ORIGINAL RESEARCH

Surface Demineralization in Enamel and Dentin Exposed to Mono-Species and Dual Species Biofilm of *Streptococcus Mutans and Candida Albicans:* In-Vitro Tooth Model Study.

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Abstract

Aim: To investigate the surface demineralization on enamel and dentin when exposed to monospecies and dual species biofilm of *Streptococcus mutans (S.mutans),* and *Candida albicans (C.albicans)* through an in-vitro tooth model study.

Materials and Methods: Eight teeth sections (4x4x2mm) were divided into 5 groups. One tooth specimen in Group 1 (Brain Heart infusion) and Group 2 (Lactic acid) respectively and two specimens each in Group 3 (*S.mutans* mono species), Group 4 (*C.albicans* mono species) and Group 5 (*S.mutans and C.albicans* dual species). *S. mutans* ATCC 25175 and *C. albicans* ATCC 14053 were used. Inoculation of the test organism was done only on the first day of the experiment and media was replaced every 48 hours for 30 days. Harvesting was done after Day 30. Specimens were analyzed on Environmental Scanning electron microscope (FEI, Quanta 200).

Results: The dual species biofilm consisted of a diffuse, thick, densely packed meshwork of *S.mutans* and *C.albicans* with a predominance of *C.albicans* on the enamel surface, while on the dentin surface the biofilm meshwork was localized, thinly populated with *C albicans* and with predominance of *S.mutans*. The size of the *S.mutans* ranged from 788.7nm to 1.27µm and *C.albicans* was 1.12 µm to 4.34 µm respectively. The mono species biofilm of *C.albicans* produced lesser demineralization as compared to *S.mutans*. Enhanced demineralization was noted with dual species biofilm, more in dentin as compared to enamel.

Conclusion: Less surface demineralization in enamel was noted as compared to dentin when exposed to dual species biofilm. The dual species colonization indicates a positive finding of curbing dental caries in enamel.

Keywords: enamel, dentine, dual species biofilm, S.mutans, C.albicans

Introduction

The human mouth supplies plenty of nutrients which provide a favorable environment for the growth and development of complex microbiota in the oral cavity. This complex microbiota co-exists in equilibrium, which is important for the maintenance of oral health.[1,2] The occurrence of oral diseases is a consequence of disturbance in the equilibrium of co-existence among microbiota, where the dominance of pathogenic species escorts the onset and progression of most common oral diseases *viz*, periodontitis and dental caries.[3-5] According to the World Health Organization (WHO), oral diseases are considered a major public health problem due to their high incidence, causing discomfort, pain and defacement, economic impact, and most importantly negative influence on the health of the affected person.[6]

Dental caries, the most prevalent human oral disease, is caused by microbial biofilms developed on the tooth surface. Microbial biofilm is a complex community harboring microbe enclosed by the self-produced network of exopolymer matrix which enhances the severity of caries.[7,8] In such a community, widespread inter-species synergistic communications take place, where one microbe creates a niche for other microbes and promotes its colonization and progression.[9] Despite advancements in cariology research in the past years, tooth decay remains a serious problem worldwide. Approximately, 490 million children have dental caries of primary

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teeth, mainly early-childhood caries (ECC) and around 2.5 billion people have dental caries of permanent teeth.[10,11]

Bacterium-fungus communication contributes to the transition from a healthy to an unhealthy situation (diseased) in a human host niche. Within the dental plaque biofilm, gram-positive bacterium *Streptococcus mutans (S. mutans)* has been considered as the most effective microbe for the progression of dental caries. *S. mutans* with its glucans polysaccharide can adhere strongly to teeth in addition, this bacterium produces acid and survives in acidic conditions, which ultimately triggers the disbanding of hydroxyapatite of tooth dentin and enamel.[12-14] Since a long time *S. mutans* have been reported as the major causative agent of dental caries, interestingly recent reports demonstrated a promising role of *Candida albicans (C.albicans)*, the fungal species, in connection with *S. mutans*.[7,15,16] *C. albicans* is the most commonly detected fungal microbe on human mucosal surfaces, and it often causes polymicrobial biofilms on soft tissue and prosthetic surfaces.[17,18] Like *S. mutans*, this fungal microbe, is also a natural colonist of the human oral cavity but in immune-compromised patients. Also, with alternations in the host environment conditions, this opportunistic fungus can quickly become pathogenic causing different infections, most commonly known as oral candidiasis.[19] *C. albicans* is generally referred to as a dimorphic species since it grows both as yeast and filamentous cells. This property helps the fungus in its pathogenesis and ability to develop biofilm.[8,20] Biofilm of this fungus is largely composed of mannans and glucans (β-1-3 and β-1-6-glucan) polysaccharides which form a complex mesh around the fungal cells.[21,22]

Interestingly, different clinical studies report that a higher number of *C. albicans* is often detected, with *S. mutans*, in dental-caries biofilm from children infected with Early childhood caries.[23,24] There are only three studies[22,25,26] examining how these two different microbes act together with each other and produce demineralization. However, they have checked the biofilm effect for only 72-96 hours. Given these limitations, a study was designed to investigate the surface demineralization on enamel and dentin when exposed to mono species and dual species biofilm of *S. mutans* and *C. albicans* for a period of 30 days through an *in vitro* tooth model study.

Materials and Methods

This is an in vitro tooth model study. Before, beginning the study, approval has been obtained from the Scientific Committee and Institutional Ethics committee (DPU/752(19)/2017) .

Table 1: Description of the groups

Group 1 (Negative control)	BHI* media
Group 2 (Positive control)	0.1M lactic acid (pH-5)
Group 3	*S.mutans* + BHI*+1% sucrose
Group 4	*C albicans* + BHI*+1% sucrose
Group 5	*S.mutans* + *C.albicans*+BHI*+1% sucrose

*2% BHI: Brain Heart Infusion (30ml)

Culture condition

S. mutans ATCC 25175 and *C. albicans* ATCC 14053 were routinely grown in sterile Brain Heart Infusion (BHI, Hi-Media Lab) and Sabouraud dextrose broth (Hi-Media Lab), respectively. The stock solution of both the cultures was kept at -80ºC in 40% (v/v) glycerol.

Biofilm architecture visualization

Freshly extracted five human teeth samples (without evidence of caries) were obtained and stored in formalin. These were cleaned and subjected to sectioning using a diamond cutter. Sections were obtained in such a way that they had equal portion of dentin and enamel, respectively (4x4x2mm). A total of 8 teeth sections were obtained (Sample size based on convenience sampling). The samples were sterilized using gamma irradiation with a dose of 14.5kGy. Gamma irradiation was done using a cobalt 60 source with 60Gy per min. Five separate groups were made as described in Table 1. One tooth specimen in Groups 1 and 2 respectively and two samples each in Groups 3-5 respectively. Inoculation of test organism i.e. *S. mutans* ATCC 25175 and *C. albicans* ATCC 14053 were done only on the first day of the experiment (Number of cells at optical density 1.0 at 660nm corresponds to 3 x 107 cells per ml) and media was replaced every 48 hours for 30 days. No renewal was done for control groups. Contamination of media was checked every day using gram staining. Harvesting was done after Day 30. Specimens were mounted on aluminum stubs with double sided carbon tape and directly analyzed on Environmental Scanning electron microscope (FEI quanta 200) at an accelerating voltage of 20kV with a working distance of 10mm. Each sample was first scanned for lesions before treatment and after treatment and biofilm formation post treatment at 40×, 1000×, 2000× and 4000× at the dentin and enamel portion.

Results

Surface biofilm on the tooth sample after Day 30 is depicted in Figure 1. No growth is seen on tooth samples of negative (1) and positive control (2) respectively. The mono species biofilm(3) shows diffuse distribution of *S. mutans* in the form of clusters and chains on the enamel surface, while localized distribution is seen on the dentine surface. The mono species biofilm (4) shows localized colonies of *C.albicans* in the spore form on both enamel and dentin surfaces. The dual species biofilm (5) consists of diffuse, thick, densely packed meshwork of *S.mutans* and *C.albicans* with a predominance of *C.albicans* on the enamel surface, while on the dentin surface the biofilm meshwork is localized, thinly populated with *C.albicans* and with a predominance of *S.mutans*. The size of the *S.mutans has* ranged from 788.7nm to 1.27µm, while the size for *C.albicans* was 1.12 µm to 4.34 µm.

Pictorial description of surface demineralization (Figure 2) indicates that the negative control samples (1) showed no evidence of demineralization and Positive control samples (2) showed a maximum amount of surface demineralization in both enamel and dentin. Comparatively, the mono species biofilm of *S mutans(3)* produced more demineralization as compared to *C.albicans (4).* Overall, the demineralization observed in dentin was more than in enamel.[3,4] Enhanced demineralization was noted with dual species biofilm (5), more in dentin as compared to enamel.

Discussion

Tooth decay is the most common defect, that is developed from bacterial biofilm. The biofilm that causes caries, is the interaction between organisms within the consortia rather than specific pathogens.[9,10,13] In the present study, the emphasis was on the surface demineralization caused by *S. mutans* and *C. albicans* biofilm formation, as these oral pathogens are regularly noticed in tooth decay, especially in children's plaque with ECC[24,27,28]. The physical interactions between *S. mutans* and *C. albicans* in the presence of sucrose have been associated with a mutual relationship in which the adherence of both microbes on the entire dental surfaces are stimulated and can enhance the aggressive onset of the carious lesions.[10]

An animal model of co-infection established that *C. albicans* in the oral cavity potentially enhances *S. mutans* colonization on teeth surface and oral tissue.[9] It was demonstrated that *C. albicans* derived exopolysaccharide enhanced *S. mutans* proliferation within the biofilm. The results of the present study indicate that the combined colonization of *S. mutans* and *C. albicans*, in the dentine and enamel region, form a mesh-like network of both microbial cells. This result is in accordance with the previous literature[28] which showed that *S. mutans* grown in association with *C. albicans* on dental surface formed more homogeneous and denser biofilm and *C. albicans* cells located around clusters of *S. mutans* cells.

Dual species biofilm was associated with a greater amount of demineralization as compared to mono species biofilm is reported in the previous studies.[22,25] In the present study the dual species biofilm produced more demineralization in dentine as compared to enamel. This finding is also in consensus with the earlier studies in literature.[22,25,26]

In a biofilm tooth model experiment[22] using bovine dentine slab exposed to human saliva, analysis of a 96 hour biofilm showed that *C. albicans* mono species biofilm did not

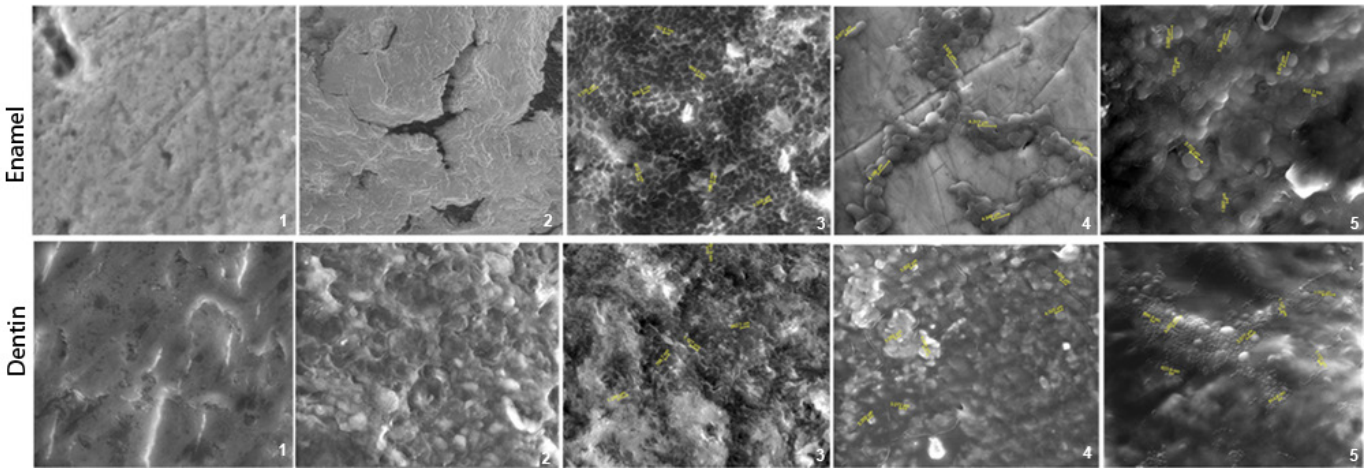

Fig. 1: Environmental Scanning Electron Micrographs showing the *in vitro* adherence (Magnification 4000X)
Foot note: 1. Negative control; 2. Positive control; 3. Mono species biofilm of *S.mutans*; 4. Monospecies biofilm of *C. albicans*; 5. Dual species biofilm of *S.mutans* and *C.albicans* .

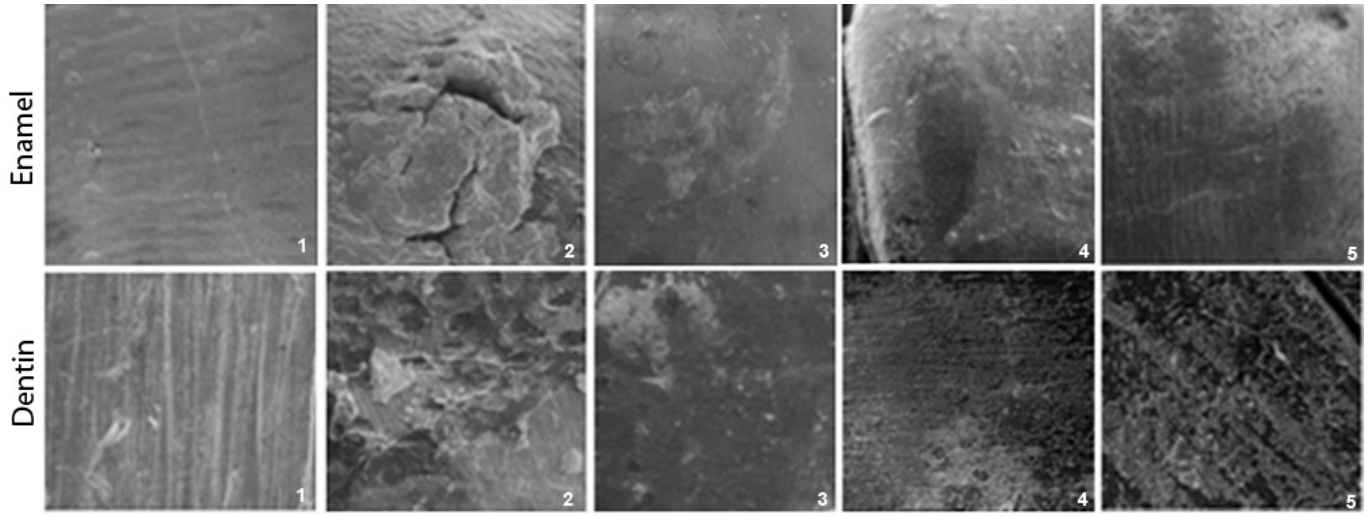

Fig. 2: Surface demineralization at the end of 30 days (Magnification 40X)
Foot note: Exposed to:1. BHI; 2. Lactic acid; 3. Mono species biofilm of *S.mutans*; 4. Mono species biofilm of *C. albicans*;5. Dual species biofilm of *S.mutans* and *C.albicans* .

demineralize dentine, but dual species biofilm caused increased demineralization of dentine. It was reported that *C.albicans* caused dentine demineralization due to lowering of pH by the *S. mutans* biofilm. A higher acidogenicity was due to increased production of lactic acid in the dual species as compared to mono species biofilm. The dual species biofilm had more volume and high quantity of insoluble exopolysaccharides which changed the biofilm matrix composition and structure. These changes provided a virulent factor for the formation of a cariogenic biofilm. The dual species film was more porous which could have been due to the larger surface area to volume ratio of *C. Albicans* cells.

Contrastingly, the dual species biofilm is reported to behave differently in enamel. A study[25] using the in-vitro biofilm model, assessed enamel carious lesion on the bovine enamel slab by investigating the surface micro-hardness, transversal microradiography and pH after 72 hours of biofilm formation. The results were as follows: Candida single species caused lesser change in surface microhardness as compared to *S. mutans* and dual species. The medium pH was recorded as 4.5, 7.5 and 6.1 for *S.mutans* and *C.albicans* mono species and dual species respectively. It was reported that *C. albicans* reduce the cariogenic potential of *S. mutans* biofilms; increase the pH of the medium and thus show low enamel demineralization potential.

Additionally, one another study[26] used an *in vitro* biofilm model and assessed the medium pH, lactic acid production capacity, and calcium release from hydroxyapatite disk exposed to 72 hours dual species biofilm of *S.mutans* and *C. albicans*. The results indicated that *C.albicans* is not a cariogenic micro-organism. It can metabolically drive alkalization within the biofilm by consuming lactic acid which in turn decreases demineralization and reduces the cariogenic capacity.

The findings of the literature reports[22,25,26] are confirmed by the present study. However, there are differences in the compared studies. The biofilm assessed in the present study is 30 days old versus the 72-96 hours mature biofilm reported in the literature.[22,25,26] Also, in previous reports, bovine tooth slab and hydroxyapatite disc have been used in contrast to the human tooth used here. However, results are comparable as human and bovine enamel and dentine show similarity.[29]

This is a novel study that has assessed demineralization in both enamel and dentine surfaces using dual species biofilm on human teeth. However, two important lacunae can be pointed out. Firstly, the outcome measures have been recorded only once after 30 days and periodic assessment would have been more confirmatory. Secondly, the results are based on pictorial (subjective) assessment and no objective measurements were made. Based on the results of the study, future research can be planned. Firstly, to confirm and verify the results established with a bigger sample size; Secondly using objective markers to study the biofilm; and finally investigating the potential if any, of this dual species biofilm as anti-caries agent. However, *C. albicans* cannot be introduced into a healthy mouth to get the dual species biofilm benefit, it can be proposed that other non-pathogenic fungal species should be pooled with *S. mutans* and this combination should be tested for efficacy.

Conclusion

The results of the present study indicate less surface demineralization in enamel as compared to dentine when exposed to dual species biofilm of *S.mutans and C.albicans*. Also, the dual species colonization indicates a positive finding of curbing dental caries in enamel.

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