinical and microbiological difference between furcation and non-furcation sites. Over the course of 52 weeks of observation, a higher and more varied post-operative microbiological count was found within furcation defects (Loose et al., 1988). It's interesting to note that while there was a negative correlation found between baseline microbiological values and CAL changes in molar furcation sites, the microbiological data showed a limited positive correlation with post-debridement PPD reduction and with CAL gain for non-furcation sites. In other words, furcation sites exhibiting higher initial microbiological counts during the 1-year follow-up demonstrated less favourable CAL gain. Additionally, this relationship holds true for baseline PPD, indicating that high preoperative microbial counts may make adequate debridement of molar furcation sites less possible (Loos et al. 1988). Additionally, there was a significant correlation found between the mean postoperative PPD and the mean postoperative

microbiological counts for molar furcation sites. At the 1-year follow-up, the furcation sites with the worst post-operative PPD change also had higher microbial counts (Loos et al., 1988). With regards to non-molar sites, this relationship was not seen. Furthermore, at the end of the 52-week follow-up, the probing data indicated a slight rebound trend for the furcation sites. It is important to note that these differences could be caused by limited technologies in laboratory methods or by limited amount of subgingival plaque sampling and the analysis that followed (Loos et al., 1988).

2.4 Aims

Previous literature was not able to clearly highlight the microbiological features of molars with furcation involvement, due to the limited technology used for the furcation microbiome as the phase-contrast microscopy (Loos et al., 1988). However few studies offered as marginal outcome interesting conclusions about furcation microbiome, stating how this may differ from non-furcation defects, and

is characterised by an overall higher count of microorganism, in particular anaerobic (Loos et al., 1988; Queiroz et al., 2017).

Therefore, the lack of evidence in previous periodontal literature required more than a single investigation to further investigate the dysbiotic environment in furcation sites.

2.4.1 Hypotheses flow-chart

The scientific approach towards a new research question should always start from a process of hypothesis generation that should be later tested in different scenarios. Therefore, the first step of this project was to design a cross-sectional study entitled 'Microbiological and molecular profile of furcation defects in a population with untreated periodontitis' (Chapter 3). This study helped this author to proceed with an explorative analysis of furcation microbiome in participants with untreated periodontitis to generate a hypothesis to evaluate on following studies. The nature of this investigation included a broad and detailed analyses

of sub-gingival plaque and its correlation with gingival crevicular fluid collected markers from furcation sites, non-furcation sites, and healthy sites. The aforementioned result was used to generate a primary hypothesis that was tested on two longitudinal studies: 'Clinical and patient-reported outcomes in grade III furcation defects: a randomised feasibility trial ' (Chapter 4), and 'Survival of molars with degree III periodontal furcation involvement following non-surgical or surgical therapy: a multicentre single-masked superiority randomised controlled trial' (Chapter 5) (Figure 2.4).

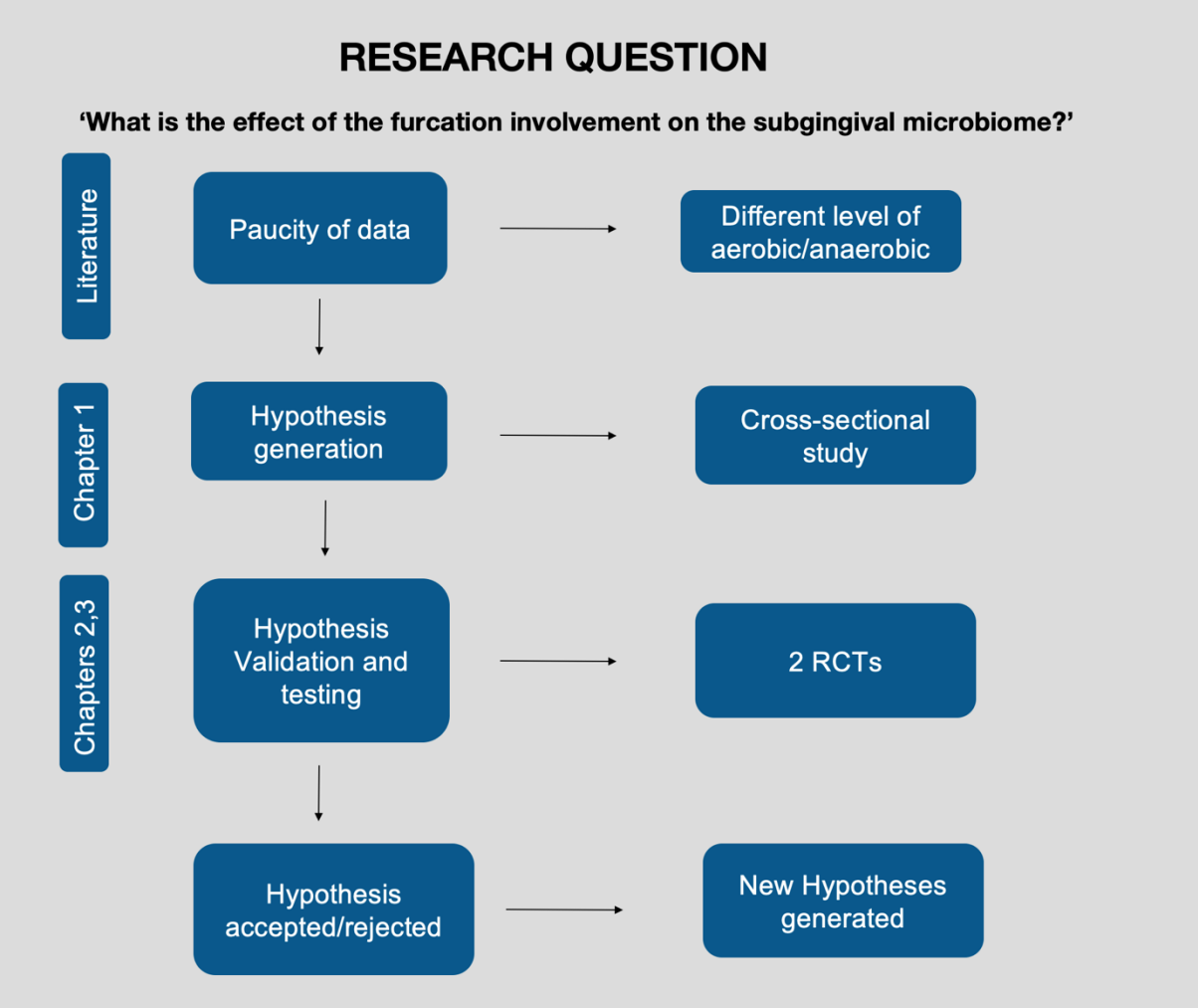


Figure 2.4 Hypothesis generation flowchart

To answer the research question "What is the microbiome in periodontal furcation lesions?', due to the lack of data in literature, a preliminary hypothesis was generated and tested with a cross-sectional design study in participants

with untreated periodontitis, subsequently validation, the same hypothesis was tested on two randomised controlled clinical trials.

2.4.2 Primary aim

To compare the level of aerobic, anaerobic, and facultative genera in III-degree furcation sites treated with surgical or non-surgical approach.

2.4.3 Secondary aim

To assess alpha and beta diversity, relative abundance, gingival crevicular fluid biomarker levels and its correlation with microbiological factors in furcation, non-furcation sites and healthy sites.

To assess clinical outcomes as PPD, CAL, and REC and alpha and beta diversity, relative abundance between furcation sites treated with surgical approach and

furcation sites treated with a non-surgical approach, and their matched healthy sites.

2.5 Methods

The commonly used methods for subgingival plaque collection include the curetting method and the paper point method (Hartroth et al., 1999; Jervøe-Storm et al., 2007). Both techniques are reliable and widely used. However, the curetting method could mainly collect attached plaque on the surface of teeth or implants, while the adsorption method could collect only unattached bacteria in periodontal pockets(H. Lu et al., 2022). It is not clear from previous periodontal literature whether one method was better than the other for the subsequent analysis of subgingival plaque (Jervøe-Storm et al., 2007). Therefore, for the three investigations included in this project, the curettage method was preferred. The reason was related to the intention of this author to analyse not only the unattached subgingival plaque but also the attached plaque. Furthermore, with

the curetting method it was possible to clinically visualise the sample on the tip of the curette, and in case of not enough ‘material’ collected a second attempt was possible. However, it is recommended by this author to use curettes with thin blades, and long terminal shanks to access deep pockets, allowing less tissue distension with less risk of trauma. It needs also to be considered that it could be very difficult to insert a curette in furcation sites without damaging the soft tissue.

2.5.1 Sub-gingival plaque sampling procedure

In all three studies, sub-gingival plaque process from collection to analyses followed detailed standard operative procedures. Sub-gingival plaque samples also known as the 'Material’ was collected from patients in accordance with the patient consent form and the patient information sheet and included all tissue samples or other biological materials. Furthermore, the custodian of the ‘Materials’ was ensured that the use of the above ‘Material’ was for the use of this study only. After ethics approval for the study has expired, the sub-gingival

plaque samples/'Materials' will be disposed of in accordance with the Human Tissue Act 2004, and any amendments thereto, or transferred to a licensed tissue bank.

2.5.2 Sub-gingival plaque collection protocol

All worktop surfaces were thoroughly decontaminated with 1% Virkon before and after each sample collection. It was double checked that each participant was assigned with a study ID and the consent form was accurately signed. The clinician collecting samples was protected full PPE. The clinician needed to visualise the sites of collection in agreement of exclusion and inclusion criteria and carefully identified on the periodontal chart and confirmed on available radiographs. Label the collection tube with the patient code number. The supragingival plaque, calculus and food residue were carefully removed with a standard scaler, the sites isolated from saliva with cotton rolls and gently dried. A sterile curette needs to be inserted on the assigned aspect of the tooth and below the gingival margin. Only the subgingival plaque was collected. After a

single stroke, the content was placed in the collection tube. This procedure needed to be repeated for the control site, the content was collected with a different sterile curette and placed in a different coded collection tube. The collection tube was firmly closed and placed in the ice box. Samples were taken to Guy's Tower, Guy's Hospital, floor 17, labelled, and stored in the -80°C HTA2 freezers. The sample storage location was documented in the Brady/Pro-Curo system account created for each study. The sample log was filled with the details of samples collected. Follow-up samples were collected according to the study protocol. Informed consent was enduring throughout the patient's dental treatment and any follow-up appointments. Prior to collecting samples, the patient was asked to verbally confirm they are happy to continue to donate samples. A protocol was also taken in place for impossibility of plaque collection due to local factors as bleeding, suppuration, swelling and pain. If for any reason the sample collection was not completed for any of the aforementioned reason, a new appointment was offered to the participant in no more than 7 days to proceed with a new collection procedure. In relation to the collection method, a

previous study analysed the difference in terms of community composition after the collection of dental plaque using a scaler or a soft brush, concluding that no statistical differences in DNA concentrations or community composition at the phylum and operational taxonomic unit levels between different dental plaque collection were detected (Luo et al., 2016). As limitation of this method, the author reports that sterile curettes were used for the collection of all samples, however the sterility was not verified with a buffer solution before the collection of the samples.

2.5.3 Sub-gingival plaque storage

Sub-gingival plaque storage was carried out centrally at the Microbiology and salivary research laboratories, Floor 17 Tower Wing, Guy's Hospital, King's College London Faculty of Dentistry, Oral & Craniofacial Science. A sample collection log was prepared and kept with the study documentation in three separate study folders. Samples were anonymised by using labels including only sequential centre, patient numbers followed by TS (for test sites) and CS (for

control sites) and by the study time point (for example C01-01-TS-bl indicating centre 01, patient 01 and baseline appointment). Samples taken, were transferred immediately to the freezer in the Floor 17 laboratory according to standard operating procedures (SOP). Samples collected at the other centres were transferred to the designated laboratory in 1 batch at the end of the study or in 2 batches, according to SOPs and subject to Material Transfer Agreement (MTA) between the institutions involved. The date the samples were sent to the Laboratory, time of collection and temperature/conditions at which it was sent were recorded in the sample collection log to ensure their integrity and viability is not compromised.

2.5.4 Samples Sequencing

The microbiology complexity of the subgingival plaque has been reported since 1630 when Van Leeuwenhoek performed the first microscopic examination of this sample (M. Tal, 1980). Although the correlation between gum disease and subgingival plaque is well known (Loe et al., 1965), new methods and technology

are able to offer a more detailed prospective on the role of the subgingival plaque microbiota in the initiation and progression of periodontitis (Hajishengallis & Lamont, 2012). In particular, next-generation sequencing (NGS) technologies have greatly progressed the exploration of the oral microbiome's role (Zhou et al., 2019), however understanding the effects of collection method, storage media and conditions, sample processing and DNA extraction methods is essential to properly interpret the results of NGS (Luo et al., 2016).

2.5.5 DNA extraction: analysis of different procedures

The DNA extraction method is a complex procedure aimed to isolate DNA yields from subgingival plaque to attempt the sequencing of the oral community. Two methods are available for the DNA extraction: enzymatic or mechanical bead beating approach; the main difference relies on the way the initial cell disruption is performed: enzymatic or mechanical bead beating approach (Wu et al., 2014). The role of the extraction method on the yield and quality of DNA extracts is still controversial, with some investigations reporting no differences among different

methods (Wu et al., 2014; Zhou et al., 2019) and other reporting a better outcome for the mechanical bead beating approach (Sohrabi et al., 2016). In line with this, the comparison of the bacterial profile detected from four common microbial DNA kit extraction did not show any significant difference among the DNA extraction approaches (Vesty et al., 2017). The most interesting aspect regarding the DNA extraction process for dental plaque is the storage media. A previous investigation reported as same samples, held in different media and at different temperature, reported minimal changes on microbial composition and structure, but significant differences in terms of microbial diversity (Luo et al., 2016). Similarly, a recent investigation reported as a specific Bead solution used as storage vial may preserve the oral microbiota for longer periods of time, up to six months and it is and is compatible, with either a bead-beating or non-bead beating DNA extraction method (Zhou et al., 2019). Bead solution contains guanidine salts that are a protein denaturant and nucleic acid protector and is recommended by the Human Microbiome Project for storage of oral microbiome samples (Zhou et al., 2019). Moreover, the same author reported how Bead

solution was more effective as storage medium compared to the ethanol, commonly used for stabilisation and DNA preservation. Therefore, in line with the previous findings (Zhou et al., 2019) this author decided to use a bead-beating DNA extraction process using the bead solution as storage of the sample microbiome.

2.5.6 DNA extraction protocol

The reagent and protocol were based on the manufacturer's instructions of the DNeasy PowerSoil Pro Kit (Qiagen Inc., USA). The Equipment needed were a Microcentrifuge (17,000g), sterile filter pipettes tips, fastPep-24 Bead Beater, thermomixer Heat block at 70°C. During each session of DNA extraction, it was possible to extract 24 plaque samples for an average duration of 7 hours. Before starting the procedure, a checklist was followed to ensure that all reagents were at the room temperature, all samples were in the screw lid tubes. Furthermore, all reagents needed to be reconstituted correctly with no precipitates formed. If Furthermore, it was detrimental to make sure that the Thermomixer Heat block

was switched on for 70°C, and microcentrifuge was switched on and ready to use on 4°C. Twenty-four plaque samples were defrosted and labelled twice, once on the lid and once on a separate lab sheet, to prevent misidentification in case the original label was damaged. The samples were then centrifuged at 17,000 x g for 15 minutes, after which the supernatant was carefully removed without disturbing the pellet. A specific powder was added to the pellet of each sample, followed by the addition of 800 µl of lysisi buffer (solution CD1). The tubes were vortexed for 5 seconds, ensuring that the lids were tightly secured. The samples were then heated at 70°C for 10 minutes and subsequently placed on ice to cool. For homogenization, the FastPrep-24 bead-beater (or similar equipment) was employed, running the samples for 4 minutes at 6.5 m/s, with 1 minute of homogenization followed by 1 minute on ice to prevent overheating, while taking care to avoid tube breakage. Post bead-beating, the samples were centrifuged at 15,000 x g at 4°C for 1 minute. Approximately 500-600 µl of supernatant was transferred to a clean 2 ml microcentrifuge tube. To this, 200 µl of a solution to precipitate proteins (solution CD2) was added, and the mixture was vortexed for

5 seconds before being centrifuged again at 15,000 x g at room temperature for 1 minute. Taking care not to disturb the pellet, 700 µl of the supernatant was transferred to a new 2 ml microcentrifuge tube. Next, 600 µl of a binding buffer solution (solution CD3) was added, and the samples were vortexed for 5 seconds. The lysate (650 µl at a time) was loaded onto a filtered centrifuge tube called MB Spin Column and centrifuged at 15,000 x g at room temperature for 1 minute, with the flow-through being discarded. This process was repeated until all the lysate was processed, usually requiring two rounds per sample. The filter was then placed into a clean 2 ml collection tube, and 500 µl of ethanol-acetic solution (solution EA) was loaded onto it before centrifugation at 15,000 x g for 1 minute. The flow-through was discarded, and the filter was placed back into the same collection tube. Subsequently, 500 µl of a wash-buffer solution (solution CD5) was added to the filtered tube and was centrifuged at 15,000 x g for 1 minute. The flow-through was discarded, and the filter was transferred to a new 2 ml collection tube. The samples were centrifuged at up to 16,000 x g for 2 minutes, and each filter was then placed into a new 1.5 ml microcentrifuge tube.

A break of 15-30 minutes was given to allow the ethanol to evaporate. After the waiting period, 50–100 µl of low-salt buffer solution (solution CD6) was carefully added to the center of the white filter membrane in the centrifuge tube, with the pipette held vertically to ensure precise delivery. A final centrifugation at 15,000 x g for 1 minute was performed, and the filter discarded. The final elute, containing 100 µl of DNA, was retained in the final tube, labeled according to the initial sample label, and stored at -80°C, as solution CD6 does not contain EDTA, ensuring the DNA's stability. The quantity and quality of the extracted DNA were assessed using a Nanodrop 2000C UV-Vis spectrophotometer (Nanodrop Technologies) and a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.). The average sample DNA concentration detected by the Qubit analysis was 13.6 (17.07) ng/µl with an input available in 2.5 ul(ng) of 277.96 (273.12)ng/µl. All samples with a DNA concentration higher than 60 ng/µl were normalised with a dilution process. Nanodrop analysis revealed average A260/280 of1.83 (1.23) and A260/230 of A260/280 with a concentration of 10.4 ng/µl.

2.5.7 Bacterial 16 rRNA analysis

Once the extraction process was completed for all samples, metagenomic analysis was performed by analysing the prokaryotic 16S ribosomal RNA gene (16S rRNA). This analysis was conducted at the University College of London, Zayed Centre genomics laboratory by a team of applications Specialist in NGS leaded by the Head of Sequencing. The analysis was delivered in two batches: the first one delivered in July 2023 including the plaque samples collected from participants recruited in the project entitled: 'Microbiological and molecular profile of furcation defects in a population with untreated periodontitis' (Chapter 3). The second batch was sent for analysis in March 2024 and included the remaining samples from the other two projects: Clinical and patient-reported outcomes in grade III furcation: a randomised feasibility trial ' (Chapter 4), and 'Survival of molars with degree III periodontal furcation involvement following non-surgical or surgical therapy: a multicentre single-masked superiority randomised controlled trial' (Chapter 5). The 16S rRNA gene is approximately

1,500 bp long and contains nine variable regions interspersed between conserved regions, variable regions of this gene are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations. Which 16S rRNA region to sequence is an area of debate, and the region of interest might vary depending on things such as experimental objectives, design, and sample type. The protocol used for this project was based on a method for preparing samples for sequencing the variable V3 and V4 regions of the 16S rRNA gene. This protocol can also be used for sequencing other regions with different region-specific primers. This protocol combined with a benchtop sequencing system, on-board primary analysis, and secondary analysis provided a comprehensive workflow for 16S rRNA amplicon sequencing.

2.5.8 16s analyses workflow summary

The 16s analysis was conducted by an external lab: the UCL Genomics, UCL Great Ormond Street Institute of Child Health Zayed Centre for Research into

Rare Disease in Children, London. The protocol followed strictly the steps presented by 16S Metagenomic

Sequencing Library Preparation (Pichler et al., 2018).

- Amplicon primers–The protocol included the primer pair sequences for the V3 and V4 region that create a single amplicon of approximately ~460 bp. The protocol also included overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters.
- Library preparation –Several the steps need to be followed to amplify the V3 and V4 region and using a limited cycle PCR, add Illumina sequencing adapters and dual-index barcodes to the amplicon target. Using the full complement of Nextera XT indices, up to 96 libraries can be pooled together for sequencing.
- MiSeq Sequencing: Using paired 300-bp reads, and MiSeq v3 reagents, the ends of each read are overlapped to generate high-quality, full-length reads of the V3 and V4 region in a single 65-hour run. The MiSeq run output

was approximately > 20 million reads and, assuming 96 indexed samples, generated > 100,000 reads per sample, commonly recognized as sufficient for metagenomic surveys.

To sum up:

Bacterial 16S rRNA gene region V3–V4 in samples was amplified using following primer sequences.

Forward Primer:

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG- 3'

Reverse Primer:

5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

-3'.

Amplicons were then barcoded using IDT for Illumina unique dual indexes (UDIs), multiplexed. Batch one was loaded at 8pm, batch two at 4pm, both

batches spiked with 10% PhiX and sequenced using the Miseq V3 600 cycle kit in the 300 bp paired end read method on the Illumina Miseq platform (Illumina, San Diego, CA, USA). All sample concentrations were measured using the HS DNA Qubit at the initial sample stage, amplicons, and final libraries. A selection of amplicons and final libraries were QC'd on the TapeStation, to assess quality and peak size. Most of the prep was performed manually, but some bead clean ups were performed on the Hamilton Star liquid handler.

2.5.9 Sequence Analysis and Taxonomic Classification

Raw Illumina reads were sent as 'fastq' file through a shared platform called 'Globus Connect'. All reads were quality filtered, trimmed, a Q-score>20 for ambiguous bases recovered in the overlapping region and up to 2 ambiguous bases allowed in the overlap. Sequences were analysed using the DADA2 1.18 pipeline based on qiime2 platform (qiime2-amplicon-2023.9) to cluster them into amplicon sequence variants (ASVs) and the HOMD V15.1 database to classify each ASV at the genus level. Alpha diversity was analysed with the R package

Phyloseq 1.44.0 using the Simpson and Shannon indices as estimators of richness and diversity, respectively. Statistical differences of these estimators based on the variables of the study were assessed using the Kruskal–Wallis test. For beta diversity, principal coordinates analysis (PCoA) of Bray-Curtis, dissimilarity was conducted with the Phyloseq package, and differences among the defined groups were assessed using the PERMANOVA test implemented in the adonis function of the vegan 2.6.4 package. Differential abundance of the identified taxa was studied using Wilcoxon rank sum test for pairwise comparison and Kruskal–Wallis test for each group, filtering the differences by p values smaller than 0.05 a log2-fold change (L2FC) absolute value higher than two and by their base mean value, not considering ASVs with a base mean value pertaining to the lowest quartile. Significance values have been adjusted by the Bonferroni correction for multiple tests. The association between the patterns of bacterial genera and the GCF molecular profile was assessed using Spearman correlation, correlation coefficients greater than 0.2 or smaller than -0.2 were labelled as significant at the 5% level. Aerobic, anaerobic and facultative

metabolism were also manually assigned to each genus using the Human Oral Microbiome Database (HOMD), and relative percentages per each group calculated. Statistical analysis was completed using Kruskal–Wallis test for 3 groups, filtering the differences by p values smaller than 0.05.

3 Chapter: Microbiological and molecular profile of furcation defects in a population with untreated periodontitis.

3.1 Introduction

The inflammatory processes involved in periodontitis makes the subgingival microenvironment more susceptible to disruption, leading to the development of periodontal pockets. This, in turn, influences the selection of pathogenic microbiological profiles. Several bacterial and anti-bacterial cell types, signalling molecules, and inflammatory products have been found in the gingival crevicular fluid (GCF), indicating that the dysbiosis that results in these periodontal pockets promotes the host-response cascade (Pellegrini et al., 2017). Currently conventionally any kind of periodontally-diseased site, including those affected by vertical bone loss (infrabony), horizontal bone loss (suprabony) and those with furcation involvement (FI), is referred to as a periodontal defect. Despite the lack of direct microbiological and histopathological data, this assumes that FI are just an extension of periodontal pockets (Al-Shammari et al., 2001b; Glickman, 1950). Even with supportive periodontal care (SPC), FI is linked to an increased risk of tooth loss, making it a special therapeutic challenge (Nibali, Sun, et al., 2017; Trullenque-Eriksson et al., 2023). When compared to single-rooted teeth, less

efficient mechanical removal of subgingival plaque in poorly accessible furcation areas leads to increased microbial colonization and a limited response to periodontal treatment (Gill et al., 2022a). There is little information on the composition of subgingival biofilm in periodontal furcation defects. Following subgingival debridement, furcation areas were found to have greater overall microbiological counts and anaerobic counts than non-furcation sites (NF) (Loos et al., 1988). Before surgical treatment, 16s rRNA analysis of 39 molar furcation areas revealed that the microbiological composition of FI and "severe" periodontal lesions was similar, but the core microbiome of FI was less "representative" than that of other periodontal defect types (Queiroz et al., 2017). Likewise, the molecular profile gathered in periodontal defects with various anatomical configurations has been studied using GCF. Interestingly, when compared to healthy sites (HS), both infrabony defects and suprabony defects exhibit noticeably higher concentrations of markers of inflammation, connective tissue degradation, and repair/regeneration in GCF (Koidou et al., 2020; Santamaria et al., 2023). Nevertheless, infrabony and suprabony defects could

not be distinguished upon analysis of GCF marker molecular profile. It is unknown how furcation involvement affects the molecular profile and subgingival microbiome of GCF. Thus, the main goal of this study was to perform a taxonomic analysis of the subgingival microbiota of FI in patients with untreated periodontitis in comparison to non-furcation defects (NF) and healthy sites (HS) The development of an AI-validated clustering model to highlight potential microbial interactions among communities found in various periodontal defects and the identification of any molecular differences in GCF among FI, NF, and HS were the secondary goals.

3.2 Material and methods

This chapter has been re-drafted as paper for the Journal Clinical of Periodontology and in June 2024 accepted for publication.

3.2.1 Study population

The East of England-Cambridge East Research Ethics Committee provided the NHS UK Research Ethics Service approval (reference 20/EE/0241), which allowed for the inclusion of eligible participants in King's College London Oral, Dental, and Craniofacial Biobank. Each participant gave written consent to participate in the Biobank. The Biobank Management Committee gave permission for access to the samples and data used in this investigation (REF007). The current investigation's cross-sectional design was planned to use the STROBE checklist. The following inclusion criteria were set: i) age 18-75, ii) stage III-IV periodontitis (Tonetti et al., 2018), iii) at least one maxillary or mandibular tooth excluding third molars with: a) one molar with a FI site with degree II/III horizontal FI (at least between 2 roots for maxillary molars), class B-C vertical FI (bone loss up to the middle third of root cones), probing pocket depths (PPD) >5mm in the furcation area, not readily accessible for self-performed oral hygiene *(test site)*; b) one non-furcation periodontal defect site on a molar with no signs of FI *(positive control)* (PPD >4mm within 1mm of PPD on

test site, radiographic defect depth ≥3mm); c) and one periodontally HS *(healthy control)* (PPD<4mm, with no previous signs of radiographic bone loss and no bleeding upon probing. In cases with multiple periodontal defects, sites with the deepest PPD were included. In the absence of symmetrical contralateral molars, neighbouring teeth were chosen (in order other molars, second premolars, first premolars, canines). iv) mobility < degree II, v) absence of any ongoing endodontic pathology, vi) absence of an adjacent tooth with unfavourable periodontal prognosis (as judged by the study clinician). Exclusion criteria were: i) current self-reported cigarette smoking or vaping (or smoking or vaping in the previous 5 years), ii) history of diabetes (confirmed by chair-side Hb1Ac test), hepatic or renal disease, or other serious medical conditions or transmittable diseases, iii) anti-inflammatory or anticoagulant therapy during the month preceding the baseline exam, iv) history of conditions requiring prophylactic antibiotic coverage prior to invasive dental procedures, v) systemic antibiotic therapy in the preceding 3 months, vi) self-reported pregnancy or lactation, vii) other severe acute or chronic medical or psychiatric condition, viii) periodontal

treatment within the last 12 months, ix) previous surgical periodontal performed to the study site.

Recruitment of participants took place from September 2022 to March 2023, and 57 consecutive participants who met the previously mentioned requirements were enrolled. A single calibrated examiner (author PS) measured six periodontal parameters per participant at each of the following sites/tooth: full mouth probing pocket depth (PPD), recession (REC) of the gingival margin from the cement-enamel junction (CEJ), bleeding on probing (BOP), and tooth mobility. The Bland-Altman test and the Kappa agreement coefficient analysis were used to obtain the intra-examiner calibration after PPD and REC were measured twice in 15 minutes on five subjects who were not part of the current study. A co-efficient of repeatability less than ± 2mm in 90% of the cases was considered acceptable. I It was suggested the Kappa result be interpreted as follows: values ≤ 0 as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement. The results showed an almost perfect coefficient of agreement (92%

SE0.01 p=.001). Furcation defects were classified according to Hamp et al., for the horizontal loss of attachment in the furcation area (Hamp et al., 1975) and in agreement with Tarnow and Fletcher (Tarnow & Fletcher, 1984) for the vertical loss of attachment, using Nabers and UNC-15 periodontal probe respectively. Radiographs were obtained based on clinical need and were employed to validate the FI classification as well as the NF's radiographic defect depth (≥3 mm).

3.2.2 Sub-gingival plaque sampling

Ahead of sample collection, the clinician needed to visualise the sites of collection in agreement of exclusion and inclusion criteria and carefully identified on the periodontal chart and confirmed on available radiographs. Label the collection tube with the patient code number. Then, supra-gingival plaque was carefully removed, the site isolated with cotton wool rolls and gently dried. Sub-gingival plaque samples were collected preferably from the buccal surface of FI sites; alternatively, either mesial or distal furcation sites in maxillary molars, or

lingual in mandibular molars were sampled if the buccal furcation was not degree II/III. A sterile curette was then carefully inserted to the bottom of the pocket and removed with a single stroke and the content placed in TE buffer solution (Cat No. 93283 Sigma-Aldrich) and stored at -80ºC until the time of analysis. Only the subgingival plaque was collected. After a single stroke, the content was placed in the collection tube. This procedure needed to be repeated for the control site, the content was collected with a different sterile curette and placed in a different coded collection tube. The collection tube was firmly closed and placed in the ice box. Samples were taken to Guy's Tower, Guy's Hospital, floor 17, labelled, and stored in the -80°C HTA2 freezers. The sample storage location was documented in the Brady/Pro-Curo system account created for each study. The sample log was filled with the details of samples collected. Follow-up samples were collected according to the study protocol in the same site were the baseline collection was completed. Protocol was described in detail in sections 2.5.1, 2.5.2, and 2.5.3.

3.2.3 DNA Extraction, Sequencing and Taxonomic Analysis

The subgingival plaque analysis followed the protocol described in Chapter 2.

3.2.4 AI-based model for Microbiome Clustering

16S-rRNA Seq data were then analyzed using an autoencoder, which is a type of artificial neural network known for its efficiency in reducing dimensionality while preserving critical information. The autoencoder was designed with variable encoding dimensions, learning rates, and activation functions, optimized through a hyperparameter search over specified ranges. The model's architecture included one input layer, multiple hidden layers (based on the number of layers parameter), and an output layer mirroring the input layer's dimensionality. The Adam optimizer and binary cross-entropy loss function were used for training the model. The TensorFlow and Keras libraries facilitated the model's implementation and training.

3.2.5 Clustering and Cluster Validation

Post-dimensionality reduction, KMeans clustering was applied to the reduced features to identify distinct microbial communities within the periodontal dataset. The number of clusters was determined through iterative testing, with silhouette score, Davies-Bouldin Index, and Calinski-Harabasz Index serving as metrics for evaluating clustering performance. These metrics provided insight into cluster cohesion, separation, and validity. Performance metrics such as silhouette score, Davies-Bouldin index, and Calinski-Harabasz index are commonly used to evaluate the quality of clustering algorithms in microbiome data analysis. Silhouette Score measures how similar an object is to its own cluster when compared to other clusters. It ranges from -1 to 1, where a score close to 1 indicates that the object is well matched to its own cluster and poorly matched to neighboring clusters, suggesting a good clustering. Davies-Bouldin Index evaluates the average similarity between each cluster and its most similar cluster, relative to the average dissimilarity between points in different clusters. A lower Davies-Bouldin index indicates better separation between clusters, with

values closer to 0 indicating better clustering. Calinski-Harabasz (also known as the variance ratio criterion) measures the ratio of between-cluster dispersion to within-cluster dispersion. A higher Calinski-Harabasz index suggests better-defined clusters, with larger values indicating better clustering. These performance metrics are useful for assessing the effectiveness of clustering algorithms in grouping microbiome samples based on their microbial composition. By comparing different clustering methods or parameter settings, researchers can determine which approach yields the most meaningful and biologically relevant clusters. However, it is important to note that while these metrics provide quantitative assessments of clustering quality, they do not capture the biological relevance of the clusters. Therefore, biological interpretation of the clustering results is essential to ensure that the identified clusters reflect meaningful microbial communities or patterns in the data.

3.2.6 Feature Importance via Random Forest Classification and Visualisation

The feature importance of microbial taxa in differentiating between the identified clusters was ascertained using a Random Forest classifier after clustering. In order to gain a deeper biological understanding of the data, it was imperative to determine which microbial features were most indicative of the particular cluster assignments. In order to facilitate visual interpretation, principal component analysis (PCA) was used to reduce the dimensionality of the dataset in order to plot it in three dimensions and visually distinguish the clusters. These visualizations were created using Matplotlib and Seaborn, which depict the spatial distribution of the samples and the clustering result.

3.2.7 Software and Libraries

These analyses were conducted using Python (version 3.7), leveraging libraries such as pandas, scikit-learn, TensorFlow, Keras, matplotlib, and seaborn for data manipulation, machine learning modeling, and visualization.

3.2.8 GCF collection

Sterile Periopaper strips (OraFlow Inc.) were used to gather GCF prior to sampling plaque. Strips were gently inserted into the gingival sulcus for 30 seconds until a slight resistance was felt. This was done after saliva and supra-gingival plaque were carefully removed to reduce the risk of sample contamination. The periopaper was then put inside the Periotron machine (OraFlow Inc.) the value recorded in Periotron units to estimate the volume of GCF absorbed. A sterile Eppendorf microtube was used to store the strip at -80°C. Every Periotron measurement was preceded by a calibration reading that was conducted using blank periopaper according to manufacturer's instructions.

3.2.9 GCF-Laboratory analysis

According to previous studies, 18 GCF markers were selected as inflammatory markers: interleukin-1α (IL-1α), interleukin1-β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-17 (IL-17), interferon-gamma (IFN-γ); connective tissue degradation markers: fibroblast activation protein alpha (FAPα), matrix

metalloproteinase-8 (MMP-8), matrix metalloproteinase-3 (MMP-3); and repair/regeneration markers: bone morphogenic protein-2 (BMP-2), platelet-derived growth factor-AA (PDGF-AA), sclerostin (SOST), receptor activator of nuclear factor kappa-Β (RANKL), epidermal growth factor (EGF), osteopontin (OPN), derived growth factor-BB (PDGF-BB), tissue inhibitor metalloproteinase-1 (TIMP-1) and vascular endothelial growth factor (VEGF) (Koidou et al., 2020, 2022; Santamaria et al., 2023).

The GCF elution and analysis protocol that our group presented in a recent study (Santamaria et al., 2023) was closely adhered to. After defrosting each periostrip, which represented a distinct sample, a sterile microcentrifuge tube containing 50μL of PBS/protease inhibitor cocktail (PBS with protease inhibitors 1X, Complete ULTRA tablets, Mini; EDTA-free, Sigma-Aldrich) was used to elute each periostrip collected prior to the GCF analysis. The tube was then centrifuged twice at 11,000g for 15 minutes at 4°C up until a total volume of 100μL was reached. Following the removal of the Periostrip, the samples were aliquoted into two sterile tubes, each holding 50μL, and kept at 80°C until analysis. Each sample

was analysed in duplicate using a Luminex bead-based multiplex immunoassay (Luminex, R&D systems, Minneapolis, MN) (Koidou et al. ,2022). A specially designed 18-plex kit was created by diluting the GCF 1:4 with assay diluent. Each 50-µL sample was reconstituted in a separate well containing an antibody microparticle cocktail sealed with a foil plate sealer on magnetic beads and incubated on a horizontal orbital microplate shaker for two hours. The biotin antibody cocktail was added after washings, and then incubated for an hour as previously documented. Following the washing, a 30-minute incubation period was required for streptavidin-phycoerythrin (Streptavidin-PE). The samples were prepared for analysis with a Luminex MAGPIX analyser (Luminex; R&D systems). Before reading, the Luminex analyser machine was calibrated and cleaned in compliance with the manufacturer's instructions.

3.2.10 GCF statistical analysis

The GCF volume was calculated from the Periotron units using the formula y = a+bxc, where a is 0 for intercept, b is 135 for serum, c is 0.834, and y is the

periotron score in units (Ciantar & Caruana, 1998). GCF volume, PPD and PPD+REC, and the GCF markers between FI (test), NF and HS, concentrations (pg/ml) and total amounts (pg/30s) are described as means and standard deviations (SD). Non-parametric tests were used for the analysis since GCF markers and volume were not normally distributed. The differences between FI, NF and HS for each of the GCF markers' concentrations were obtained with the Independent-samples Kruskal-Wallis Test. Significance values were adjusted by the Bonferroni correction for multiple tests.

3.2.11 Sample size /power calculation

IL-1 alpha levels of infrabony and HS found in a prior study (Santamaria et al. 2023) were used to calculate the sample size for the GCF marker analysis. by). The study required a minimum of 21 patients to detect a difference of 200.11 (pg/ml) in IL-1 alpha between FI and HS, as the significance and power levels were set at 5% and 90%, respectively. 28 participants (3 samples each) were included in the GCF analysis to account for the possibility of multiple GCF

markers in some samples as well as the multiple GCF markers in the investigation. For the plaque analysis, a convenience sample of 57 participants was chosen in order to account for potential low DNA quality and/or sample contamination due to the dearth of prior studies.

3.3 Results

Fifty-seven participants were included in the current project, with an average age of 55 years and BMI of 28.6. The majority of participants were male (58%) and average high level of plaque and bleeding score, 37.57% and 33.56% respectively.

Patient-level	Average
Age	54.9 (11.54)
BMI	28.6 (4.12)
Biological Sex Male Female	 33 (58%) 24 (42%)

FMPS (%)	37.57 (9.67)
Full mouth PPD (mm)	3.89 (0.34)
Full mouth REC (mm)	0.41 (0.14)
Full mouth PPD+REC (mm)	4.3 (0.47)
Full mouth BOP (%)	33.56 (8.23)

Table 3.1 Demographics and clinical parameters of included cases

The table presents the means (SDs) except for biological sex (frequency). Abbreviations: BMI, body mass index; BOP, bleeding on probing; FMPS, full mouth plaque score; PPD, pocket probing depth, REC, gingival recession.

Overall , 57 (28 GCF) sites were sampled per each group, periodontal sites had higher PPD and PPD+REC than HS ($p<.05$) but there was no discernible variation between FI and NF. The PPD+REC average for the FI was 7.78 (1.36) mm, whereas the NF's average was 7.71 (1.33) mm. HS comprised five canines, ten premolars, fifteen additional molars, and twenty-seven contralateral molars.

Table 3.1 includes demographic information and general periodontal features.

Table 3.2 displays plaque site-specific periodontal clinical characteristics of the 57 included participants. Table 3.3 shows the GCF site-specific periodontal clinical characteristic of 28 included participants.

Site-level		PLAQUE	
	Furcation sites (FI n=57)	Non-Furcation sites (NF n=57)	Healthy sites (HS n=57)
Average PPD	6.54 (1.11)	6.70 (1.19)	2.35 (0.47)*
Average PPD+REC	7.78 (1.36)	7.71 (1.33)	2.71 (0.89)*
Average REC	1.24 (0.97)	1.01 (1.02)	0.36 (0.23)*
BOP (+:-)	57:0	57:0	0:57
Mobility Degree 1 (yes:no)	18:39	10:47	2:55
Horizontal Furcation involvement II:III	34:23		
Vertical Furcation involvement B:C	38:19		
Furcation Molars			
Maxillary:Mandibular	37:20		
First: Second	31:26		

Table 3.2 Periodontal clinical data summarised by plaque-site level

All data reported as means (standard deviations). * Indicates statistical significance difference between site ($p < .05$). PPD, probing depth; PPD+REC, clinical attachment level; GR, gingival recession; BOP, bleeding upon probing; GCF, gingival crevicular fluid.

Site-level		**GCF**	
	Furcation sites (FI n=28)	**Non-Furcation sites (NF n=28)**	**Healthy sites (HS n=28)**
Average PPD (mm)	6.46 (1.02)	6.46 (1.17)	2.48 (0.58)*
Average PPD+REC (mm)	7.77 (1.47)	7.50 (1.41)	2.89 (0.75)*
Average REC (mm)	1.31 (1.01)	1.04 (1.03)	0.41 (0.51)*
BOP (+:-)	28:0	28:0	0:28
GCF volume (µL)	0.75 (0.32)	0.61 (0.26)	0.33 (0.26)*
Mobility Degree 1 (yes:no)	8:20	5:23	1:28
Horizontal Furcation involvement II:III	15:13		

Vertical Furcation involvement B:C	12:16
Furcation Molars	
Maxillary:Mandibular	18:10
First: Second	20:8

Table 3.3 Periodontal clinical data summarised by GCF-site level

All data reported as means (standard deviations). * Indicates statistical significance difference between site ($p < .05$). PPD, probing depth; PPD+REC, clinical attachment level; REC, gingival recession; BOP, bleeding upon probing; GCF, gingival crevicular fluid.

3.3.1 Microbiome analysis

The Shannon indexes was 3.25 (0.43) for NF, 3.09 (0.33) for FI and 2.66 (0.27) for HS, while the Simpson index was 0.9 (0.03), 0.91 (0.07) ,0.85 (0.02) for NS, FI and HS respectively. Both indices revealed significant differences in bacterial diversity and richness between diseased and healthy sites (Figure 3. 1 and Figure 3.2). While no significant differences were found between FI and NF defects (Shannon p=0.85, Simpson p=0.64), higher richness and diversity were seen in FI

and NF compared to HS (Shannon p=6.0 x10-4, Simpson p= 3.8 x 10-3). Bray-Curtis PCoA plots were used to evaluate the distribution of the microbial composition (Figure 3.3). When analysing each group individually, more microbial variability was detected in HS (adonis p value = .001, R2 = 0.81) compared with the diseased groups (FI: p value = .001, R2 = 0.55, NF: p value = .001, R2 = 0.47).

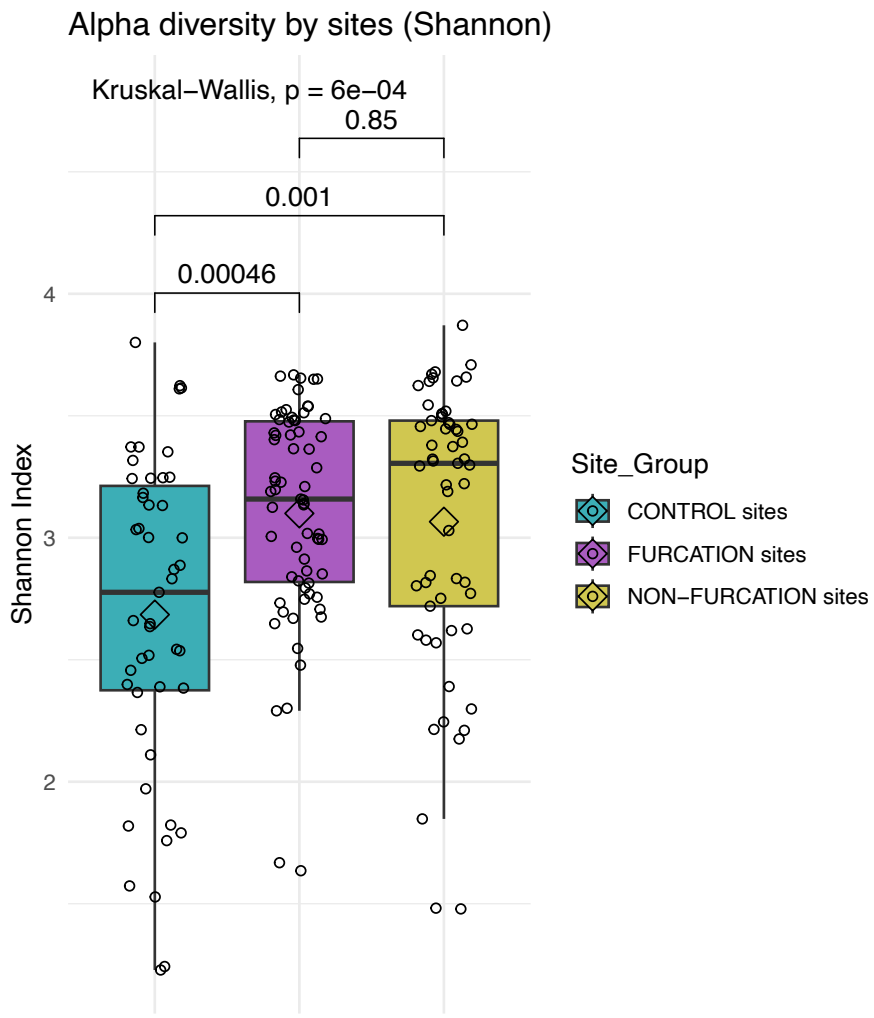


Figure 3.1 Alpha diversity with Shannon index

Alpha diversity with Shannon index of the microbiota grouped by furcation sites, non-furcation and healthy sites.

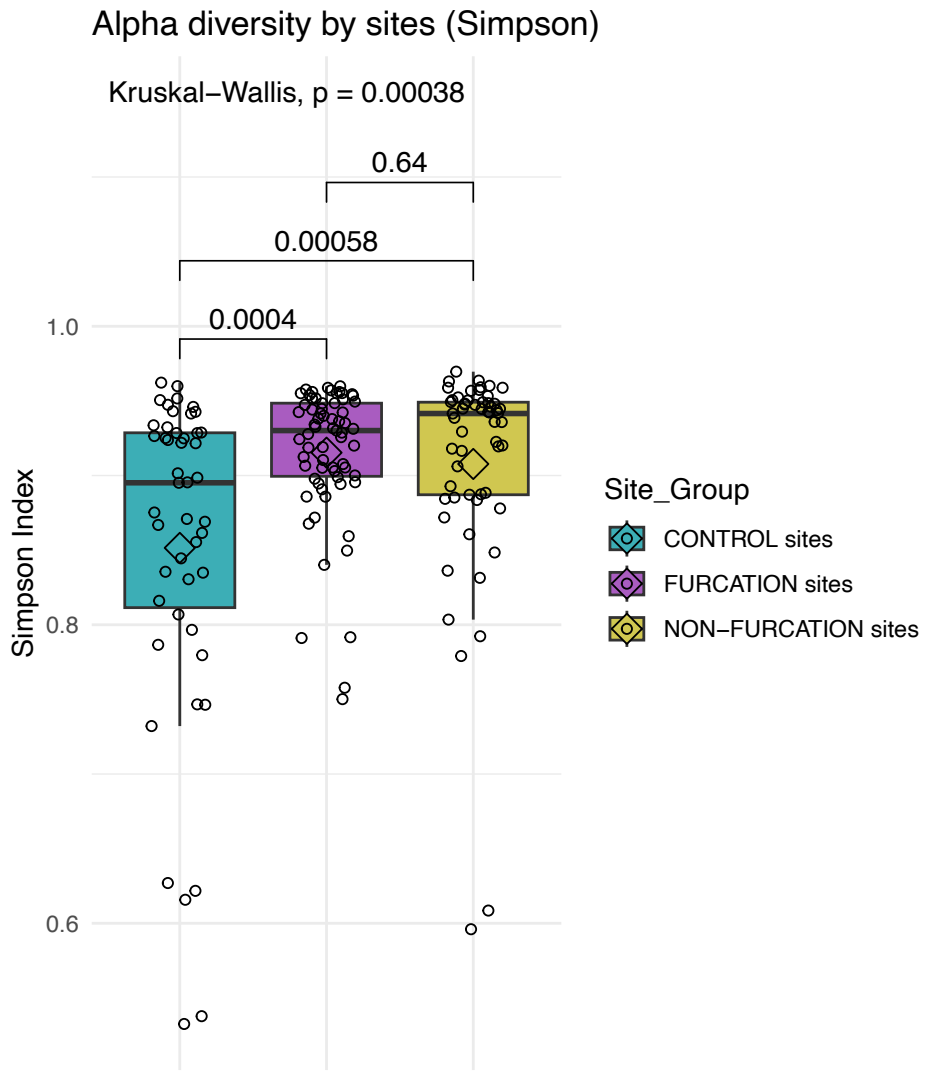


Figure 3.2 Alpha diversity with Simpson index

Alpha diversity with Simpson index of the microbiota grouped by furcation sites, non-furcation and healthy sites.

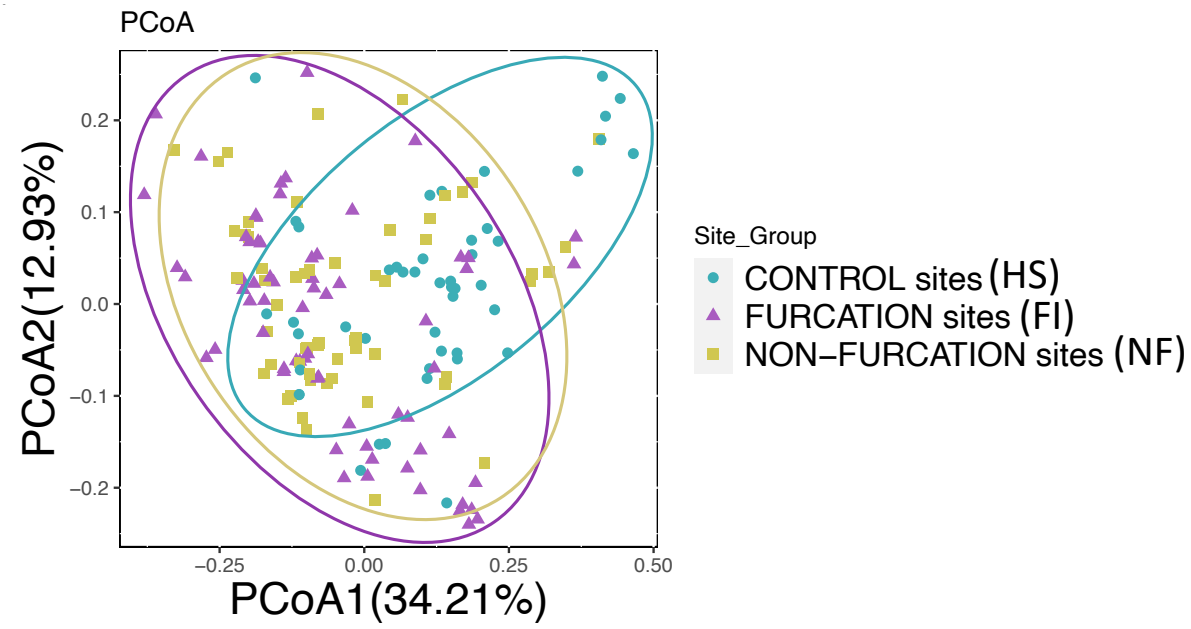


Figure 3.3 Principal coordinates analysis of Bray-Curtis

Principal coordinates of the microbiome structure of the subgingival samples, grouped by furcation sites, non-furcation and healthy sites.

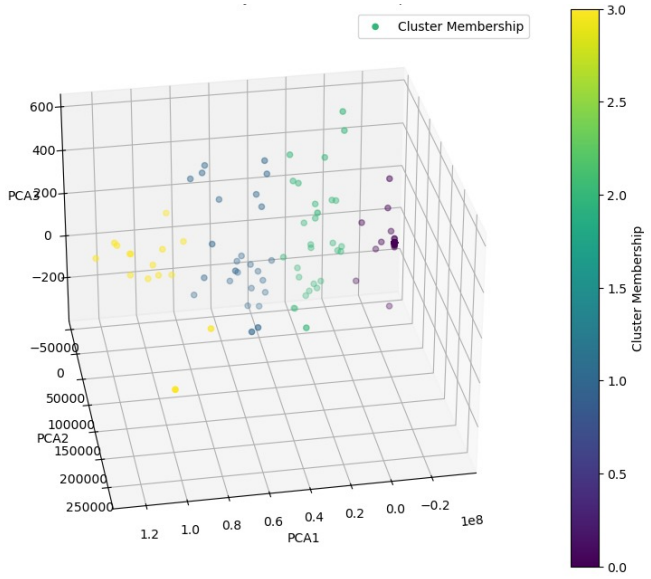
3.3.2 AI-model clustering analysis

The AI-optimised autoencoder configuration included a 32-dimensional encoding layer, a learning rate of 0.1, two layers with 'relu' activation, and a batch

size of 128, effectively categorizing the samples into four distinct microbiome clusters (Figure 3.4) with a Silhouette score of 0.807, a Davies-Bouldin Index of 0.406, and a Calinski-Harabasz Index of 1626.63, implying well-defined separate clusters (Figure 3.4a). The relative percentage of sites showing cluster 0 in FI and NF groups was approximately double (68.42% and 73.68%) when compared with HS (35.09%). On the other hand, for clusters 1 and 2, HS (26.32%, 28,07%) showed a relative percentage more than double when compared with FI (8.77%,8.77%) and NF (10.52%, 14.3%). Cluster 3 was more represented by HS (10.53%) and FI (14.4%) when compared with NF (1.75%). The ten most represented genera are reported in Table 3.4 showing highly predominant periodontal pathogens in cluster 0 compared with cluster 1,2 and 3.

Random Forest classification underscored specific microbial taxa as pivotal in distinguishing between clusters, (Figure 3.4b)

a)


Cluster Membership
PCA3
PCA2
PCA1
1e8
Cluster Membership

b)

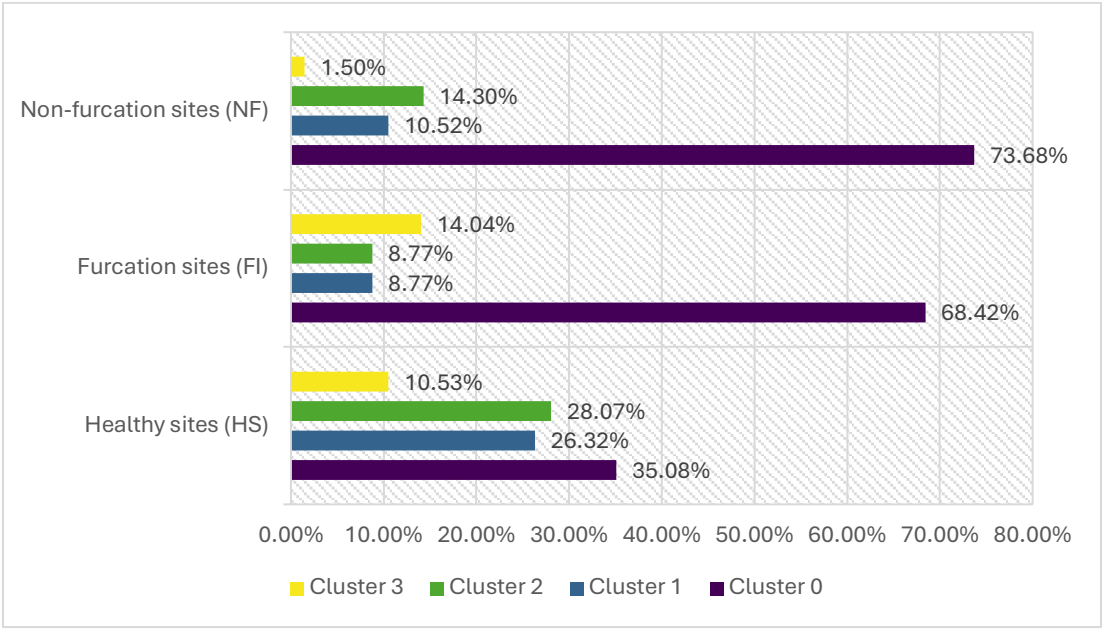

Non-furcation sites (NF)
1.50%
14.30%
10.52%
73.68%
Furcation sites (FI)
14.04%
8.77%
8.77%
68.42%
Healthy sites (HS)
10.53%
28.07%
26.32%
35.08%
0.00% 10.00% 20.00% 30.00% 40.00% 50.00% 60.00% 70.00% 80.00%
Cluster 3
Cluster 2
Cluster 1
Cluster 0

Figure 3.4 AI-model clustering analysis

a) Principal coordinates analysis for three-dimensional plotting, enabling visual discrimination of the four clusters. b) Relative percentage of each cluster divided per group (non-furcation sites (NF), furcation sites (FS), healthy sites (HS)).

Cluster 0	Cluster 1	Cluster 2	Cluster 3
Tannerella	*Porfiromonas*	*Prevotella*	*Clostridiales*
Treponema	*Kingella;*	*Fusobacterium*	*Actinobacillus*
Fusobacterium	*Treponema*	*Parvimonas*	*Cardiobacterium*
Schwartzia	*Selenomonas*	*Rothia*	*Veillonella*
Porphyromonas	*Fusobacterium*	*Clostridiales*	*Prevotella*
Dialister	*Tannerella*	*Tannerella*	*Rothia*
Prevotella	*SHD-231*	*Leptotrichia*	*Actinomyces*
Selenomonas	*Neisseria*	*Cardiobacterium*	*Campylobacter*
Campylobacter	*Campylobacter*	*Campylobacter*	*Veillonella*
Granulicatella	*Cardiobacterium*	*Dialister*	*Dialister*

Table 3.4 Ten most represented genera per each cluster

3.3.3 Distribution of genera between groups

152 bacterial genera were found in all of the samples; 34 genera accounted for slightly more than half of the sequences found in each group site (Figure 3.5). Relative abundance of *Streptococcus* (p=1.7 x 10-4), *Rothia* (p=9.6 x10-5), *Neisseria* (p= 0.002), and *Lautropia* (p = 5.8 x10-7) was statistically higher in the HS group when compared to the FI and NF groups. Periodontal defects (FI + NF) were associated with higher relative abundances of *Selenomonas* (p = 0.00053), *Treponema* (p = 2.2 x 10-4), TG5 (p = 7.9 x 10-5), *Tannerella* (p = 4.1 x 10-5), and *Fusobacterium* (p = 3.1 x 10-5). Seven genera in total displayed statistically significant variations between FI and NF. In contrast to FI, NF sites had higher concentrations of *Neisseria* (p=0.002), *Fusobacterium* (p=0.032), and *Cardiobacterium* (p=0.009) (Figure 3.6). Whereas FI sites had significantly higher concentrations of *Olsenella* (p=0.008), *Atopobium* (p=0.04), *Actinomyces* (p=0.002), and *Moryella* (p= 0.02) (Figure 3.7).

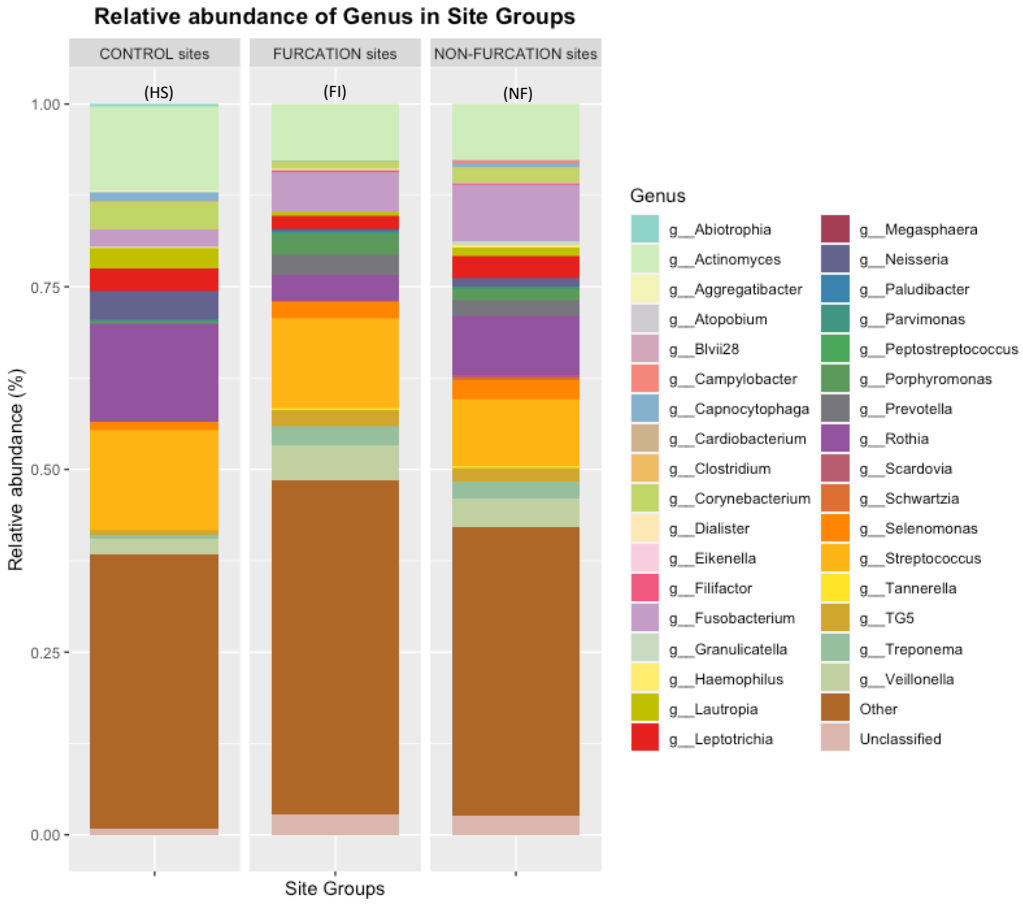


Figure 3.5 Relative abundance difference of genera

Relative abundance difference of the genera significantly higher in NF versus FI.

P-values: * indicates values below the significance level of 0.05, ** below the significance threshold of 0.001.

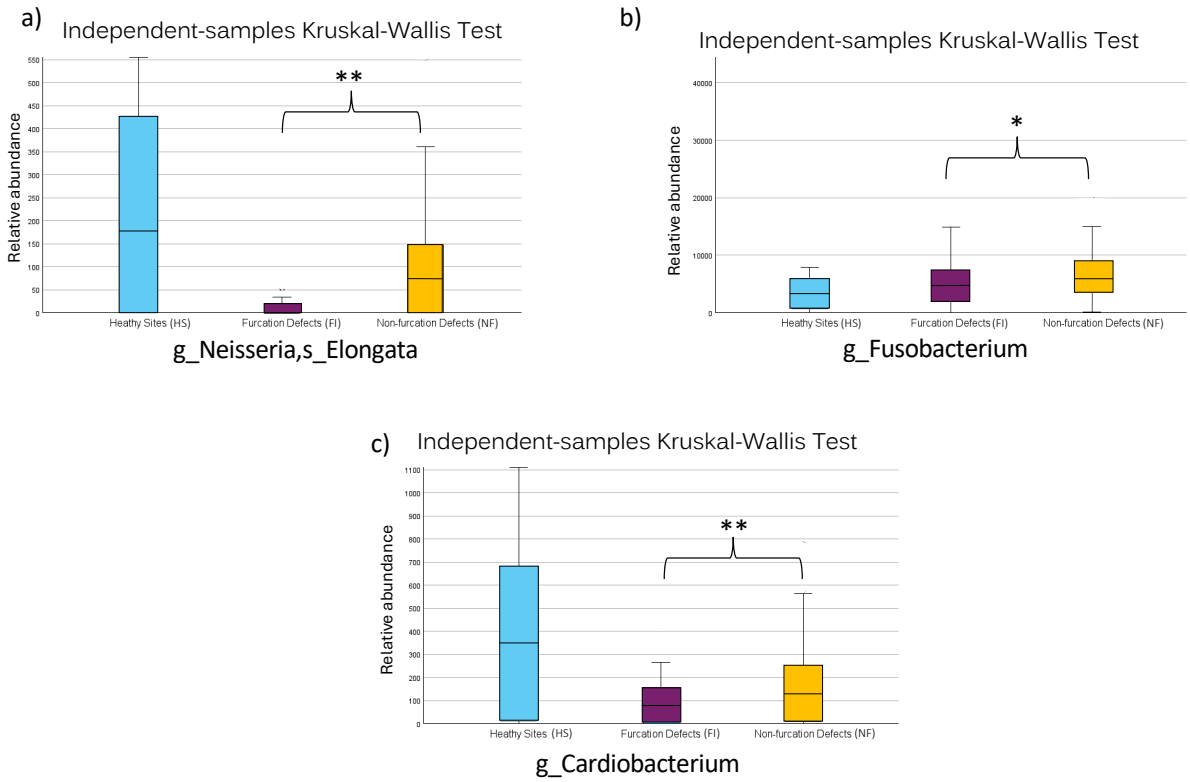


Figure 3.6 Relative abundance difference of genera higher in NF

Relative abundance difference of the genera significantly higher in NF compared to FI. P-values: * indicates values below the significance level of 0.05, ** below the significance threshold of 0.001.

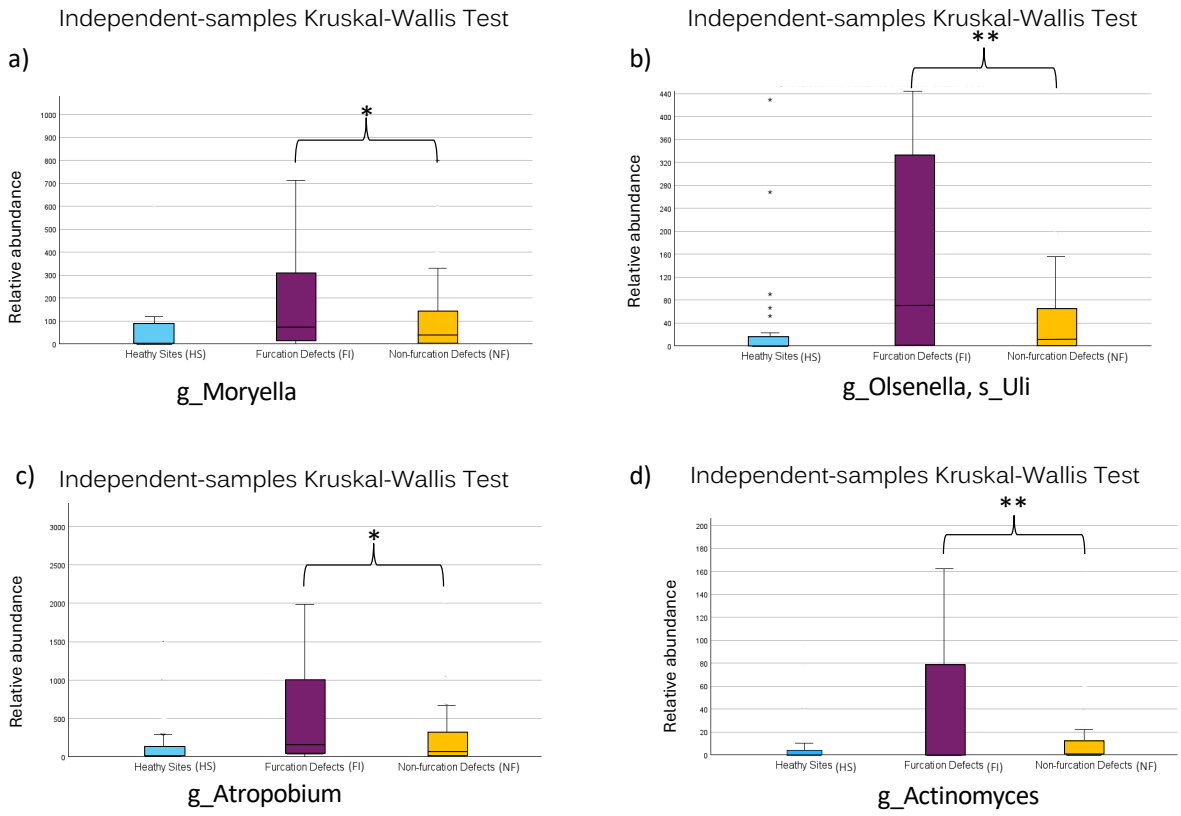


Figure 3.7 Relative abundance difference of the genera higher in FI

Relative abundance difference of the genera significantly higher in FI compared to NF. P-values: * indicates values below the significance level of 0.05, ** below the significance threshold of 0.001.

3.3.4 Aerobic metabolism distribution between groups

Each genus identified was categorized based on their oxygen consumption, grouping in aerobic, anaerobic, and facultative bacteria Figure 3.8a, b, and c. The

relative percentage of each strain within each group (FI, NF, HS) was then determined, showing a higher percentage of facultative ASVs in the HS sites compared to NF sites ($p=0.0022$), with no significant difference between NF and FI sites ($p=0.08$). Anaerobic genera were found to be significantly higher in both FI and NF sites ($p=2.2x10-5$, $p=7.4x10-6$) compared to HS sites. In contrast, aerobic genera were statistically lower in both FI and NF sites compared to HS sites ($p=5.9x10-6$, $p=2.2x10-5$), and FI sites had significantly lower levels than NF sites ($p=0.0018$).

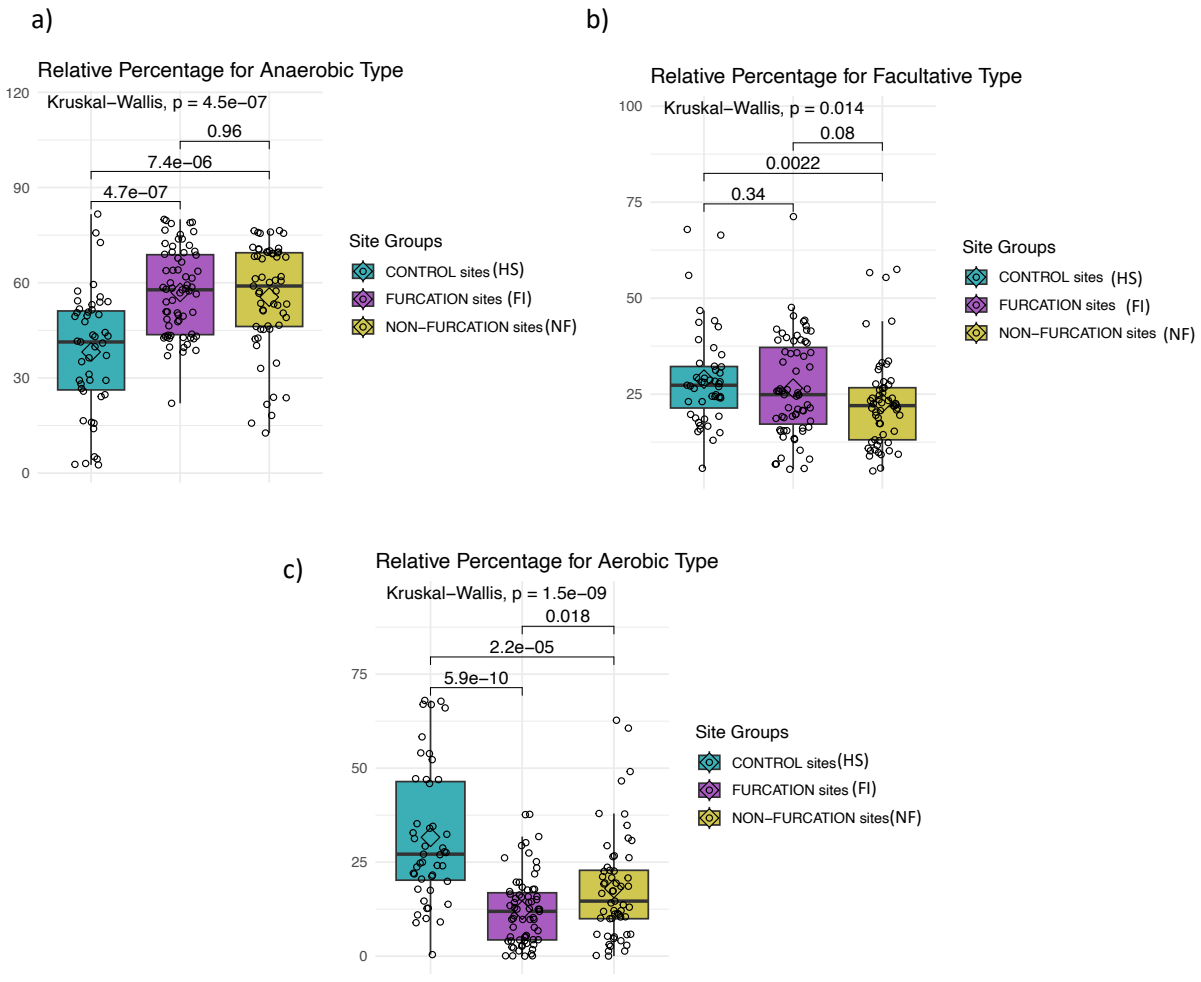


Figure 3.8 Relative percentage of aerobic, anaerobic and facultative taxa

Percentage of aerobic, anaerobic and facultative taxa., grouped by furcation sites (FI), non-furcation (NF) and healthy sites (HS)

3.3.5 GCF volumes at sampled sites

While there was no statistically significant difference in the GCF volume between FI and NF, both types of periodontal defects had a significantly higher GCF volume when compared to HS. Table 3.3 shows the following: FI mean: 0.75 (0.32)µl, NF mean: 0.61 (0.26)µl, HS: 0.33 (0.26)µl, ($p = .0001$).

3.3.6 GCF biomarker levels

Within the range of the immunoassays, all 18 markers included in the Luminex analysis were detectable (Table 3.5 a,b). When HS (n=28) and periodontal defects (FI+NF, n=56) were compared, periodontal defects showed statistically significant higher ($p<0.05$) GCF levels of IL-1α, IL-1β, IL-6, FAP-α, MMP-3, MMP-8, PDGF-AA, PDGF-BB, SOST, EGF, TIMP-1, and VEGF. BMP-2, RANKL, INF-γ, IL-10, IL-17, and OPN did not significantly differ. Table 3.5b shows the GCF levels of IL-6 ($p=0.0001$), MMP-3 ($p=0.003$), MMP-8 ($p=0.008$), BMP-2 ($p=0.008$), SOST ($p=0.0001$), EGF ($p=0.03$), and TIMP-1 ($p=0.015$) in FI that were significantly elevated when compared with NF.

	Healthy Sites (HS)		Furcation Sites (FI)		Non-furcation Site(NF)		Δ NF-HS	Δ FI-HS	Δ NF-F
	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	P-value	P-value	P-value
FAP-α	28	1.72 (4.12)	28	14.95 (32.6)	28	6.87 (10.67)	0.002**	0.0001***	0.262
IL-1α	28	201.01(596.34)	28	504.22 (734.54)	28	484.04 (813.96)	0.096	0.0001***	0.514
IL-17	28	0.74 (0.96)	28	0.76 (0.73)	28	0.85 (0.56)	0.010*	0.161	0.238
PDGF-AA	28	0.83 (1.51)	28	2.79 (5.66)	28	1.57 (1.48)	0.003**	0.0001***	0.268
RANKL	28	7.29 (10.74)	28	5.65 (6.82)	28	6.36 (7.41)	0.619	0.712	0.543
INF-γ	28	4.85 (4.48)	28	6.14 (4.77)	28	5.13 (3.85)	0.314	0.015*	0.153
IL-1β	28	85.57 (144.86)	28	336.52 (343.76)	28	218.01 (280.29)	0.005**	0.0001***	0.071
IL-10	28	5.41 (12.79)	28	2.98 (2.96)	28	4.12 (8.81)	0.113	0.01*	0.322

OPN	28	615.01 (918.36)	28	632.18 (498.39)	28	652.82 (579.62)	0.631	0.921	0.756
PDGF-BB	28	0.84 (0.62)	28	0.89 (0.39)	28	1.19 (0.58)	0.0001***	0.069	0.743
VEGF	28	15.93 (13.56)	28	50.84 (36.45)	28	31.07 (20.78)	0.003**	0.0001***	0.067
BMP-2	28	3.62 (1.1)	28	4.37 (1.12)	28	3.6 (1.28)	0.951	0.010*	0.008**
IL-6	28	2.96 (4.05)	28	11.57 (6.69)	28	5.07 (5.64)	0.0001***	0.0001***	0.0001***
MMP-8	28	5273.06 (9605.83)	28	32158.12 (23160.67)	28	17244.08 (1964.88)	0.005**	0.0001***	0.008**
SOST	28	6.53 (9.21)	28	21.72 (10.79)	28	8.98 (11.42)	0.524	0.0001***	0.0001***
EGF	28	6.92 (14.09)	28	12.72 (11.83)	28	9.09 (10.8)	0.38	0.0001***	0.031*
MMP-3	28	14.84 (17.41)	28	91.80 (90.01)	28	61.93 (118.89)	0.026**	0.0001***	0.003**
TIMP-1	28	424.77 (309.51)	28	818.24 (707.51)	28	860.85 (661.62)	0.013*	0.005**	0.015**

Table 3.5 GCF cytokine marker concentrations (pg/ml)

GCF cytokine marker concentrations (pg/ml) in diseased (furcation defects and non-furcation defects), and periodontally healthy sites section. Data reported as means (standard deviations). * Indicates statistically significant differences between sites $p<0.05$, ** $p<0.001$ and *** $p< 0.0001$.

3.3.7 Correlation of molecular biomarkers with subgingival microbiota

Figure 3.9 shows the Pearson correlation between molecular biomarkers and the 50 most represented taxa in FS, NF and HS. Overall, there was a positive correlation (>0.2) between molecular biomarkers and genera classified as 'red' and 'orange' complex, and a negative correlation (<0.2) with genera classified as 'green' and 'yellow' complex, as reported in Chapter 2 a correlation coefficient greater than 0.2 or smaller than -0.2 were labelled as significant at the 5% level (Schober et al., 2018).

Inflammatory markers as IL-1α, IL-1β were positively correlated to ASV10 (*Porphyromonas*), ASV13-ASV1 (*Treponema*), ASV117 (*Parvimonas*), ASV31 (*Fusobacterium*), ASV51 (*Paludibacter*), ASV45 (*Filifactor*), ASV22 (TG5), ASV57 (*Dialister*), ASV101 (*Peptostreptococcus*), ASV9 (*Selenomonas*), ASV61 (*Prevotella*), and negatively associated with ASV84 (*Rothia*). MMP-3. MMP-8 and FAP-α showed a positive correlation with ASV1 (*Treponema*), ASV61 (*Prevotella*), ASV57 (*Dialister*), ASV22 (*TG5*), and a negative correlation with ASV28 (*Lautropia*), and ASV83 (*Propionibacterium*). PDGF-BB and VEGF showed a

negative correlation with ASV28 (*Actinomyces*), and a positive correlation with ASV22 (*TG5*), ASV57 (*Dialister*), ASV101 (*Peptostreptococcus*), ASV1-ASV13 (*Treponema*), ASV17 (*Capnocytophaga*), ASV40 (*Tannerella*). RANKL was the only biomarker to show a negative correlation with ASV10 (*Porphyromonas*), ASV13-ASV1 (*Treponema*), ASV117 (*Parvimonas*), ASV31 (*Fusobacterium*), ASV22 TG5, ASV57 (*Dialister*), ASV101 (*Peptostreptococcus*), ASV9 (*Selenomonas*),and ASV61 (*Prevotella*).

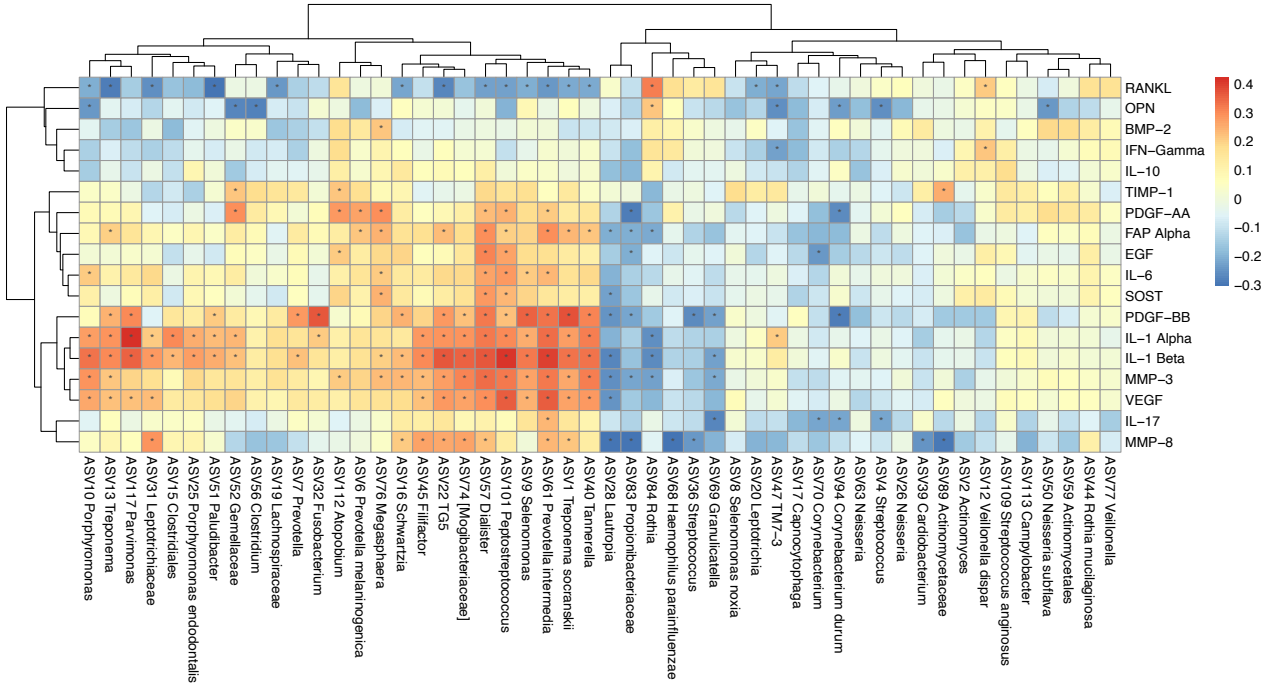


Figure 3.9 Heatmap of the correlation

Correlation between 18 biomarkers and bacterial genera detected in furcation, non-furcation sites and healthy sites

3.4 Discussion

The distinct anatomical features of periodontal furcation defects increase susceptibility to plaque accumulation, progressive bone loss, and an increased relative risk of tooth loss. The precise microbiological profile and molecularly driven inflammatory events that cause this increased rate of tissue destruction are, nevertheless, poorly understood. This is the first study to compare the GCF milieu of the furcation microbiome with that of NF and HS. While more microbial diversity and richness were detected in periodontal sites when compared with HS, the microbiome of FI contained a reduced aerobic component, suggesting that the intricate anatomical configuration of furcation defects may be a significant factor in the formation of the subgingival biofilm in patients with untreated periodontitis. These results are consistent with another study that found that, in comparison to non-molar sites, FI displayed richer and less aerobic counts of microorganisms. This finding may be related to the challenges of achieving complete debridement of the furcation pockets (Loos et al. 1988). Moreover, the authors contended that the limitations of the anaerobic culturing, phase-contrast microscopy, and plaque collection methods employed in the analysis could also have an impact on the results (Loos et al. 1988; Pihlstrom et

al., 1985). Similar to the results of this study, which found different genera and a less aerobic component between FI and NF, previous 16s rRNA analysis of FI comparing two surgical procedures concluded that the furcation microbiome appeared to be different to that from interproximal lesions (Queiroz et al., 2017). According to previous studies, the subgingival microbiome's richness and diversity were higher in FI and NF when compared to HS, suggesting that the addition of late colonizers to the subgingival biofilm expands the pathogenetic community as a whole as a component of the dysbiosis (Arredondo et al., 2023; Griffen et al., 2012). Curiously, the current study found that periodontal pathogens can also be found in HS in patients who have periodontitis. This finding may be connected to the intraoral translocation of periodontal pathogens from periodontal pockets to healthy sulcus when the diseased-free sites become colonized (Lourenço et al. 2014). It is unclear if a higher proportion of pathogenic microorganisms in HS can lead to dysbiosis and subsequently bone loss (Lourenço et al., 2014). Both types of periodontal defects had significantly higher representation of genera that are commonly associated with periodontitis, and the robustness of the microbial analysis presented here was confirmed by the elevated levels of genera previously associated with periodontal health as

Streptococcus, Rothia, Neisseria, and Lautropia (Cai et al., 2021; Socransky et al., 1998). Only a select few genera: *Olsenella, Atropobium, Moryella, and Actinomyces* (Abusleme et al., 2013; Paster et al., 2001; Vielkind et al., 2015), which have been linked to periodontitis, were more prevalent in the furcation region than in the NF. However, it can be speculated that only few genera that separate FI from NF may not fully account for the intricacy of the mechanisms that underlie the greater rate of progression and tooth loss that occurs in FI when compared to other periodontal defects. One could argue that the anatomical structure of furcation defects could choose a distinct microbiological community; in fact, the heterogeneous anatomy of furcation sites could influence environmental features and determine the diversity of microbes (Queiroz et al. 2017).

This is, as far as we know, the first study to use an AI-based model to group the microbiological profile of patients with periodontal defects and assess the relationship between the morphology of the defects and these microbial profiles. Large volumes of microbiome data were processed by the AI-based model, which was able to spot patterns in the data that conventional methods had missed. Specifically, k-means clustering was able to find patterns in the

microbiome data without the need for labelled training data, which facilitated investigation and the discovery of new microbial interactions. Four distinct clusters, ranging from 0 ('more dysbiotic' taxa) to 3 ('less dysbiotic' taxa), could be successfully distinguished by the model. A tendency was noticed differentiating between healthy and diseased locations solely based on microbiological resemblances in the unlabelled data. In particular, in eighty percent of the cases, a site exhibiting a cluster 0 was linked to the microbiological profile of a periodontal defect. It's interesting to note that cluster 0 was found in both healthy and periodontal disease sites, indicating that the same pathogenic cluster that causes periodontal defects may potentially exist in sites without any radiological or clinical signs of illness (Lourenço et al. 2014). Furthermore, while FI and NF were more predominant in the cluster 0, HS showed an almost homogenous distribution among the four clusters emphasizing that the absence of clinical signs of disease should not exclude the presence of a dysbiotic environment. The use of an AI-based model to group the microbiological profile of patients with periodontal defects offers a nuanced understanding of the microbial landscape within the oral cavity and its potential link to periodontal health. This comprehensive analysis elucidates the complex microbial

interactions in the oral environment, highlighting the potential for targeted therapeutic strategies in periodontology based on microbial composition. The study also revealed some intriguing results about what seem to be special molecular characteristics in the GCF of FI. It's interesting to note that, despite the slightly higher GCF volume in furcation defects, no difference was found when compared to matched periodontal defects. This suggests that, rather than affecting the quantity of GCF, the inflammatory process hosted in FI affected its composition. This finding could be explained by a prior study (Barros et al., 2016) that discovered the GCF volume represents the general degree of inflammation rather than an effect that is site-specific. Additionally, a negative correlation was found with genera typically linked to periodontal health and a positive correlation with molecular biomarkers and well-known periodontal pathogen genera. These findings showed a linear correlation between some microbial genera and biological parameters, suggesting that some genera could be able to flourish in conditions dictated by particular host characteristics. Nonetheless, when interpreting the earlier results, potential confounders linked to host-microbiome interactions, hypothetical undetected microbial interactions, sample collection, and processing techniques should be taken into account. Interestingly, the FI

group was found to be associated with an elevated level of a number of molecular biomarkers, including IL-6, which was strongly linked to deep pocket depths and severe periodontal inflammation (Silva-Boghossian et al., 2013), and MMP-3 and MMP-8 which are associated with turnover of periodontal connective tissue collagen (Barros et al., 2016). It was also noticed that levels of RANKL were negatively correlated with the majority of keystone periodontal pathogens including *Porphyromonas, Prevotella, Tannerella,* and *Treponema.* RANKL is involved in regulating osteoclast differentiation, essential for the complete differentiation of osteoclast precursor cells and plays a critical role in periodontal bone resorption. A previous investigation suggested that significant higher RANKL levels were detected in GCF of patients with periodontitis compared to healthy patients or with gingivitis (Bostanci N et. al, 2007). A further investigation reported how upregulated RANKL levels were related to the level of *P.gingivalis* in periodontal tissues. (Nawarat Wara-aswapati et al. 2017). According to a different study, *P.gingivalis* increased PGE2 production, that can upregulate the RANKL level in bone marrow stromal cells. Authors concluded that PGE2-RANKL-*P.gingivalis* mechanism could help to explain why PGE2 might play a role in mediating the induction of RANKL by *P.gingivalis* (Reddi et al, 2006).However, our

findings seemed to be in contrast with previous literature, suggesting that probably the analysis of GCF was not the most appropriate investigation to reflect the correlation between RANKL level, keystone pathogens and periodontitis.

A range of cellular cementum deposits, islands, droplets, enamel projections, and multiple concavities seen in varying combinations are among the anatomical characteristics of FI (Roussa, 1998; Svärdström & Wennström, 1996b). This complex morphology has a significant effect on plaque accumulation, favouring the retention of bacterial deposits and resulting in a more heterogeneous microbiome and less aerobic environment (Loos et al., 1998), Furthermore, it is possible that the elevated levels of biomarkers associated with inflammation, tissue degradation, and repair found in gingival crevicular fluid of furcation defects reflect the intricate microbial challenge and provide indirect evidence of the ways in which host-microbial interactions in periodontal defects might be different in relation to different anatomical configurations . This study's strengths include its novelty resulting from a thorough microbial and molecular analysis of furcation defects and the use of a single operator for DNA extraction, sample collection, and clinical examinations. To ensure that these results are applicable to other populations, we recommend

validating them on distinct cohorts and considering the potential impact of ethnic and geographic differences on the microbiota (Arredondo et al., 2023). The primary limitation of this study is that the genus-level analysis of microbiome might not adequately capture how finer variations within species may differ among samples or experimental conditions. A second drawback is that the study's cross-sectional design only allowed for single time point capture of microbiological features. A strict linear relationship between genera and biological markers is assumed by the Pearson correlation, which is one of the additional limitations associated with the exploratory nature of the statistical analyses. Future studies should examine the furcation microbiome in various populations following debridement and surgical procedures in order to gain a deeper understanding of the intricate and complex mechanisms underlying the more rapid progression of attachment loss in furcation defects.

In conclusion, the study in this chapter demonstrated that notably reduced aerobic bacterial levels in conjunction with elevated host-mediator levels could signify distinct markers of periodontal aetiopathogenesis within the furcation region. This suggests that the intricate anatomic structure of the furcation may

promote a microbial composition associated with a distinct pro-inflammatory and tissue turnover molecular profile.

4 Chapter: Clinical and patient-reported outcomes in grade III furcation defects: an interim analysis of a randomised feasibility trial.

4.1 Introduction

In Chapter 3, it was reported how molars with grade II/III FI with untreated periodontitis showed some dissimilarities in terms of microbiological features compared to other periodontal defects. Therefore, it was speculated that the complex anatomical configuration could potential select a specific microbiological environment hosted in the furcation, partially explaining the higher risk of tooth loss for molars with advanced furcation involvement. However, the difficulty related to the peculiar anatomy of the furcation area represents a clinical challenge even when consolidated therapeutic techniques are delivered for the treatment of these defects. This suggests the hypothesis that the microbiological environment could be a detrimental factor for tooth loss even when specific treatments try to cope with the complexity of the furcation defects. The goals of furcation therapy are the same as those of all periodontal therapy: to preserve the dentition while offering function and aesthetics throughout life (Goldberg et al., 2001). In fact, once an FI is established, the available treatment options include non-surgical maintenance, resective and regenerative approaches or extraction (Nibali et al. 2016).

Therefore, therapeutic approaches to furcation defects can be divided into three broad categories (Nibali, 2018):

- Conservative: Conservative treatment here refers to any surgical or non-surgical method that is aimed at debridement of the furcation area and that does not have regenerative or root separation purposes. These techniques may sometimes involve interventions aimed at modifying the anatomy of the tooth or the supporting structures. Conservative treatments include local antibiotics, scaling and root planing, open flap debridement, osteo-odontoplasty; tunneling and root amputation.
- Resective: Resective treatments, on the other hand, include root separation, root resection, root amputation.
- Regenerative: Finally, regenerative techniques include guided tissue regeneration, regeneration with enamel matrix derivate, bone grafting and growth factors.

Table 4.1 shows guideline specific therapeutic options for each horizontal degree of FI (Sanz et al., 2020), the first recommendation was that periodontal therapy is recommended in molars with class II and III furcation involvement and residual pockets. Furcation involvement is no reason for extraction. Class II furcation on

mandibular teeth and class II buccal furcation on maxillary teeth should be treated with periodontal regenerative surgery, regeneration of furcation can be performed with enamel matrix derivative alone or bone-derived graft with or without resorbable membranes. In class III furcation defects, and maxillary interdental class II or multiple class II defects, no guidelines were offered but only an open recommendation stating to consider non-surgical instrumentation, open flap debridement, tunnelling, root separation or root resection.

Horizontal Furcation lesion	Guidelines	Recommendation
Degree I	Supportive periodontal therapy	
Degree II on mandibular teeth and buccal on maxillary teeth	Periodontal regenerative surgery	
Degree III, maxillary interdental class II or multiple class II defects	Not available	Nonsurgical instrumentation, open flap debridement, tunneling, root separation root resection

Table 4.1 The EFP S3-level clinical practice guideline

Different treatment modalities are presented according to different horizontal degrees of furcation lesions.

Despite the availability of multiple treatment options, research has demonstrated that non-surgical therapy is often ineffective for these teeth (Loos et al., 1989), and the likelihood of any other treatment becoming feasible decreases with the severity of the FI, with degree III FI proving to be the most difficult due to the low effectiveness of regenerative therapy (Pontoriero & Lindhe, 1995). The greatest therapeutic challenge is specifically with molars that have grade III furcation involvement, as there are currently no established recommendations for the best course of action for these teeth (Huynh-Ba et al., 2009). In spite of the knowledge of these clinical difficulties and the fact that FI is a relatively common finding in patients with periodontitis, there are actually only sparse data available specifically for teeth with class III FI (Araújo & Lindhe, 1998; Becker et al., 1988). Furthermore, there are no data in the literature to help explain the complexity of this periodontal defect from a microbiological standpoint. One could argue that the intricate structure of the furcation area, home to a varied and hardy bacterial community, is a major contributor to the increased risk of tooth loss in molars

with grade III furcation involvement. One theory is that the complex anatomy of root surfaces and the small, difficult-to-reach gaps in furcation zones offer an environment that is favourable for the development and maturation of bacterial biofilms. As was previously reported, it is challenging to successfully eradicate harmful bacteria from this biofilm since it is frequently resistant to both traditional mechanical debridement and antibiotic treatments (Matia et al., 1986; Parashis & Mitsis, 1993). These microorganisms' enduring presence may worsen periodontal inflammation and hasten tissue deterioration, raising the possibility of progressive bone loss. Interestingly, the interplay between microbial persistence and host response in grade III furcation-involved molars underscores the increased risk of tooth loss even when surgical procedures are delivered (Nibali et al., 2018).

Hellden (Hellden et al., 1989) evaluated the long-term prognosis of tunnel preparations performed in a large number of teeth with advanced periodontal furcation defects. Tunnel preparation is a procedure usually delivered in grade III furcation defects to expose the furcation area in the supragingival environment and offer the chance to both patient and clinician to access effectively with standard oral hygiene procedures. In particular, tunnel preparations were

delivered on 156 molars with grade III FI. Results after approximately 3 years showed that an overall of 40 teeth (39%) showed caries. In particular, 10 out of 40 teeth were extracted, 7 teeth needed a second surgery, and the remaining 23 (15.4%) were reported to show an 'initial or established caries'. The authors also concluded that there was no relationship between caries development and length of the observation time (Hellden et al., 1989). These findings were subsequently supported by a further investigations that confirmed root caries, which in general frequently occurs in maintenance patients, to be the main reason for loss of molars with furcation tunnels during supportive therapy (Eickholz et al., 1991; Little et al., 1995a; Reiker et al., 1999). Rudiger (Rüdiger et al., 2019), also confirmed that caries was the main reason of tooth loss in tunnelled molars and conducted microbiological analyses to study the microbiological environment of furcation defects treated with tunnel techniques. Therefore, it is clear that clinicians do not have precise guidelines for the treatments of grade III FI but just open recommendations (Sanz et al., 2020). The clinical data available on advanced furcation defects highlighted that these teeth have a high risk of being lost for root caries (Nibali et al., 2018). No randomised-controlled clinical trial (RCT) have been published comparing the clinical and microbiological

outcome of surgical and non-surgical treatment of molars with degree III (Eickholz et al., 2021). Therefore, the primary aim of this feasibility study was to investigate treatment options for grade III FI molars, by comparing the level of aerobic, anaerobic, and facultative genera in III-degree furcation sites treated with surgical or non-surgical approach.

4.2 Materials and methods

This study was a 6-month interim analysis of a single-centre, single-masked, randomized controlled feasibility trial

4.2.1 Ethics approval

Ethics approval was obtained by the HRA and Health and Care Research Wales (HCRW) Ethics Committee (reference 21/EE/0256) and the study was conducted according to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects. The study did not raise any significant ethical issues as it did not involve experimentation using a novel treatment, but rather, its application to advanced cases. It was made clear to patients from the beginning that a proportion of the treated teeth may still

maintain an 'unfavourable' prognosis after treatment and may need extraction at some point. The study was registered on clinicaltrials.gov on 24th Jan 2022 (reference ID 23012022). Each patient gave written consent to take part in the study.

4.2.2 Procedure for Obtaining Informed Consent

When a potentially suitable new periodontitis patient was seen in new patient clinics, the assigned hospital consultant informed him/her of the study and ask if the patient was interested in discussing this with a member of the research team. A member of the research team then approached the patient, provided more information about the study procedures and gave them an information sheet about the study. If the patient agreed to take part, they were offered with a baseline appointment. If they needed more time to consider participation, they were contacted within 1 week to enquire about their willingness to take part in the study and to give them the opportunity to ask any questions about the study. If all inclusion/exclusion criteria could not be verified at the new patient clinic appointment, a review appointment prior to baseline was offered. Informed consent followed the King's College London SOPs and was conducted by staff trained in taking consent.

4.2.3 Potential Risks or Burdens for Research Participants

The clinical procedures performed were mainly standard routine procedures. No risks or burdens were expected from the basic periodontal examination and treatment. Minor pain or discomfort may follow the sub-gingival debridement and it can be easily controlled by using 0.2% chlorhexidine gluconate solution rinse and, if needed, a paracetamol dose of 2 x 500 mg up to 4 times a day for the first two days. Some of the teeth receiving treatment had a very questionable to hopeless prognosis, owing to extensive periodontal disease. Therefore, it was possible that, should symptoms deteriorate, they could have need extraction at some stage during the course of the study or after study completion. This was explained to the participating patients as well as to the referring dentists as part of obtaining their informed consent. Participants were advised that, following surgical periodontal treatments, tooth sensitivity, tooth mobility and developing root caries were the most common risk. Therefore, for participants undergoing this procedure, detailed oral hygiene and the use of appropriate topical fluoride treatments were offered. With all treatments, standard procedures and protocols were carried out. If any signs of disease worsening or general deterioration over

the study period were detected, participants were treated with the standard required therapy (surgery, antibiotics, or tooth extraction, depending on the clinical presentation).

4.2.4 Research team contribution

The original idea of this project was developed by Luigi Nibali (LN) that was also the principal coordinator of the study. Priya Bahal (PB) in agreement with LN designed the study protocol and requested the ethics approval for this project, PB was also the care provider for 15 out of 20 participants and was the coordinator of the randomisation process, clinical data entry, and statistical analysis of clinical parameters. Zainab Malaki (ZM) provided treatment to the remaining 5 out of 20 participants. Pasquale Santamaria (PS) was secondarily involved during the ethics approval process in October 2021. PS applied for the Capacity and Capability assessment from the NHS R&D department at the Guy's Dental Hospital in London, United Kingdom, and registered the study on clinicaltrial.gov. PS delivered the initial periodontal therapy for most patients according to the EFP Step 1 and Step 2 guidelines (Sanz et al., 2020) outside the study protocol and recruited 20 participants in the study. PS was also the clinical

examiner and responsible of collection, storage, DNA extraction and analysis of plaque samples. The clinical results from this study were used by PB for the final dissertation of her Master of Clinical Dentistry in Periodontics, while the microbiological results were used by PS as part of the current PhD thesis.

4.2.5 Patient population

Patients referred to the periodontal department of Guy's Dental Hospital in London, United Kingdom were screened to identify potential study participants. An intra-oral examination, a full-mouth periodontal probing, and a comprehensive medical and dental history were all part of the comprehensive periodontal examination. Periapical radiography was used for the radiographic examination. After a periodontal diagnosis was made, participants who met the study's inclusion and exclusion requirements received a written patient information sheet outlining the protocol and an invitation to take part in the research.

Inclusion criteria were: i) age 18-70, ii) diagnosis of Periodontitis stage III or IV, grade A, B or C (Tonetti et al., 2018), iii) presence of ≥1 tooth with furcation involvement grade III (Hamp et al., 1975) degree B or C (Tarnow & Fletcher, 1984) without any restorative problems, mobility < degree III (as examined by the study

clinician), iv) absence of any ongoing endodontic pathology (as examined by the study clinician) and v) able to consent to study participation. Subjects were excluded from the study if they were i) smoking (any current or in past 5 years), ii) medical history including diabetes or hepatic or renal disease, or other serious medical conditions or transmittable diseases, iii) history of conditions requiring prophylactic antibiotic coverage prior to invasive dental procedures, iv) anti-inflammatory or anticoagulant therapy during the month preceding the baseline exam, v) systemic antibiotic therapy during the 3 months preceding the baseline exam, vi) history of alcohol or drug abuse, vii) self-reported pregnancy or lactation, viii) other severe acute or chronic medical or psychiatric condition or laboratory abnormality that according to the investigator may increase the risk associated with trial participation, ix) non-surgical periodontal therapy performed to the study site within the last 6 months, x) previous surgical periodontal performed to the study site. Valid, informed consent was obtained from all the subjects to be entered in the study.

4.2.6 Clinical examination

All participants had undergone a course of professional mechanical plaque debridement prior to entering the study. Following consent, at baseline, self-reported patient medical and smoking histories were checked. Clinical parameters were assessed by gentle probing using a UNC-15 periodontal probe and a Nabers probe for furcation involvement.

The study 'test site' was chosen as a non-adjacent molar tooth which demonstrated a grade III furcation involvement, with mobility <2 and no endodontic pathology. A 'healthy site' was chosen among the other teeth (ideally another molar, if available) which fulfilled the criteria of demonstrating periodontal probing depths <4mm and not bleeding on probing at the screening visit. Before beginning the study, a UNC-15 periodontal probe and a Nabers probe for furcation involvement were used to gently collect the clinical parameters.

4.2.7 Sample size calculation

No power calculation was required because there were no prior trials in periodontal literature. For this study, a convenience sample of twenty participants

was chosen in order to get preliminary data for a larger trial. Data on the recruitment rate, participants' willingness to be randomised, and the frequency of "responders" after various treatments were specifically analysed before setting the sample size of participants.

4.2.8 Withdrawal / dropout of subjects

Participants were withdrawn from the study if no longer willing or able to attend the study visits or if medical reasons make it not appropriate for them to continue in the study (e.g., pregnancy). If any periodontal clinical additional procedure was needed (outside the study protocol), this was carried out as clinically necessary according to the study investigators and will be documented in the case report forms. If a patient was withdrawn from the trial, reasons for withdrawal and any follow-up information collected was recorded. Subjects were not replaced if they dropped out during the study period.

4.2.9 Randomisation and allocation concealment

Participants were assigned a subject number in ascending order after being found eligible for the study, and baseline data were taken. Using a computer-generated table, participants were randomly assigned to receive either open flap

debridement or non-surgical periodontal treatment (www.sealedenvelope.com). To avoid an uneven distribution of the two treatments, simple randomization was employed to create the randomization tables. In this feasibility study, there was no patient characteristic stratification applied during the randomization process. Twenty different pieces of paper were printed with the name of the treatment item after that the randomization table was ready. These were kept in numbered envelopes that the study therapists alone could access. When a subject was prepared for treatment 1, the operator proceeded to open the numbered envelope and made the necessary arrangements for the treatment to be administered. Participants were advised of the treatment they would receive in advance of their appointment, and valid consent was obtained.

4.2.10 Subgingival plaque sampling

Sub-gingival plaque was collected from each participant at the start of their baseline, 6-month and 12-month visits from the distal aspect of the test and control site at the same visits. Collections, processing and analysis followed the protocols described in Chapter 2.

4.2.11 Intra-examiner calibration

Following initial training, the examiner performed repeated examinations on 10 subjects for PPD, REC and FI with at least 15 minutes' separation. Upon completion of all measurements, inter-examiner repeatability for PPD measurements was assessed. The calibration for continuous variables (PPD and REC) was analysed with the Bland–Altman graph (Figure 4.1) and by calculation of Kappa agreement coefficient (Kappa=0.91), considering a coefficient of repeatability less than ±2 mm in 90% of the cases was considered acceptable.

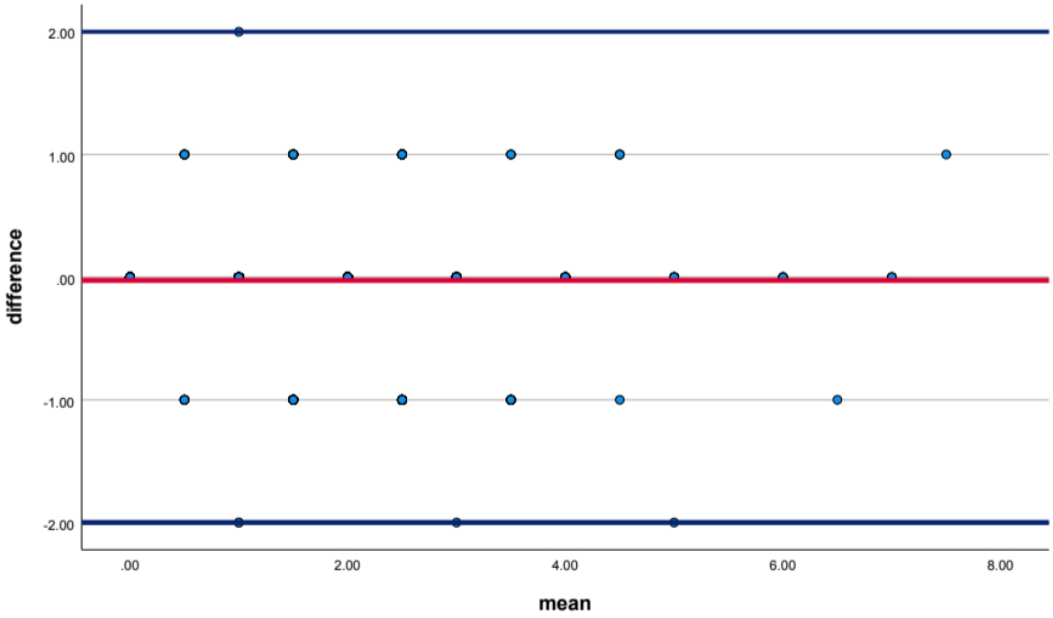


Figure 4.1 Bland-Altman analysis of examiner calibration.

This analysis assessed the agreement between two sets of measurements taken by the same examiner, highlighting the mean difference (-0.1886) and limits of agreement (lower -0.0345; upper -0.0032 with 95% confidence interval of difference)

4.2.11.1 Non-surgical periodontal therapy

After the baseline screening exam, at least two weeks had passed before beginning non-surgical periodontal therapy. Under local anaesthesia (2 percent lidocaine hydrochloride 1:80,000), a standard cycle of non-surgical periodontal therapy was completed on the test tooth. Together with supra- and subgingival mechanical instrumentation of the root surface, oral hygiene advice was given. This therapy was administered by one of the two study therapists (ZM and PB) distinct from the study examiner (PS) utilizing a Cavitron ultrasonic scaler and Gracey curettes. The visit's treatment time was noted.

4.2.11.2 Open flap debridement

The patients were given a local anaesthetic (2 percent lidocaine hydrochloride with 1:80,000 adrenaline) and had intrasulcular incisions made to reflect full

thickness flaps on the buccal and lingual/palatal aspects. Following flaps reflection, the granulation tissue surrounding the tooth was scraped away using a curette or other appropriate scaling tool, being careful not to damage any root surfaces. When needed, Gracey curettes and piezoelectric/ultrasonic tools with thin tips were used to perform a thorough debridement of the furcation area. 4/0 vicryl rapid (resorbable) simple interrupted sutures were used to close the surgical site. Participants received comprehensive instructions on how to take care of the site after surgery. Treatment time was recorded for the visit.

4.2.12 Post-treatment reviews

At one week, one month, and three months after treatment, participants were re-evaluated. During these visits, early healing was assessed, plaque control was reviewed, oral hygiene recommendations were reaffirmed, and adverse events or change in medical histories could be noted. Suture removal for participants who underwent OFD was finished during the one-week visit.

4.2.13 Reassessment examinations

Reassessment visits were completed by the study examiner (P.S.) at 6-months after treatment. During these appointments, the same clinical measurements that were taken at baseline were recorded (FMPS, FMBS, PPD and REC).

4.2.14 Statistical analysis

Data from all included patients were entered into a spreadsheet and proofread for entry errors. Approximately 10% of the data were entered in duplicate to check for accuracy. Access to data entry was restricted to study investigators. Data analysis was performed using R project. A descriptive analysis was carried out at baseline and 6 months.

4.3 Results

In this section are presented clinical and microbiological results at baseline and 6-month after treatment.

4.3.1 Patient flow

The participant recruitment started in June 2022 and was completed by March 2023. Study visits started in June 2022 and were completed by March 2024. Twenty of the twenty-one people who passed the eligibility screening were

invited to participate in the study. Ten participants were randomly assigned to NSPT, and the remaining ten underwent OFD on the test teeth (Figure 4.2). At the 6 months, all participants returned for the review visit.

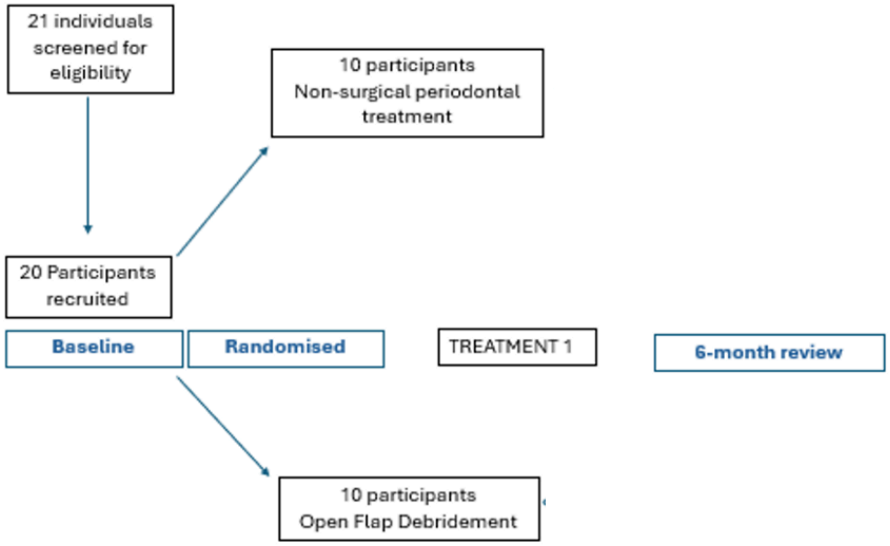


Figure 4.2 Study flow-chart

4.3.2 Baseline characteristics

The baseline demographic and clinical characteristics of every patient involved in the study are listed in Table 4.3.

The average age of participants was 56 with the average BMI being 27.7. Approximately 55% of the participants were female. 40% of the participants were

Caucasian with the remaining 60% being divided equally between participants of Asian or Afro-Caribbean ethnicity. All participants were diagnosed with either stage III C or IV C periodontitis, and in terms of extent, there was an equal distribution of generalised and localised cases with an average number of pocket probing depths ≥5mm, was 20.4.

		Participants (n = 20)	NSPT (n=10)	Surgical Treatment (ST)(n=10)
Age		56 ± 10.1	58 ± 7.2	54 ± 13.1
Biological Sex	**Male**	9 (45%)	4 (40%)	5 (50%)
	Female	11 (55%)	6 (40%)	5 (50%)
Ethnicity	**Caucasian**	8 (40%)	5 (50%)	3 (30%)
	Asian	6 (30%)	3 (30%)	3 (30%)
	Afro-Caribbean	6 (30%)	2 (20%)	4 (40%)
Periodontitis Stage	**III**	15	7	8
	IV	5	3	2
Grade	**B**	0		

C	20	10	10	
Local: Gen	10:10	4:6	6:4	
FMPS (%)	20.4	21.2	19.6	
FMBS (%)	10.3	11.3	9.4	
Study sites				**Δ NSPT-ST P value**
PPD (mm)	7.05 ± 2.04	6.8 ± 2.73	7.3 ± 2.7	NS
CAL (mm)	9.35 ± 2.7	9.02 ±1.89	9.68 ±2.1	NS
REC (mm)	2.3 ± 2.63	2.22 ±1.3	2.38 ±1.3	NS

Table 4.2 Baseline demographic and clinical characteristics

Clinical characteristics (both full-mouth and selected study test sites) at baseline and 6-months are reported in table 4.4. A considerable decrease in PPD and CAL and a decrease in gingival recession was detected in the study sites. Table 4.4 also report data relative to the study sites at baseline and 6 months according to treatment allocation (non-surgical therapy vs. OFD). At the baseline, no significant difference was detected between NSPT (6.8 ±2.73) and OFD (7.3 ± 2.7) for PPD. At 6 months, there was a greater reduction ($p<0.01$) in PPD at the test sites when a surgical procedure (3.55 ± 1.4) was performed compared to NSPT (5.4 ± 1.3).

Clinical Measurements	Baseline	6 months	Δ 0 - 6 months P value
Study Sites			
PPD (mm)	7.05 ± 2.04	5.25 ± 1.77	<0.001
CAL (mm)	9.35 ± 2.7	9.65 ± 2.6	<0.01
Recession (mm)	2.3 ± 2.63	5.75 ± 2.95	<0.001
Control sites			
PPD (mm)	3.3 ± 1.36	3.05 ± 1.29	NS
CAL (mm)	4.53 ± 1.8	4.29 ± 1.98	NS
Recession (mm)	1.23 ± 1.37	1.41 ± 1.53	NS
NSPT			
PPD (mm)	6.8 ± 2.73	5.4± 1.3	<0.01
CAL (mm)	9.02 ±1.89	8.32 ±0.87	<0.01
REC (mm)	2.22 ±1.3	2.92 ±0.98	<0.01
Surgical Treatment			
PPD (mm)	7.3 ± 2.7	3.55 ± 1.4	<0.01
CAL (mm)	9.68 ±2.1	7.5 ±1.87	<0.01

REC (mm)	2.38 ±1.3	3.95 ±1.7	<0.01

Table 4.3 Clinical characteristics at baseline and 6-month visit

4.3.3 Microbiome analysis

The Shannon index at baseline was 3.57 (0.34) for the NSPT group, 2.75 (0.15) for the surgical group and 1.75 (0.34) for HS. The Shannon index at the 6-month visit was 3.76 (0.43) for the NSPT group, 3.15 (0.12) for the surgical group and 1.87 (0.28) for HS. No significant difference between NSPT and surgical therapy groups both at baseline and 6-month after treatment was detected (Figure 4.3 and Figure 4.4 respectively). However, both test groups (NSPT and Surgical therapy) showed a significant higher Shannon diversity compared to HS, both at baseline (p=0.001) and 6 months after treatment (p=0.01). P Bray-Curtis PCoA plots were used to evaluate the distribution of the microbial composition at baseline (Figure 4.5), 6-month visit (Figure 4.6), and per each group at baseline-6-month follow-up (Figure 4.7). When analysing each group individually at the baseline, more microbial variability was detected in HS (adonis p value = .001, R2 = 0.45) compared with the diseased groups (NSPT p value = .001, R2 = 0.09, Surgical Therapy p value = .001, R2 = 0.17. When analysing each group individually 6 months after treatment, more microbial variability was detected in

HS (adonis p value = .001, R2 = 0.48). NSPT being the least variable (adonis p value = .001, R2 = 0.011) while Surgical therapy group being in the middle (adonis p value = .001, R2 = 0.24).

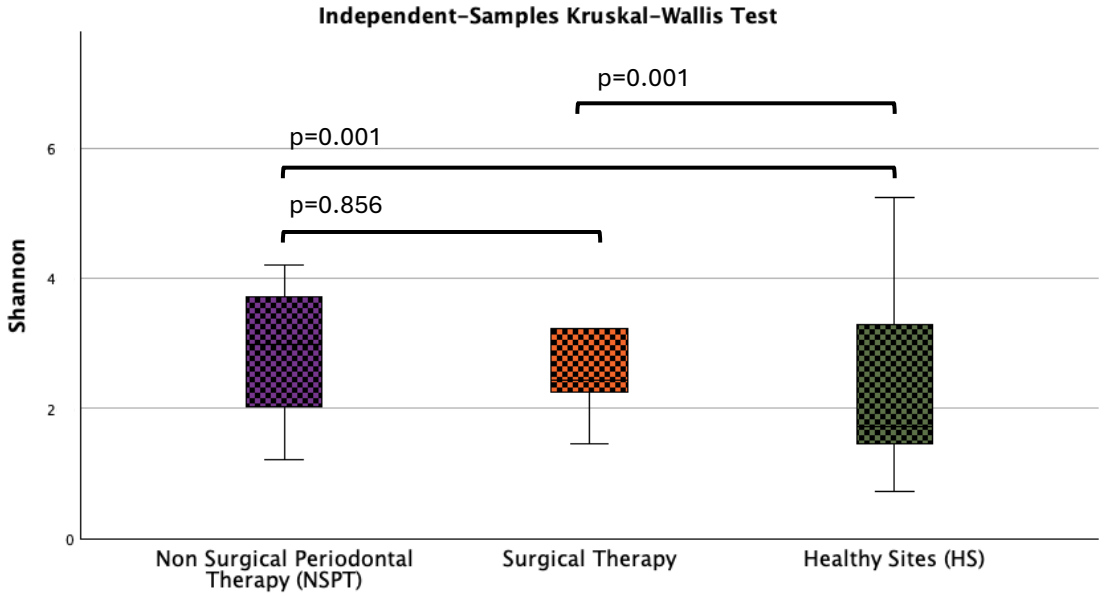


Figure 4.3 Shannon Index for the three groups at baseline

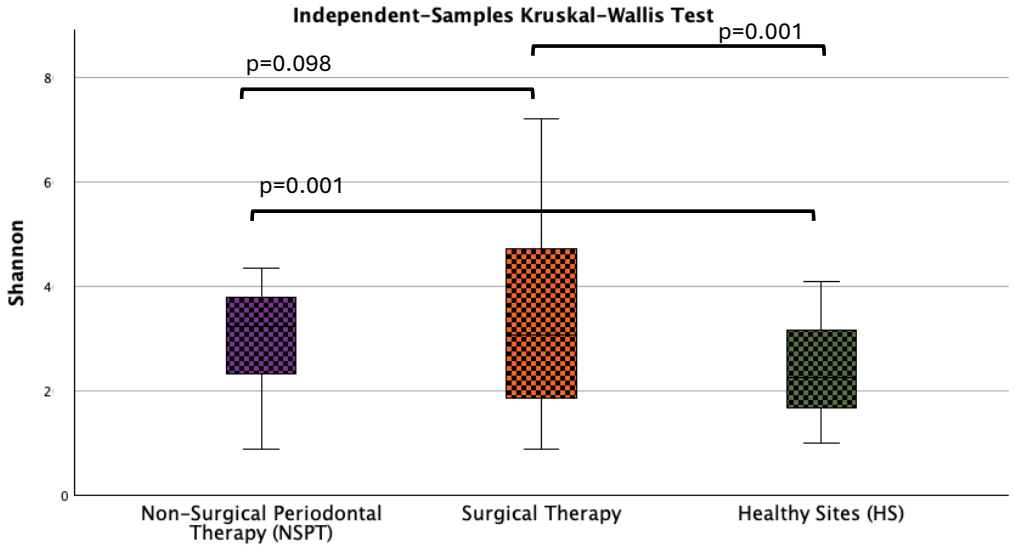


Figure 4.4 Shannon Index for the three groups at 6-month visit

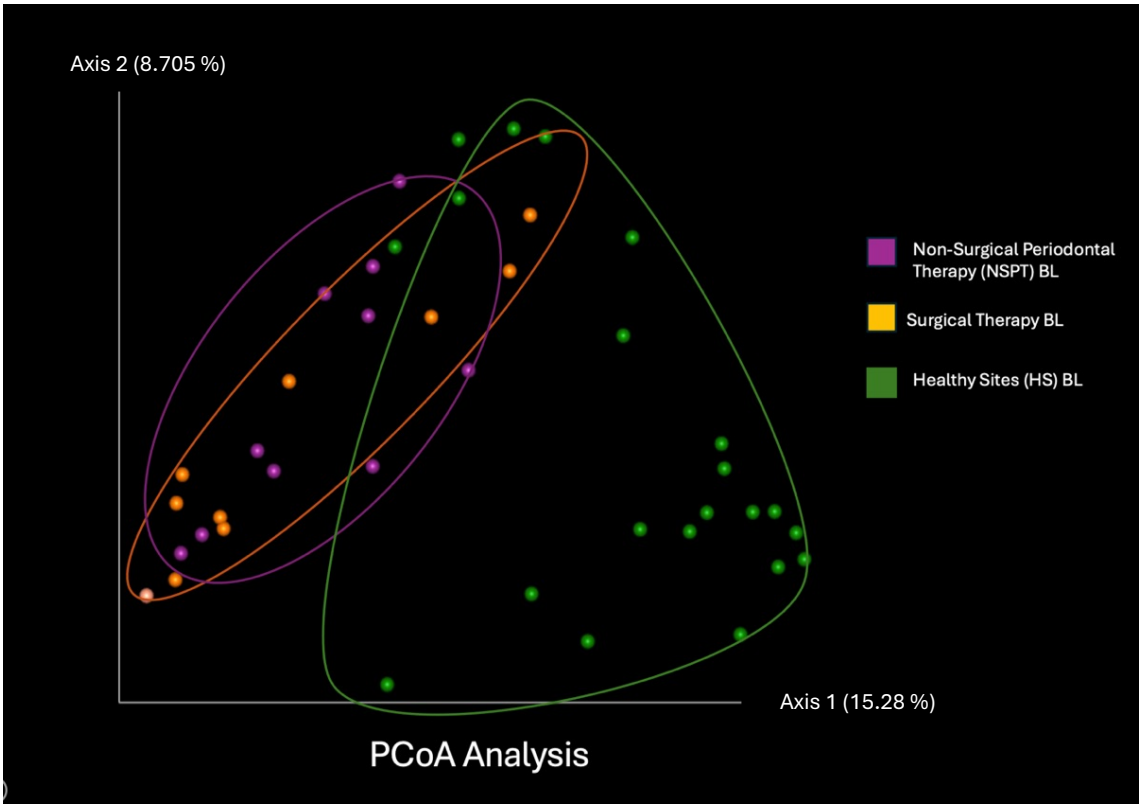


Figure 4.5 PCoA for the three groups at baseline

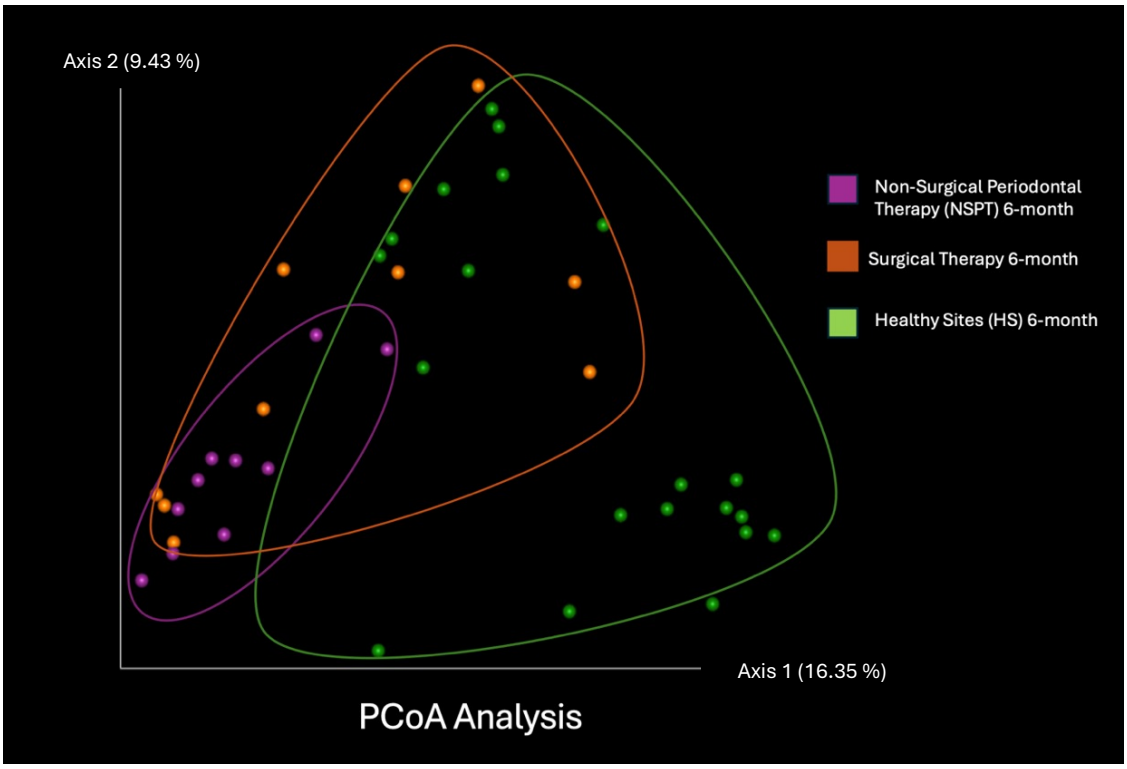


Figure 4.6 PCoA for the three groups at 6-month Visit

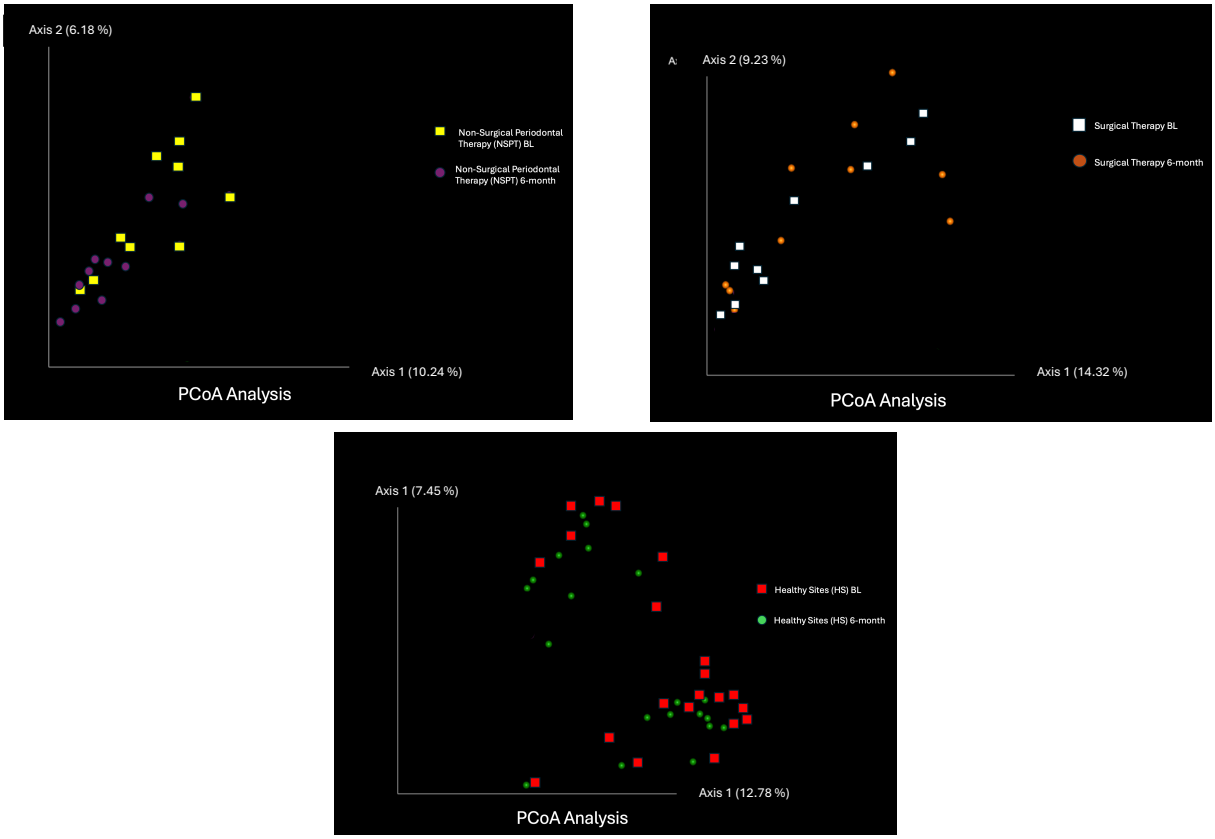


Figure 4.7 PCoA per group at baseline and at 6-month visit

4.3.4 Distribution of genera between groups

198 bacterial genera were found in all the samples at baseline and follow-up visit (6-month). Seven genera in total displayed statistically significant variations between NSPT and Surgical groups 6-month after treatment. No significant difference was detected for *Streptococcus mutans* between surgical group and NSPT group at baseline. At 6 months, the surgical group significant showed a higher level of *Streptococcus mutans* (p= 0.01) compared with baseline, while no significant difference was detected between baseline and 6-month for the NSPT group. The surgical showed a significant increase of *Streptococcus mutans* (p=

0.01) compared with NSPT group at 6-month follow-up (Figure 4.7). Other genera showed higher levels in the surgically treated group: *Streptococcus oralis* (p=0.01), *Streptococcus gordonii* (p=0.003), and *Kingella* (p=0.001), *Granulicatella* adiacens (p=0.02), and *Prevotella denticola* (p=0.001), *Rothia mucillaginosa* (p=0.004) (Figure.4.8)

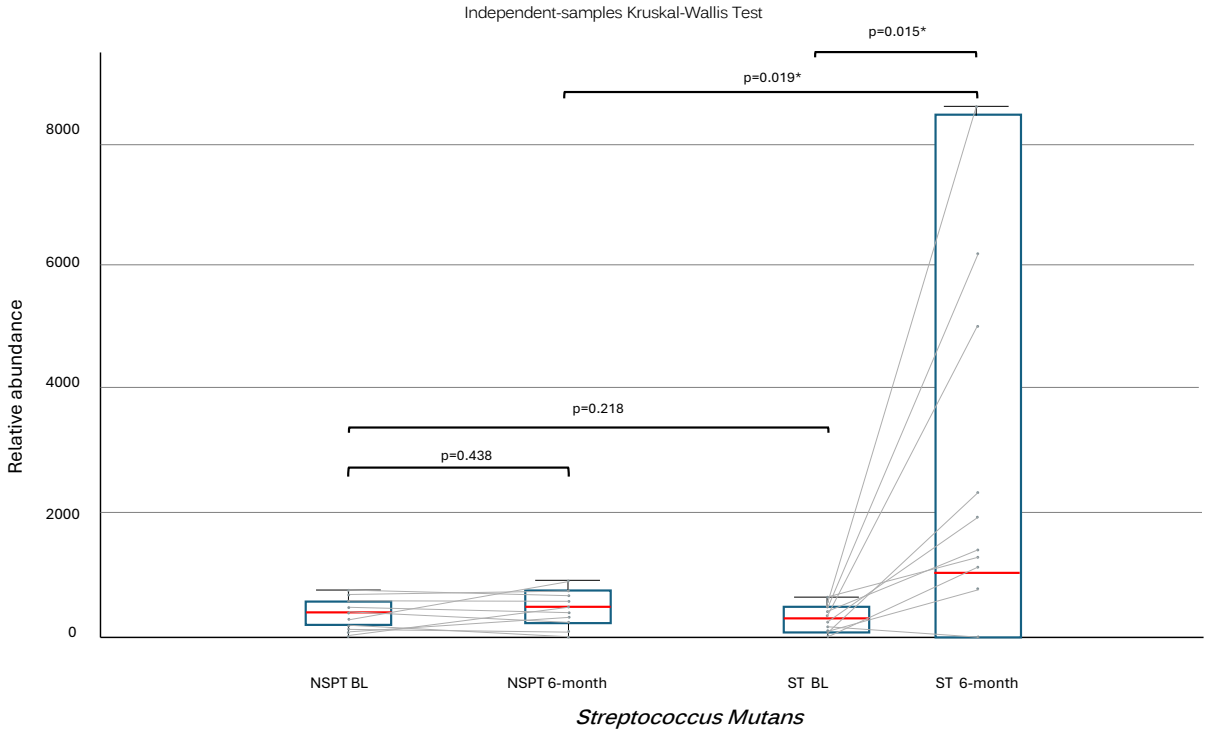


Figure 4.8 Relative abundance of streptococcus mutans at baseline and 6-month visit

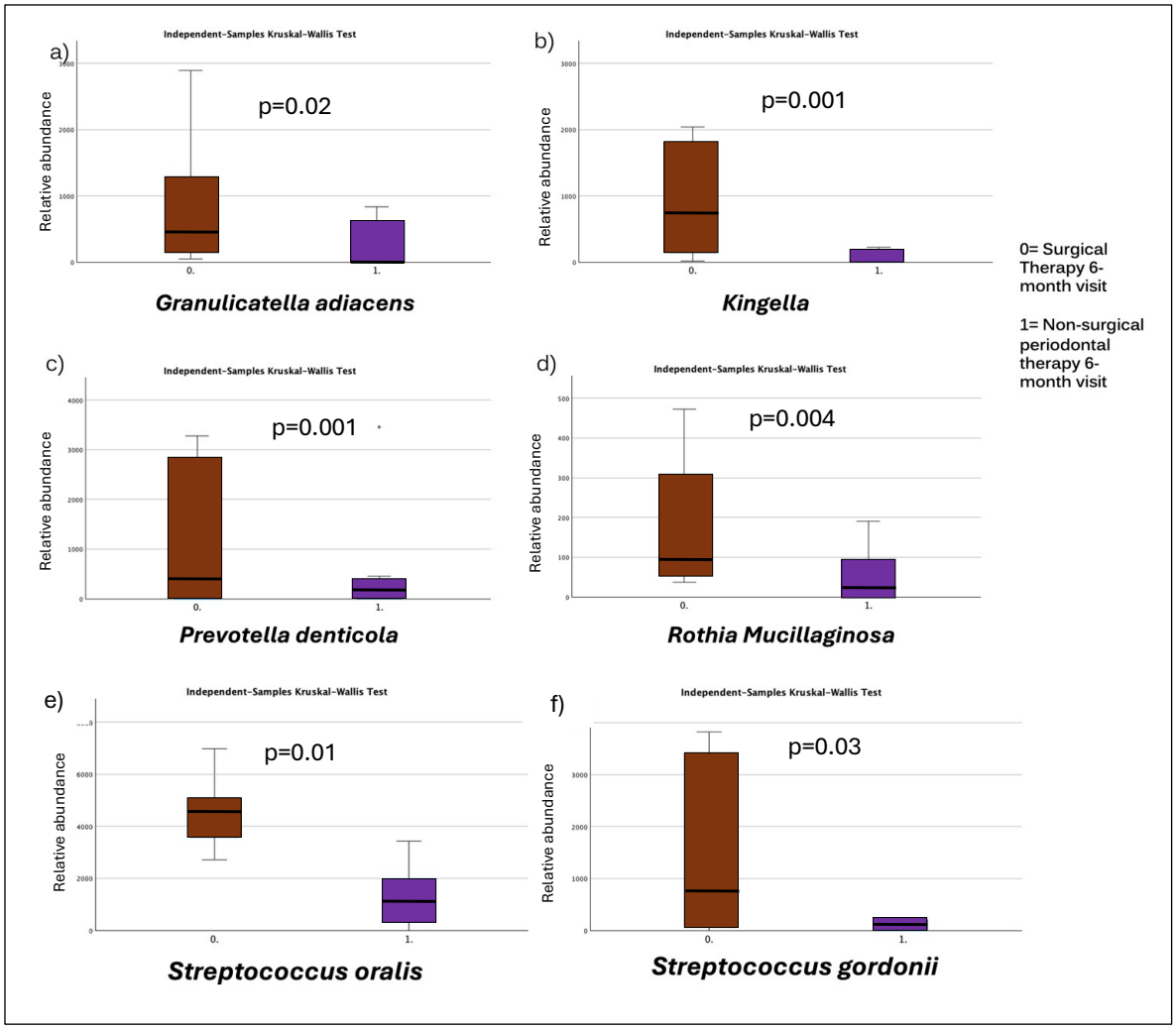


Figure 4.9 Relative abundance among other genera at the 6-month visit

4.3.5 Aerobic metabolism distribution between groups

As in Chapter 3, each identified genus was categorized based on their oxygen consumption, grouping in aerobic, anaerobic, and facultative bacteria (Figure 4.9, and 4.10). The relative percentage (%) of each strain within each treatment group (surgical treatment, and NSPT) at baseline and 6-month after treatment was then determined, reporting no significant differences in aerobic (NSPT: 9.2%, Surgical treatment: 11.1%), anaerobic (NSPT: 57.1%, Surgical treatment: 53.5%)

and facultative level (NSPT: 33.6%, Surgical treatment: 35.2%) between the two groups at the baseline (Figure 4.9). A higher percentage of aerobic level in the surgical group (Surgical treatment: 26.7%) compared to NSPT (NSPT: 19.24%) group at 6-month visit (p=0.03). Anaerobic (NSPT: 44.9%, Surgical treatment: 37.1%) and facultative genera (NSPT: 35.8%, Surgical treatment: 36.2%) were found to be not significantly different between the two treatment groups at 6-month (p=0.69, p=0.49 respectively) (Figure 4.10).

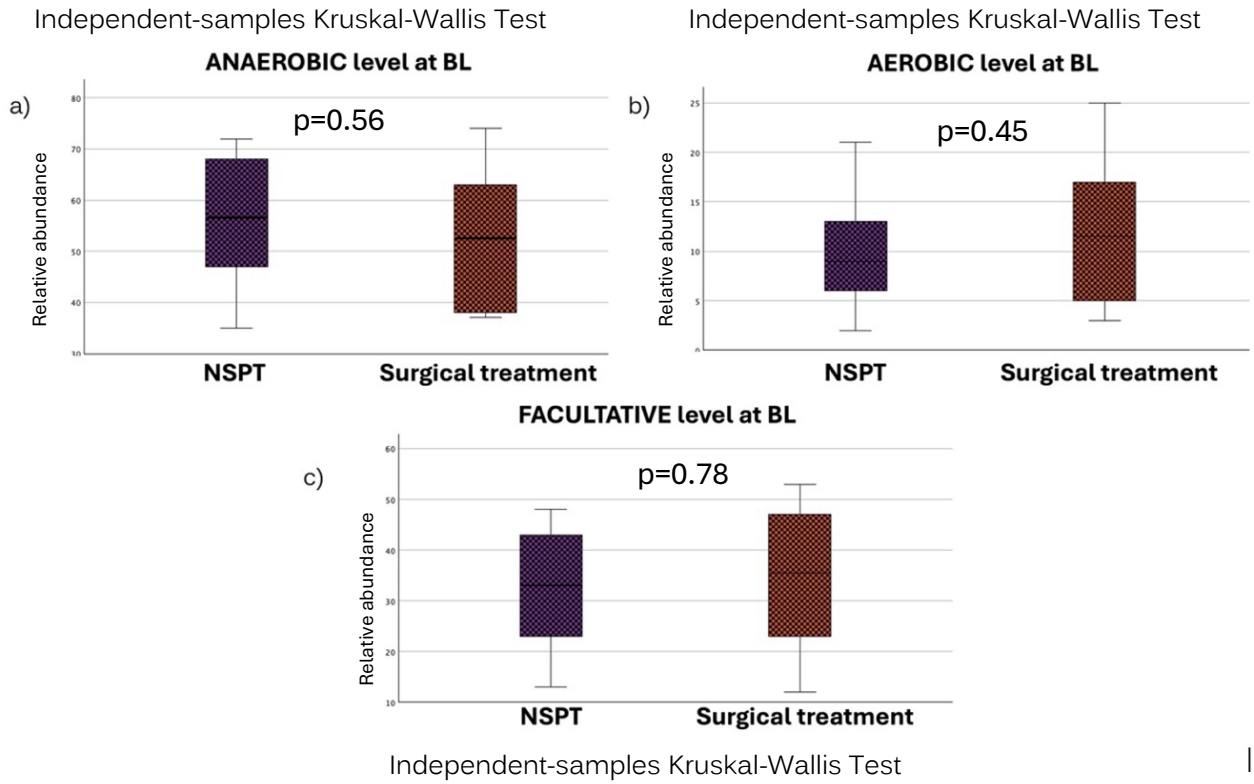


Figure 4.10 Anaerobic, aerobic, facultative levels at baseline

Relative percentage of a) anaerobic bacteria levels b) aerobic bacteria levels between c) facultative bacteria levels in Test (Surgical) and Control (NSPT) group at baseline.

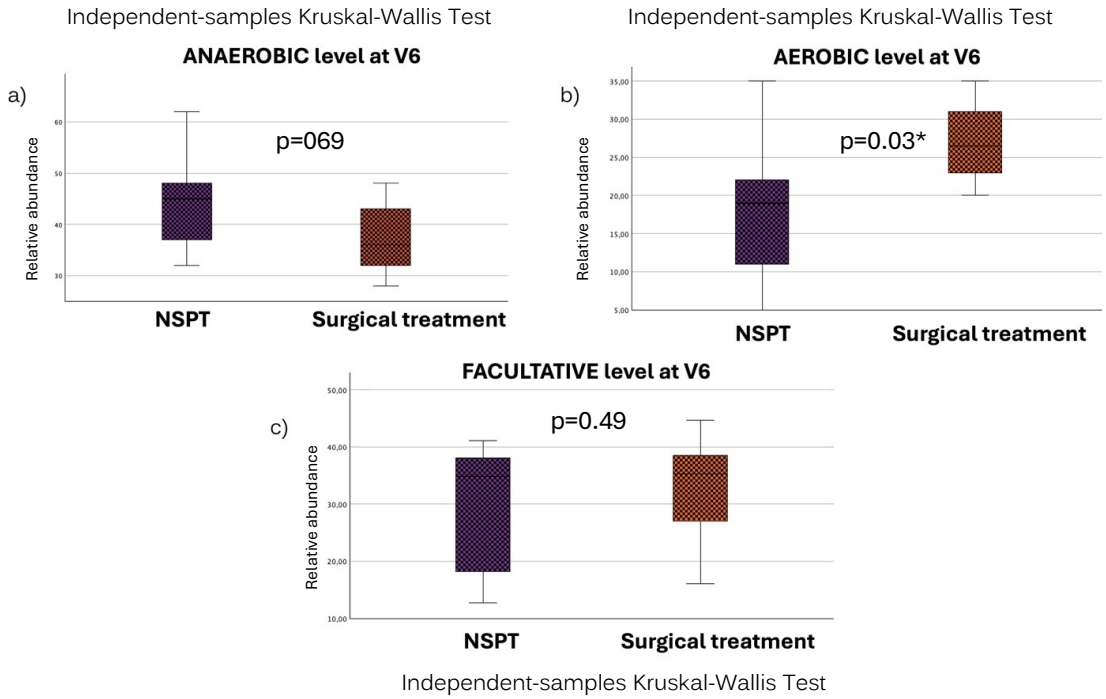


Figure 4.11 Anaerobic, aerobic, facultative levels at 6-month visit

Relative percentage of a) anaerobic bacteria levels b) aerobic bacteria levels between c) facultative bacteria levels in Test (Surgical therapy) and Control (NSPT) group at 6 months.

4.4 Discussion

The subgingival plaque analysis presented in the current chapter offered some interesting results in relation to the significant higher level of aerobic genera detected in the test group, where participants were randomised to receive a surgical approach as OFD or tunnel technique. It is clear that in the test group, a greater gingival recession, and pocket depth reduction were observed with a subsequent greater exposure of root surface. This result is in line with previous literature (Badersten et al., 1987; Buchanan & Robertson, 1987) that reported that both OFD and apically positioned flap may result in an greater gingival recession and periodontal pocket depth reduction when compared to NSPT (Lang, 1983). An interesting study (Shi et al., 2018) characterised the subgingival bacterial biodiversity of periodontal pockets with different probing depths, demonstrating how different subgingival microbiological profiles correspond to different level of probing depth, with an increase of obligate anaerobic in the deepest area of periodontal pockets. Therefore, it can be speculated that as consequence of the periodontal pocket reduction after the surgical treatment, the periodontal sites were more accessible to oxygen, shifting the microbial balance, and favouring the proliferation of aerobic bacteria over anaerobic/facultative species. Furthermore, it was also highlighted how specific streptococci species showed

statistically higher level in the surgical-treated group compared to non-surgical treated group. In particular *Streptococcus mutans* that is well-known for its association with dental caries (Van Houte, 1994). Rüdiger et al. (Rüdiger et al., 2019) reported that, in molars treated with tunnel technique, the finding of root caries was associated with a higher levels of *Streptococcus mutans,* a higher presence of other streptococci genera as *Streptococcus sanguis* indicated lower risk of caries development (Emilson et al., 1988). Apart from *Streptococcus mutans,* other streptococci were showed increased levels in surgically treated sites compared to the NSPT group as *Streptococcus oralis* and *Streptococcus gordonii*. It was reported that bacteria derived from the genus Streptococcus are the first inhabitants of the oral cavity (Abranches et al., 2018). Oral streptococci generate a vast array of adhesive molecules that facilitate their effective colonization of various oral tissues. Additionally, they are remarkably adept at fermenting carbohydrates to produce acids as byproducts of the process. Less acid-tolerant species, like *Streptococcus oralis* and *Streptococcus gordonii*, produce a lot of alkali and play a significant part in the oral cavity's acid-base physiology. Other cocci as *Rothia*, and Granulicatella, defined as normal oral commensals, were also detected in higher level in the test group. Interestingly,

other bacteria as genera *Kingella* (facultative aerobic) and *Prevotella* (obligate anaerobic), often associated with a pathogenetic flora, were also significantly detected in the surgically treated group. It can be argued that since not all test teeth showed a complete resolution of pre-existing periodontal pockets, higher level of facultative/anaerobic bacteria may be the result of interaction between this residual periodontal pocket and the intricate anatomic structure of the furcation area.

In general, these results should be interpreted with caution due to the small sample size and single-centre design of this project. Furthermore, limitation bias due to subgingival plaque collection and analysis should be also considered.

To conclude, this project confirmed the hypothesis that molars with furcation involvement treated with a surgical approach showed a greater PPD reduction compared to the control group. This clinical outcome seemed to impact on the selection of a specific subgingival microbiome characterised by higher levels of aerobic bacteria with significant levels of *Streptococcus* genera.

5 Chapter: Survival of molars with degree III periodontal furcation involvement following non-surgical or surgical therapy: a multicentre single-masked superiority randomised controlled trial.

5.1 Introduction

The previous chapter drew stimulating conclusions on how more extensive analyses about grade III furcation defects from a microbiological perspective may be crucial in preventing tooth loss due to the complex interplay between periodontal pathogens and the host's immune response. This is in line with the Consensus of the European Federation of Periodontology, where it was stated that new studies should focus their hypothesis on treatment of molars with grade III FI. As was previously mentioned, grade III furcation defects result in a difficult-to-clean tunnel-like passage (Matia et al., 1986). This environment becomes a reservoir for pathogenic bacteria even after routine periodontal procedures, which can result in ongoing inflammation and infection. From a microbiological standpoint, a previous investigations tried to understand indirectly the correlation between furcation microbiome and FI (Eickholz et al., 2021). Eickholz (Eickholz et al., 2021) concluded that systemic antibiotics adjunctive to subgingival instrumentation were associated with enhanced tooth survival in teeth with class III FI. Therefore, understanding the specific bacterial species and their biofilm formation within these defects is essential. Destructive periodontitis can result from these infections because they can elicit an immune response from the host

and avoid common periodontal therapies (Nibali, 2018). Moreover, studying the furcation microbiome, in patients with untreated periodontitis and after receiving a suitable treatment, may shed light to understand better the pathogenic process in generating furcation lesions. In fact, for many years furcation involvement has been considered as merely the extension of a periodontal pocket into the interradicular area of bone in multirooted teeth, however further investigation is needed to clarify the etiopathogenesis of this unique periodontal defect (Nibali, 2018). By analysing the microbial communities associated with grade III furcation defects, researchers and clinicians can improve mechanical debridement techniques, preventive care protocols, and targeted antimicrobial therapies. Moreover, the microbial insights can aid in predicting the progression of periodontal disease and the effectiveness of treatment strategies. With the use of this microbiological evidence, individualized treatment programs can be created, lowering the chance of tooth loss and enhancing overall oral health results. Interestingly, a recent investigation aimed to evaluate the impact of furcation status on the risk for molar loss (Trullenque-Eriksson et al., 2023). Copious data on furcation molars were collected from the Swedish Quality Registry for Caries and Periodontal diseases, confirming that advanced furcation

involvement resulted in 2-3 times higher risk of tooth loss. 'Do not consider extraction as the first treatment option of teeth displaying advanced furcation involvement' is the main conclusion of this study and it is supported by the high external validity of using a national registry (Trullenque-Eriksson et al., 2023). Therefore, the microbiological study of grade III furcation defects is vital for developing effective interventions to reduce the risk of extracting teeth assigned with poor/questionable prognosis that can be retained in-situ if appropriate guidelines are clearly followed. With the use of this information, individualized treatment programs can be created, lowering the chance of tooth loss and enhancing overall oral health results. Multi-centre research can provide more networking opportunities, resource sharing between centres, and larger sample sizes (Payne et al., 2011). When compared to single-centre studies, multi-centre research makes it possible to improve clinical work's reproducibility, generalizability, and availability of clinical translation (Hunniford et al., 2019). In the medical and dental fields, the outcomes of multi-centre studies are crucial for determining best practices for interventional procedures as well as for assessing the efficacy of interventional tools. Moreover, a microbiological analysis conducted on samples derived from different geographical area may

also investigate if there is a significant difference between the relative abundance and prevalence of such bacteria when comparing subjects of different ethnicities or geographical locations as reported by previous literature (Haffajee et al., 2004; Teles et al., 2013). In fact, despite the presence of a specialized core of periodontitis-associated bacteria, some studies have found differences that might exist between the microbiota of different geographical origins both in healthy and periodontal patients. Arredondo and associates (Arredondo et al., 2023) reported that the subgingival microbiome, whether in healthy or diseased individuals, can vary greatly depending on the subjects' country of origin. However, a core of bacterial genera linked to periodontitis was found to be conserved across all of the countries, whereas genera linked to healthy status were more diverse. To what extent these results will also affect the furcation microbiome is not known, as it is actually unknown what is the gold standard therapy for advanced furcation defects. No RCTs, comparing the two most valid treatment options: periodontal non-surgical treatment and open flap debridement, have been so far conducted. Therefore, the overall primary aim of this study was to assess the survival of molars with advanced FI at 5 years after surgical or non-surgical periodontal therapy. However, this PhD thesis presents

an interim analysis evaluating the microbiological environment differences before and after the two different periodontal treatment, reported in the study protocol as secondary outcome.

5.2 Material and Methods

5.2.1 Study Design

This is an interim analysis of a single-masked multicentre, randomised controlled trial testing surgical vs. non-surgical treatment for molars with furcation degree III, carried out in 5 centres across the UK, Spain, Germany, Sweden and Italy.

5.2.2 Ethics approval

The study is registered on clinical trials with the following number NCT05237401. The coordinating centre was the Periodontology Department of King's College London (KCL), London, United Kingdom. An overall of five centres obtained ethics approval from the relevant local ethics committee. For the London centre with reference HR/DP-21/22-25910 permitted by the Research Ethics Committee of

King's College London, Frankfurt centre with code 2021-244 obtained from the Ethics Committee of the Department of Medicine at Goethe University, Malmo centre with code 2021-00292 granted by the Stockholm medicine ethics committee, Santiago de Compostela centre with code 2021/448 obtained the Ehtics Committe 'de la investigación con medicamentos' of Galicia, Turin (Italy) with number 00132/2022 by the Bioethics Committee of Turin University.

5.2.3 Patient population

All patients included in the present analyses have been recruited from Periodontal Clinics starting from February 2022 until July 2023 at the 5 participating centres:

- United Kingdom (Ravenscourt Dental Practice, London)
- Germany (Periodontology Clinic, University Hospital of Frankfurt)
- Spain (Periodontology Department, Dental School, Santiago de Compostela)
- Sweden (Periodontology Department, Dental School, Malmo)
- Italy (Periodontology Department, Dental School, Turin)

When a potentially suitable patient was identified in the clinics, the dentist (private practice in UK) or hospital consultant informed him/her of the study and asked if the patient was interested in discussing this with a member of the research team. Then a member of the research team approached the patient, provided more information about the study procedures and gave them an information sheet about the study. If the patient agreed to take part, they were offered a baseline appointment. If they needed more time to consider participation, they were contacted by phone by a member of the periodontology research team within 1 week to enquire about their willingness to take part in the study and to give them the opportunity to ask any questions about the study. If all inclusion/exclusion criteria could not be verified at the new patient clinic appointment, a review appointment prior to baseline was offered. Each subject was explained that the duration of the study is approximately 5 years and gave written consent to take part in the study.

The following inclusion criteria were considered for patient screening:

- Minimum of 12 teeth present

- Diagnosis of Severe Periodontitis stage III or IV (Tonetti et al., 2018)

· At least one maxillary/ mandibular molar with: i) degree III horizontal furcation involvement (at least between 2 roots for maxillary molars) (Eickholz & Walter, 2018) class B and C vertical furcation involvement (bone loss up to the middle third of root cones) (Tarnow & Fletcher, 1984), iii) residual probing pocket depths > 5 mm in furcation area, iv) maximum mobility degree I (Laster et al., 1975) and v) not already accessible for self-performed oral hygiene.

· Received a course of non-surgical periodontal therapy within the past six months.

The exclusion Criteria were based on both patient- and molar-related factors:

Patient-related criteria:

· Full mouth plaque score > 30%

· A course of antibiotics within the past 3 months

· Pregnant/lactating women

· Relevant medical history as evaluated by the examining clinician which may have the potential to affect periodontal surgical treatment

· Individuals on long-standing (2 or more years) supportive periodontal care (SPC) management plans

Molar-related factors:

· Ongoing endodontic pathology affecting the furcation involved molar, as judged by the examining clinician

· Previous periodontal surgical treatment to the furcation affected molar within the previous 5 years

· Endodontically treated molar tooth without a full coverage restoration

· 'Unrestorable' molar tooth (lacking adequate tooth structure to provide a restoration) as deemed by the examining clinician.

· The presence of occlusal dysfunction as assessed by the examining clinician

5.2.4 Randomisation Procedures

Randomisation for test or control treatment was carried out between baseline (visit 1) and visit 2. The randomisation service 'Sealed envelope' was used in each centre for randomisation and to ensure allocation concealment. A sealed envelope was enclosed with the patient's notes by personnel not directly involved in the study. The therapist was informed about treatment allocation by opening the envelope at the beginning of the treatment appointment.

For each centre patients were allocated to one of the two treatment groups using a randomisation list that was previously prepared by the study statistician. For important influencing factors ('centre') stratified randomisation was carried out as recommended (Kernan et al., 1999), especially in case of small sample sizes (Kernan et al., 1999). No stratification by age was carried out, because for small sample sizes, stratification was limited to one or very few factors (Kernan et al., 1999) and because the influence of age is limited by the study design and was adjusted for in statistical analyses. Thus, random permuted block sizes of 4 was employed within centres. Randomisation lists for each centre were generated by a statistician, who will not perform the statistical analyses. The examiners, who assess the clinical variables, were blinded with regards to the patients' treatment

assignments. The surgeon could not be blinded since they musted handle the different devices, but they did have no access to the data collection sheets or the group allocation. The assistant to the surgeon could not be blinded since she/he had to handle the different devices, but she/he had no access to the data collection sheets or the group allocation. The patients were not blinded to the treatment provided. All items of the CONSORT 2010 guidelines were followed in order to further minimise bias. Since the main study is still ongoing, it was not possible to break the randomisation process and compromise the blinding for the interim analysis conducted in the current study. Therefore, the same research member who previously prepared the randomisation list (PS) received the data from each centre and grouped each participant to treatment group A and treatment group B according to different treatment approach received and preserving the blinding protocol still in place. This interim analysis was requested by the coordinating centre (London) and approved by the KCL Research Ethics Committee with reference number LRM-23/24-25910.

5.2.5 Clinical periodontal examination

Dichotomous (no/yes) full mouth plaque scores (FMPS) (Guerrero et al., 2005) were recorded, identifying tooth surfaces revealing the visual presence of plaque following the use of plaque-disclosing tablets. Periodontal measurements were taken by the calibrated examiners at six sites per tooth using a manual University of North Carolina (UNC-15) periodontal probe. The following periodontal measurements were taken full mouth at 6 sites per tooth: probing pocket depth (PPD), recession of the gingival margin from the cemento-enamel junction (CEJ), dichotomous (no/yes) bleeding on probing (BoP) (Ainamo & Bay, 1975). Recession was recorded as a negative number if the gingival margin is above CEJ (or the assigned reference e.g. restorative margin); and as a positive number (incl. 0) if margin is on (0) or below CEJ (>0). Further, tooth mobility (no/yes and degree 1, 2 or 3 (Laster et al., 1975), horizontal furcation involvement using a Nabers probe (no/yes and degree 1, 2 or 3) (Eickholz and Walter, 2018) and finally vertical furcation involvement (no/yes and class A, B or C) measured with a UNC-15 probe were recorded. Clinical attachment levels (CAL) will be calculated as PPD + recession. The amount of keratinized gingiva by the test furcation was

recorded as the distance in mm from the gingival margin and the muco-gingival junction (MGJ).

5.2.6 Periodontal treatment

Participants included in this interim analysis needed to attend at least 5 Visits from the baseline to 4-month after the periodontal treatment (see flowchart in Appendix 1). All patients received a comprehensive including steps one and two of the periodontal therapy protocol (Sanz et al., 2020) that involves oral hygiene instructions, risk factor control and supragingival instrumentation.

5.2.6.1 Non-Surgical Periodontal treatment (NSPT)

Half of the study participants (controls) were randomised to receive continued non-surgical periodontal treatment (NSPT). All treatments were carried out by the same expert therapist in each centre, including oral hygiene. The protocol for NSPT was as follows: participants received local anaesthesia, then thorough debridement of the root surface was completed to the depth of the periodontal pocket and of the furcation lesion, by using sonic scaler (KaVo Sonicflex, KaVo,

Germany), piezo-electric/ultrasonic devices with specific thin and delicate tips (such as Cavitron, Dentsply Sirona, US) and/or curettes.

5.2.6.2 Surgical Periodontal treatment/ Open Flap Debridement (OFD)

The other half of the study participants (test) were randomised to receive surgical periodontal treatment in the form of open flap debridement (OFD). The aim of the surgery was to achieve thorough debridement of the furcation area and (if possible) improve accessibility for patient-performed hygiene in the furcation area. The protocol for this procedure was based on the study by Sallum et al. 2005 and is as follows: patients received local anaesthesia and intrasulcular incisions were made on the buccal and lingual/palatal aspects in order to reflect full-thickness flaps. After reflection of the flaps, the granulation tissue around the tooth was removed with the non-cutting edge of a suitable scaling instrument such as a curette, taking care not to damage the root surfaces. Thorough debridement of the furcation area was carried out by using diamond coated inserts, sonic scaler (KaVo Sonicflex, KaVo, Germany), piezo-electric/ultrasonic devices with specific thin and delicate tips (such as Cavitron, Dentsply Sirona, US) and/or curettes. Osteoplasty (removal of non-supporting bone), if needed,

was carried out, as judged by the operator. The surgical site was closed with resorbable or non-resorbable sutures. Participants were given detailed post-operative instructions regarding care for the site after surgery, including rinsing with Chlorhexidine, and they returned one week later for removal of the sutures and a review of the surgical site. Treatment time was completed for each visit.

In some cases, the study therapist deemed it necessary to perform a tunnelling procedure in the affected furcation lesion. This applies to cases when it was judged that furcation anatomy (furcation entrance, root divergence, length of root trunk), position in the mouth and patient manual dexterity were conducive to potential good access for self-performed future cleaning of the furcation area. The protocol for tunnelling (Nibali et al. 2019b) is detailed below: local anaesthesia was administered as necessary. In cases of large amounts of keratinized gingiva, a scalloped incision was performed by the furcation entrance, followed by the removal of the secondary flap after intra-sulcular incisions, to expose the furcation area. In cases with a limited amount of keratinized gingiva, intra-sulcular incisions was carried out, followed by a split-thickness flap associated with lateral buccal relieving incisions, to then apically reposition the flap by periosteal suturing. Following flap elevation and removal of

granulation tissue, a probe and/or inter-dental brush was used to test accessibility to the furcation area. In the presence of bony ridges, if necessary, these were removed using an appropriate round bur, such as a Waerhaug diamond bur or Piezosurgery inserts or bone files (Sugarman/Schluger). The aim was to obtain an ideal distance from the fornix to the bone crest of around 5–6 mm. After completion of scaling and root planing, the flaps were repositioned by sutures, ensuring bone coverage. Sutures anchoring the flap to the periosteum were performed in case of an apically repositioned flap. When possible, a suture was placed through the furcation.Some of the included maxillary molars could show additional FI on the same tooth (for example, grade III FI buccal to mesial and grade I, II or III distal). In this occurrence, the other furcation was treated according to judgment by the treating clinician. We anticipated that, based on inclusion criteria, the majority of cases may have multiple grade III FI. In case of multiple grade III FII, the deepest bone loss within the furcation radiographically assessed was treated.

5.2.7 Examiner Calibration

Following initial training exercises involving all centres and the preparation of specific standard operative procedures (SOPs) for clinical examination, a repeatability exercise was performed by five examiners one for each centre. This involved repeated examinations on 10 subjects measuring PPD, gingival recession and furcation involvement with at least 15 minutes separation between each subject. Upon completion of all measurements, the calibration for continuous variables (PPD and REC) was analysed with the Bland–Altman graph and by calculation of Kappa agreement coefficient. The resulting coefficient of agreement reported in table 5.1 was significant for each centre considering a coefficient of repeatability less than ±2 mm in 90% of the cases was considered acceptable. For FI, an Intraclass correlation analysis (ICC) was performed using the Cohen's Kappa for qualitative variables, obtaining an ICC > 0.93 in each centre. Inter-examiner calibration was not possible due to practical reasons.

Recruiting centres	Kappa coefficient of agreement for PPD and REC.	Intra class correlation coefficient (ICC) for FI.
London (UK)	91.3%	0.95
Santiago (Spain)	97.3%	0.93
Malmo (Sweden)	93.1%	0.94
Frankfurt(Germany)	92.4%	0.94

Table 5.1 Intra-examiner calibration per each recruiting centre

5.2.8 Sub-gingival plaque sampling, processing and analysis

Subgingival plaque samples were collected at the baseline and 4 months after the treatment. The collection, analysis and processing were carried-out according to the protocol described in Chapter 2. The subgingival plaque was collected in each participant from:

· Test site (TS): preferably buccal surface of furcation site; alternatively, mesial furcation site in maxillary furcation if buccal furcation is not degree III

· Healthy site (CS): ideally, symmetrical contralateral site in case of no furcation involvement. Alternatively, another site with PPD<4mm and no bleeding on probing will be chosen and recorded.

5.2.9 Follow-up Procedures

All sites in the mouth of participants received the required periodontal treatment by a trained therapist (periodontist/dentist/hygienist) throughout the duration of the study, as judged by the examining clinician. It was likely to involve supportive periodontal therapy, including supra- and sub-gingival debridement, polishing, oral hygiene re-enforcements and motivation but it may also involve more advanced periodontal treatment including surgical options. If any participant-related acute medical or dental problems arise, these were managed in the appropriate manner in line with routine clinical practice. Further details on the study protocol are in Appendix II.

5.2.10 Coordination between centres

Owing to the multicentre nature of the study, every effort was made to facilitate protocol compliance and coordination between centres. Centres were located in

different countries in Europe and were part of a community of practice named Furcation European Research Group (FERG), which had a key role in the delivery of this project. This group was composed by Investigators who have intensely worked in the topic of periodontal furcation research before and had an extensive track record in this field (see Appendix 3). The importance of this group is crucial for the successful completion of this study, as the multicentre nature of the study will improve generalisability and the possibility to recruit a large number of patients in a relatively short period of time.

The FERG is coordinated by the Periodontology Unit at King's College London, Guy's Hospital. The activities planned to coordinate this project were:

- Production of a 6-monthly newsletter, to be circulated to all participating centres, with an update about the current trial and any other activities related to research on periodontal furcation involvement (example in Appendix III).

- Write-up and review of the current protocol.

- Organisation of training and calibration of examiners, following common SOPs.

- Coordinated meetings every 6 month to monitor the progress of the current trial, improve adherence to the protocol and ensure data quality assurance. The aim of these meetings was also to develop a cohesive team trusting one another's skills and engaging researchers in a coordinated process that contributes to achieving study goals.

- Develop ideas for lectures or symposia about furcation involvement when appropriate.

- Coordinated data analysis and interpretation of current trial.

5.2.11 Sample size calculation

This investigation presents results from an interim analysis, while the samples size was originally calculated on the primary outcome that was the tooth survival after 5 years. Assuming 70% at 5 years for access flap/tunnelling + SPC vs. 40% for SPC only (Nibali et al. 2016). A two-sided log rank test with an overall sample size of 135 subjects (67 in the control group and 68 in the treatment group) achieves 80% power at a 5% significance level to detect a difference of 30%, with the proportion surviving in the control group being 40% and the proportion

surviving in the treatment group being 70%. Each centre needed to contribute with 5 to 20 cases.

5.2.12 Statistical analysis

Data from all patients were entered into a spreadsheet and proofed for entry errors. The database was imported into statistical software package IBM SPSS® 27.0. The statistical significance level was set to 5% throughout with per-protocol analysis reported. Continuous, normally distributed variables are reported as means and standard deviations. Non-parametric test was used to detect significant changes between baseline and 4 months for all clinical outcome using the patient as unit of analysis. Descriptive data was presented by pre-specified prognostic baseline factors (age, FMPS, CAL).

5.3 Results

5.3.1 Patient flow

From February 2022 to July 2023 an overall of 22 participants were recruited in the study (13 in the London centre, 1 in the Malmo centre, 3 in the Frankfurt centre, 5 in the Santiago centre). One participant recruited in the London centre

was excluded after the baseline and before the treatment visit due to a serious medical illness that required hospitalisation (brain abscess). A total of 21 patients completed successfully the 4-month review by December 2023. The delivery process of the samples started in January 2024, samples were delivered from January to February 2024 to the London centre, Department of Host Microbiome Interaction, Floor 17, Guy's Hospital London following ensuring that a temperature of -20°C was maintained during the delivery time. The only centre not able to meet the deadline and sending samples was the Malmo centre due to university restrictions in sending biological samples abroad. In the end, 20 participants were included in the present analysis with an overall of 80 plaque samples (20 test sites and 20 healthy sites at the baseline, and 20 test sites and healthy sites at 4-month after the treatment). Ten participants received to Treatment A, and the remaining ten underwent Treatment B on the test teeth, in accordance with protocol. Twelve participants were from the London centre, 5 from the Santiago centre, 3 from the Frankfurt centre.

5.3.2 Baseline characteristics

The baseline demographic and clinical characteristics of every patient involved in the study are listed in Table 5.2.

	Patients (n = 20)
Age	63.47 ± 8.2
Gender	
Male	10
Female	10
Ethnicity	
Caucasian	14
Asian	2
Afro-Caribbean	3
Mixed/ Other	1
Not reported	-
Periodontal diagnosis- Stag	
II	0
III	18
IV	2
Periodontal diagnosis- Grade	
A	0
B	0
C	20
Periodontal diagnosis- Extent	
Localised	4
Generalised	16
FMPS	22.2 ± 4.17 %
FMBS	10.5 ± 7%

Table 5.2 Baseline demographic and clinical characteristics at patient level

The average age of participants was 63. Fifty per cent of the participants was female. Fourteen participants were Caucasian with the remaining 6 being distributed between participants of Asian, mixed or Afro-Caribbean ethnicity. All participants were diagnosed with either stage III C or IV C periodontitis, and in terms of extent, there was a majority of generalised periodontitis. At the baseline, plaque and bleeding level were 22.2% and 10.5% respectively. Among the test site group, 12 were first and maxillary molar, and 8 second and mandibular molars. For the test sites, the mesio-palatal furcation site was the most represented (n=10), followed by lingual (n=5), buccal (n=4) and disto-palatal (n=1). For the healthy site group, 13 were either first or second molars, 3 were third molars, and 4 second premolars. Twelve were maxillary teeth, the mesial aspect was the most represented, followed by buccal, lingual and distal sites (Table 5.3).

	Test sites (n = 20)
Tooth type	
First Molar	12
Second Molar	8
Arch	
Maxilla	12
Mandible	8
Site	
Buccal	4
Lingual	5
Mesio-Palatal	10
Disto-Palatal	1
Mobility	
0	13
I	7
II-III	0
	Healthy sites (n = 20)
Tooth type	
First Molar	7
Second Molar	6
Third Molar	3
Second Premolar	4
Arch	
Maxilla	12
Mandible	8
Site	
Buccal	4
Lingual	1
Mesio-Palatal	14
Disto-Palatal	1
Mobility	
0	20
I-II-III	0

Table 5.3 Baseline demographic and clinical characteristics at site-level

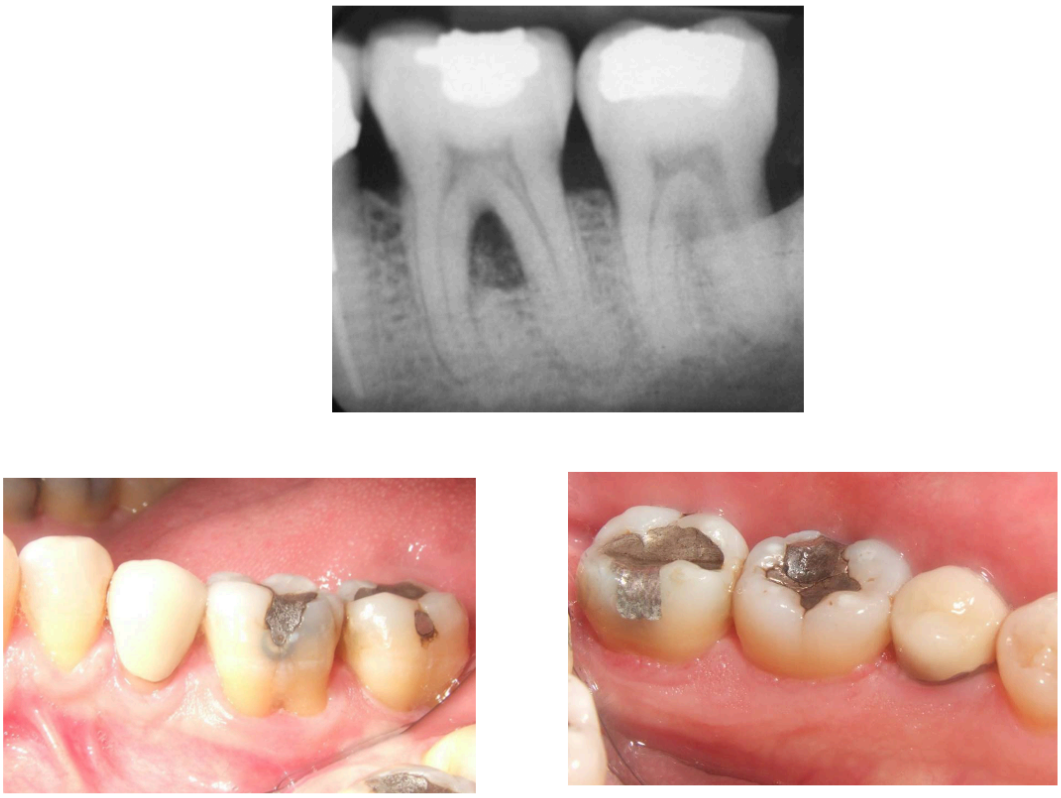


Figure 5.1 Example of a molar included in the study

Radiographic and clinical pictures of a mandibular furcation-involved molar recruited in the study

5.3.3 Clinical results: test *versus* healthy group

In the test group, a significant decrease of PPD and CAL ($p < 0.001$) and significant increase of REC ($p < 0.05$) was observed between baseline and 4-month after

treatment, while no significant differences were observed between baseline and 4-month post treatment for the healthy group. (Table 5.4)

Clinical Measurements	Baseline	4-months	P value
Study Sites (1 per participant)			
PPD (mm)	6.35 ± 0.87	3.7 ± 1.56	<0.001*
CAL (mm)	9.17 ± 1.81	7.45 ± 1.82	<0.001*
Recession (mm)	2.82 ± 2.41	3.75 ± 1.68	<0.05*
Healthy sites (1 per participant)			
PPD (mm)	2.55 ± 1.12	2.6 ± 1.23	0.543
CAL (mm)	4.45 ± 1.57	4.8 ± 1.77	0.532
Recession (mm)	1.9 ± 1.24	2.2 ± 1.78	0.432

Table 5.4 Clinical Parameters of Test and Healthy groups

The table shows the longitudinal measurements for test and healthy groups of PPD, CAL and REC in mm observed between baseline and 4 months after the treatment.

5.3.4 Clinical results: Treatment A group *versus* Treatment B group.

No statistically significant difference was detected at baseline between Treatment A (n=10) and Treatment B (n=10) for PPD, CAL and Recession. Comparing the group that received Treatment A between baseline and 4-month visit, it is evident that PPD, CAL (decrese) and REC (increase) showed a significant change from baseline ($p<0.01$, $p<0.001$, $p<0.05$ respectively). Similarly, in the group where Treatment B was delivered, a significant decrease was observed for all three clinical parameters (PPD, $p < 0.001$; CAL and REC, $p < 0.01$) (Table 5.5).

Clinical Measurements	Baseline	4-months	P value
Treatment A (n=10)			
PPD (mm)	6.6 ± 1.14	4.5 ± 1.66	<0.01*
CAL (mm)	9.5 ± 1.94	7.9 ± 1.74	<0.001*
Recession (mm)	2.9 ± 1.78	3.4 ± 1.76	<0.05*
Treatment B (n=10)			
PPD (mm)	6.3 ± 1.36	3.4 ± 1.32	<0.001*
CAL (mm)	8.9 ± 1.66	7.5 ± 1.77	<0.01*

Recession (mm)	2.6 ± 1.88	4.1 ± 1.12	<0.01*

Table 5.5 Clinical Parameters of Treatment A and Treatment B

The table shows the longitudinal measurements of PPD, CAL and REC in mm observed grouped according to treatment received (A or B), between baseline and 4 months after the treatment.

5.3.5 Microbiome analysis

The Shannon index at baseline was 2.84 (0.12) for the Treatment A group, 2.73 (0.21) for Treatment B group and 1.68 (0.15) for HS. The Shannon index at the 4-month visit was 2.58 (0.23) for the Treatment A group, 2.47 (0.45) for Treatment B group and 1.75 (0.28) for HS. No significant difference between each treatment groups was detected both at baseline and 4-month after treatment (Figure 5.2 and Figure 5.3 respectively). However, both diseased sites (Treatment A and Treatment B) showed a significant higher Shannon diversity at baseline (p=0.001) and at the 4-month visit (p=0.01) compared to HS. Bray-Curtis PCoA plots were used to evaluate the distribution of the microbial composition at the baseline (Figure 5.4) and 4-month visit (Figure 5.5) and per each group between baseline and 6-month visit (Figure 5.6). When analysing each group individually at the baseline, more microbial variability was detected in HS (adonis p value = .001, R2

= 0.29) compared with the diseased groups (Treatment A: p value = .001, R2 = 0.18, Treatment B: p value = .001, R2 = 0.21). When analysing each group individually 4 months after treatment, more microbial variability was detected in HS (adonis p value = .001, R2 = 0.36). Treatment A being the least variable (adonis p value = .001, R2 = 0.07) while Treatment B group being in the middle (adonis p value = .001, R2 = 0.23).

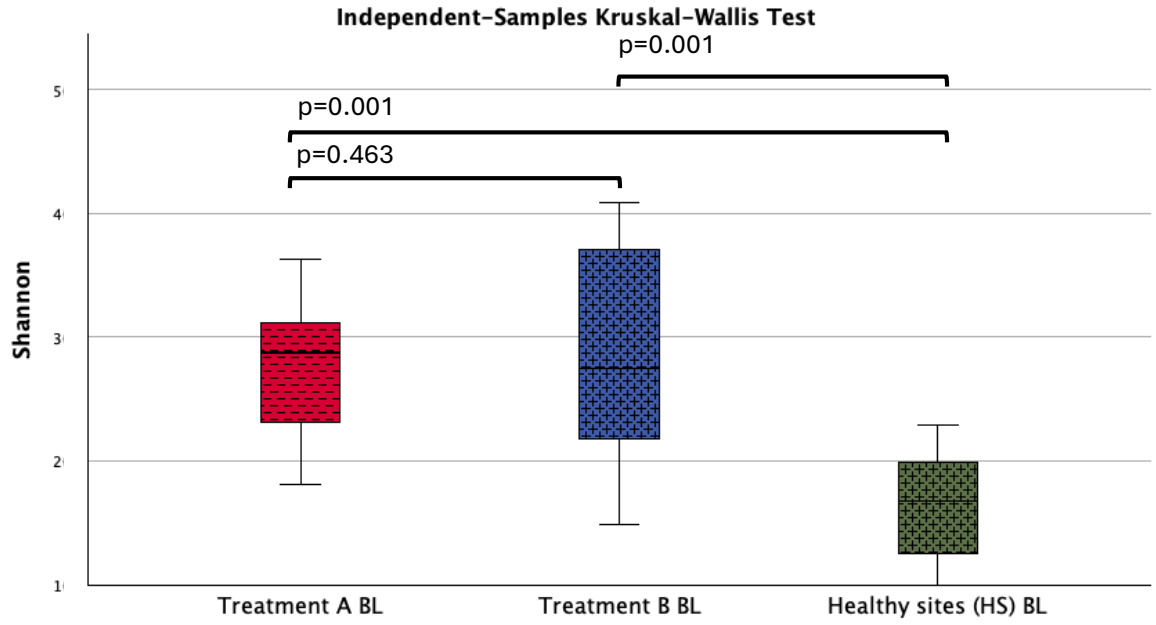


Figure 5.2 Shannon Diversity of diseased and healthy sites at baseline

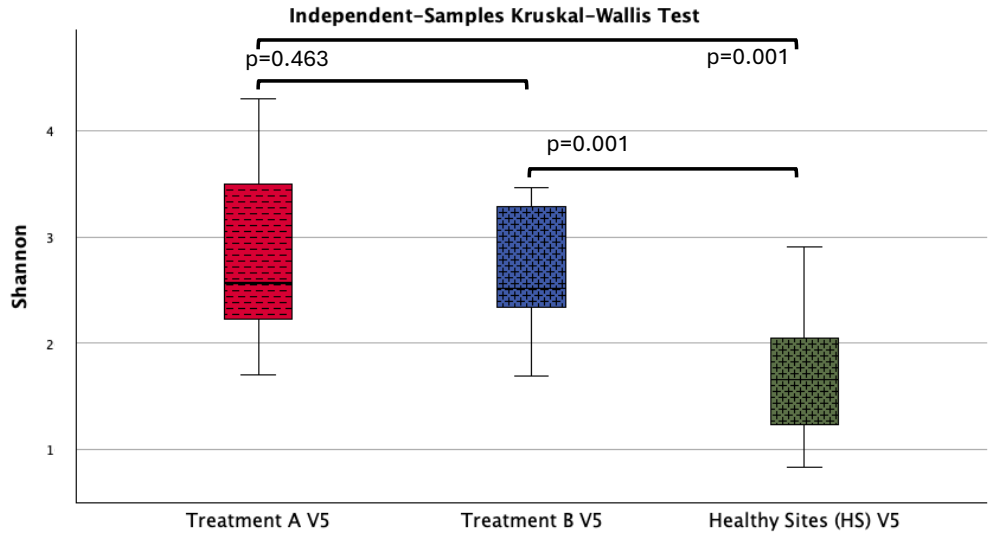


Figure 5.3 Shannon Diversity of diseased and healthy sites at 4-month visit

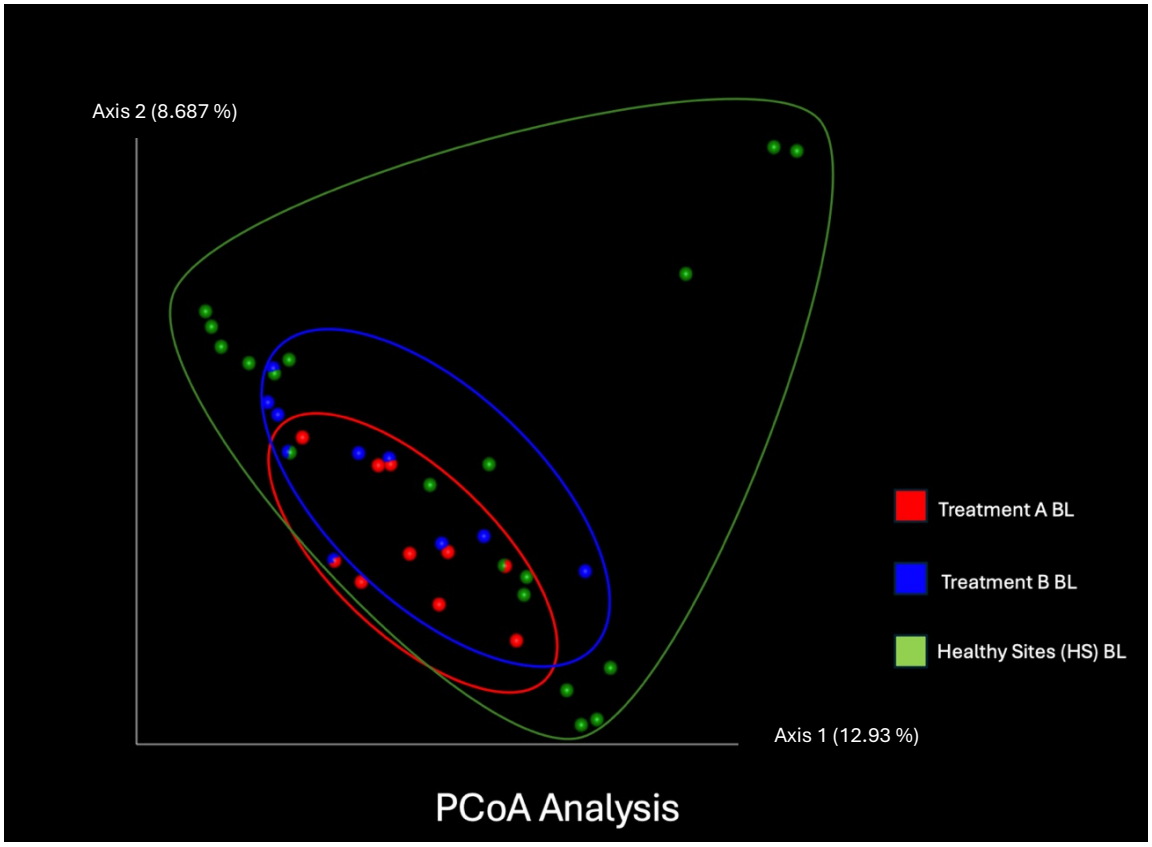


Figure 5.4 PCoA for the three groups at baseline

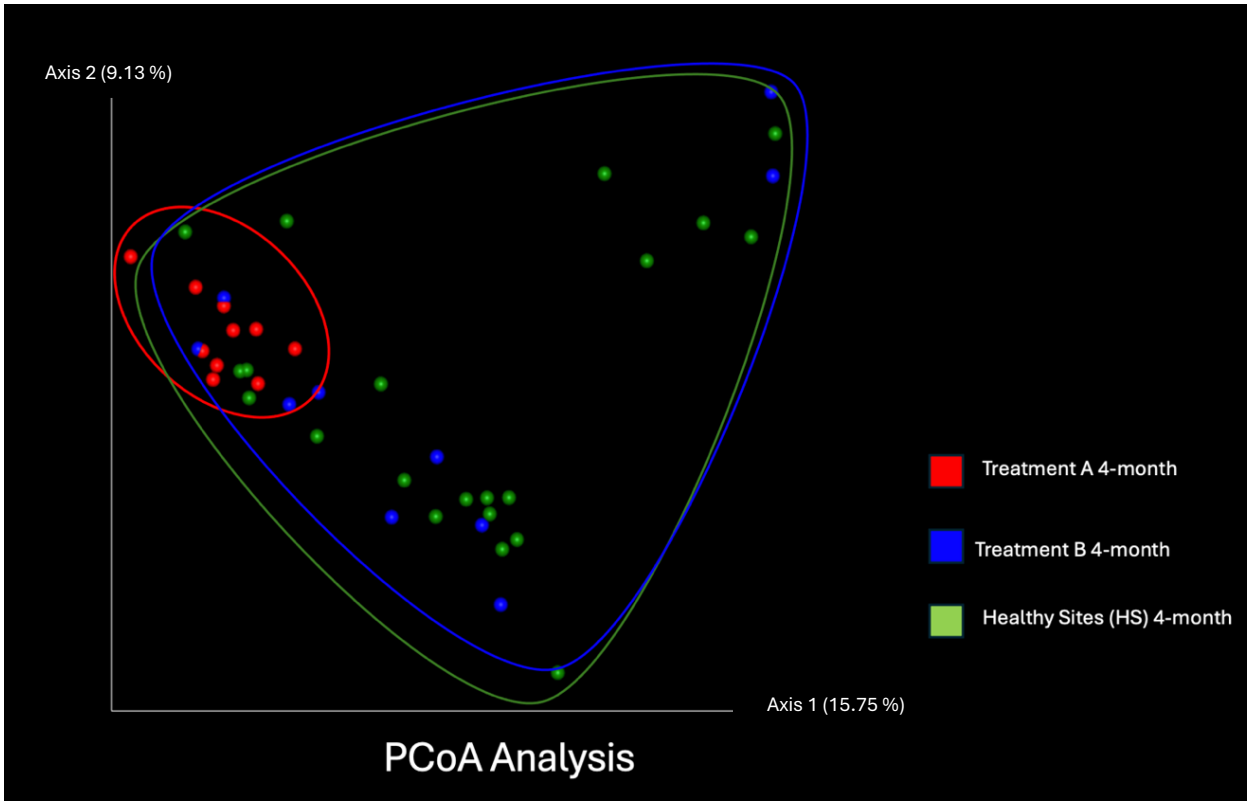


Figure 5.5 PCoA for the three groups at 4-month visit

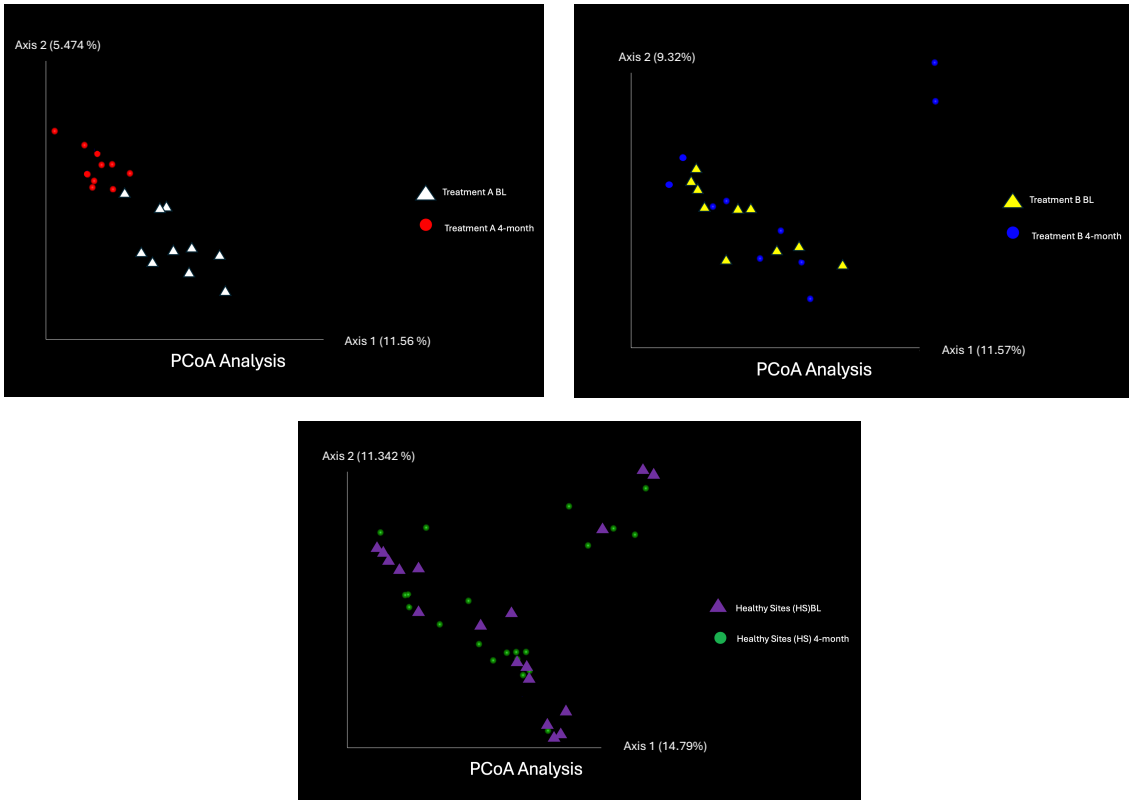


Figure 5.6 PCoA per group at baseline and 6-month visit

5.3.6 Distribution of genera between groups

164 bacterial genera were found in all the samples at baseline and follow-up visit (4 months after the treatment). Two species in total displayed statistically significant variations between Treatment A and Treatment B at 4 months after the treatment. Treatment B group showed higher concentrations of *Cardiobacterium valvarum* compared to Treatment A ($p<0.001$) (Figure 5.6), while *Fusobacterium periodonticum* showed statistically higher levels in Treatment A group compared to Treatment B ($p=0.004$) (Figure.5.7). Genera streptococcus

level was not statistically significant between Treatment A and B at V5, but a greater level of streptococcus was detected in HS compared to Treatment A (p=0.03), while no significant difference of *Streptococcus* level was detected between Treatment B and HS (p=0.66) (Figure 5.8).

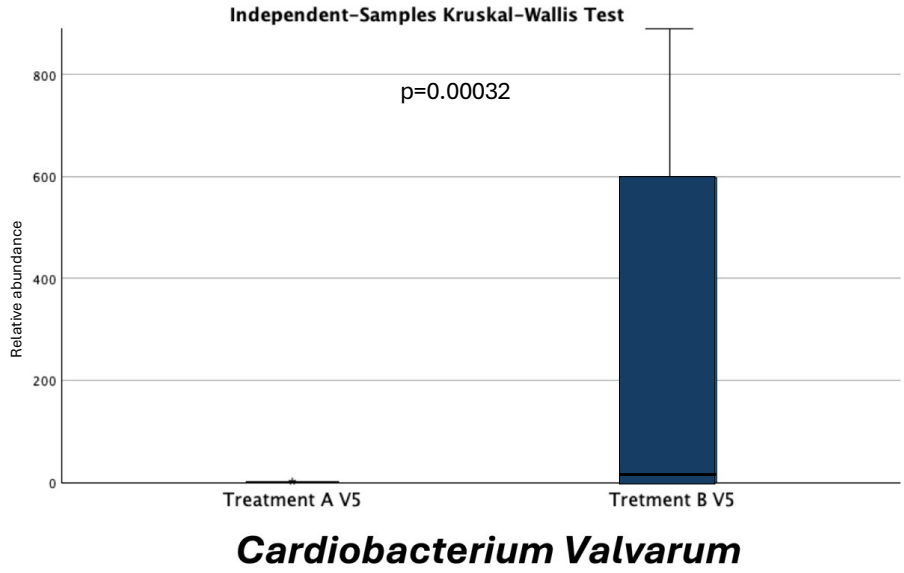


Figure 5.7 Relative abundance of *Cardiobacterium* genera between groups

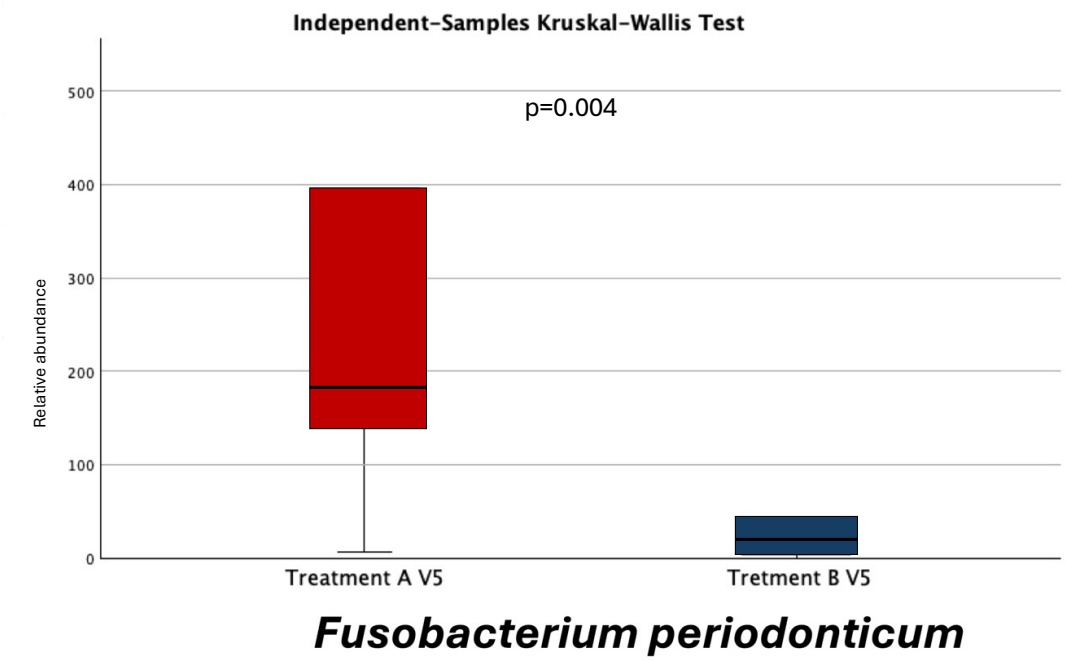


Figure 5.8 Relative abundance of genera *Fusobacterium* between groups

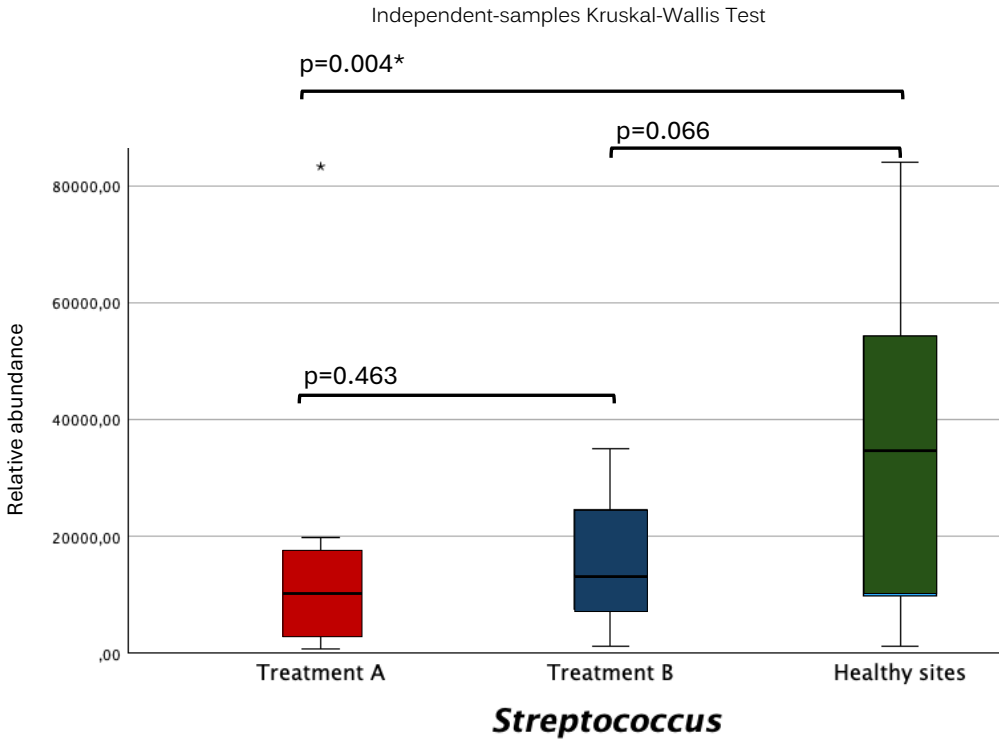


Figure 5.9 Relative abundance of genera *Streptococcus* between groups

5.3.7 Aerobic metabolism distribution between groups

As in Chapter 3 and 4, each genus identified was categorized based on their oxygen consumption, grouping in aerobic, anaerobic, and facultative bacteria (Figure 5.9 and 5.10). The relative percentage (%) of each strain within each treatment group (Treatment A, and Treatment B) at 4-month visit was then determined, reporting no significant differences in aerobic (Treatment A: 12.7% ,Treatment B: 14.3%), anaerobic (Treatment A: 54.1%, Treatment B: 51.4%) and facultative level (Treatment A: 33.2% , Treatment B:34.3%) between the two groups at the baseline. No significant difference was detected for anaerobic level between Treatment A (32.2%) and Treatment B (38.6%) (p=0.54). Lower level of aerobic bacteria in Treatment A (28.6%) group compared to Treatment B (34.2%) at borderline statical significance (p=0.05) was detected; and facultative genera were found to be statistically increased in the Treatment A group (39.1%) compared to Treatment B (27.2%) at 4 months (p=0.02).

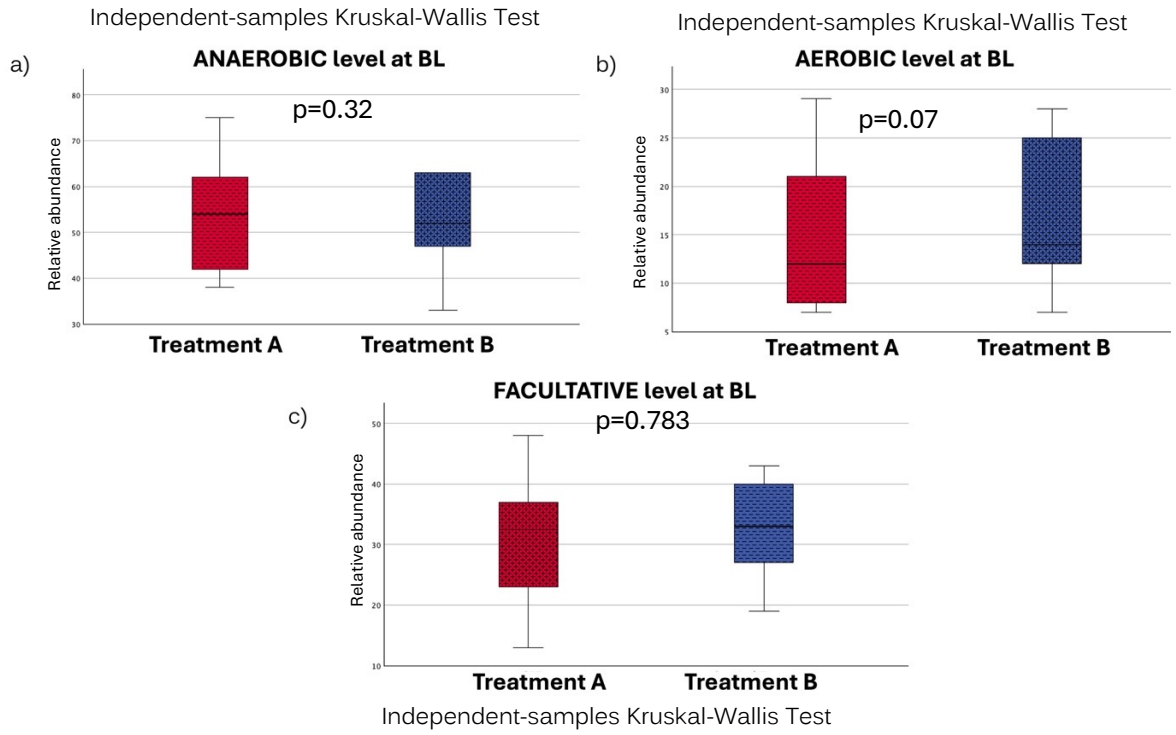


Figure 5.10 Anaerobic, aerobic, facultative levels at BL

a) anaerobic bacteria levels b) aerobic bacteria levels between c) facultative bacteria levels in Treatment A and Treatment B groups at 4 months after treatment.

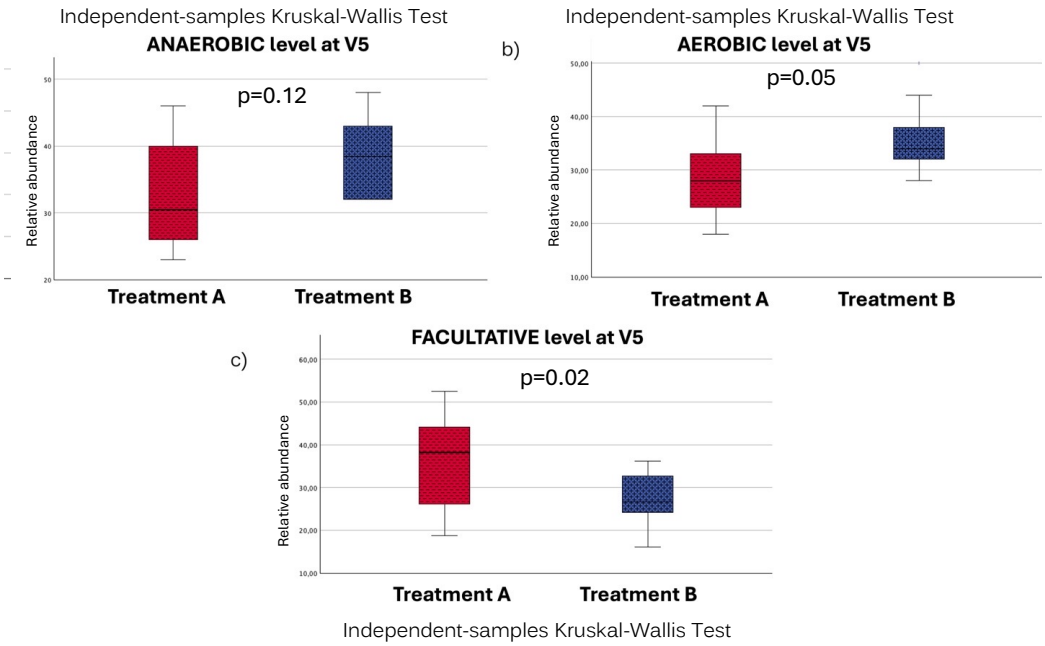


Figure 5.11 Anaerobic, aerobic, facultative levels at 4-month visit

a) anaerobic bacteria levels b) aerobic bacteria levels between c) facultative bacteria levels in Treatment A and Treatment B groups at 4 months after treatment.

5.4 Discussion

This chapter presented a multicentre project where two different treatment modalities were delivered on degree III FI sites with a 5-year follow-up. The current results derived from an interim microbiological analysis that aimed to preserve the randomisation code and the blindness across centres. Therefore, treatment assignment was kept anonymised and described as Treatment A or B. The clinical results showed that in the Treatment A group a mean PPD of 4.5 mm persists at 4 months, while in the Treatment B an average healthy gingival crevice (PPD=3.4mm) was obtained. These clinical results reflected in the

microbiological profile detected in the furcation area. In particular, a lower level of aerobic was detected in the Treatment A group compared with Treatment B group. This difference shows a borderline of statistical significance ($p=0.05$), presumably not statistically significant due to the relatively small sample size (Hackshaw & Kirkwood, 2011). No significant difference between the two treatment groups was found for anaerobic level of bacteria, in line with a previous report (Loos et al., 1988), that showed similar anaerobic counts in furcation sites before and after being treated. A new finding emerged from these current analyses in relation to the facultative level of microorganisms, in particular statistically higher levels of facultative bacteria were detected in the Treatment A group. Facultative bacteria show unique metabolic versatility, which allows to thrive in varying oxygen conditions. In relation to their ability to proliferate in both aerobic and anaerobic environments, these bacteria are particularly adaptable to the fluctuating conditions within periodontal pockets (Abdulkareem et al., 2023). Moreover, these bacteria play a critical bridging role in plaque formation, facilitating the coexistence of aerobic and anaerobic bacteria (Abdulkareem et al., 2023). Their significant presence in Treatment A group may represent a transitional zone within the biofilm, which on one side may supports the survival

of aerobic bacteria associated with eubiotic subgingival flora, but on the other side they may favour a microbiological shift towards an anaerobic dysbiotic environment. This result was also in contrast with the one reported in Chapter 4, where no difference was detected between surgical and non-surgical therapy for the facultative level. However, in agreement with previous literature (Mombelli, 2018), even periodontally treated sites are subject to recolonisation with a maturation of subgingival biofilm that can change over time, therefore the different timepoint of subgingival plaque collection (6 months in Chapter 4, and 4 months in Chapter 5) may play a crucial role in the different level of facultative detected between these two projects. Moreover, patient's level of oral hygiene, and the patterns of periodontal microorganism distribution throughout the oral cavity all influence the degree and rate of recolonisation (Mombelli, 2018). Interestingly, one of the bacteria that was significantly expressed in Treatment A compared to Treatment B group was *Fusobacterium periodonticum,* firstly reported in 1983 (Slots et al., 1983) and associated with periodontitis. While *Cardiobacterium valvarum* was the species with significant higher abundance in Treatment B group, this species was first reported in 2004 (Han et al., 2004) and associated with endocarditis as part of the HACEK group (Han & Falsen, 2005), it

is unclear whether it plays a role in periodontitis (Han & Falsen, 2005), however it is a normal resident of the oral flora (Washio et al., 2019).

Few limitations may be raised regarding the present project: first, with only 20 participants distributed across 4 centres, the sample size is relatively small. This significantly limits the statistical power of the study, making it difficult to detect significant differences or generalize findings to a larger population. The involvement of multiple centres can introduce variability due to differences in sample collection techniques, handling procedures, and storage conditions, even with standardized protocols. Such variability can affect the interpretation of findings particularly pertinent in microbiological studies, where the country of origin also had a significant impact on the microbiota and is therefore an important factor to consider when describing subgingival bacterial communities (Arredondo et al., 2023).

In summary, interim analysis of this multicentre trial confirmed the hypothesis that different periodontal treatments affect aerobic, facultative composition in the subgingival microbiome of furcation area. However, the anatomical modifications in terms of residual periodontal pocket following these periodontal

treatments can play a crucial role in generating a unique environment that can drive the subsequent recolonisation of periodontal pockets.

6 Chapter: Final discussion and future directions

6.1 Evidence summary

Exploring the subgingival microbiome in relation to furcation defects in untreated periodontitis and after surgical and non-surgical therapy was the main aim of the present work. A summary of the key results can be found in Table 6.1 (Study 1, 2 and 3). In study 1, periodontal untreated molars with degree II and III furcation involvement (FI) were compared to non-furcation (NF) sites. This was the first study to profile periodontal furcation defects from a microbiological and inflammatory standpoint using conventional and AI-based analyses. The AI-based model helped to identify 4 different clusters (from 0 'more dysbiotic' to 1 less 'dysbiotic'). A combination of clusters 0>3>1>2, 0 (more represented taxa) to 2 (less represented taxa) was more frequently detected in FI sites, while the combination of 0>2>1>3 occurred mainly in NF. In terms of GCF markers, the elevation in several molecular biomarkers such as IL-6, MMP-3, MMP-8, BMP-2, SOST, EGF, TIMP-1 in furcation sites may reflect a unique molecular signature of the inflammatory process in the furcation area compared with the other periodontal defects. The most interesting results of this project was the significant lower detection of aerobic level in furcation sites compared to NF defects . It can be speculated that the significantly lower aerobic levels combined

with increased levels of host mediators may represent specific signatures of periodontal etiopathogenesis in the 'untreated' furcation area, implying that the anatomical complexity of the furcation may interact with a specific microbial profile to drive a unique molecular response. A dysregulation of aerobic, anaerobic, facultative levels was also observed in both longitudinal studies (Study 2, and study 3). In particular, higher aerobic levels were found after FI defects were treated with surgical treatment (Study 2) or with Treatment B (Study 3) compared to the corresponding control groups (NSPT, Treatment A). A greater reduction in PPD was noted at follow-up in both the surgical therapy group (Study 2) and Treatment group B compared to the corresponding control groups (NSPT, Treatment A).Therefore, it can be argued that the depth of the periodontal pockets may have an influence on the regulation of aerobic, anaerobic and facultative levels in the subgingival plaque of the furcation area, with the aerobic value progressively increasing and the depth of the periodontal pockets decreasing. This correlation between clinical and microbiological factors was not observed for the Shannon diversity in both Study 2, and Study 3. Regardless of treatment provided and remaining PPD, both treated groups demonstrated similar Shannon diversity. This may be related to changes in the abundance of

specific pathogenic species rather than changes in overall diversity. It may also be a signal that the microbial community is converting to a non-pathologic composition pattern in the control groups (NSPT, Treatment A) as well, however this result is not yet clinically evident due to the short follow-up period (6 months or less 4 months). Interestingly, few specific bacteria were elevated in the surgically treated group (study 2) compared to NSPT as *Streptococcus gordonii, Streptococcus oralis, Streptococcus mutans, Granulicatella,* and *Rothia.* Also in study 3, *Cardiobacterium valvarum* was elevated in Treatment B group compared to Treatment A group. These results confirmed the hypothesis of a relationship between anatomical changes in the furcation area such as pocket reduction and microbiological profile. Test groups with greater PPD reduction showed a microbiological profile with higher numbers of aerobic, which are normally associated with healthy plaque biofilm. Interestingly, the relative abundance and percentage of anaerobic bacteria was similar between the surgical therapy group and the NSPT group, the same trend was noted between Treatment A and Treatment B.

Due to the greater PPD reduction and stronger aerobic profile at furcation sites treated with surgical therapy, it can be argued that this treatment may provide

the best clinical and microbiological outcome compared to NSPT. However, this author suggests to also take into account the lack of a difference in the anaerobic bacteria detected between NSPT and surgical therapy at 6-month visit, which could indirectly indicate that the surgical treatment (presumably Treatment B) resulted in a faster clinical outcome, reflected in a different microbiological composition, in aerobic cocci predominate. On the other hand, NSPT (presumably Treatment A) offered a slower healing process with less PPD reduction, lower aerobic level and higher facultative levels. Therefore, the conclusion about which treatment is best in case of grade III furcation involvement is not clear with this microbiological analysis performed at short-term follow-up.

		UNTREATED PERIODONTITIS		
		FI sites (FI)	Non-Furcation defects (NF)	Δ FI *vs* NF p value
Study 1	Diversity	3.09 (0.33)	3.25 (0.43)	NS
	Genera	Moryella* Olsenella* Atropobium** Actinomyces*	Cardiobacterium* Neisseria*Fusobacterium*	p<0.01
	Aerobic level (%)	10.1%*	13.7	p<0.01
	PPD (mm)	6.54 (1.11)	6.70 (1.19)	
	GCF volume (µl),	0.75 (0.32)	0.61 (0.26)	NS
	GCF markers	IL6* MMP3* MMP8* BMP2* SOST* EGF* TIMP1*	-	p<0.01
	AI-clustering (%)	0>3>1>2	0>2>1>3	-
		S3-LEVEL CLINICAL PERIODONTAL THERAPY		
6-month after therapy		FI sites treated non-surgically (NSPT)	FI surgical therapy	Δ NSPT *vs* Surgery p value
Study 2	Diversity	3.76 (0.43)	3.15 (0.12)	NS
	Streptococci	-	*S.oralis*S.gordonii*S.mutans**	p<0.01
	Other Genera	-	*Kingella* G.adiacens**	p<0.01
	Aerobic level (%)	19.24	26.7	p<0.05
	PPD mm	5.4 (1.3)	3.55 (1.4)	p<0.01
4-month after therapy		FI sites Treatment A	FI sites Treatment B	Δ A *vs* B p value
Study 3	Diversity	2.58 (0.23)	2.47 (0.45)	NS
	Other Genera	Fusobacterium*	Cardiobacterium*	p<0.001
	Aerobic level (%)	28.6	34.5	p=0.05
	PPD mm	4.5 (1.66)	3.4 (1.32)	p<0.01

Table 6.1 Summary of microbiological, molecular and clinical findings

6.2 Clinical implications

A dysregulation in levels of aerobic bacteria and presence of specific bacteria as *S. mutans* was observed after surgical therapy (Study 2) and Treatment B (Study 1). In this section, clinical implications of this microbiological finding were presented.

Previous literature is in line with our microbiological findings, suggesting that furcation defects showed a different microbiome profile compared to non-surgical defects (Queiroz et al.,2017). Furthermore, anaerobic counts were still higher at FI sites than at NF sites even when NSPT was performed (Loos et al. 1988). Therefore, it was generally accepted that molars with grade III FI were treated with a tunneling technique when appropriate to expose the root vault in the oral cavity and remove the remaining periodontal pocket. Several studies highlighted that most common adverse event on tunnelled molars with FI is the development of root caries, often leading to tooth loss (Hellden et al., 1989; Little et al., 1995; Rüdiger et al. 2019).

A greater gingival recession and the consequent root exposure are common traits after surgical periodontal therapy, especially if the aim is to surgically create a passageway through the furcation area to facilitate self-performed oral hygiene

and professional maintenance. Once the root cementum is exposed to the oral environment, it can experience a faster process of demineralization compared to the enamel due to its higher critical pH (Hoppenbrouwers et al., 1987). The root cementum is also defined by a greater content of organic matrix and carbonate and magnesium hydroxyapatite compared with enamel, contributing to its higher solubility (Dung & Liu, 1999) and making the root surfaces more vulnerable to dental caries once exposed to the dental biofilm.

In study 2, it was demonstrated that 6 months after surgical treatment, a higher level of *S. mutans* was detected in the sub-gingival biofilm of furcation defects. This bacterium shows a strong ability to adhere to rough surfaces (Yu et al., 2016), as demonstrated by a previous investigation that studied the adhesion forces between *S. mutans* and different surface roughness of zirconia. The author (Yu et al., 2016) concluded that among all tested surfaces: coarse, medium, and fine; the roughest surface had a positive influence on the *S. mutans* initial adhesion force and early attachment.

Indeed the anatomical configuration of the exposed root furcation area such as grooves, enamel projections, and bridges may represent the perfect niche to drive the adhesion and the accumulation of a caries-oriented biofilm (Bollenl et

al., 1997). Therefore, it can be speculated that the typical acidogenic flora associated with dental caries may start to colonise the residual periodontal pocket and exposed root surface in a surgically treated molar with advanced FI, early as 6 months after treatment.

The correlation between dental caries and periodontitis is still controversial. It was commonly accepted that dental caries and periodontitis specifically affect different types of individuals since there are characterised by different microbial dental-associated biofilms (Sanz et al., 2017). However, a recent systematic review demonstrated that periodontitis was associated with the presence of root carious lesions, indicating that patients with periodontitis with roots left exposed by disease process and/or its treatment may benefit from preventive measures as fluorides (Romandini et al., 2024). It remains unclear whether an association with dental caries may also exist for gingival recessions not associated with periodontitis, although they affect only a minor proportion of the population. Similarly, future research should profile the supragingival and subgingival biofilm at different probing pocket depth levels in the furcation area, trying to investigate to what extent the gingival recession following periodontal therapy may affect

the furcation microbiome and the correlation between caries and molars with advanced FI.

6.3 Microbiological profile and periodontal prognosis

A personalized approach to the treatment of periodontal patients includes the creation of a tailored treatment plan and the use of prognostic systems as a tool to predictably define a long-term supportive protocol according to the fourth step of periodontal treatment (Sanz et al., 2020). From a microbiological perspective, it is generally accepted that a predominance of obligate anaerobic bacteria in the subgingival microbiome of periodontal defects is usually associated with a 'poor' periodontal prognosis (Charalampakis et al., 2013). In studies 2 and 3, a reduction in anaerobic levels was noted in both the test and control groups after treatment, suggesting that both periodontal treatments (surgical and non-surgical; A and B) were able to improve the furcation microbiome of one to shift the pathogenetic anaerobic profile to a less dysbiotic profile, which lead to an improvement in the periodontal prognosis of these teeth. These results are consistent with a previous investigation (Rüdiger et al., 2019) which described how a lack of periodontal pathogens was detected in tunnelled mandibular

molars, noting that the microbiological profile consisted mainly of streptococci and lactobacilli, with a resulting higher risk of root caries. Generally, a ‘poor’ or ‘unfavourable’ prognosis is assigned to molars with advanced furcation involvement (Graetz et al., 2011; Kwok & Caton, 2007; McGuire & Nunn, 1996; Nibali et al., 2017). Indeed, clinicians are often focused on offering 'strategic' extraction of grade III FI molar as one of potential treatment options. Unexpectedly, the current clinical prognostic systems overestimate the rate of tooth loss since the majority of teeth, including molars with furcation defects, assigned with 'poor' prognosis are actually retained during supportive periodontal care (Saydzai et al., 2022). In addition, the concept of 'strategic' extraction of teeth with 'poor' prognosis has been wisely reviewed in the last two decades. In the past, it was commonly accepted that elective or strategic extractions were the best solution for dealing with compromised dentition, and an earlier elimination of the dentition was nevertheless recommended to preserve the bone volume necessary for implant placement (Kao, 2008). Nowadays, the management of a compromised dentition has found a more cautious approach, deferring strategic extractions as long as possible (Kao et al., 2022). Comparably, the S3 guidelines published by the European Federation of

Periodontology (Sanz et al., 2020), reinforce the concept that extractions for grade III FI should be deferred to a later stage. Therefore, given the favourable PPD reduction and less dysbiotic microbiological profile achieved with both treatments tested, clinicians should have more confidence in their ability to treat and maintain these teeth in long-term. In addition, in agreement with previous longitudinal studies that demonstrated a good survival rate of treated molars with advanced furcation involvement (Nibali, Krajewski, et al., 2017; Ross & Thompson, 1978), patients should be also motivated toward the possibility of preserving in function molars with grade III FI rather than being discouraged by assigning a 'poor' prognosis. The current microbiological results support the thesis that not only molars with furcation involvement should not be extracted (Sanz et al., 2020), but also a grade III furcation involvement should not be used as the sole parameter for assigning a “poor” prognosis.

6.4 Conclusion

To conclude, a total of 331 subgingival furcation plaque samples were analysed across three different projects: a cross-sectional study and two randomised controlled clinical trials. Within its limit, an interaction between aerobic levels in

subgingival microbiome and furcation area was evident, suggesting lower levels of aerobic in untreated furcation defects, and higher levels of aerobic when the furcation defect was treated with a surgical procedure. The combination of complex anatomical configuration of furcation defects and residual periodontal pocket depth may play an essential role in driving the microbiological recolonisation of the furcation area.

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8 Appendices

Chapter 4

Appendix I

Protocol procedure

	Visit (V) Number										
Protocol Procedures	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11
	Baseline (BL) measurements	Treatment I	1-week post treatment I review	1-month post BL review	3-months post BL review	6 months post BL review	Treatment II	1-week post treatment II review	7 months post BL review	9 months post BL review	12 months post BL review
	Day 0	2 ± 2 weeks from BL	1 week after visit 2	8 ± 2 weeks from BL	16 ± 2 weeks from BL	28 ± 2 weeks from BL	32 ± 2 weeks after BL	1 week after visit 7	36 weeks ± 2 after BL	44 weeks ± 2 after BL	56 weeks ± 2 after BL
Verification Inclusion/Exclusion Informed Consent	x										
Medical History (MH)	x	x	x	x	x	x	x	x	x	x	x
Dental History (DH)	x	x	x	x	x	x	x	x	x	x	x
Demographicsx Height and Weight (BMI)	x										
Periodontal measurements (PPD, CAL, FMBS, mobility, furcation involvement)	x					x					x

Plaque assessment (FMPS)	x			x	x	x			x	x	x	
PROMS (OHIP-14)	x					x					x	
Saliva collection	x				x	x				x	x	
GCF collection	x				x	x				x	x	
Subgingival plaque (bacteria) collection	x					x					x	
Clinical photographs (facial, left and right buccal, upper and lower occlusal views)	x					x					x	
Periodontal treatment according to allocation		x	x				x	x				
Oral Hygiene Instructions (OH)	x	x	x	x	x	x	x	x	x	x		
Randomisation	x					x						
Prophylaxis / supragingival scaling	x			x	x	x			x	x		
Furcation specific questionnaire	x					x					x	

Study procedures

Schedule of Treatment for each visit

Visit 1 - Baseline (day 0)

- Informed Consent, medical/dental history and demographics
- Clinical photos (facial, left and right buccal, upper and lower occlusal).
- Record concomitant medications
- Record any former smoking history
- Body composition measurement (BMI) (height, weight, waist circumference)
- Demographic and socio-economic information (self-administered questionnaire)
- PROMS (self-administered OHIP-14 and furcation-specific questionnaire)
- Saliva sampling
- GCF sample collection from test and control sites
- Microbiological sample collection (subgingival plaque) from test and control sites
- FMPS recording

- Periodontal assessment with recording of full mouth PPD, periodontal pocket depths (PPD), clinical attachment levels (CAL), bleeding on probing / bleeding score (FMBS), mobility and furcation involvement.
- Patient homecare routine will be discussed and directed at maintenance of plaque levels on a daily basis to a level <20% (including oral hygiene instructions with the use of standard brand tools for interproximal cleaning)
- Subjects will be scheduled for the first treatment visit
- Randomisation
- Prophylaxis / supragingival scaling

Visit 2 – Treatment I (4 +/- 2 weeks from Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- Patient homecare routine will be discussed and directed at maintenance of plaque levels on a daily basis to a level <20% (including oral hygiene instructions with the use of standard brand tools for interproximal cleaning)

- Study treatment I
- Post-treatment regime will be outlined according to protocol

Visit 3 - 1 Week Post treatment I Review (1 week after Visit 2)

- Update medical/dental history and record adverse events and/or concomitant medications
- Patient homecare routine will be discussed and directed at maintenance of plaque levels on a daily basis to a level <20% (including oral hygiene instructions with the use of standard brand tools for interproximal cleaning)
- Removal of sutures if surgical procedure completed

Visit 4 - 1 Month Evaluation (8 +/- 2 weeks from baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- FMPS recording

- Patient homecare routine will be discussed and directed at maintenance of plaque levels on a daily basis to a level <20% (including oral hygiene instructions with the use of standard brand tools for interproximal cleaning tools)
- Oral hygiene re-enforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival scaling

Visit 5 - 3 Months Evaluation (16 +/- 2 weeks from Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- FMPS recording
- Oral hygiene re-enforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival scaling
- Saliva sampling
- GCF sample collection from test and control sites

Visit 6 - 6 Months Post Evaluation (28 +/- 2 weeks from Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- Periodontal assessment consisting of recording of full mouth periodontal pocket depths (PPD), clinical attachment levels (CAL), bleeding on probing / bleeding score (FMBS), mobility and furcation involvement.
- Oral hygiene re-enforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival scaling
- PROMS (self-administered OHIP-14 and furcation questionnaire)
- Clinical photos
- Saliva sampling
- GCF sample collection from test and control sites
- Microbiological sample collection (subgingival plaque) from test and control sites

- FMPS recording
- Reassessment. Reallocation for further treatment depending on response to treatment I

Visit 7 – Treatment II (32 weeks +/- after Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- Study treatment II
- Patient homecare routine will be discussed and directed at maintenance of plaque levels on a daily basis to a level <20% (including oral hygiene instructions with the use standard brand of tools for interproximal cleaning)
- Post-treatment regime will be outlined according to protocol

Visit 8 – 1-week post Treatment II Evaluation (33 weeks +/- 1 after Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications

- Oral hygiene re-enforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival scaling
- Removal of sutures if surgical procedure completed

Visit 9 – 7 Months post baseline Evaluation and Maintenance (36 weeks +/- 2 after Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- FMPS recording
- Oral hygiene re-enforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival scaling

Visit 10 – 9 Months post baseline Evaluation and Maintenance (44 +/- 2 weeks after Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- Saliva sampling
- GCF sample collection from test and control sites
- FMPS recording
- Oral hygiene re-enforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival scaling

Visit 11 – 12 Months post baseline Evaluation (56 +/- 2 weeks after Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- Saliva sampling
- GCF sample collection from test and control sites
- FMPS recording

- Periodontal assessment consisting of recording of full mouth periodontal pocket depths (PPD), clinical attachment levels (CAL), bleeding on probing / bleeding score (FMBS), mobility and furcation involvement.
- Oral hygiene re-enforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival scaling
- Microbiological sample collection (subgingival plaque) from test and control sites
- PROMS (self-administered OHIP-14 and furcation questionnaire)
- Clinical photographs

Chapter 5

Appendix I

Participants flow at the 6-month review

At the 6-month review, six of the ten participants who underwent NSPT were deemed responders at the 6-month review, while the remaining four were

classified as non-responders based on the success criteria. Three people were non-responders and seven people who had undergone OFD were responders at the six-month review. Following the 6-month review, the seven participants who responded to OFD and the six who responded to NSPT could begin low-level monitoring, which involved supportive periodontal treatment administered one and three months later.

Protocol procedure

Protocol Procedures	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7 to 18	Visit 19
	Baseline (BL)	Treatment	1-week visit	1-month visit	4-month visit	8-month visit	12-month and every 4 months thereafter visit	5-year follow-up visit
	Week 0	4± 1 weeks from BL	5± 2 weeks from BL	8± 2 weeks from BL	20± 2 weeks from BL	36± 3 weeks from BL	52± 3 weeks from BL	61 months from BL
Verification Inclusion/Exclusion Informed consent	x							
Medical/Dental History & Updates/ Demographics	x	x	x	x	x	x	x	x
Height and Weight and waist measurements	x							x
Periodontal measurements	x				x	x	x	x
Plaque assessment	x	x	x	x	x	x	x	x
Randomisation		x						
PROMs	x			x		x	x*	x
Clinical photographs/videos	x	x	x	x		x	x*	x
Radiographs	x							x
Periodontal treatment (according to study arm)		x	x					
Supportive periodontal therapy				x	x	x	x	x
Sub-gingival plaque collection	x				x		x*	x

* to be done only yearly (visit 10,13,and16)

From Visit 7 to 19, participants can be fully re-assessed at least every 12 months according to clinical requirements.

Study procedures

Schedule of Treatment for each visit

Visit 1 - Baseline (day 0)

- Informed Consent, medical/dental history and demographics
- Record concomitant medications
- Body composition measurement (height, weight, waist circumference)
- Periodontal assessment with recording of full mouth PPD, gingival recession, bleeding on probing (BOP), tooth mobility and furcation involvement, followed by confirmation of overall treatment planning
- Long cone periapical radiographs with paralleling technique (if radiograph taken in previous 3 months is not available)
- Clinical photographs
- Sub-gingival plaque sampling
- Oral hygiene instructions
- PROMs (self-administered questionnaire)

Visit 2 - Treatment (intervention) visit

- Medical/dental history update and record adverse events and/or concomitant medications
- Randomisation
- Clinical photographs
- Study treatment according to randomisation
- Post-treatment regime will be outlined according to protocol

Visit 3 - 1 Week Post-Intervention and Further Treatment (5 ± 2 weeks from Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- Study treatment of remaining sites (if needed)
- Clinical photographs
- Post-treatment regime will be outlined according to protocol
- Full mouth plaque score (FMPS) recording (6 sites per tooth)

Visit 4 – 1-Month Post-Intervention and Maintenance (8± 2 weeks from Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- PROMs (self-administered questionnaire)
- Full mouth plaque score (FMPS) recording (6 sites per tooth)
- Oral hygiene reinforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival polishing
- Clinical photographs

Visit 5 - 4 Months Post Intervention and Maintenance (16± 2 weeks from Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- FMPS recording

- Sub-gingival plaque sampling
- Periodontal assessment consisting of recording of full mouth (6 sites per tooth) PPD, recession, BOP, mobility (tooth level) and furcation involvement (for each multirooted tooth)
- Oral hygiene reinforcement, full mouth supra- and sub-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival polishing

Visit 6 - 8 Months Post Intervention Re-evaluation and Maintenance (28 ± 3 weeks days from Baseline)

- Update medical/dental history and record adverse events and/or concomitant medications
- PROMs (self-administered questionnaire)
- Clinical photographs
- FMPS recording
- Periodontal assessment consisting of recording of full mouth PPD, recession, BOP, tooth mobility and furcation involvement

- Oral hygiene reinforcement, full mouth supra- and sub-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival polishing

Visit 7 to 18 - 12 Months Post Intervention Maintenance Visit (54 ± 3 weeks from Baseline) and every 12 months thereafter until 5-year follow-up

- Update medical/dental history and record adverse events and/or concomitant medications
- FMPS recording
- Periodontal assessment consisting of recording of full mouth PPD, recession, BOP, tooth mobility and furcation involvement
- Oral hygiene reinforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival polishing
- PROMs (self-administered questionnaire)

- Clinical photos*
- Sub-gingival plaque sampling

Visit 19 - 5-year follow-up

- Update medical/dental history and record adverse events and/or concomitant medications
- FMPS recording
- Periodontal assessment consisting of recording of full mouth PPD, recession, BOP, tooth mobility and furcation involvement
- Oral hygiene reinforcement, full mouth supra-gingival debridement to remove new accumulations of plaque or calcified deposits and supra-gingival polishing
- PROMs (self-administered questionnaire)
- Body composition measurement (height, weight, waist circumference)
- Clinical photographs
- Sub-gingival plaque sampling

- Long cone periapical radiograph of test sites with paralleling technique
- Study completion and further allocation for treatment (if needed) or discharge the patient for periodontal maintenance

Treatment chair time will be recorded for each visit.

Appendix III

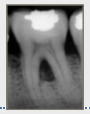
Example of Newsletter

Issue 4 2023

FERG NEWS

Furcation European Research Group Newsletter

The Fornix Corner



Important updates & information

Successful Ethical Approvals

Congratulations to the Turin University for obtaining the ethical approval. We have now 5 centres that are actively recruiting patients for this prestigious project. The remaining teams are all closing in on this lead & hopefully anticipate to be in the same position in the very near future ...

Recruitment update

London team has recruited 7 participants, Santiago de Compostela centre has managed to include 6 patients, Malmo team has managed to recruit two participants, followed by the Frankfurt Team who recruited one patient and is screening 2 more patients.

Current affair

A belated welcome to the fourth edition of FERG News. I am sure this edition will give you an interesting highlight on our ambitious project.

First and foremost, we would like to send our support to those individuals who have directly or indirectly been affected by the Turkey-Syria earthquake.

Next March, Rimini (Italy) will host the 21 International Conference of the Italian Society of Periodontology (SIdP), We hope to meet all of you there in this amazing venue to have an update on how to reduce invasiveness without compromising the clinical outcomes.

What's new

We are delighted to announce that the FERG will include 1 new center in this project: the Sapienza University of Rome. A warm welcome to this team, please find below a link for a shared drive that we have set-up which includes the most up to date versions of the documents required for recruitment (protocol, CRF, patient information sheet, consent form, questionnaires).

https://drive.google.com/drive/folders/1wmWZG1YWPl_EccmQHlnmhA7vmgrbgPnW?usp=sharing

Other important info

We are planning a collective meeting with all centres next 29th March at 3.00 pm (UK time), this will be an opportunity to discuss about challenges and future prospectives of this project. Let's hope all of you will be present, an invitation link will be sent to all centres shortly.

Furcation involvement and tooth loss - A registry-based retrospective cohort study.

Anna Trullenque-Eriksson et al. J Clin Periodontol. 2022.

This investigation is quite interesting and aimed to evaluate the impact of furcation status on the risk for molar loss.

To this end, data on 381,450 subjects; 2,374,883 molars with furcation involvement were collected from the Swedish Quality Registry for Caries and Periodontal diseases Data on dental and periodontal status were extracted for the subsequent 10-year period. Furcation involvement degree 2-3 resulted in 2-3 times higher risk of tooth loss.

Do not consider extraction as the first treatment option of teeth displaying advanced furcation involvement is the main conclusion of this study and it is supported by the high external validity of using a national registry.

Please see below some baseline X-rays of molars that are currently included in our project from different centres.