MATERIALS

1. ATCC strain of E. faecalis – 29212
2. ATCC strain of F. nucleatum – 25586
3. Anaerobic jar (Becton Dickinson)
4. Confocal microscope (Carl Zeiss)
5. Composite resin (Spectrum Dentsply)
6. Chisel
7. 2% Chlorhexidine (ASep RC)
8. Coagregation buffer
9. Diamond disk
10. Distilled water
11. 17% EDTA
12. E. faecalis RT-PCR kit. (Helini biomolecules)
13. 96 well Elisa plates
14. F. nucleatum RT-PCR kit. (Helini biomolecules)
15. Fluorescein diacetate (Sigma)
16. 1.5 ml ependroff tubes
17. Gates Glidden drills (No 2 to 5)
18. Gas pak anerobe system (Becton Dickinson)
19. Lactose (Sigma)
20. L-Arginine (Sigma)
21. L-Lysine (Sigma)
22. Micro pipettes
23. N-Acetyl-Galactosamine(Sigma)
24. Propidium iodide (Sigma)
25. PCR
26. Side-vented needle(Ultradent)
27. Silicon carbide grinding paper
28. Slow speed handpiece (NSK.Japan)
29. 3% sodium hypochlorite
30. Thioglycolate medium (Oxoid hampshire, UK)
31. Test tubes
32. Ultrasonic bath
FLOW CHART

PHASE I

PRE ANALYTICAL PHASE I

Teeth Samples

Tooth preparation  \[\rightarrow\]  Target bacterial preparation

Enterococcus faecalis  \[\rightarrow\]  Fusobacterium nucleatum

Inoculated in teeth samples as mono species biofilm and dual species biofilm-21 days in an anaerobic medium

ANALYTICAL PHASE I

A. Inoculated teeth samples with mono species biofilm and dual species biofilm for 21 days

Enterococcus faecalis n-15  \[\rightarrow\]  Fusobacterium nucleatum n-15  \[\rightarrow\]  Enterococcus faecalis & Fusobacterium nucleatum n-15

Dual Species Biofilm

Phase 1A

Confocal microscope

IA.a Depth of penetration
IA.b Biovolume of bacteria in dentinal tubules

Phase 1B

Visual coaggregation assay
Smear-Confocal microscope

Coaggregation of Enterococcus faecalis & Fusobacterium nucleatum

Data analysis and interpretation
**PHASE II**

**PRE ANALYTICAL PHASE II**

Teeth Samples → Tooth preparation → Target bacterial preparation →

- Enterococcus faecalis
- Fusobacterium nucleatum

Inoculated in teeth samples as mono species biofilm and dual species biofilm-48 hrs in a anaerobic medium

**ANALYTICAL PHASE II**

**Control (E. faecalis & F. nucleatum) & Fusobacterium nucleatum (n-15)** → **3% Sodium hypochlorite (n-15)** → **2% Chlorhexidine (n-15)** →

**PHASE II A - CONFOCAL**

- Biovolume of bacteria-CLSM

**PHASE II B - Molecular probe assay – RT PCR**

- Quantification by molecular probe assay- RT-PCR

**Data analysis and interpretation**
PHASE III

Preanalytical Phase III

Enterococcus feacalis & Fusobacterium nucleatum

Inoculated in teeth samples as dual species biofilm- 48 hrs in an anerobic medium

Control (n=15)  Lactose (n-15)  N-acetyl-D-Galactosamine (n-15)  L-Arginine (n-15)  L-Lysine (n-15)

Analytical phase III

Phase III A(n-120)  Phase III B(n-120)

3%NaOCl  2% Chlorhexidine

Stained with FDI and PI  Dentin chips-gates glidden drills (size 4&5)

Confocal microscope  molecular probe assay-RT-PCR

Biovolume and quantity (Copy number) of bacteria – pretreated with coaggregation inhibitors (sugars & aminoacids) followed by 3% Sodium hypochlorite & 2% Chlorhexidine using CLSM & molecular probe assay(RT-PCR)
MATERIALS AND METHODOLOGY

3.1. SOURCE OF DATA:
Study Source: 375 Premolars extracted for periodontic or orthodontic purpose

3.2. METHODS OF COLLECTION OF DATA:

1. Inclusion criteria:
   - Premolar teeth with single root canal
   - Non carious premolar teeth with complete and intact root end closure.

2. Exclusion criteria:
   - Carious teeth
   - Teeth with multiple root and root canals
   - Teeth with fractured root or incomplete root end
   - Teeth with any other developmental anomaly.

3.3. STUDY SAMPLE DESIGN: Simple random sampling.

3.4. SAMPLE SIZE: Carious, intact premolar teeth having single root canal.
Analysis: Input: Output: A priori: Computer required sample size Effect size $f = 0.4954182$ $\alpha \text{ err prob} = 0.05$ Power $(1-\beta \text{ err prob}) = 0.8$
Number of groups = 3
Noncentrality parameter $\lambda = 11.0447637$
Critical $F = 3.2199423$
Numerator df = 2
Denominator df = 42
Total sample size = 45
Actual power = 0.8263435
3.5. STUDY DESIGN: Experimental in vitro study

3.6. PREPARATION OF TOOTH SPECIMENS FOR CONFOCAL MICROSCOPE:

Single-rooted lower premolar human teeth were collected under a protocol approved by the ethics committee of Sri Ramachandra University (IEC-NI/12/MAR/27/13). A root segment with a length of about 7 to 8 mm was prepared by sectioning the root tip and the crown at 2 to 3 mm below the cementoenamel junction (9). Each root canal was enlarged to a size of a Gates Glidden bur #2 (70mm). Irrigation was performed with 1 mL 3% NaOCl solution after each instrument change with a 27-gauge side vented needle (Ultradent). The smear layer was removed using 17% EDTA. The teeth were washed with 5 ml of distilled water in ultrasonic bath for 10 minutes and stored in sterile water for 1 week to remove any residual chemical compound. Superficial longitudinal grooves were made in the buccal and lingual surface to facilitate the fracture of the specimens with chisel into two half segments and then flattened with silicon carbide grinding paper. Nail varnish was applied to avoid penetration of bacteria into the dentinal tubules other than the root canal. The specimens were sterilized by autoclave for 15 minutes at 121°C.

3.7. PREPARATION OF TOOTH SPECIMENS FOR POLYMERASE CHAIN REACTION:

A root segment with a length of about 7 to 8 mm was prepared by sectioning the root tip and the crown at 2 to 3 mm below the cementoenamel junction (9). Each root canal was enlarged to a size of a Gates Glidden bur #2 (70mm). Irrigation was performed with 1 mL 3% NaOCl solution after
each instrument change. The smear layer was removed using 17% EDTA. The teeth were washed with 5 ml of distilled water in ultrasonic bath for 10 minutes and stored in sterile water for 1 week to remove any residual chemical compound. Root canal was sealed with resin composite in the apical end. Nail varnish was applied to avoid penetration of bacteria into the dentinal tubules other than the root canal. The specimens were sterilized by autoclave for 15 minutes at 121°C.

1.8. INNOCULATION OF DENTIN SAMPLES:

The strains F. nucleatum American Type Culture Collection (ATCC) 25586 and E. faecalis ATCC 29212 were anaerobically incubated at 37°C in 10 ml of a thioglycolate medium (Oxoid Ltd, Basingstoke, Hampshire, UK) supplemented with 0.5 mg/l vitamin K and 5 mg/l hemin. For dentin infection, samples were transferred into 96 well plates for confocal microscope evaluation. 200 μl of E. faecalis suspension and F. nucleatum suspension was inoculated in 15 samples each (group A -15) (group B-15) and 100μl of E. faecalis and 100μl of F. nucleatum were inoculated together in 15 samples (group C -15) for 21 days (1A &B) and 48 hrs for all the other samples. For molecular probe assay samples were transferred into in 1.5 ml eppendorf tubes and 100μl of E. faecalis and 100μl of F. nucleatum were inoculated together in 15 sampleseach for 48 hours in anaerobic condition. The anaerobic environment was produced by activating GasPak Anaerobe System with indicator (Becton-Dickinson) in anaerobic jars (Becton-Dickinson Microbiology Systems).

3.9. TREATMENT GROUPS:
The root samples were randomly selected and divided into four groups (n=15), as described:

PHASE I A : 45 Samples

<table>
<thead>
<tr>
<th>Group</th>
<th>Biofilm Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>Enterococcus faecalis monospecies biofilm</td>
</tr>
<tr>
<td>Group B</td>
<td>Fusobacterium nucleatum monospecies biofilm</td>
</tr>
<tr>
<td>Group C</td>
<td>E. faecalis &amp; F. nucleatum dual species biofilm</td>
</tr>
</tbody>
</table>

I Aa : Depth of penetration into the dentinal tubules
I Ab : Biovolume of bacteria in the dentinal tubules
PHASE I B: Visual coaggregation assay in to the dentinal tubules & Bacterial smear.

Visual Coaggregation Assay

Coaggregation assays were performed as described by Cisar in coaggregation buffer (CAB; 150mM NaCl, 1mM Tris, 0.1 mM CaCl₂, 0.1 mM MgCl₂ • H₂O (pH 7.5). Cells were pelleted and re-suspended in CAB to a final concentration of 2x10⁹ cells. Coaggregation assay was done by mixing 200μl of each strain to a total volume of 400μl in a reaction tube. Once the second partner strain was added, reaction mixtures were immediately vortexed for 5 seconds and incubated for at least 10 min prior to evaluation using a visual scoring system ranging from 0 to 4. A coaggregation score 0 denotes there is no change in turbidity and no visible coaggregates. Score +4 denotes maximum coaggregation and large coaggregates settled immediately, leaving a water-clear supernatant. Score+3 denotes formation of large settling coaggregates but a slightly turbid supernatant. Score+2 denotes definite coaggregates were visible but did not settle immediately. Score +1 indicated finely dispersed coaggregates in a turbid background.

Bacterial coaggregation – smear: Overnight cultures of E. faecalis were used to prepare the smear. A loopful of culture was spread evenly on a clean glass slide and air-dried. The smear was heat fixed and slides were stained with Propidium Iodide (PI) and the slides were viewed under confocal microscopy.

PHASE II

<table>
<thead>
<tr>
<th>Group A</th>
<th>E. faecalis &amp; F. nucleatum dual species biofilm (control-no treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group B</td>
<td>3% Sodium hypochlorite</td>
</tr>
<tr>
<td>Group C</td>
<td>2% Chlorhexidine</td>
</tr>
</tbody>
</table>
IIA: (45 Samples) Bio-volume of bacteria before and after disinfection with 3% Sodium hypochlorite and 2% Chlorhexidine – Confocal Microscope

IIB: (45 Samples) Quantitative and percentage of remaining bacteria after disinfection with 3% sodium hypochlorite and 2%chlorhexidine – Molecular probe assay-RT-PCR

Phase III

III A. (120 Samples)
- A1: (n-60) Biovolume of bacteria pretreated with coaggregation inhibitors followed by 3% sodium hypochlorite – confocal microscope.
- A2: (n-60) Quantitative and percentage of remaining bacteria pretreated with coaggregation inhibitors followed by 3% sodium hypochlorite - Molecular probe assay-RT-PCR.

III B. (120 Samples)
- B1: (n-60) Biovolume of bacteria pretreated with coaggregation inhibitors followed by 2% chlorhexidine.- confocal microscope.
- B2: (n-60) Quantitative and percentage of remaining bacteria pretreated with coaggregation inhibitors followed by 2% chlorhexidine – Molecular Probe Assay-RT-PCR.

GROUP A was divided into 5 groups

<table>
<thead>
<tr>
<th>GROUP I</th>
<th>3% Sodium hypochlorite (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP II</td>
<td>100 mM Lactose (sugar) followed by 3% NaOCl</td>
</tr>
<tr>
<td>GROUP III</td>
<td>100 mM N-Acetyl galactosamine (sugar) followed by 3 NaOCl</td>
</tr>
<tr>
<td>GROUP IV</td>
<td>100 mM L Arginine(amino acid) followed by 3% NaOCl</td>
</tr>
<tr>
<td>GROUP V</td>
<td>100 mM L– Lysine(amino acid) followed by 3% NaOCl</td>
</tr>
</tbody>
</table>
GROUP B was divided into 5 groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP I</td>
<td>2% Chlorhexidine (control)</td>
</tr>
<tr>
<td>GROUP II</td>
<td>100mM Lactose (sugar) followed by 2% Chlorhexidine</td>
</tr>
<tr>
<td>GROUP III</td>
<td>100mM N-Acetyl galactosamine (sugar) followed by 2% Chlorhexidine</td>
</tr>
<tr>
<td>GROUP IV</td>
<td>100 mM L-Arginine (amino acid) followed by 2% Chlorhexidine</td>
</tr>
<tr>
<td>GROUP V</td>
<td>100 mM L-Lysine (amino acid) followed by 2% Chlorhexidine</td>
</tr>
</tbody>
</table>

100 mM of sugars and amino acids was placed in the root canal in anerobic condition for 60 mins. Final irrigation protocol was followed. Group A samples were irrigated with 5 mL of 3% NaOCl for 1 minute and group B samples were irrigated with 5 ml of 2% Chlorhexidine for 1 min followed by distilled water.

3.10. STAINING FOR CONFOCAL MICROSCOPE:

After the disinfection protocol, dentin segments were washed with PBS to remove nonadherent bacteria of the root canal walls and then stained with 50 μl fluorescein diacetate (FDA; Sigma) and 50 μl propidium iodide (PI, Sigma). The specimens were immediately analyzed by inverted Carl zeiss confocal microscope (CLSM) using a 20 X magnification oil lens. The 9 Z stack images were obtained by using sections of 1 μm step size. For an objective analysis, the CLSM images were exported to the Image J software in order to quantify the amounts biovolume of bacteria.

3.11. DENTIN COLLECTION FOR MOLECULAR PROBE ASSAY:

After incubation, the samples were rinsed 3 times with 10mL of sterile PBS. The root canal of each tooth sample was again enlarged with sterile #3 Gates-Glidden burs (0.9 mm diameter) and #4 Gates Glidden (400μm), dentin chips were collected into 1.5 mL of sterile PBS. DNA was extracted from the prepared samples and then evaluated with real-time PCR. The tooth samples were stored in a freezer at -20°C until the DNA was extracted.
POLYMERASE CHAIN REACTION:

Enterococcus faecalis PCR Kit components (Helini biomolecules)

<table>
<thead>
<tr>
<th>Component</th>
<th>No. of Reactions</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe PCR Master Mix - 8µl/reaction</td>
<td></td>
<td>200µl</td>
<td>2X200µl</td>
</tr>
<tr>
<td>Taq Enzyme Mix - 2µl/reaction</td>
<td></td>
<td>50µl</td>
<td>2 X 50µl</td>
</tr>
<tr>
<td>Enterococcus faecalis Primer Probe Mix - 2.5µl/reaction</td>
<td></td>
<td>65µl</td>
<td>2 X 65µl</td>
</tr>
<tr>
<td>Internal Control Primer &amp; Probe Mix [IC PP Mix] - 2.5µl/reaction</td>
<td></td>
<td>65µl</td>
<td>2 X 65µl</td>
</tr>
<tr>
<td>Internal Control Template [IC template] - 5µl/reaction</td>
<td></td>
<td>125µl</td>
<td>2 X 125µl</td>
</tr>
<tr>
<td>Water, Nuclease Free</td>
<td></td>
<td>6ml</td>
<td>6ml</td>
</tr>
<tr>
<td>Enterococcus faecalis Positive template [QS1]</td>
<td></td>
<td>125µl</td>
<td>125µl</td>
</tr>
</tbody>
</table>

Fusobacterium nucleatum PCR Kit components (Helini biomolecules)

<table>
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<tr>
<th>Component</th>
<th>No. of Reactions</th>
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<td>2 X 50µl</td>
</tr>
<tr>
<td>Fusobacterium nucleatum Primer Probe Mix - 2.5µl/reaction</td>
<td></td>
<td>65µl</td>
<td>2 X 65µl</td>
</tr>
<tr>
<td>Internal Control Primer &amp; Probe Mix [IC PP Mix] - 2.5µl/reaction</td>
<td></td>
<td>65µl</td>
<td>2 X 65µl</td>
</tr>
<tr>
<td>Internal Control Template [IC template] - 5µl/reaction</td>
<td></td>
<td>125µl</td>
<td>2 X 125µl</td>
</tr>
<tr>
<td>Water, Nuclease Free</td>
<td></td>
<td>6ml</td>
<td>6ml</td>
</tr>
<tr>
<td>Fusobacterium nucleatum Positive template [QS1]</td>
<td></td>
<td>125µl</td>
<td>125µl</td>
</tr>
</tbody>
</table>
Positive control for the PCR

For copy number determination and as a positive control for the PCR set up, the kit contains a positive control template. This was used to generate a standard curve of copy number / CT value. Each time the kit is used, at least one positive control reaction was included in the run. The quantitation standards are defined as copies/μl. The following equation has to be applied to convert the values determined using the standard curve into copies/ml of sample material:

\[
\text{Result (copies/μl) x Elution Volume (μl)} = \frac{\text{Result (copies/ml)}}{\text{Sample Volume (ml)}}
\]

As a matter of principle, the initial sample volume should be entered in the equation above.

Negative control FOR THE PCR

To confirm the absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNAse/DNAse free water was used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples was repeated. Later internal control template is added during DNA purification and The Internal control is detected through the HEX channel and gives a CT value of 26 +/- 3. Standard values were established

DETECTION MIX:

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe PCR Buffer Mix</td>
<td>8μl</td>
</tr>
<tr>
<td>Taq Enzyme Mix</td>
<td>2μl</td>
</tr>
<tr>
<td>Fusobacterium nucleatum Primer Probe Mix [Fuso PP Mix]</td>
<td>2.5μl</td>
</tr>
<tr>
<td>Internal control Primer Probe Mix</td>
<td>2.5μl</td>
</tr>
<tr>
<td>Purified DNA sample</td>
<td>10μl</td>
</tr>
<tr>
<td>Total reaction Volume</td>
<td>25μl</td>
</tr>
</tbody>
</table>
**Amplification Protocol**

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq enzyme activation</td>
<td>15min</td>
<td>95°C</td>
</tr>
<tr>
<td><strong>Denaturation</strong></td>
<td>20sec</td>
<td>95°C</td>
</tr>
<tr>
<td><strong>Annealing/Data collection</strong></td>
<td>20sec</td>
<td>56°C</td>
</tr>
<tr>
<td>Extension</td>
<td>20sec</td>
<td>72°C</td>
</tr>
</tbody>
</table>

**RESULTS WERE OBTAINED**

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Negative Control</th>
<th>Positive Control</th>
<th>Internal Control</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Bacterial DNA detected.</td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Bacterial DNA Not detected or Beyond detection limit.</td>
</tr>
<tr>
<td>Positive Or Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Experiment failed. PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.</td>
</tr>
<tr>
<td>Positive Or Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Experiment failed. Reagent contamination. Repeat testing using fresh reagents.</td>
</tr>
</tbody>
</table>
**Figure 8:** Tooth samples – 96 Well Plate

**Figure 9:** Prepared tooth sample for confocal microscope
Figure 10: Prepared tooth sample for RT-PCR

Figure 11: Anerobic jar

Figure 12: Anerobic bag
Figure 13: Gas Pak with indicator

Figure 14: Confocal microscope
**Figure 15:** Laser control

**Figure 16:** Lactose

**Figure 17:** N-Acetyl-D-Galactosamine
Figure 18: L-Arginine

Figure 19: L-Lysine

Figure 20: Milli-Q-Water

Figure 21: Weighing machine
Figure 22: PCR