

**FLUORESCENCE AND BACTERIAL MICROBIOME OF
DENTIN IN DEEP CARIOUS LESIONS**

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ABSTRACT

Leslie H. Trippe: Fluorescence and bacterial microbiome of dentin in deep carious lesions
(Under the direction of Andrea Ferreira Zandona)

Purpose: To determine if the presence or absence of fluorescence as clinically detected by fluorescence-aided caries excavation (FACE) correlates with dentin bacterial microbiome diversity, as assessed by 16S rRNA amplicon sequencing and to compare and contrast traditional tactile dentin caries detection with fluorescence. **Materials and Methods:** Unidentified, extracted human, carious teeth considered discarded waste were obtained from a dental facility. Providers unrelated to the research study supplied the teeth - NHSR (Not Human Subjects Research). Included teeth had a carious lesion two-thirds into the dentin, verified by a radiograph (post extraction), and fluoresce red using FACE technology (SIROInspect, Sirona, Bensheim Germany). Fluorescing carious dentin was assessed using the traditional visual-tactile method, and comparisons made before and after cavity preparation. Red fluorescing (RF) sites were sampled with a sterile spoon excavator, and dentin characteristics evaluated clinically via traditional visual/tactile methods. Once RF dentin was removed, a second sample of carious dentin with pink fluorescing (PF) dentin was obtained with a sterile spoon excavator. After excavation with sterile round burr to non-fluorescing (NF) dentin, the dentin characteristics were evaluated again by visual-tactile method, and a third sample was collected with a new sterile slow speed round burr. Samples were transported on dry ice for analysis of bacterial microbiome to the UNC Microbiome Core Facility. **Results:** Out of 134 extracted teeth collected, 21 fit the inclusion criteria, yielding 61 samples which were subsequently analyzed. RF samples

had a higher number of observed OTUs (Operational Taxonomic Units), at 154, followed by PF 109 and (NF) 100. Regarding tactile assessments: RF carious dentin was primarily 'soft', but also had readings of 'leathery' or 'hard' tactically, and the NF dentin was assessed as 'hard' 100% of the time by both examiners. The rank correlation chi-squared statistic for the association of fluorescence and tactile was highly statistically significant ($p < 0.001$) for each examiner strongly suggesting an association between fluorescence and tactile assessment. However, approximately one-third of the tactile assessments of hard dentin still displayed some level of fluorescence, either PF or RF. **Conclusion:** The sampled fluorescing (RF, PF) and NF carious dentin layers displayed diverse bacterial taxa that varied in the *proportion* of bacterial species. Tactile assessments correlated with RF, PF and NF approximately two-thirds of the time, as one-third of the time hard dentin displayed fluorescence.

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LIST OF ABBREVIATIONS

CCR	Complete Caries Removal
CDA	California Dental Association
CDD	Caries Detection Dye
CFU	Colony Forming Units
CMCR	Chemomechanical Caries Removal
DEJ	Dentin Enamel Junction
DNA	Deoxyribonucleic Acid
FACE	Fluorescent Aided Caries Excavation
KHN	Knoop Hardness Number
LIF	Laser Induced Fluorescence
NF	Non Fluorescing
OTU	Operational Taxonomic Unit
PCR	Partial Caries Removal
PEARL	Practitioners Engaged in Applied Research and Learning
PF	Pink Fluorescing
RDT	Remaining Dentin Thickness
RF	Red Fluorescing
SW	Stepwise

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Dental Caries is defined as the progressive demineralization of susceptible tooth surfaces due to the metabolic activities that occur in dental plaque (i.e. the biofilm). The four basic ingredients required for caries lesion formation are: susceptible tooth surface, bacteria, fermentable carbohydrates and time. The term dental caries refers to the *process* while a dental caries lesion describes the '*result of this process*' of actual dissolution of the hard tissues (enamel, dentin, cementum), which occurs due to acidic bacterial byproducts of carbohydrate fermentation, followed by the loss of the protein matrix. This dynamic process can be acute or chronic, causing significant mineral loss with or without sensitivity or pain recognition from an individual.[1, 2] Once there is significant mineral loss, a cavitation may become apparent. Management of a cavitated caries lesion typically involves excavation of the involved tooth structure and preparation of a cavity to receive a restorative material. This dental caries excavation process is very subjective as it usually involves removing all presumably infected and affected dentin until tactile sensation indicates that firm and intact tooth structure is reached. Traditionally only hard dentin was considered clean, healthy dentin, acceptable as supporting tissue underneath a restoration. This process is referred to as complete caries removal (CCR).

The evolving understanding of the dental caries process and of the histopathology of caries lesions has led to a paradigm shift in dental caries management as it relates to caries excavation. The focus has moved away from removing all of the *affected* dentin, referred to

as ‘inner carious dentin’, and towards disease management following tooth preserving principles. [3, 4] [5, 6] New restorative materials allow for more conservative cavity preparations and can rely on enamel bonding rather than the compact packing of restorative materials for a seal. Compounding evidence indicates that when a good seal is present the caries lesion arrests, even if bacteria are still present. [7] [8] [9] [10] Recent data with reverse-capture checkerboard analysis indicate that excavating to hard dentin does not assure a ‘*sterile*’ dentin, as bacteria are present in all dentinal layers in deep caries lesions.[11] Thus, it is essential that the correlation between the biofilm microbiome content and dentin characteristics are better understood. This understanding has the potential to translate to a more accurate and clinically applicable method to differentiate between *infected*, now referred to as ‘outer carious dentin’ and inner carious dentin, a critical need in dentistry.

1.2 Sound Dentin:

Dentin is a bone-like matrix that is porous, has a yellow-hue and is made of 70% inorganic materials, 20% organic materials and 10% water by weight. Due to its elastic properties, it functions as a supporting tissue for enamel and aids in prevention of enamel fracture. It comprises most of the tooth structure and, in teeth with a viable pulp, it continues to form after tooth eruption and throughout its life via the activity of odontoblasts. [12] Dentin structure encompasses microtubules (dentinal tubules), which house the odontoblast processes and dentinal fluid. The microtubules are surrounded by a highly mineralized peri-tubular zone and intertubular dentin between the microtubules. In the coronal dentin the dentinal tubules extend from the dentinal enamel junction (DEJ) to the outer wall of the pulp, while in the root portion they extend from the cemento-enamel junction (CEJ) to the outer wall of the pulp. The organic phase of dentin includes collagen

(fibrillary Type 1) and non-collagenous proteins. The inorganic phase of dentin consists of apatite crystals, mainly hydroxyapatite and non-crystalline amorphous calcium phosphate.[13] Because the microtubules follow the tooth anatomy, their diameter is smaller in the outer dentin compared to the inner dentin, which results in more compacted dentinal tubules. Therefore, in the inner dentin, the microhardness has been reported as 72.53 (Knoop Hardness Number- KHN) in outer (superficial) dentin and 65.05 KHN in deep dentin.[14]

1.2.1 Dentin Caries Development and Process:

Dental caries is a diet regulated and biofilm modulated multifactorial disease. There is strong evidence indicating that a diet high in fermentable carbohydrates will modify the microbial biofilm, favoring acidogenic and aciduric bacteria. Organic acids, such as acetic, lactic and propionic acid are produced through a carbohydrate fermentation process, [15] causing a drop in the oral biofilm pH. Breakdown of enamel is reported to occur when the pH drops to ~5.5, while for dentin breakdown occurs when pH drops to ~6.4, and depends on the level of fluorapatite present. However, the critical pH below which enamel and dentin dissolves is not constant but rather a range which is inversely proportional to the concentrations of calcium and phosphate in the saliva and plaque fluid.[16] Fejerskov *et al*, refers to caries as the '*result*', which occurs after a shift in the ecology and metabolic activity of the biofilm. The consequence of this shift is an imbalance of enamel and dentin mineral content which can create tooth structure dissolution.[1] Carious lesions may initiate in the enamel and progress into dentin [17, 18], or may initiate in exposed dentin. The initiation and progression of dental caries is attributed to not only the type of microorganisms present, but also the quantity of acid producing bacteria. *Streptococcus mutans* is a considered a major player in caries initiation because this type of bacteria is

highly aciduric, feeds off a high sucrose diet and produces an insoluble glucan, which promotes further bacterial adhesion. Simon-Soro, *et al* reported that particular bacteria are abundantly present during the initial stages of the enamel carious process; however, at the dentin level more proteolytic bacteria are involved. *Lactobacillus*, *Prevotella* and *Streptococcus* are reported to increase in number in the deeper dentin carious lesions, whereas *Neisseria*, *Capnocytophaga* and *Fusobacterium* significantly decrease in numbers.[19].

1.2.2 Histopathology of Carious Dentin:

A carious lesion in dentin was described by Fusayama to have three layers: outer, inner and sound dentin. The outer layer, or the most superficial part of the carious lesion is characterized as being non-remineralizable in contrast to the inner carious dentin layer which can be remineralized. These layers may further be described and categorized into 'zones'. The outer carious dentin layer is also called the zone of destruction and contains dentin which has been decomposed due to the action of acid (which results in mineral loss) and proteolytic enzymes (which result in loss of the dentin collagen matrix). This zone has also been described as the 'necrotic' or 'contaminated' zone, and relates to soft non-remineralizable dentin that is heavily infected with bacteria. (Figure 1) Collagen in this zone has been irreversibly denatured and thus, can no longer function as a frame work for remineralization. The inner carious dentin comprises the zone of bacterial penetration and the translucent zone. The zone of bacterial penetration can be identified by the destruction of odontoblastic processes, further leaving the tubules empty with the ability of bacteria to invade. In a slowly developing lesion tubular sclerosis may be observed. Tubular sclerosis can be defined as one of the initial defense mechanisms of the pulp-dentin complex, by which there is deposition of minerals from the intertubular dentin into the dentin tubules, causing gradual occlusion. This gradual occlusion changes the optical properties of the

dentin and when visualized under a microscope it appears translucent. This translucent zone is a zone of demineralization which has also been described as the 'transparent' zone, and is argued that this area may tactically feel either firm or leathery and can ultimately remineralize.[20, 21] In some slowly developing lesions the tubular occlusion and further mineralization of the intratubular dentin is able to form a highly mineralized layer that appears dark clinically and transparent under the microscope. Sound hard dentin and tertiary dentin may also be present adjacent to the pulp chamber.

Shovelton in 1968 was already reporting that demineralization precedes bacterial invasion. He stated that in 'hard' dentin post caries excavation, only 64% was technically free of bacteria. Ultimately he concluded that softening dentin generally precedes the invading organism, however even in the deepest parts some infected dentinal tubules may remain.[22] Angker, *et al.* correlated the mechanical properties and mineral content of carious dentin. Their study concluded that in a progressing dentin carious lesion, when there is a reduction in mineral content, evidence of deterioration of mechanical properties exists. Hardness and elastic modulus were discovered to be an exponential function of its mineral content.[23]

Recommendations have been made to leave the inner carious dentin intact, and only remove the outer carious dentin layer of greatly softened and discolored dentin, as this layer is considered non-remineralizable where the collagen is irreversibly denatured. [24, 25] This has been referred to as *partial caries excavation* or more recently, *selective caries excavation*. The goal of these procedure is to remove only the irreversibly demineralized dentin (outer dentin caries) which is in contrast to the traditional notion of *complete caries removal*.

1.3 Complete Caries Removal

Traditionally, when managing a carious lesion at any depth, the dental provider was to remove all of the inner and outer carious dentin to not only prevent further tooth destruction by the cariogenic activity, but also to provide a sound base of dentin for a restoration.[26] Dentists have been taught to remove all of the softened dentin in order to eliminate the inner carious dentin tissue, assuming that both the biofilm and the respective microorganisms within this layer of dentin is what drives the carious process. Many are still taught to not only remove all soft outer and inner carious dentin, but also to remove stain at the dentin-enamel junction (DEJ).[17] Current approaches recognize that, as the caries removal process approaches the pulp, the potential for an unnecessary pulpal exposure becomes a concern. The dental provider must then decide if they wish to attempt an indirect/direct pulp capping procedure, or perform a pulpotomy or pulpectomy. Every dental procedure has inherent risks, which may negatively impact the pulpo-dentinal complex, and potentially lead to further treatment such as a root canal, or even extraction. In light of this clinical reality, Schwendicke has described the caries removal process as ‘*initiating a cascade of re-interventions*’, which ultimately compromises long term pulp vitality.[27]

It has long been suggested that pulp exposures were a result of ‘too vigorous removal of affected dentin’, or inner carious dentin. If a tooth is symptomless, and responds well to vital pulp testing, stained dentin (inner carious dentin) can be left over the pulp as long as it is ‘reasonably hard’.[6] Furthermore, Kidd concludes that biologically, it may be potentially harmful to even attempt to remove all of the inner carious dentin, and emphasizes the placement of a well-sealed restoration.[6] As Tomes stated back in 1859 “*It is better that a layer of discolored dentine should be allowed to remain for the protection of the pulp rather than run the risk of sacrificing the tooth*”. [28] The end point in the caries

removal process (i.e. when is the carious dentin clinically hard enough?) is very subjective and varies widely according to where the dentist received training, and that individual's interpretation of when softened, inner carious dentin tissue may remain directly adjacent to the pulp.

Regardless of what type of caries excavation method is utilized, CCR or a type of selective caries removal process, the dentist clinician still must determine at what point to cease the removal of dentin. Currently, the most common criterion used to guide the caries excavation is by means of visual/tactile perception. The visual determination of the presence of carious tooth structure is often led by variations in the color of the dentin, and the tactile sensation is accomplished through the use of a dental probe or explorer where the assessments are based on hardness or texture. These methods are inherently subjective; vary between dentists in clinical practice as well as dental researchers and dental educators. These variations inevitably produce different sizes of cavities, and potentially carry significant clinical implications. [29] The pulpal health may be jeopardized if too much dentin is removed, and the overall strength of the tooth is compromised structurally, leaving minimal remaining dentinal thickness (RDT).

Ricketts *et al.*, through a Cochran Oral Health Group review, analyzed current operative management techniques, and concluded that both stepwise (SW) and partial caries removal (PCR) procedures reduced the incidence of pulp exposure in symptomless, vital carious primary and permanent teeth. The authors further stated that complete caries removal techniques have clear disadvantages, including damage to the pulp and weakening of tooth structure.[30] However, in clinical practice as evidenced by a recent PEARL (Practitioners Engaged in Applied Research and Learning) network study, the preferred management of deep caries lesions is complete caries removal. In a survey of clinicians in

the PEARL network using case studies, 62% percent of those responding (85/92) indicated that, in a case where pulp exposure may be expected, they would remove all outer and inner carious dentin (CCR) and only 18% would consider selective caries removal.[31]

Financial consequences of dental caries is estimated to be the fourth-most expensive disease to treat by the World Health Organization (WHO). In the United States, as reported by the American Association of Endodontics, there are over 41 thousand root canals performed each day at a cost of US\$34 million annually. It is likely that many pulp exposures and subsequent endodontic treatments may be avoided if a more conservative approach of caries removal is chosen.[27, 32]

1.4 Incomplete Caries Removal

Kidd and Ferjerskov ask the question, “what drives this caries process?”, and wondering if it is exclusive to the biofilm entering from the cavity surface or possibly from the bacteria which are present *within* the lesion? They suggest that the progression of the lesion can be arrested by the sealing of any remaining bacteria, which may exist in the cavity, by restoring the tooth properly, thus allowing the ongoing removal of the cariogenic biofilm by routine daily hygiene. [20] The sealing of any remaining bacteria, which may exist in the inner carious dentin, is referred to as Incomplete Caries Removal, and may be approached in two different ways: 1) Stepwise Excavation (SW), or 2) Selective Caries Removal (SCR). SW is performed by removing the outer carious dentin layer or the zone of destruction/contamination of the caries lesion, leaving soft residual caries, placing a medicated liner/base and then a *provisional* therapeutic restoration, which has to be removed and replaced with a permanent restoration in 6 weeks to 3 months. SCR, previously called incomplete or partial caries removal, uses a similar protocol with removal

of necrotic, disorganized dentin to tactile 'hardness' along the peripheral walls of the cavity preparation, leaving soft dentin over the pulp, placement of an indirect pulp cap and a *permanent*, well-sealed restoration. In both protocols only the **outer carious dentin** is removed, while the **inner carious dentin** (zones of bacterial penetration and translucent zone) are preserved prior to placement of a permanent restoration. The outer carious dentin is characterized by necrotic, disorganized and irreversibly demineralized dentin tissue, while the inner carious dentin is characterized by vital, organized, and remineralizable dentin. Ample evidence shows that sealing inner carious dentin will lead to lesion arrest, [7] [8] [9] and preservation of pulp vitality, thus decreasing the need for endodontic treatment [32-34].

This conservative approach preserves tooth structure, which limits the need for more complex, costly procedures. High caries risk patients particularly reap great benefits from the resulting health-gains and cost-savings. [8] [27] Selective caries excavation relies on accurate differentiation between outer and inner carious dentin, which is not straightforward clinically. Clinicians rely on tactile sensation to determine if characteristics of the dentin indicate inner carious dentin (hard, firm or leathery) or outer carious dentin (very soft, disorganized). This uncertain diagnosis, compounded with the concern that leaving bacteria behind would allow its proliferation and cause lesion progression, has led to low acceptance of selective caries excavation. [31] [35] [36] However, studies have shown that the total colony forming unit (CFU) counts of common caries pathogens were gradually reduced during the dentin partial excavation. [7] [37] [38] Maltz *et al* conducted a multicenter randomized clinical trial (RCT) to determine the primary outcome of pulp vitality utilizing SW and SCR with a 3 year follow up. At 3 years, the success rates were 91% and 69% for SCR and SW respectively. The types of failures were similar, as both groups reported pulpitis and pulpal necrosis as the primary failure mode. The author

attributed part of the success rate of SCR over SW was due to the low completion rate of SW treatments.[8] The fact that SW is a two-stage procedure is alone a risk for failure, as the dentist must rely on patient compliance to return for the second stage procedure. Additionally the risk of pulp exposure may be increased by a re-entry. In regards to cost effectiveness, Schwendicke found that SCR, especially in high risks groups was associated with lower costs than other caries removal strategies. He concluded that the SCR technique retained teeth and their vitality longer with lower out of pocket costs to the patient.[27] In a systematic review, Kidd concluded that procedures which utilize selective caries removal in symptomless primary and permanent teeth, reduce the risk of pulpal exposure. Furthermore, Kidd also suggested that SCR would be the preferable treatment over the traditional CCR to reduce the risk of carious exposure, and that any microorganisms which remained were insignificant.[28]

1.5 Caries Detection and Removal Techniques

Banerjee *et al.* observed that at present there are no clear, objective and reproducible histological markers available to differentiate the outer carious dentin that should be removed, from the remineralizable inner carious dentin which can remain.[39] Iain Pretty describes our current visual and tactile assessment evaluation techniques as ‘the most ubiquitous caries detection system’, using features such as color and texture, which are qualitative in nature. [40]

Presently, in clinical practice, when intervention is necessary to restore a carious tooth, the lesion is excavated removing soft outer carious dentin until the remaining dentin is hard to touch and stain free. The question to the dental practitioner is ‘how much dentin must be removed from the cavity to ensure the success of a restoration without unnecessary removal of hard tooth structure?’[5] A systematic review and network meta-analysis was carried out by Schwendicke to assess the clinical evidence supporting described excavation

criteria during caries removal. Their systematic search sought to identify research studies which evaluated the excavation of cavitated primary or permanent teeth, and described the methods utilized to assess caries removal endpoints in different groups. Only 28 of 375 studies met the inclusion criteria. Color and moisture were not included in the review. Categories included: tactile hard, tactile soft, chemo-mechanical caries removal (CMCR), Er-YAG laser and non-stainable. 'Tactile hard' was described as: dentin remaining on the pulpal floor was not sticky to probing and did not exert any tug-back. 'Tactile soft' was described as: remaining dentin was not hard, but leathery or sticky to probing and did exert tug-back. With regards to risk of complications, they determined that the removal of all softened biomass until only hard or non-stainable dentin remained was clinically ineffective. Furthermore, in the studies that were reviewed, it was found that performing CCR had no advantage over removing only soft outer carious dentin. From all the assessed strategies, leaving softened (sticky/leathery) dentin proximal to the pulp seemed the most beneficial, reducing post-operative risks and treatment time. They concluded by stating that the number of remaining bacteria was insignificant relative to long term clinical success of a restoration, post-operative complications and patient comfort.[41]

1.5.1 – Caries Detection Dye

There have been several efforts to develop strategies which would assist the dental provider in the differentiation of the layers found in dentinal caries lesions, and to aid in the removal of only the superficial, non-remineralizable or "heavily infected" layer of carious dentin. One of the first attempts was the caries detection dye (CDD). Caries detection dyes (CDD) were first described by Stao and Fusayama in 1976. They sought to discover a caries removal process which would guide the dental clinician by using 0.5% basic fuchsin-propylene glycol to stain outer carious dentin only, leaving the deeper layer of

unstainable dentin which could be remineralized. Their ultimate goal was to assist the practitioner in removing only the infected, superficial layer which could not be remineralized, and thus producing a smaller cavity preparation. The results of their study indicated that bacteria was only present in the fuchsin-stained areas, and not present in the excavated side of the extracted teeth they evaluated. They concluded that CDD staining was considered to be a reliable clinical guide for the complete removal of deteriorated dentin, and bacteria without causing failure to remove the ‘*infection*’ completely.[42] Although this dye was developed with the intention of linking the fuchsin-stainable first layer with the presence of bacteria, it is not specific for bacteria. Anderson and Charbeneau in 1985 performed a bacteriologic study of a caries detection dye which revealed that a 0.5% solution of basic fuchsin in propylene glycol showed no statistically significant effect on the viability of the bacteria most commonly associated with carious dentin. Their study contradicts Fusayama where he described the fuchsin dye as preceding bacterial invasion, as they were still able to identify colony forming units(CFU) in the non-stained dentin.[43] In 1994 Yip *et al* discovered that the dyes appear to neither stain the bacteria, nor to delineate the bacterial front, but actually in fact stain demineralized organic matrix. His study concluded that the circumpulpal dentin and the dentin comprising the DEJ may be more susceptible to the dye staining due to the higher proportion of organic matrix present at these sites, and that the dyes actually lack the specificity for accurate detection of carious dentin.[44] An *in-vitro* study which was conducted by Banerjee and Kidd sought to compare and contrast different methods of caries excavation. The results of their study confirmed that the dye will actually stain clinically sound as well as carious dentin, and thus lead to over-preparation of the cavities when compared to those prepared using traditional tactile assessments.[45]

1.5.2 Fluorescence Based Technology

Fluorescence is a phenomenon by which an object is excited by a particular wavelength of light and the fluorescent (reflected) light is of a larger wavelength. Fluorescence occurs as a result of the interaction of the wavelength illuminating the object and the molecules in the object. Natural tooth structure has long been described as having natural fluorescence. However, demineralization will result in the loss of 'auto-fluorescence' which may be quantified using caries detection methods based on the differences in fluorescence between sound and carious tooth structure. Optical caries detection methods are based on the observation of the interaction of energy that is applied to the tooth, or that which is emitted from the tooth. The demineralized tooth structure will be filled with mainly bacteria and water, leading to distinct optical changes that may be measured and quantified with advanced detection methods.[40, 46] König and Schneckenburger in 1994 reported that several microorganisms were producing orange-red fluorophores or bacterial porphyrins, as by-products of their metabolism. Therefore, determining the orange-red fluorescence in dental hard tissues may be a good way to mark the 'zone of bacterial invasion'. [47] DIAGNOdent (Kavo, Biberach, Germany) utilizes Laser Induced Fluorescence (LIF) technology by means of a semiconductor laser (655nm wavelength) for caries detection and reportedly is able to detect bacterial porphyrins. However, since residual dentin, post caries excavation is often stained, DIAGNOdent is not useful to determine the end point of caries removal.[48]

Lennon *et al* obtained cultures of varying species of microorganisms, which are commonly associated with dental caries, and subjected them to fluorescence imaging to determine the ability of the microorganism to fluoresce red. Their results indicated that *Lactobacillus*, *Actinomyces* and *Prevotella* all fluoresced red, whereas *F. nucleatum* and the *Streptococcus* organisms (*sobrinus*, *mutans*, *salivarius*, *oralis*) all fluoresced green (Figure

2). They concluded that under optimal excitation and emission conditions, the use of fluorescing technology and the presence of red fluorescence would be a good indicator of the presence and activity of bacteria in dentin caries.[49] (Figure 3) In another study, Lennon *et al* compared the specificity and sensitivity of a new Visible Fluorescence (VF) method to detect residual caries, post excavation. This *in-vitro* study utilized three different dentin caries evaluation techniques (tactile, DIAGNOdent, and CDD) to evaluate the residual caries after use of the VF, a fluorescent aided caries removal device. Visible Fluorescence (a type of Fluorescence Aided Caries Excavation - FACE) was carried out with a xenon charged lamp, and a blue band-pass filter to generate excitation of the samples. This research used extracted, carious teeth and evaluated them histologically post-caries excavation utilizing confocal microscopy and imaging analysis. Visual Fluorescence had the highest sensitivity, specificity and percent correct score over the methods evaluated, and was found to be significantly more sensitive and specific than CDD or tactile assessment. [50]

1.5.3 – Caries Removal by Chemical Means

Two different types of chemomechanical caries removal (CMCR) systems which will be reviewed are: Carisolv™ (Medi Team Dental AB, Sweden), and Papacarie® (Brazil). Carisolv™, which is a 5% gel comprised of NaOCL, is marketed and sold by Troll Dental, and claims to assist the dental provider in removing “necrotic, decayed, dentin leaving healthy tooth structure unaffected”. Using conventional caries removal as a control, Azrak performed an *in-vivo* study using CMCR, Carisolv™ on primary teeth with brown, softened carious dentin. Forty-two teeth were treated, 21 with traditional methods of excavation and 21 using Carisolv™. Two samples were acquired per tooth, one pre-excitation and one post-excitation, then transferred to agar plates for determination of the total viable bacterial counts and for lactobacilli specifically. Their results indicated that both methods

of caries excavation showed a statistically significant reduction in microorganisms, however at the level of ‘sound’ dentin there were still viable organisms present. Pretreatment colony forming units (CFU) were in the range of 10^6 , and post caries removal, the CFU dropped to 10^3 or 10^2 in 90-95% of the samples.[51] Hamama *et al* conducted a meta-analysis of mean caries excavation time, which reviewed different caries excavation methods focusing on CMCR. Their analysis of caries excavation time revealed that use of the Carisolv™, Papacarie®, or hand excavation methods (8.12, 6.36 and 6.98 minutes respectively) required more than double the time that of the rotary instrumentation (2.99 minutes).[52] Papacarie® means ‘eating caries’, and is a product that is produced in Brazil for use as a CMCR. Papain is a proteolytic enzyme, which has bacteriostatic and anti-inflammatory characteristics and, according to manufacturer claims, is an agent which will not damage healthy tissue. Motta *et al* performed a very similar split-mouth design study, n-40 samples of carious dentin before and after caries excavation using low-speed bur as the control, and Papacarie® in the study group. Although their results were not statistically significant between the control and test group, there was a notable reduction of *Streptococcus*, *Lactobacilli* and overall total bacterial counts when comparing all groups (rotary and Papacarie®) in the pre-excavation to the post-excavation samples.

1.6 Microbiome of Caries

The ‘specific plaque hypothesis’ was originally formulated due to the observation, by Loesche, that specific microorganisms were cultured in association with dental caries.[53] *Streptococcus mutans*, *Lactobacillus* and *Actinomyces* species were the microorganisms that dominated the caries process, and subsequently chemotherapeutic agents and strategies began to emerge which would eliminate or suppress these particular bacteria.[54] The main causative factor for the initiation of dental caries is directly related to the

bacteria's ability to produce acid, which causes demineralization of the outer layer of the tooth. Takahashi and Nyvad reported that, although *Streptococcus mutans* is identified, the main oral microbiota which are significantly evident in the initiation of caries are *S. sanguinis*, *S. oralis* and *S. mitis*. This group represents the 'non-mutans streptococci', and are genetically different from *S. mutans*. They further state that caries lesion development is dominated by the non-mutans streptococci and Actinomyces and, with further progression of the lesion, the biofilm changes to become dominated by *Lactobacillus*, *Prevotella*, and *Bifidobacterium*. [55] In multiple studies, it was discovered that there is an overall 'lack of involvement' of *Streptococcus mutans* in many carious lesions, and that caries has been found to occur in the absence of *S. mutans*. These studies support a 'mixed/non-specific microbial hypothesis' revealing a diverse population of bacteria to include *Bifidobacterium*, *Propionibacterium*, *Veillonella* and *Selenomonas*. [56-58] In 1985 Hoshino evaluated carious dentin lesions from both shallow and deep layers and was able to isolate the predominant microorganisms in each group. He discovered the majority of the bacteria from both layers were obligate anaerobes, with the predominant genera: *Propionibacterium*, *Eubacterium*, *Arachnia*, *Lactobacillus*, *Bifidobacterium* and *Actinomyces*. [59] Previously, the microbiome which was identified in carious dentin was acquired through means of bacterial culturing processes. However, current technologies allow for the identification of bacteria in mixed populations. Sequencing of 16s rRNA amplicon have allowed us to understand that the oral biofilm is truly a 'complex ecosystem', composed of over 1200 microorganisms including *Actinomyces*, *Lactobacillus*, *Veillonella* and *Bifidobacterium*. [19, 20, 55] Kianoush *et al* isolated DNA from 110 dentin samples according to layers, with layer 1 being the most superficial to layer 5 which was the deepest part of the lesion to investigate the 'core' microbial population which is associated with dentin caries. The dominant microorganisms which were discovered in dentin caries were

Firmicutes, Actinobacteria and Bacteroidetes, which accounted for 95% of the sequences.[57] In a recent California Dental Association (CDA) article, Tanner *et al* compiled a visual reference, which displays the microbiome associated with initial enamel caries and dentinal caries. The predominant species in the initial enamel lesion were: *Actinomyces*, *Veillonella* and non-mutans streptococci. And the microorganisms associated with progression into the dentin were: *Lactobacillus*, *Bifidobacterium* and *Veillonella*. [60]

CHAPTER 2: MANUSCRIPT

The Use of FACE Technology for Detecting Infected Dentin In Deep Dentinal Lesions, an *In-Vitro* Study

2.1 Introduction

For many decades, prior to placement of a restoration, all carious dentin tissue had to be removed until hard dentin was present. This meant that only hard dentin was considered clean, healthy dentin, which was acceptable as supporting tissue underneath a restoration. In deep lesions, caries removal to hard dentin often led to pulp exposures, frequently with poor prognosis.[61, 62] The evolving understanding of the dental caries process and the histopathology of caries lesions has led to a paradigm shift on dental caries management as it relates to caries excavation. The focus has moved away from removing all of the inner carious dentin and towards disease management following tooth-preserving principles.[4, 63] In selective caries excavation (a.k.a. stepwise excavation, or partial caries removal), only the outer carious dentin or zone of destruction is removed, while the inner carious dentin is preserved prior to placement of a permanent restoration. The outer carious dentin is characterized by necrotic, disorganized and irreversibly demineralized dentin tissue, while the inner carious dentin is characterized by vital, organized, and remineralizable dentin. Selective caries excavation minimizes the risk of pulp exposure, allows the pulp to react and produce tertiary dentin, and therefore improves the prognosis of the tooth. Ample evidence shows that sealing inner carious dentin will lead to lesion

arrest [7, 9, 32], and preservation of pulp vitality, thus decreasing the need for endodontic treatment. [32-34] This conservative approach preserves tooth structure, while also being cost effective by preventing more complex procedures. Patients with high caries risk particularly reap great benefits from the resulting health-gains and cost-savings. [8, 27] Selective caries excavation relies on accurate differentiation between outer and inner carious dentin, which is not straightforward clinically. Clinicians rely on tactile sensation to determine if dentin characteristics indicate inner carious dentin (hard, firm or leathery dentin) or outer carious dentin (very soft, disorganized dentin). This uncertain diagnosis, compounded with the concern that leaving bacteria behind would allow its proliferation and cause lesion progression, has led to low acceptance of selective caries excavation. [31, 35, 36] However, studies have shown that the total colony-forming units (CFU) counts of common caries pathogens were gradually reduced when dentin was partially excavated. [37, 38, 64] Yet, there is no clear clinical definition of a threshold where dentin is no longer considered outer carious dentin and inner carious dentin. Therefore, a more accurate and clinically applicable method to differentiate outer carious and inner carious dentin is greatly needed in dentistry.

Fluorescence-aided caries excavation (FACE) has been proposed to allow removal of outer carious dentin without removing inner carious dentin.[5, 65, 66] However, there is very limited data available to support this concept. Therefore, the first aim of this study was to determine if the presence or absence of fluorescence, as clinically detected by FACE, correlates with dentin bacterial microbiome diversity. The secondary aim was to determine if the presence or absence of fluorescence, as clinically detected by FACE, correlates with the traditional tactile clinical assessment of dentin characteristics. The hypothesis was that there will be a greater bacterial species diversity in the fluorescing dentin as compared

to the non-fluorescing dentin, and that dentin which probes leathery or hard will have no fluorescence as detected by FACE.

2.2 Materials and Methods

Unidentified teeth extracted for clinical reasons (N=134), considered discarded waste, by providers who are unrelated to the research study were collected for this project-NHSR (Not Human Subjects Research – IRB#15-3183). Freshly extracted teeth were rinsed with deionized water and immediately placed in individually numbered containers and subsequently on dry ice for storage. The teeth were then transported to -80° Celsius refrigeration to maintain the stability of microorganisms' DNA, through collection, transport and storage processes. Samples were collected at three levels of fluorescing dentin which further represented layers of carious dentin: superficial Red Fluorescing (RF) prior to any excavation, middle Pink Fluorescing (PF) after removing RF only, and inner No Fluorescing (NF). (Figure 4)

Inclusion criteria – Selected extracted carious permanent human teeth met the following inclusion criteria: evidence of caries that extended approximately 2/3 into the dentin, and at least 1mm of dentin between the caries and pulp, verified radiographically post extraction. Caries had to fluoresce red with the use of SIROInspect (Sirona, Bensheim, Germany). (Figure 5 and Figure 6)

Exclusion criteria – Teeth with root caries, secondary caries and/or caries which was less than 1mm from the pulp were excluded. Teeth that had pulp exposures during the excavation were also excluded.

Radiographs were made of the extracted teeth to evaluate, measure and record the proximity of the caries to the pulp (>1mm)(Figure 7). Two calibrated examiners participated in the data collection. Sirona's SIROInspect which utilizes FACE technology was used to assess fluorescence. Lab collection packets which were used for dentin caries sampling, (Figure 8) were put together and contained the following:

Sterile High-speed/Low-speed Handpieces

3 sterile explorers

2 sterile spoon excavators

2 sterile high-speed round burs

1 sterile low-speed round bur

Air/water syringe tip*

Air/water syringe plastic cover

3 patient bibs

Plastic bonnet for water collection bowl

*used deionized water in the air/water syringe and high speed handpiece

(Table 1)

Experimental procedures were as follows:

Step 1 - Visual and tactile assessment with a sterile explorer of the carious lesion was independently recorded by both examiners.

Step 2 – Second examiner used SIROInspect with FACE technology to collect dentin from a red fluorescing axial wall. An area which was fluorescing red was sampled using a sterile spoon excavator, (pre-caries excavation-Sample A, Figure 9).

Step 3 – Excavation of caries: Only dentin which was fluorescing red utilizing the SIROInspect was removed with high speed hand-piece and sterile round bur. Excavation was terminated when there was no red fluorescing dentin in the prepared cavity.

Step 4 – After the first layer of red fluorescing carious dentin was removed, excavated dentin was reassessed by both examiners to determine and record visual-tactile evaluation. The layer of pink fluorescing carious dentin present was sampled with a sterile spoon excavator, (mid-excavation – Sample B)

Step 5 – All remaining pink fluorescing dentin was excavated with a high speed handpiece and sterile round bur. Both examiners reassessed and recorded tactile sensation using sterile explorer. (Figure 10)

Step 6 – Second examiner used the SIROInspect to verify that no remaining pink fluorescing dentin was present and sampled an area post caries excavation using a sterile slow speed handpiece and sterile round bur, post-excavation – Sample C, Figure 11)

Three samples per tooth specimen were obtained, and placed in individual sample containers. The individual samples after being stored in -80° Celsius were subsequently transported on dry ice to the UNC Microbiome Core Facility for microbiome analysis.

16S rRNA amplicon Illumina sequencing + HOMINGS:

Bacterial DNA extraction was performed using QIAmp DNA extraction kit (QIAGEN) and total dsDNA content was quantitated using Quan-iT PicoGreen dsDNA Quantitation kit (Invitrogen). Sequencing libraries were prepared by PCR amplification using the HotStar HiFidelity Polymerase kit (QIAGEN, Valencia, CA) with 7.5 mM MgSO₄

and primers (Integrated DNA Technologies) directed against the 16S rRNA V3– V4 region [67, 68] was designed to incorporate Illumina compatible sequencing adaptors. PCR products were purified using AMPure XP reagent (Beckman Coulter, Indianapolis, IN), and quantified by Quanti-IT Picogreen dsDNA 1 kit (Invitrogen). For MiSeq library preparation, the Nextera XT Index kit was used. Illumina sequencing was performed in a MiSeq instrument (Illumina, San Diego, CA) operating Real Time Analysis software (RTA) version 1.17.28. Raw sequencing data files were processed using the open-source software pipeline Quantitative Insights into Microbial Ecology (QIIME) version 1.8 [69] and Operational taxonomic units (OTUs) were clustered using QIIME implementation of UCLUST and compared with *HOMINGS* data at 98.5% sequence similarity. Phylogenetic and non-phylogenetic alpha and beta diversity metrics was recorded and compared at the 10,000 rarefaction depth (Figure 12).

2.3 Statistical Analysis

Regarding our first aim, to determine whether fluorescence was correlated with the dentin microbiome within genera, statistical analysis was performed on aggregated totals of expression counts. Expression counts obtained from all samples of different extracted teeth, producing 18,453 Operational Taxonomic Units (OTUs) with known taxonomy. Fluorescence was treated as categorical and analyses were conducted on genus instead of OTUs. In particular, we summed the counts relating to expression over the OTUs within taxa defined at the genus level for each sample. Analysis was provided by the Mantel-Haensel (MH) mean score Chi-square statistic using standardized modified rank scores for the stratified tables of fluorescing by genus expression with teeth as strata. These tests were based on the within-tooth ranks (1, 2 and 3 in the absence of ties, with mid-ranks for ties) of the counts for the red, pink and non-fluorescing sites. For each genus, the mean

ranks were computed over the 21 teeth to allow comparison and reporting of the relative expression levels according to fluorescence. Finally, false discovery rate (FDR; Benjamin and Hochberg, 1995) and family-wise error rate (FWER; Hochberg, 1988) methods, as described in Preisser et al. (2011), were applied to the p-values of the stratified MH mean score statistics for exploratory and confirmatory testing, respectively, to identify any statistically significant differences among genera. An overall error rate of 0.05 was used in each procedure.

In regards to the second aim, the unweighted and weighted kappa statistics were computed to assess agreement between the two examiners with respect to their tactile evaluations. Kappa statistics were computed for the agreement between fluorescence status and tactile score for each examiner; this statistic is of interest to the extent that correlation is expected between the two measures in the sense that layer A (RF) has soft dentin, layer B (PF) has leathery dentin, and layer C (NF) has hard dentin. A complimentary analysis is provided by the Mantel-Haensel Correlation Chi-Squared Statistic using standardized modified rank scores (SAS PROC FREQ option “scores=modrfit”) for the stratified tables of fluorescence by tactile (for each examiner) with tooth as strata. Specifically, the rank correlation statistics test the null hypothesis of no association between fluorescence and tactile score.

2.4 Results

Out of 134 extracted carious teeth that were collected, 21 fit into the inclusion criteria and were subsequently used in our study. The 113 teeth that were excluded presented with root caries, secondary caries, caries which was <1mm from the pulp, or pulpal exposure during the caries excavation. Of the 21 teeth, 19 teeth produced 3 samples

each, with 2 teeth only yielding 2 samples – giving a total of 61 samples which were analyzed by the UNC Microbiome Core Facility. Two of the teeth did not present with a ‘Sample B’ due to not revealing an intermediate layer of PF, therefore - once the RF was removed, NF was evident and sampled. Therefore only Sample A, and Sample C were obtained for these two teeth.

The 16S rRNA amplicon sequencing analysis revealed 18,453 Observational Taxonomic Units (OTUs). Total number of reads was 10,268,174 with a median of 120,223 and a mean of 151,002 per sample. The microbiota discovered in the various layers of fluorescing dentin varied greatly in composition. The red fluorescent portion showed a higher number of observed OTUs (154); followed by the pink fluorescing (109) and the no fluorescing (100) (Table 2). The *t*-statistical analysis confirmed a significant difference between RF and NF ($p=0.003$), as well as between RF and PF ($p=0.003$). No statistically significant difference was detected between PF and NF ($p=0.843$) (Table 2). **Red fluorescing (RF)** carious dentin had a predominance of: *Prevotella* (11.7%), *Selenomonas* (8.4%), *Veillonella* (7.6%) and *Propionibacteria* (6.4%)(Table 3). The **Pink fluorescing (PF)** dentin had a predominance of: *Propionibacterium* (18.9%), *Coriobacteriaceae* (18.0%) *Eubacterium* (10.2%) and *Lactobacillus* (9.0%)(Table 4). The **Non fluorescing (NF)** dentin had the predominant microorganisms: *Eubacterium* (15.9%), *Corynebacterium* (10.4%), *Actinomyces* (9.1%) and *Streptococcus* (7.3%) (Table 5).

There were 230 genera identified after collapsing data within genera and 229 among them are informative (one non-informative genus had only zero counts in all 61 tooth sites). A total of 62 among 229 genera were detected as statistically significant by FDR error control, and under strong error control, there were 29 significant genera by FWER at the

significance level of 0.05. Microorganisms which were predominant amongst the three layers (RF, PF, and NF) are highlighted in Table 11. . These 29 genera were differentially expressed among Red, Pink and No fluorescing. Among these, 20 genera had increased counts for RF, 2 for PF and 7 were most pronounced under NF. Thus, in the context of these significant genera that were differentially expressed across fluorescence (table 11), the hypothesis seems to be supported with greater “diversity” among RF.

Regarding our secondary aim, which was to determine if the presence or absence of fluorescence as detected by FACE correlates with the clinical assessment of dentin characteristics, we assessed the following among the 61 samples: Hard – 32, Leathery – 15, Soft – 14 – assessments obtained by examiner one; Hard – 31, Leathery – 19, Soft – 11 – assessments obtained by examiner two. Alpha diversity values found in the tactile assessments were remarkably similar to the values noted in the RF, PF, and NF samples. The mean number of OTUs identified were as follows: soft dentin – 157, leathery- 115 and hard – 113. The *t*-statistical analysis also confirmed a significant difference between soft and leathery dentin ($p= 0.006$) and soft and hard dentin ($p= 0.003$). Whereas, differences between hard and leathery dentin in regards to tactile assessments were not significant ($p= 1.00$) (Table 6). The rank correlation chi-squared statistic for the association of fluorescence and tactile was highly statistically significant ($p < 0.001$) for each examiner, strongly suggesting an association between fluorescence and tactile assessment. The agreement between examiners’ tactile scores is “Substantial” with a kappa of 0.78. While the agreement between fluorescence and Examiner 1’s tactile score was “Substantial” with Kappa of 0.62, agreement between fluorescence and Examiner 2’s tactile score was “Moderate” with Kappa of 0.56 (Tables 4 and 5). The tactile assessment of soft, leathery and hard dentin did not perfectly correlate with dentin fluorescence, despite the high level

of inter-examiner agreement. RF carious dentin was primarily 'soft', but also had readings of 'leathery' or 'hard' tactically. Approximately one-third of the tactile assessments of hard dentin still displayed some level of fluorescence, either pink or red. Similar findings were found with pink fluorescing carious dentin, but the non-fluorescing was assessed as 'hard' 100% of the time by both examiners.

Principal Coordinate Analysis (PCoA) of Unifrac distance matrices is a method often applied to visualize similarities or dissimilarities of research findings, by trying to find a main axis through the data. Each component in the PCA (Principal Component Analysis) represented one of the 61 samples, and were placed in three different axis', PC1, PC2 and PC3. The points along each axis were also separated by RF, PF and NF characteristics and identified by the colors Red, Pink and Yellow. This PCoA analysis which was used to observe the Beta diversity, assisted in further describing the differences *between* the samples (Table 6). The Beta diversity plot showed each sample, and the clustering of the groups according to RF, PF, and NF with distinct separation among them in relation to bacterial content.

2.5 Discussion:

One question which has been repeatedly researched over the decades is whether or not bacteria remains in 'hard' dentin, post-caries removal? The result of our study supports that yes, bacterial DNA remains in the hard dentin, however the diversity is compromised. Controversy has surrounded the subjectivity of assessing outer and inner carious dentin, and how far the clinician should excavate prior to placement of a restoration. Fusayama attempted to implement a more objective process with the Caries Detection Dye (CDD),

which proved to stain dentin which could actually be remineralized, thus creating unnecessarily larger preparations.[6, 25, 70]

The manufacturer of the SIROInspect, Sirona, reports in their brochure that FACE technology can tell the end user where the bacteria is present: “With FACE, users can see where bacterially infected dentine is located and where not”. However, they also state that “red-fluorescing dentin must be completely excavated so that as little bacterially infected dentin as possible is left behind”. [71] Our present research showed remaining DNA from multiple bacterial species even at the hard and non-fluorescing level to include *Eubacterium*, *Corynebacterium*, *Actinomyces* and even *Streptococcus*. However, bearing in mind, only the genera that are expressed significantly differentl at RF, PF and NF dentin, there is less diversity in the hard NF dentin. Although there is some overlap on the bacterial species across the different levels of fluorescence even if considering only the top predominant bacteria on RF and PF (*Propionobactirium*) and PF and NF (*Eubacterium*) the different layers (outer dentin, inner dentin and sound dentin) was able to be identified by both fluorescence and tactile sensation.

The International Caries Consensus Collaboration meeting, held in Leuven, Belgium, in February 2015, developed guidelines for the dental practitioner based on lesion depth into the dentin. These guidelines indicate that shallow or moderate lesions, which do not reach the inner third or $\frac{1}{4}$ of dentin, should be selectively excavated to firm dentin, defined by a ‘feeling of resistance’ to a hand excavator. In regards to the deep lesion, where the caries extends into the pulpal third or $\frac{1}{4}$ of dentin, it is recommended to perform selective caries removal to ‘soft’ dentin to avoid a pulp exposure. It is essential that the

peripheral enamel and dentin are prepared to 'hard' dentin prior to placement of a restoration so as to create an effectively sealed restoration.[72]

Kidd points out, however, in reviewing multiple studies on Stepwise (SW) excavation, that suggest that remaining microorganisms had become altered to a 'less cariogenic flora', and upon re-entry the dentin was dryer, harder and darker. Thus indicating a positive correlation to bacterial degradation when the nutrient supply is removed by a well-sealed restoration.[28] She also mentions that the remaining microorganism which have been 'entombed' become irrelevant due to the lack of permeability of the reparative and tertiary dentin and ultimately states that there is no clear evidence suggesting that it is deleterious to leave infected dentin.[73] Furthermore, clinical studies have found that there are no detrimental effects to the pulp by 'sealing' remaining bacteria with a proper restoration which is cleansable by the patient.[74] It is possible that there are changes in the metagenomics of the bacterial species due to the conditions of the environment and/or the interaction within species at each of the different layers that may explain the fluorescence expression (or lack of expression).

As early as the 1980's there was a change in identifying and classifying bacteria from using culturing methods to the use of molecular methods. These molecular methods are based on gene sequences which give the ability to identify bacteria in any environment examined.[60] The use of 16S rRNA sequencing methodology in evaluating species present in the oral cavity was first introduced by Paster Laboratories in 2001 where they looked at diversity among the bacteria in periodontally involved patients.[75]

Simon-Soro, *et al.*, in an *in-vivo* study, obtained dentin caries samples from 6 teeth with a sterile spoon excavator after removing the top layer of biofilm. And, carious dentin

from 6 other teeth with 'hidden' caries diagnosed through radiographs and exposed after drilling through the enamel with a water cooled high-speed handpiece. RNA was extracted and cDNA was constructed and used to amplify the 16S rRNA gene. The sequences obtained through this process were used to determine the bacterial composition. Their findings revealed *Lactobacillus*, *Shlegelella*, *Pseudoramibacter Eubacterium*, and *Atopobium* to be 'clearly associated with dentin lesions', however this was the combined conclusion of all 12 dentin samples obtained, from both open dentin lesions and hidden dentin lesions.[76] These results were similar to ours, however *Atopobium* was negligible at 0.4% in the presumed heavily infected red fluorescing dentin caries, and, *Shlegelella* was not represented and/or detected in our taxonomic results.

In another *in-vivo* study from Japan, Obata *et al.* acquired deep dentin caries samples from 32 teeth after removal of plaque, enamel and a shallow layer of carious dentin. 16S rRNA sequencing technology was utilized for microbiome classification and analysis. The results were classified into 'clusters', varying according to the abundance of *Lactobacillus*: Cluster I, II, III with high, medium and low levels of *Lactobacillus* respectively. The mean distributions among the clusters identified the genera that followed in proportion to *Lactobacillus* were: *Propionibacterium*, *Prevotella*, *Atopobium*, *Streptococcus* and *Actinomyces*. The authors suggest that the abundance of *Atopobium* were possibly considered unique to the Japanese population, further stating that 'no previous reports have reported *Atopobium* as a dominant microbiota associated with dentinal caries'.[77] Differences noted between their findings and our research study: 1) *in-vivo* versus *in-vitro*, 2) our carious lesions were exposed, and they removed layers of carious enamel and dentin prior to first sample acquisition, 3) they classified the results

into 'clusters' according to abundance of *Lactobacillus*, and 4) *Atopobium* was not a predominate microorganism discovered in our study.

In a study which was conducted similarly to ours, Lima *et al.* sought to analyze and identify the microbiota in the different layers of dentinal caries (superficial, middle and deep) through the use of a reverse-capture checker-board hybridization assay. Twenty Seven (27) patients yielded 81 samples. Their reported tooth crown conditions were: intact 11 (40.7 %) microcavitated 8 (29.6 %) cavitated 8 (29.6 %), with a mean age of 13.5. Use of the reverse-capture checker-board allowed the author to survey specific bacteria, which they selected 28 to probe with the 16s rRNA analysis. Bacteria which were prevalent in the superficial layer are as follows: *Fusobacterium*, *Lactobacillus*, *Atopobium*, and *Veillonella*. Middle layer: *Atopobium*, *Lactobacillus*, *Fusobacterium*. And, the deep layer: *Fusobacterium*, *Atopobium*, *Lactobacillus*, *Bifidobacterium* and *Streptococcus*. Their research study concluded that bacteria which dominates in the deep dentinal lesions may further be involved with pulpal damage, stating that they are in the 'front line' of the lesion. It was also suggested that the superficial bacterial layers of caries could represent historical information of the disease process, and they may provide a nutrient source as well as protection to the inner layers of the carious lesion.[10] The major differences from our study: 1) our population age was unknown; 2) our lesions were already through the enamel with exposed soft carious dentin; 3) we sampled the first very soft layer, and they discarded the first layer (Figure 13). Interesting to note that they did not probe for certain bacteria which were predominant in our study, such as: *Selenomonas*, Coriobacteriaceae and *Eubacterium*.

2.6 Limitations:

The collection of extracted carious teeth was not conducted by the same individual throughout the study process, therefore handling of the tooth specimens may have been inconsistent. Post-extraction, the carious teeth were to be rinsed with deionized water to attempt to remove any blood and/or debris. Any bioburden which may have been left on the tooth could have been represented in the microbiome analysis. Furthermore, although steps were taken to attempt to maintain a clean field, by the use of individually sterilized/packaged instruments and burs, which were changed during each step of the sampling process (A, B, C), the protocol we used to acquire the samples from the carious extracted teeth was not in a totally 'sterile' environment. Tooth to tooth variation in regards to the depth of the caries, type of tooth (incisor, premolar, molar), cariogenic history of the patient, use of fluoride, diet and hygiene practices may have impacted the microbiome results. The drawback of using a non-culturing technique, such as this DNA-based study is that the PCR step may amplify DNA from inactive or even dead microorganisms. Thus, it would become necessary to determine the viability of the bacteria which may be present in the deepest layer, by performing an additional step through culturing.[76]

2.7 Conclusion:

Within the limitations of this study, the presented protocol sought to evaluate the diversity of microorganisms at varying layers of carious dentin using FACE technology, as well as determine if fluorescence correlated with the traditional tactile method of caries assessment. It became evident that a vast array of microorganisms were present throughout the layers of carious dentin from soft to leathery to hard, as well as from RF, to PF to NF. In addition, the carious dentin layers sampled displayed a variation in *proportion* of bacterial microbiome, although still remaining quite diverse. The first hypothesis was

accepted, considering only the genera which were significantly differentially expressed in RF, PF and NF dentin, there was a higher bacterial species diversity in RF dentin as compared to PF and NF dentin. When comparing fluorescence (RF, PF, NF) with tactile assessments, approximately one-third of the hard dentin was noted as Pink Fluorescing (PF) or Red Fluorescing (RF), thus providing strong evidence to reject the second hypothesis that 'hard dentin will have no fluorescence'.

Table 1: Flowchart of sampling methods

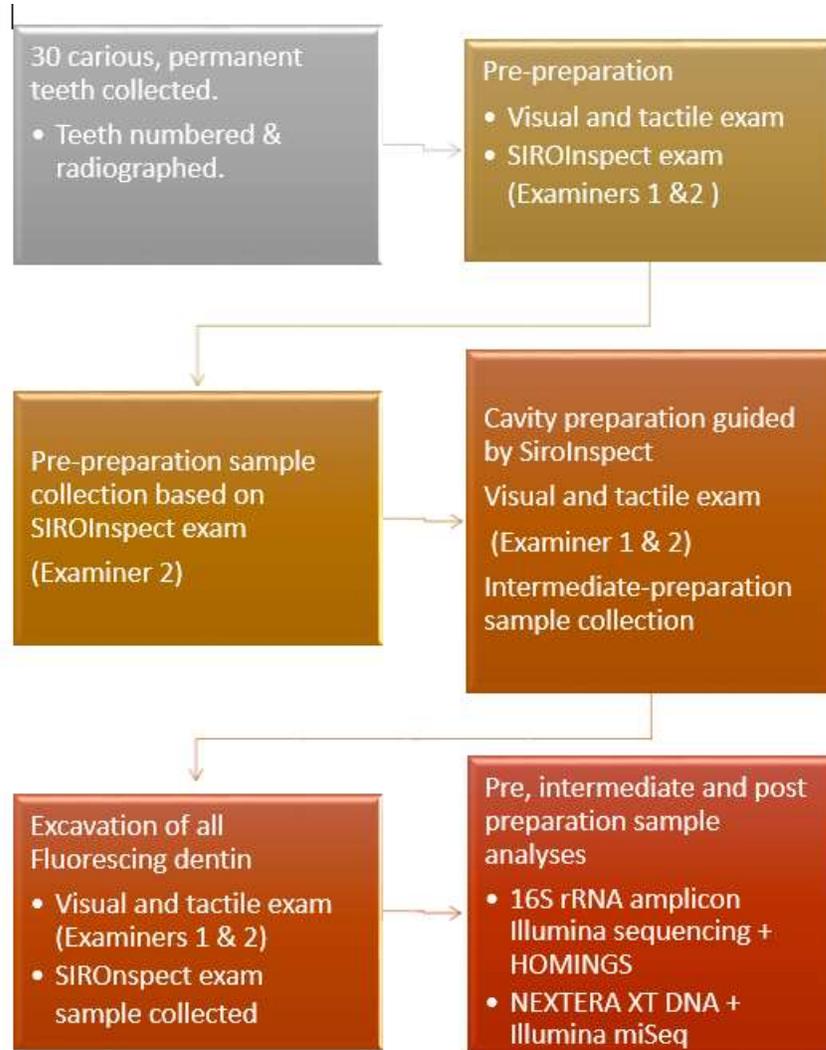


Table 2: Microbiome diversity within each sample regarding fluorescence (Alpha Diversity)

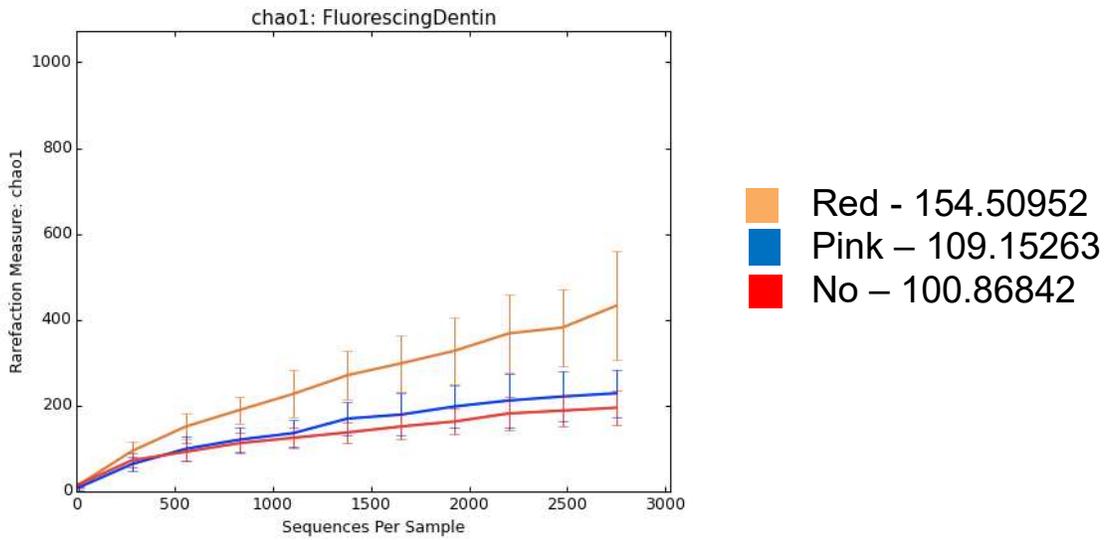


Table 3: Predominant species in RF

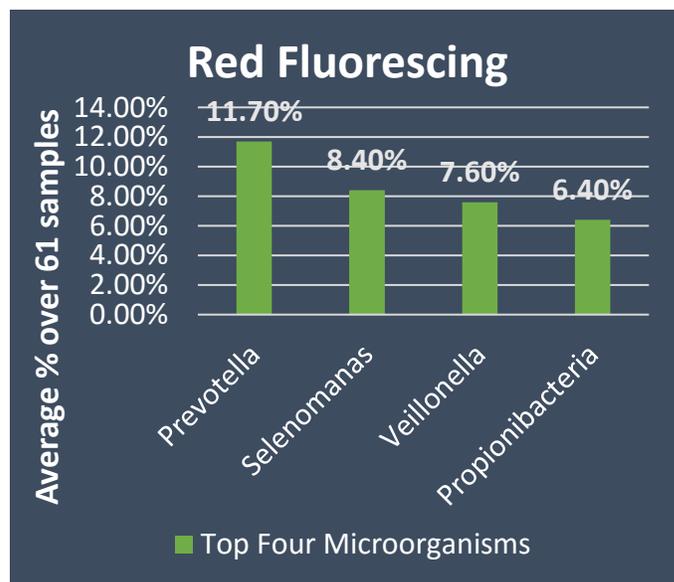


Table 4: Predominant species in PF

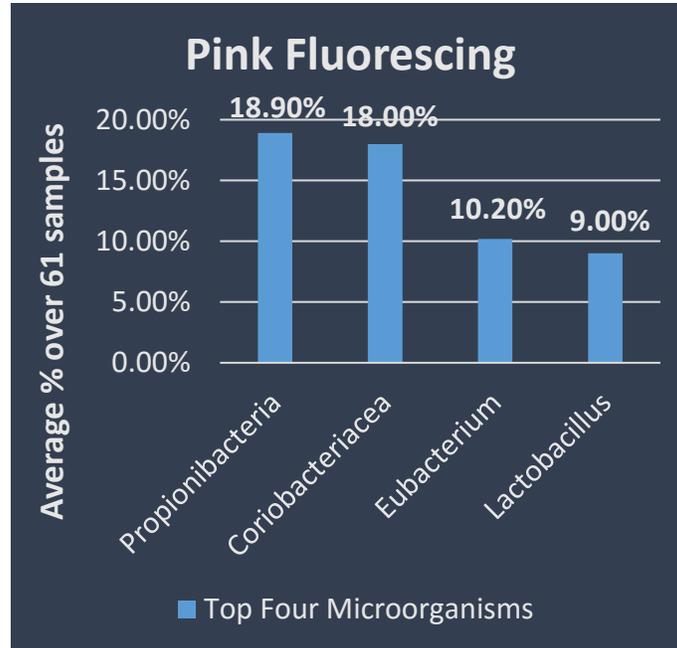


Table 5: Predominant species in NF

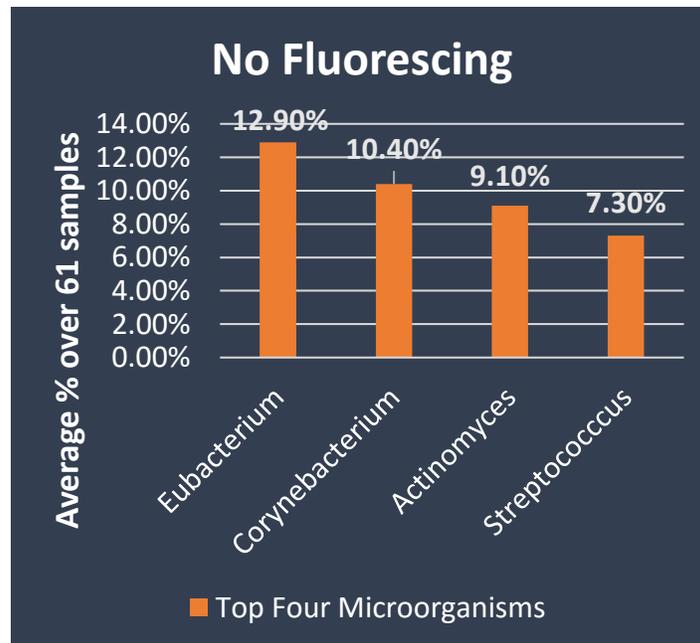


Table 6: Microbiome diversity within each sample regarding tactile (Alpha Diversity)

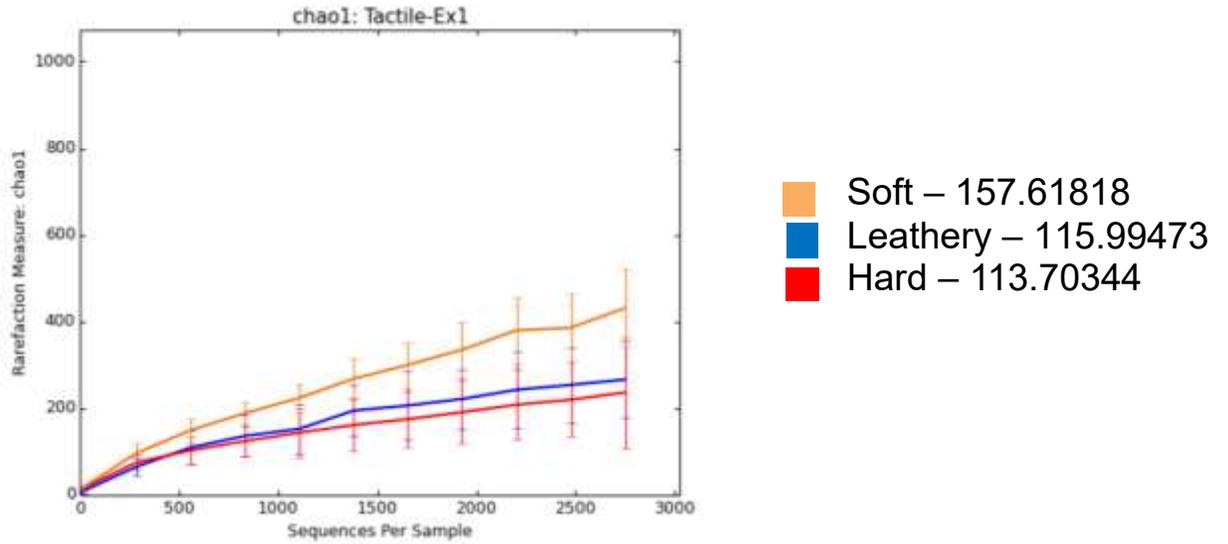


Table 7 : Tactile assessments based on Fluorescence, Examiner 1 and 2

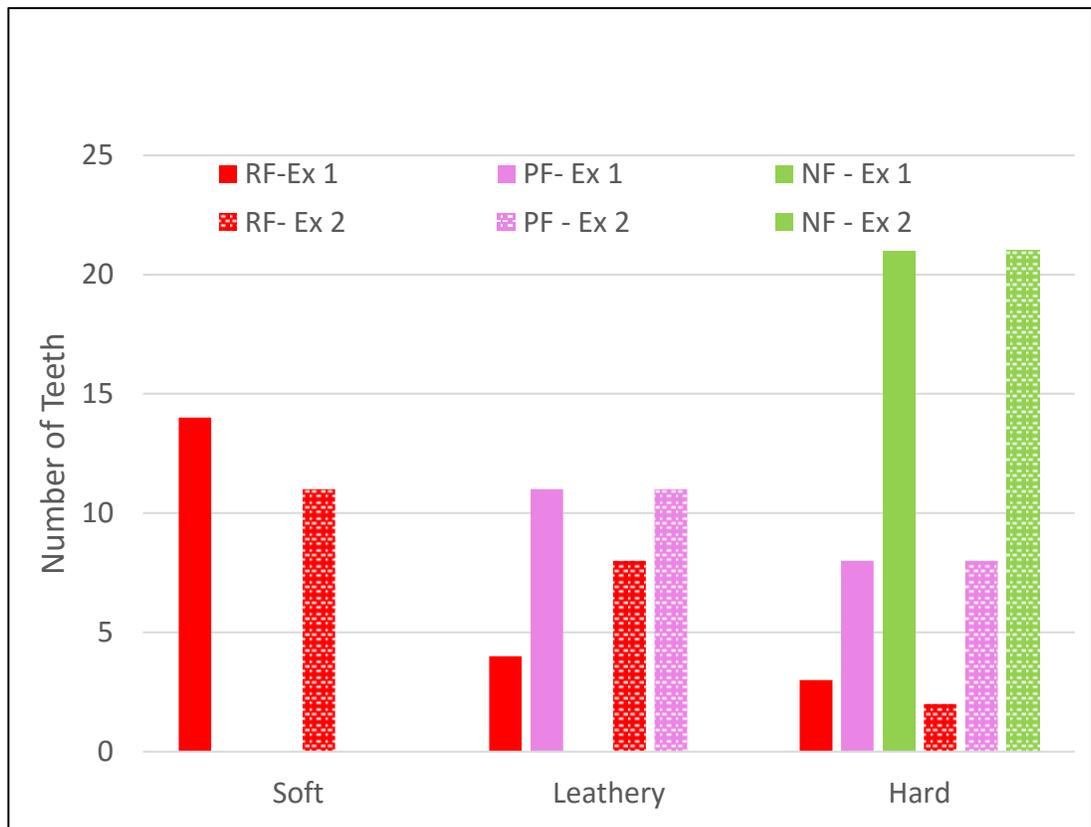


Table 8: Tactile Assessment Agreement*

*Kappa 0.79 and Weighted Kappa 0.86

Sample Size = 61

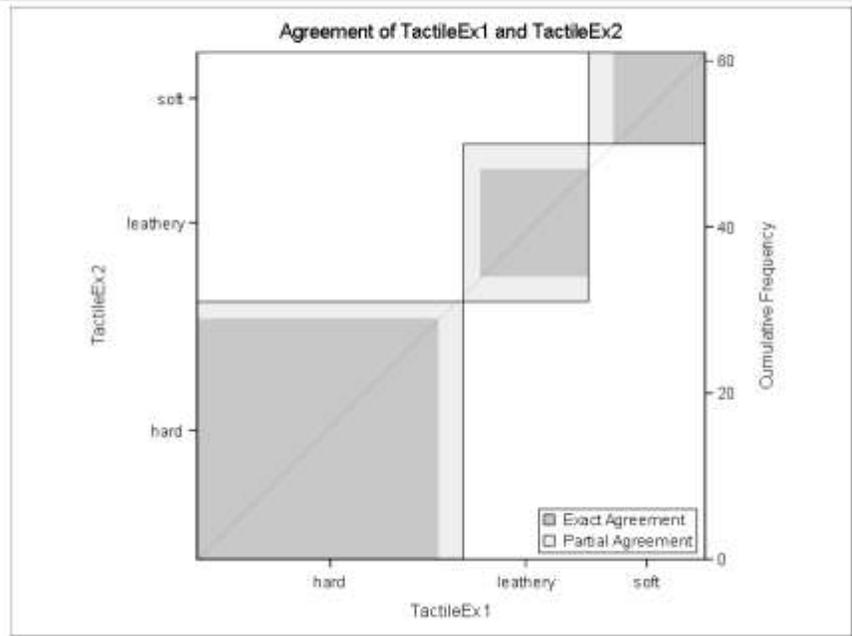


Table 9: Principal Component Analysis (PCA) Beta Diversity, between samples (n=61)

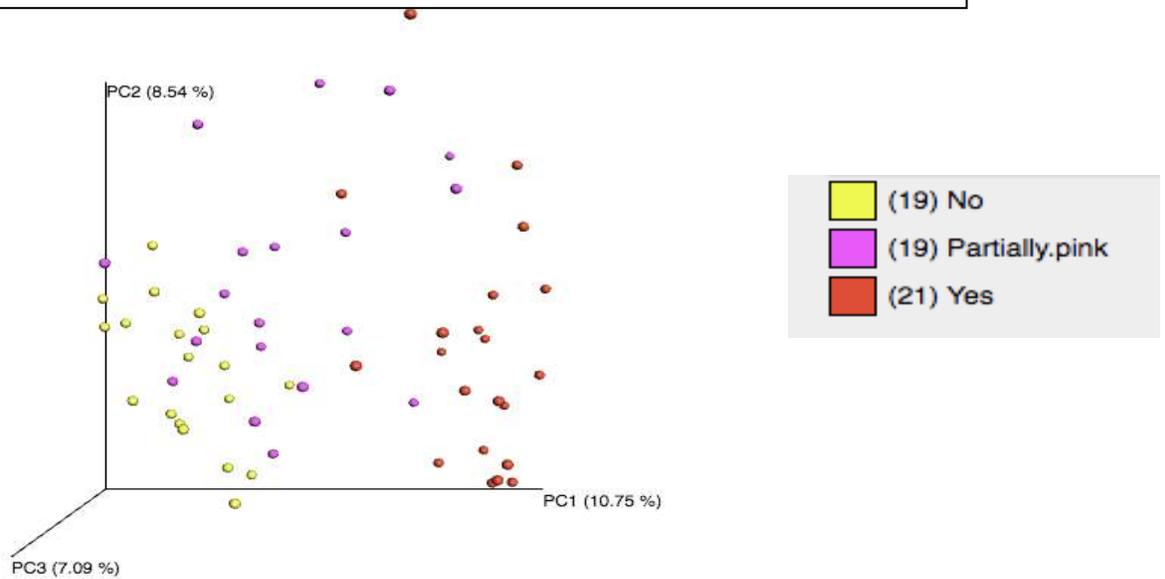


Table 10: 29 genera with most significant p-values, RF highlighted red, PF highlighted pink, and NF highlighted yellow

List of 29 genera (from FWER method) including genus number and their taxonomy ordered by statistical significance (taxa with most significant p-values appearing first)

id	genus	Kingdom	Phylum	Class	Order	Family	Genus
1207		Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	
1146		Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum
1152		Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium
1120		Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Selenomonas
1050		Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	Prevotella
1045		Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
1227		Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia
1040		Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
1117		Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megasphaera
1132		Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Bulleidia
1114		Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	
1102		Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Morvella
1061		Bacteria	Firmicutes	Bacilli			
1121		Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella
1009		Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinospicaceae	
1187		Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Campylobacter
1103		Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Oribacterium
1214		Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Pyramidobacter
1031		Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Scardovia
1138		Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Lentotrichiaceae	Lentotrichia
1136		Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
1027		Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	
1220		Bacteria	TM7	TM7-3	CW040	F16	
1028		Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
1074		Bacteria	Firmicutes	Bacilli	Gemellales	Gemellaceae	
1088		Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
1168		Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia
1124		Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Mogibacterium
1119		Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Schwartzia

Figure 1: Histological layers and clinical manifestations; adapted from Ogawa *et al*, 1983

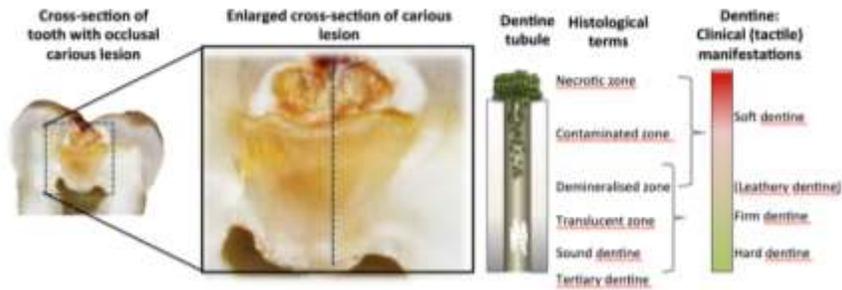


Figure 2: Fluorescence ratio - Red:Green Lennon 2006

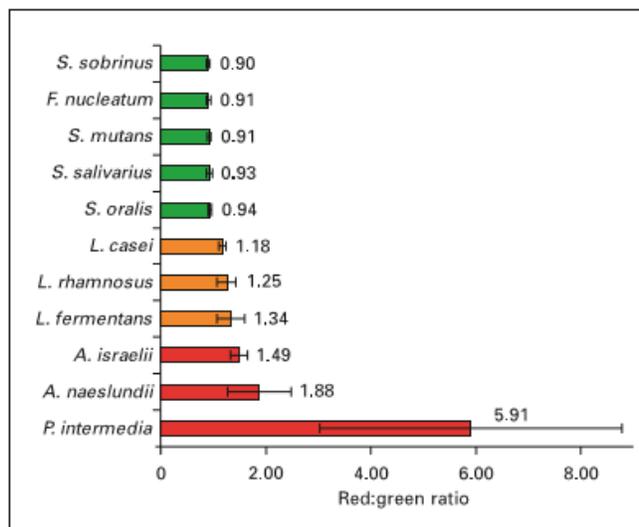


Figure 3. Fluorescence images of *P. intermedia* (**a**), *S. mutans* (**b**), *F. nucleatum* (**c**) and *Anaerolundia* (**d**) Lennon 2006

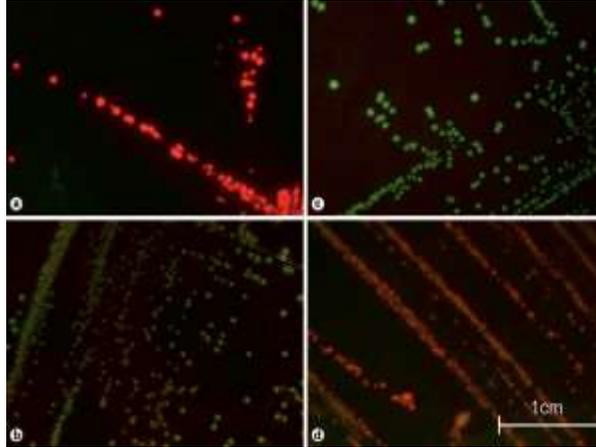


Figure 4: Illustrates layers sampled during our *in-vitro* study:

Superficial (RF) Middle (PF) and Inner carious dentin (NF).



Figure 5: SIROInspect, Sirona (Bensheim, Germany)



Figure 6: Fluorescing red through the use of SIROInspect



Figure 7: Radiograph of extracted tooth revealing deep dentin caries.



Figure 8: Lab packet for sample collection



Figure 9: Sample A, pre-excavation



Figure 10: Post-excavation tactile assessment



Figure 11: Sample C, Post-excavation



Figure 12: Microbiome Lab sequences

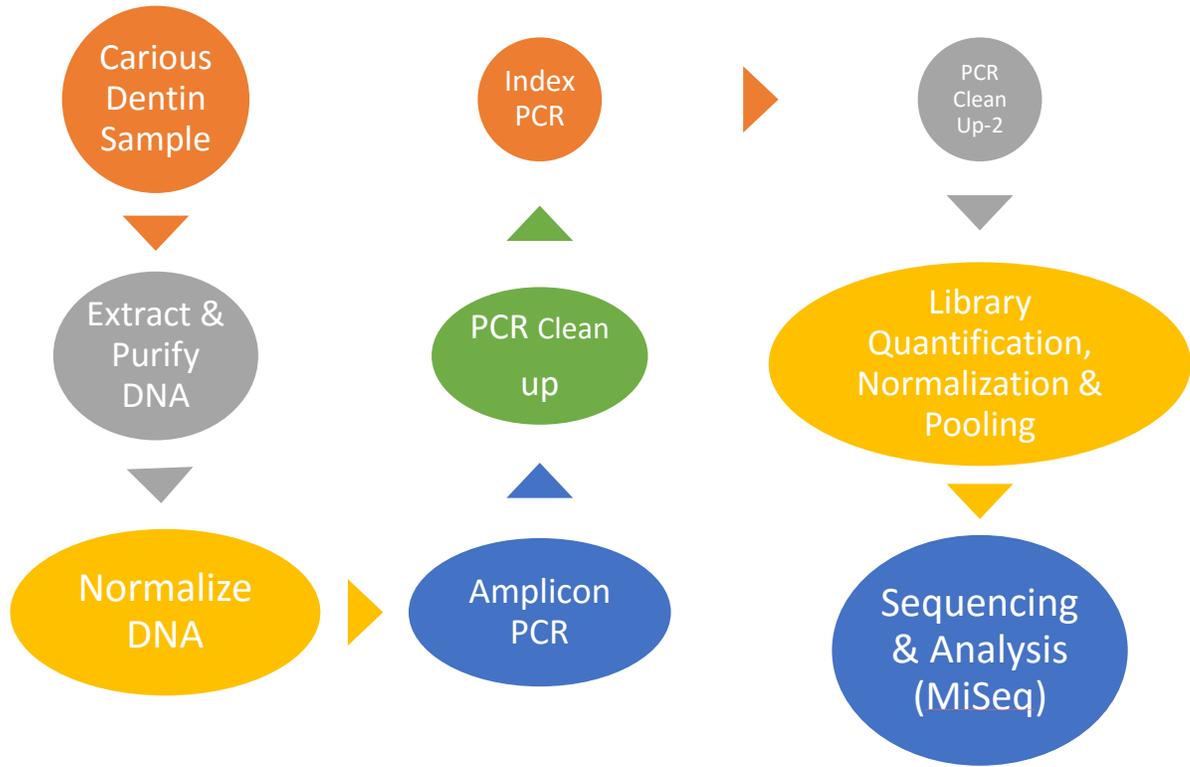
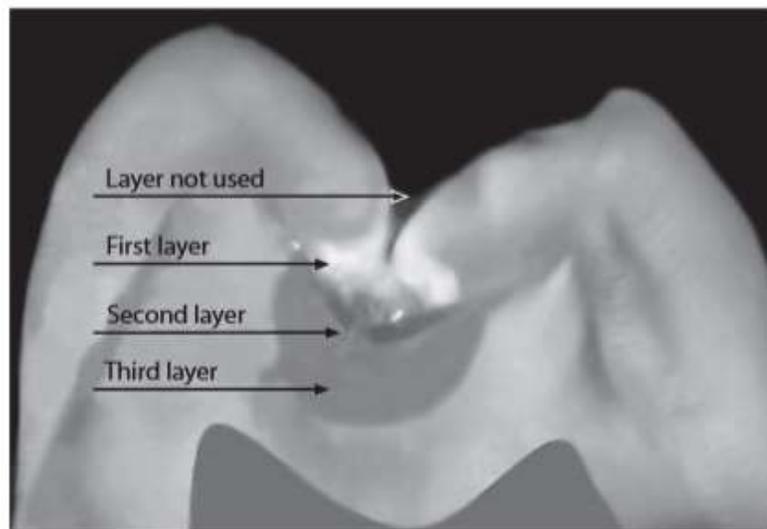


Figure 13: Illustrates layers sampled during *in-vivo* study Lima *et al*,



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