Diversity of Site-Specific Microbes of Occlusal and Proximal Lesions in Severe- Early Childhood Caries (S-ECC)

Kausar Sadia Fakhruddin
University of Sharjah
Lakshman Perera Samaranayake
The University of Hong Kong, Hong Kong Special Administrative Region
Rifat Akram Hamoudi
University of Sharjah
Hien Chi Ngo
The University of Western Australia
Horoshi Egusa (egu@dent.tohoku.ac.jp)
Tohoku University Graduate School of Dentistry

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Abstract

Background: Severe-early childhood caries (S-ECC) is a global problem of significant concern, commonly manifest as a dentinal lesion on the occlusal and proximal surfaces of the affected deciduous dentition. Although there are major ecological differences between these two niches, it is unclear whether these are reflected in the composition of their dysbiotic cariogenic microbiome. Therefore, we compared the compositional differences in the microbiota of occlusal and proximal caries lesions in S-ECC.

Methods: Deep-dentine caries samples (19-occlusal and 19-proximal) from asymptomatic primary molars of children with S-ECC (n=19) belonging to caries-code 5/6, according to ICDAS classification, were evaluated. Employing two primer pools, we amplified and compared the bacterial 16S rRNA gene sequences of the seven hypervariable regions (V2 to V4 and V6 to V9) using a next-generation sequencing based assay.

Results: Bray-Curtis dissimilarity data indicated that occlusal lesions had a more homogeneous microbial community structure than the proximal lesions with significant compositional differences at species level (p=0.01; R-value of 0.513). Together, the occlusal and proximal niches harbored 263 species, of which 202 (76.8%) species were common to both locales, while 49 (18.6%) and 12 (4.6%) disparate species were exclusively isolated from the proximal and occlusal niches, respectively. The most commonly found genera at both locales included *Streptococcus*, *Prevotella*, and *Lactobacillus*, with 33, 27, and 22 species each, respectively. In addition, *Streptococcus mutans* predominated in the proximal cavities ($p \leq 0.05$), as opposed to *Atopobium parvulum* (p=0.01) in the occlusal niches, while *Vellonella alcalescens* was present in similar proportions in both habitats ($p \geq 0.05$).

Conclusions: Distinct differences between the caries microbiota of occlusal and proximal caries in S-ECC exist. The former niche appears to provide a habitat for a more homogeneous growth of communal microbiota than the latter. This may be due to the conditions prevalent in relatively quiescent inter-proximal regions, as opposed to the occlusal regions exposed to the ebb and flow of salivary and masticatory forces, and/or the anatomical and structural differences in the two locales. The clinical implications of these findings in terms of the rate and severity of caries progression remain to be determined.

Background

The single most common chronic disease condition of childhood is considered to be dental caries which affects 60–90% of all school children [1, 2]. Severe early childhood caries (S-ECC) is an aggressive variant of dental caries. It is classified by the presence of a decayed, missing (due to caries), or filled tooth (dmft) index score of ≥4 (age 3), ≥5 (age 4), or ≥ 6 (age 5) [3]. S-ECC is a destructive disease and causes acute pain and sepsis, potential tooth loss, and poor quality of life of the affected individual. This is further exacerbated by poor nutrition and retarded school participation due to dental pain and infection

[4]. Furthermore, S-ECC is a risk factor for caries of permanent teeth [5], and affected children are more likely to develop recurrent caries [4, 6].

The aetiopathogenesis of dental caries is complex [7]. Polymicrobial plaque biofilm communities of bacteria on enamel surfaces of deciduous teeth embedded within a sumptuous extracellular matrix initiate and perpetuate the caries lesions. This is further assisted by recurrent sugar pulses derived from dietary carbohydrates frequently consumed by these children [8, 9]. Carboxylic acids produced by biofilm metabolic processes are the root cause of the dissolution of the hydroxyapatite enamel matrix [10, 11]. If unabated, this destructive process, leads to the lesion progression into coronal dentin by a diverse group of microbiota co-existing in a dynamic ecological equilibrium within the biofilm [10, 12].

It is known that the caries progression from enamel to dentine is associated with the compositional transformation of the microbiome with an assortment of proteolytic, amino-acid degrading microbes predominating in the deeper dentinal lesions, in addition to the saccharolytic acidogenic/aciduric microbes [12]. Such differences in the enamel and dentinal caries progression may be due to their histological and structural differences. Compared to enamel, dentin is less mineralized, with mineral densities for normal enamel and dentin ranging between 2,170–3,100 mg/ml and 1,290–1,530 mg/ml, respectively [13, 14]. About a third of the dentine matrix comprises easily soluble organic material - primarily collagen, constituting approximately 90% of the organic matrix [15].

The ecosystem of such cavitated deep dentinal carious lesions is unique in that the sheltered locales are almost unreachable to routine oral hygiene measures. Additionally, lack of salivary flushing mechanisms further compounds biofilm accumulation, and this, together with sucrose-rich food and their products retained over a prolonged period in deep retentive niches, provides a steady and ready nutrient source for the resident biofilm flora [16, 17].

Both streptococci and lactobacilli have long been recognized to play a critical role in dental caries, although recent work indicates clearly that fungi, predominantly *Candida* species, play a critical role, particularly in the deep cavitated caries lesions [18]. Indeed, in a recent study, we have unequivocally demonstrated the profusion of candidal species in S-ECC [19]. Furthermore, the current consensus is that caries, in general, is not caused by specific organisms, such as *Streptococcus mutans* and lactobacilli, but by a polymicrobial consortium of cariogenic species. For instance, recent studies have identified *Bifidobacterium, Scardovia, Veillonella, Granulicatella, Fusobacterium, Prevotella*, and *Actinomyces* species as major contributors to ECC [12, 20–23].

S-ECC is common on both the occlusal and proximal surfaces of teeth. While the retentive occlusal pits and fissures of enamel are the common sites of early lesion formation, interproximal surfaces too are particularly susceptible to S-ECC [24]. This may be due to the proximal habitats being protected physically and under-exposed to the regular flushing action of saliva, masticatory forces, and tongue movements compared to the occlusal habitats [24, 25]. Moreover, according to Worthington et al. (2019), none of the available toothbrushing techniques is adequate for removing or disrupting the interdental supragingival biofilm [26]. Last but not least, the detection of proximal caries lesions is challenging even

for experienced professionals unless examined with extreme care, using imaging techniques [27]. In patients with a high-caries risk, the proximal dentinal lesions progress even after receiving micro-invasive treatment [28]. Due to these intrinsic differences between occlusal and proximal caries, it is likely that they harbor a unique plaque microbiota.

We, therefore, hypothesized that the core microbiota of occlusal and proximal caries in S-ECC is likely to be fundamentally different. Therefore, this study aimed to compare the microbial profile of occlusal and proximal caries in S-ECC using a next-generation sequencing (NGS) assay. The current report, to our knowledge, is the first to describe the differences in the microbiota of occlusal and proximal caries in S-ECC.

Material And Methods

Ethics statement:

The Research Ethics Committee, University of Sharjah, approved the protocol (REC-18-02-18-03) of the study. Nineteen children, aged-48-months to 72-months, attending routine Pediatric teaching clinics at the University Dental Hospital Sharjah (UDHS), United Arab Emirates, were invited to participate in the study. Before the oral examination, verbal and written informed consent was obtained from each child participant recruited in the study.

Study Subjects and dental examination:

A complete dental examination was carried out for all healthy, cooperative participants. Children with five or more decayed teeth and at least two asymptomatic primary molars in different mouth quadrants with either occlusal or proximal caries lesions involved were selected.

Caries diagnosis

World Health Organization (WHO) criterion of decayed, missing, and filled (dmft) tooth index was used to record caries status. To ascertain the severity of cavitated lesions as either caries code-5 or code-6, according to ICDAS- caries criteria [29]. One trained pediatric dentist (KSF) conducted a clinical examination and sample collection throughout the study.

The examiner determined the severity of cavitated lesions according to ICDAS classification, viz. code -5 being a distinct cavity with visible dentin involving less than half of the tooth surface, and code -6, a distinct and extensive cavity with visible dentin affecting more than half of the surface.

The exclusion criteria were children on antibiotics over the last 4-weeks before sample collection, those wearing orthodontic appliance/s or with congenital tooth anomalies, or any likelihood of pulp exposure during the caries excavation process. Further, dentin samples from endodontically treated teeth or when gingival bleeding contaminated the cavity during the sample collection process were also excluded.

Sample collection and DNA extraction:

A total of 38 infected-dentin samples from 19 children were aseptically collected by a single trained collector (KSF) from an occlusal and a proximal, symptom-free, caries active, deep-dentin lesions belonging to ICDAS caries-code 5 and code 6.

Dentin samples were collected using a sterile spoon excavator after cleaning and drying the cavities with a prophy brush without using prophy paste, as described in a previous study [30]. The collected samples were placed in an Eppendorf centrifuge tube (1.5ml) containing 300 µl of phosphate buffered saline (PBS) and immediately frozen at -20^{o}C until further use for NGS run.

DNA extraction of the collected infected-dentin samples was performed using MasterPure™ Complete DNA and RNA Purification (Epicenter, USA), following the manufacturer's guidelines. The extracted DNA's quality and quantity were assessed using a Colibri Microvolume Spectrometer (Titertek-Berthold Detection Systems GmbH, Germany). Extracted DNA samples were considered pure if the A260/280 ratio was higher than 1.8, and the A260/230 estimates were in the range of 1-2.2. Intact dsDNAs were measured using Qubit-DNA quantitation (Qubit4 Fluorometric Quantitation, Thermo Fisher Scientific, USA) before NGS.

16S rRNA gene amplicon sequencing of the caries-dentin samples:

Bacterial samples from deep-infected carious dentin were sequenced using the Ion S5XL semi-conductor sequencing system (Thermo Fisher Scientific, USA). For the preparation of amplicons, a combination of the two sets of primers [primer set-1 V2-4-8 and primer set-2 V3-6, 7-9] (Ion 16S metagenomics kit) was used for selectively amplifying the corresponding hypervariable regions of the 16S rRNA region of bacteria [31]. For each sample, two reactions were prepared (one with each set of primers), using 'water' as negative control and 'diluted *E. coli* DNA' as negative-positive control. The amplified products were then purified using Agencourt AMPure XP Reagent, Beckman Coulter. Thus, purified amplicons were quantified using DNA highly sensitive reagents in Qubit 3 fluorometer, and ~10ng of the purified amplicon from each primer set were pooled. The library was prepared utilizing Ion Plus Fragment Library Kit (Catalog #4471252, Thermo-Fisher Scientific) as per manufacturers' instruction.

In brief, the pooled amplicons were end-repaired using end repair enzyme followed by purification using 1.8 volumes of Agencourt AMPure XP Reagent. The purified end-repaired amplicons were ligated with adapters and unique barcodes followed by nick repair. Thus, the prepared library was purified using 1.4 volumes of Agencourt AMPure reagent, and the purified library was quantified using Ion universal library quantitation kit (Thermo Fisher Scientific). The libraries were further diluted to 10 pM and pooled equally with sixteen individual samples per pool and were amplified using emulsion PCR on Ion One Touch2 instruments (OT2) followed by enrichment on Ion One Touch ES following manufacturers instruction. Thus, prepared template libraries were then sequenced on the Ion S5 XL Semiconductor sequencer using the Ion 520 Chip.

16S rRNA Data Analysis and Taxonomy assignment:

The metagenomics data was analyzed using Ion Torrent Software Suite version 5.4. Following sequencing, the individual sequence reads were filtered to remove low-quality sequences. Quality control of sequencing reads retained sequences with a length between 120 and 350 bp. All quality-approved, trimmed, and filtered data were exported as unaligned BAM files to the Ion Reporter software (version 5.10) (Thermo Fisher Scientific) [32] where the sequences from the polymicrobial dentin samples where aligned to the following databases; Greengenes (version 13.5), MicroSEQ and 16S reference library (version 2013.1) to generate the BAM files.

To calculate downstream diversity measures using alpha and beta diversity metrics, 16S rRNA operational taxonomic units (OTUs) were defined at ≥97% sequence homology. After excluding singleton reads, using UPARSE [33], OTUs were constructed by clustering reads with a minimum pair-wise identity of 97%. All clustered-quality-checked reads were then mapped to each OTU with 97% similarity using UPARSE. Using the ChimeraSlayer utility, chimeras were identified and removed from the analysis [34]. All reads were classified to the lowermost possible taxonomic rank using QIIME and a reference dataset from the HOMD [35]. Through the vegan package of R (R-Vegan 2.4-2 package), the number of OTUs was calculated following rarefaction to 3,000 reads/sample. It was employed as an index of bacterial diversity in the present study. Following this, we proceed with alpha diversity (Shannon) and beta diversity (Bray-Curtis) metrics after normalizing abundance of <1% counts.

Statistical Analysis:

To evaluate the significant differences between occlusal and proximal dentin samples, continuous variables were compared using the *t*-test. Microsoft Excel 2019 (Microsoft Office, 2019) was used for statistical calculations. A Bray-Curtis dissimilarity matrix table was used as input for Multi-Dimensional Scaling (MDS). To test whether occlusal and proximal groups samples significantly differed in their microbiota, we used the ANOSIM (Analysis of Similarities) test in R. A *p*-value of ≤0.05 was considered significant.

Results

Deep dentinal caries samples derived from 19-occlusal and 19-proximal sites from asymptomatic primary molars of 19 children (mean-age 5.1 ± 0.76 years) with S-ECC were evaluated.

Before quality filtering, the 16S rRNA gene sequencing platform yielded a total of 5,088,337-proximal and 4,366,100-occlusal reads from the sequencing run containing V2-V9 (except V5) regions of bacterial 16S rRNA gene. After the quality filtering of all sequences (i.e., the removal of chimeras and de-noising -up to OTU assignments with >97% identity), the Ion Torren sequencing platform generated an average of 288,144 read/proximal and 229,182 reads/occlusal samples, with the mean length of 219 and 177 bp, respectively.

At the phylum level, both the occlusal and proximal caries microbiota were dominated by Firmicutes followed by Actinobacteria, Bacteroidetes, and Proteobacteria in descending order of predominance. In

addition, a low proportion of Fusobacteria, Spirochetes, and Synergistetes phyla also habituated the occlusal and proximal cavities (Fig. 1).

On the other hand, the predominant genera in both the occlusal and proximal niches were *Streptococcus, Prevotella*, and *Lactobacillus*, with 33, 27, and 22 species each, respectively, (Fig. 2a-e).

A total of 253 predominantly acidogenic and acidophilic species inhabited both the occlusal and proximal eco-niches, some of which were found solely in either the occlusal or proximal cavities. For instance, both the occlusal and proximal niches harbored 263 species, of which 202 (76.8%) species were common to both locales, while 49 (18.6%) and 12 (4.6%) disparate species were exclusively isolated from the proximal and occlusal niches, respectively (Fig. 3).

S. mutans was the most prevalent species isolated, but with a significant presence in the proximal cavities ($p \leq 0.05$). In addition, both the proximal and occlusal locales had an abundance of *Veillonella alcalescens* ($p \geq 0.05$).

Significant differences in both the bacterial composition and diversity between the occlusal and proximal caries lesions were noted ($p < 0.05$). Proximal caries lesions exhibited a greater diversity in the overall microbial population compared to the occlusal locales. We observed 31 highly prevalent species in proximal caries lesions, in contrast to only three species in the occlusal lesions ($p < 0.05$).

Atopobium parvulum, belonging to phylum Actinobacteria, was the most significantly abundant resident of the occlusal in comparison to proximal cavities ($p = 0.01$). In terms of the minor abundance species, *Olsenella genomosp* and *Aggregatibacter actinomycetemcomitans*, belonging to phyla Actinobacteria and Proteobacteria, were significantly higher ($p \leq 0.05$) in the occlusal, in comparison to the proximal niche.

Proximal locales harbored several significantly prevalent caries-associated microbiota, in descending quantitative frequency: *Haemophilus influenzae* ($p=0.01$) followed by *Propionibacterium acidificiens* ($p=0.01$), *Prevotella denticola* ($p=0.01$), *P. histicola* ($p=0.01$), *Bifidobacterium dentium* ($p \leq 0.05$), *P. melaninogenica* ($p \leq 0.05$), *Leptotrichia sp.* ($p=0.01$), *Corynebacterium matrochutii* ($p \leq 0.05$), *Lactobacillus gastricus* ($p \leq 0.05$), and lastly, *P. multisaccahrivorax* ($p \leq 0.05$). Further detailed microbial analysis showed the moderate presence of *Neisseria bacilliformis* ($p=0.01$) and *Campylobacter gracilis* ($p \leq 0.05$) in the proximal cavities compared to occlusal niches.

In terms of abundance, six different species (*Lactobacillus ghanensis*, *Veillonella parvulum, Selenomonas genomosp, S. noxia, Selenomonas spp.*, and *Granulicatella adiacens*) from the most dominant phylum Firmicutes were noted in proximal caries ($p \leq 0.05$; Fig. 4a). In addition, the latter niches harbored significantly more species of Proteobacterial (*Campylobacter concisus, Aggregatibacter segnis, Cardiobacterium hominis, C. valvarum*, and *Eikenella corrodens*) and Actinobacterial (*Olsenella profuse, Scardovia inopinata, Parascardovia denticolens and Actinomyces naeslundii*) species ($p \leq 0.05$; Fig. 4b-

c). These were in addition to *Capnocytophaga granulosa* (p=0.01) and *Tanerella sp*. belonging to Phylum Bacteroidetes, which were significantly more common in the proximal cavities ($p \leq 0.05$; Fig. 4d).

Additionally, we noted a heavy presence of collagenolytic bacteria in our samples, implying the critical role they play in dentine collage digestion, in the deep caries niches. Thus, in varying proportions, both locales harbored 12 species, belonging to eight genera, namely, *Prevotella, Fusobacterium, Bifidobacterium, Streptococcus, Scardovia, Selenomonas, Veillonella* and *Aggregatibacter* that are known for their collagenolytic attributes. All species except *A. actinomycetemcomitans* was significantly higher in proximal rather than the occlusal samples (Fig. 5).

Significant diversity was noted when the overall intra and inter-individual microbial community diversity within the proximal and occlusal samples was measured using Shannon metrics for α and β diversity [36]. The mean species diversity (i.e., α diversity), namely abundance and evenness of the microbiota in the proximal habitats was significantly higher than the occlusal habitats (p <0.05), (Fig. 6a).

Similar differences were noted in the β diversity of the microbiota between occlusal and proximal cavitated-locales (Fig. 6b). Multidimensional scaling (MDS) of the Bray-Curtis dissimilarity matrix was employed to ascertain differences in microbial community makeup for each sample. The majority of occlusal samples demonstrated a more analogous microbial community structure, as manifested by their proximity to each other in the MDS plot (Fig. 6b). In contrast, in the proximal samples, a more widespread pattern was noted, with a number of outliers reinforcing the diverse nature of the microbiota of the latter niches. In other words, the proximal caries lesions spanned a multivariate space, indicating a compositional microbiome difference within a distinct habitat, as measured by MDS at the species level (stress = 0.007). Furthermore, as can be seen in the MDS plot, the latter difference was significant with a p-value of = 0.01 and a high R-value of 0.513 (Fig. 6b) [36].

Discussion

The oral cavity comprises different habitats, such as the teeth, tongue, gingival sulcus, that provide a complex ecosystem for differential microbial growth [37, 38]. The sheltered, cavitated-trenches of dentin-caries lesions, particularly in S-ECC, appear to be a unique habitat in this context [10, 39], as reinforced by the complex, polymicrobial consortia noted in the occlusal and proximal cavitated caries milieus in the present study. As far as we are aware, this is the first report to illustrate the richness and the diversity of site-specific microbes of occlusal and proximal caries lesions in S-ECC and compare the compositional differences and diversity of taxa in these two sampled sites.

Proximal caries lesions harbor a richer and diverse microbiota than occlusal caries lesions

Using established metrics, Bray Curtis dissimilarity analysis and Shannon diversity indices, the microbiota of occlusal and proximal deep dentinal caries lesions in S-ECC were found to be distinct in terms of the richness of high- or low- abundant taxa and species (Fig. 6b). Furthermore, MDS assessment

indicated clearly that caries-microbial consortia from the proximal and occlusal samples of the same patient did not typically cluster together.

This phenomenon may be due to the anatomical and structural differences of the occlusal and proximal surfaces of deciduous teeth, and/or the intrinsic ecological differences in these two localities. For instance, the occlusal niches are constantly exposed to the ebb and flow of saliva with its arsenal of immune challenges, and the masticatory forces due to the intermittent food intake accompanied by the incessant tongue movements. Further, it has a much more dynamic environment than the proximal niches in between teeth, which are more sedate and well protected from such extrinsic stresses [30, 40]. In addition, the sheltered proximal cavitated locales are almost unreachable to routine oral hygiene measures [30, 40]. Thus, it is tempting to speculate that the inherent features overarching the proximal and distal caries ecosystems may be the keys reasons for the significant diversity in the microbiota of these two sites.

S. mutans **and** Veillonella alcalescens **are the two most prevalent species recovered from all caries sites**: We noted that *S. mutans* and *Veillonella alcalescens* were the most prevalent species, amongst all evaluated caries sites. There is an ample narrative on the link between *S. mutans* and dental caries due to its superior acidogenic and aciduric potential [12, 41, 42]. Our data confirms a report by Aas et al. (2008), who also noted that the most prevalent species in either the occlusal or proximal caries of children with S-ECC are *S. mutans* [12]. Another observation that substantiates the work of the latter group (2008) [12] is the profusion of *Veillonella alcalescens*, which we noted in both the occlusal and proximal deep-dentine locales ($p \geq 0.05$). Classically, *Veillonella* spp. and *S. mutans* are known to be co-located and intimately associated with the caries process. The former is thought to nutritionally metabolize the carboxylic acids produced by streptococci, and thereby suppress the cariogenicity of the *mutans*-group of streptococci [43]. On the contrary, others have noted that *Veillonella alcalescens* and *S. mutans* in tandem produce more acids than each of the species separately [44]. *Veillonella* species also easily coaggregate with various oral microbes, including *Streptococcus* spp. [45] thus suggesting a high degree of synergism and mutualism between them [44, 46], as was noted here.

Atopobium parvulum is the most prevalent species in the occlusal caries lesions

We noted a highly significant ($p=0.01$) prevalence of a lactate-producing species, *Atopobium parvulum*, predominantly in the occlusal cavities. Previous studies have also mentioned isolation of the Genus *Atopobium* from the carious dentin of children [47, 48]. *Atopobium* is not only acidogenic but is also known to be aciduric in nature [48]. The reason why this species predominates in occlusal rather than proximal lesions is unclear but could be due to the aforementioned ecological factors, as also described by Kleinberg and Jenkins (1964) [49]. They measured the salivary flow and pH in different parts of teeth and observed contrasting pH values even on different surfaces of subjacent proximal teeth, which they surmised would impact the preferential microbial colonization and the eventual propensity for caries [50].

An abundance of acidogenic and aciduric flora in the proximal cariogenic locale: Detailed analysis of our data indicated the rich and diverse presence ($p \leq 0.05$) of acidogenic and aciduric taxons, particularly in

proximal cavitated niches, in the following order: *Propionibacterium acidificiens*, *Leptotrichia sp.*, *Bifidobacterium dentium*, and species of genera *Lactobacillus*. This is not surprising as these attributes are essential prerequisites for survival in a very low pH cariogenic niche. Others, too, have reported similar findings. Gross et al. (2012) observed *P. acidificiens* in dentinal caries lesions of children in ECC [51], while Obata et al (2014) [47] noted its avidity to dentine collagen, which may be another reason for their preponderance in deep dentinal lesions, in comparison to early enamel caries. Furthermore, Downes and Wade (2009) have described the saccharolytic attributes of *P. acidificiens* with the production of acetic, propionic, and succinic acids as end products of dietary carbohydrate metabolism [52]. Other too have confirmed the aciduric potentials of *P. acidificiens* [53, 54], a criterion essential for survival in a low pH milieu of deep caries lesions.

Akin to *P. acidificiens*, the Genus *Leptotrichia*, a recognized putative cariogen, was noted in our cohorts in significant numbers ($p \leq 0.05$). Aas et al. (2008) have also described the high prevalence of *Leptrotrichia* in deep-dentine caries of deciduous teeth [12] which are known to ferment many mono- and disaccharides to lactic acid [55].

The acidogenic and aciduric Bifidobacteriaceae, which play a contributory role in caries progression [23], was also highly prevalent in proximal cavities ($p < 0.05$). In line with our observation, Becker et al. (2002) identified *Bifidobacterium* species as the most prevalent cariogen and even outnumbered *S. mutans* in dentinal caries of children [56]. Furthermore, in a very early *in vitro* study, Van Houte et al. (1996) have shown that Bifidobacteria had the potential to reduce the pH of glucose-supplemented media, upto to < 4.2 [57], adequate for demineralization of both enamel and dentine [58].

Historically, the two major cariogens were considered to be *mutans*-group streptococci and lactobacilli, and the latter is particularly known to be found in the advancing front of the dentinal caries lesions [43]. Indeed, lactobacilli are a critical secondary pathogen in dental caries [20, 59]. Hence it is not surprising that we noted a spectrum of 22 lactobacillus species in the dentine caries samples of both the proximal and occlusal lesions. Of these, three species, *L. salivarius*, *L. gastricus*, and *L. ghanensis* stood out as significantly more prevalent in the proximal cavities ($p \leq 0.05$) (Fig. 2a).

Apart from the foregoing predominant genera, less well-known others were noted in significant proportions in the proximal cavitated niches ($p=0.01$). These were Actinobacteria belonging to *Bifidobacteriaceae*, namely *Parascardovia denticolens* and *Scardovia inopinata*. Supportive of these findings, Mantzourani et al. have also identified *Bifidobacterium dentium*, *P. denticolens*, *S. inopinata* from the caries samples of primary teeth [60]. An intriguing characteristic shared by these species is their ability to degrade complex carbohydrates, including dextran [61] which potentiates the production of demineralizing acids within the cariogenic biofilm, even in the absence of fermentable carbohydrates [61]. Furthermore, another recent study has reported the synergism in acidogenicity in dual-species biofilms of *P. denticolens, S. inopinata*, and *B. dentium*, with *S. mutans* [62]. Taken together, it is clear that such microbial consortia play a decisive role in the pathobiology of deep caries lesions, in particular.

Several other attributes of the isolates mediate cariogenicity in S-ECC

There are several major pathogenic attributes of putative cariogenic flora that make them fit for their role and thus stand out as key cariogens, especially in deep dentinal lesions. These include, apart from their acidogenic and aciduric potential, potency to adhere and colonize both enamel and dentinal surfaces, collagenolytic and proteolytic potential to degrade dentinal collagen, and ureolytic properties that assist degradation of metabolic urea of biofilm microbiota. Some or most of these attributes are noted in the predominant species in both occlusal and proximal caries in this study.

Dentin tissue is essentially a hydroxyapatite mineral crystallite collagen matrix [63]. Indeed, type I collagen comprises up to 90% of the organic material of the extracellular dentinal matrix [64]. Cariogens *S. mutans* and *B. dentium* can preferentially colonize dentin as they possess adhesins (SpaA adhesins) that mediate their attachment to collagen [65, 66], while the former has additional collagen-binding proteins Cnm and Cbm [67, 68]. Hence, the duality of key attributes, their adhesive and acidogenic nature, makes *S. mutans* eminently suitable to be a key cariogen, particularly dentinal caries.

As opposed to the richness of acidogenic cariogens such as *S. mutans*, we also noted a significant proportion of health-associated ureolytic species *C. matruchotii*, *Haemophilus parainfluenzae*, and *Actinomyces naeslundii* in varying proportions in both the proximal and occlusal samples. *C. matruchotii* had been suggested as a nucleating species of the biofilm bacterial community in several studies. Welch et al. (2016) [69] noted a multi-genus conglomerate of nine taxa structured around filamentous corynebacterial cells. *C. matruchotii*, in particular, raises the biofilm pH by using acetate and lactate produced by the acidogenic plaque microbes [69]. Furthermore, significant numbers of other ureolytic species such as *H. parainfluenzae* and *A. naeslundii* were also noted in our samples, as was earlier reported by Ma et al. (2015) in S-ECC plaque [70]. The profusion of these urease-producing taxons amongst the abundant acidogenic, aciduric microbial consortia, particularly in the proximal deep-dentine milieu, is intriguing. It may be an attempt at keeping the pH to moderate survivable levels of the biofilm community. However, further studies are required to explore their role in such consortia.

It appears that microbe-mediated acidification of the plaque biofilm is not just the prime mover of caries progression [71]. The acidic milieu, in turn, can activate endogenous dentin-embedded and salivary matrix metalloproteinases (MMPs) and cysteine cathepsins, which are thought to play a significant role in caries development [63, 72] as proposed by Takahashi and Nyvad [71].

For instance, the trio, *Prevotella nigrescens, Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans* produce both intra- and extracellular gelatinolytic proteinases that may activate latent pro-MMP-9 [73]. Furthermore, in our study, *F. nucleatum* was detected in both proximal and occlusal lesions. The latter is a core constituent of dental biofilms and plays a pivotal role in bridging microbes of early and late colonizing species [74]. Thus, despite their relatively low numbers, the synergistic impact of the proteases of the trio, *F. nucleatum, A. actinomycetemcomitans*, and *P. nigrescens*, may significantly contribute to the structural disintegration of the collagenous scaffold of the dentine, in tandem with collagenase producing other species of *Prevotella*.

Several studies have reported the high prevalence of several *Prevotella* spp., particularly in the dentine caries [21, 48, 71, 75]. Indeed, this led Teng et al. to suggest the relative abundance of a panel of seven keystone *Prevotella* spp. from caries lesions of ECC [76] [77]. Our findings, with a total of 27 Genera belonging to *Prevotella* species, with 23 species present at both the occlusal and proximal sites, and four species (*P. intermedius, P. nanceiensis, P. marshii*, and *P. fusca*) only at the proximal sites, confirm the assertions of Teng et al [76]. Others too have echoed these sentiments and surmised that the overexpression of collagenases by *Prevotella* species during proteolytic metabolism might significantly contribute to the progression of dental caries especially at the advancing dentinal front [75]. The rich aggregates of *Prevotella* Genus with such collagenolytic attributes that were recovered from our samples included *P. denticola, P. histicola*, *P. melaninogenica*, *P. multisaccharivorax*, and low prevalent, *P. nigrescens*, and *P. intermedia*. Indeed, all of these species have been previously recorded by others as isolates from cariogenic lesions [78–82] (Fig. 5). Our findings, therefore, further substantiate the view that *Prevotella* species play a leading role in proteolytic digestion and the progression of dentinal caries. However, further work is needed to ascertain the specific mechanisms by which they mediate such changes.

Collagenolytic microbes are highly prevalent in S-ECC (Fig. 5)

Although the acidogenic and aciduric attributes of *S. mutans* are well known, their ability to degrade human collagen (acid-soluble, type I) - the major constituent of dentine, by their extracellular proteases is poorly recognized. Some studies including, new metabolomic research, indicate the overexpression of collagenase gene expression in *S. mutans* in dental caries [79, 83]. As mentioned above, we had a rich harvest of *S. mutans* across all caries samples, albeit with a significant preponderance in the proximal niches. Their heavy presence in deep dentinal caries where collagen is plentiful appears to be a likely reflection of the tenacity and avidity of *S mutans* for collagen and fibronectin, as well as the abundance of peptidases and collagenases they possess [75]. Finally, in this context, a range of several other cohabitant microbes with known collagenolytic attributes [66, 79, 80, 83, 84] was also isolated from dentin eco-niches. They were, *B. dentium, S. inopinata* of the phylum Actinobacteria, *Selenomonas noxia* and *Veillonella parvulum* [79]. These consortia involved in initiation and degradation of the demineralized organic matrix of the dentinal tissues, and the activation of host-derived proteases ratifies and add credence to the ecological hypothesis of dentine proposed by Takahashi and Nyvad [71], as well as the tissue-dependent caries propagation hypothesis of Simon et al. [75] which states that while acid-producing bacteria are the prime movers of enamel penetration, the dentin degrading collagenolytic organisms which destroy deeper dentinal tissues are co-contributors in caries propagation.

Microbiota in caries lesions may act as reservoirs for other local and systemic infections

S-ECC, if not intervened and appropriately treated, is likely to have far-reaching effects, even extending to adulthood, and impact the general health of these children. In the concluding section, we briefly discuss the possible impact of our findings on the development of local or systemic disease.

Inquimbert et al. [85] and few others [86–88] have shown that early colonization of ECC lesions by periodontopathic species may be construed as a marker of periodontal disease risk later in life. In the investigation, they identified a number of periodontopathic organisms such as *Campylobacter gracilis*, *S. noxia*, and *P. intermedia* known to be associated with the initiation and progression of periodontal infection from ECC lesions [85–88].

We also noted several pathogens such as *Campylobacter concisus, Capnocytophaga granulosa, Neisseria bacilliformis*, and *Granulicatella adiacens*, implicated in the oral-systemic disease axis, amongst caries microbiota. The two former organisms (*C. granulosa, N. bacilliformis*) are implicated in abscess development and bacteremia secondary to focal infections [89, 90]. In addition, the oral *C. concisus* strains have been associated with human irritable bowel syndrome [91, 92]. Furthermore, members of the "HACEK" group bacteria, i.e., *Haemophilus sp.*, *Aggregatibacter sp.*, *Cardiobacterium hominis, Eikenella corrodens, Kingella sp.*, and *G. adiacens*, a nutritionally variant streptococcus, all known to cause bacterial endocarditis [93, 94], were also prevalent in the dentinal caries samples. Thus, it is tempting to speculate that reservoirs of these microbes within cavitated lesions of S-ECC may act as potential reservoirs that may contribute to the foregoing systemic diseases in these children.

Limitations of the study

Our study has few limitations. The current data were derived from a relatively small sample of children and needed to be confirmed in a larger cohort, ideally from another geographic locale. Further, our report encompasses species-composition of the deep caries lesions in general but does not appertain or relate to a specific depth of the lesion. Therefore, future studies are required to evaluate the microbiome composition in dentinal cavities of varying depths to clearly understand the natural history of S-ECC and the compositional variations of the microbiota during lesion progression towards the pulpal axis.

Conclusion

Distinct differences between the caries microbiota of occlusal and proximal caries in S-ECC exist. While the occlusal lesions had a more homogeneous microbial community structure than the proximal lesions with significant compositional differences at species level. *Streptococcus mutans* predominated in the proximal cavities, as opposed to *Atopobium parvulum* in the occlusal niches. An array of collagenolytic organisms were found in both occlusal and proximal lesions indicating their critical role in the pathobiology of deep dentinal caries in S-ECC. The clinical implications of these findings in terms of the rate and severity of caries progression, and the interventional approaches remain to be determined

Abbreviations

S-ECC: Severe Early Childhood Caries

ECC: Early Childhood Caries

MDS: Multidimensional scale

HOMD: Human Oral Microbiome Database

rRNA: ribosomal Ribonucleic acid

NGS: Next Generation Sequencing

OTU: Operational taxonomic unit

QIIME: Quantitative Insights Into Microbial Ecology

Declarations

Ethics approval and consent to participate

The present study was conducted under a protocol approved by the Research Ethics Committee (REC), University of Sharjah (REC-18-02-18-03).

Consent for publication

All contributing authors consented to publication

Availability of data and material

Data of the present study were evaluated and presented in this article. Additional datasets, if required, can be provided by the corresponding author on reasonable request.

Competing interest

The authors of the present manuscript have no competing interests

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Author Contributions

KSF, together with LPS and HE, performed data collation analysis and manuscript writing; HCN and RAH critically examined and revised the manuscript. All authors gave approval for the final version to be published.

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Authors' Information

[1]Department of Preventive and Restorative Dentistry, University of Sharjah, UAE. [2]Division of Molecular and Regenerative Prosthodontics, Tohoku University Graduate School of Dentistry, Sendai, Miyagi, Japan. [3]Faculty of Dentistry, University of Hong Kong, 34 Hospital Road, Hong Kong. [4]University of Western Australia, Perth, Australia. [5]Sharjah Institute for Medical Research, College of Medicine, University of Sharjah, UAE [6]Division of Surgery and Interventional Science, University College London, London, United Kingdom

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Figures

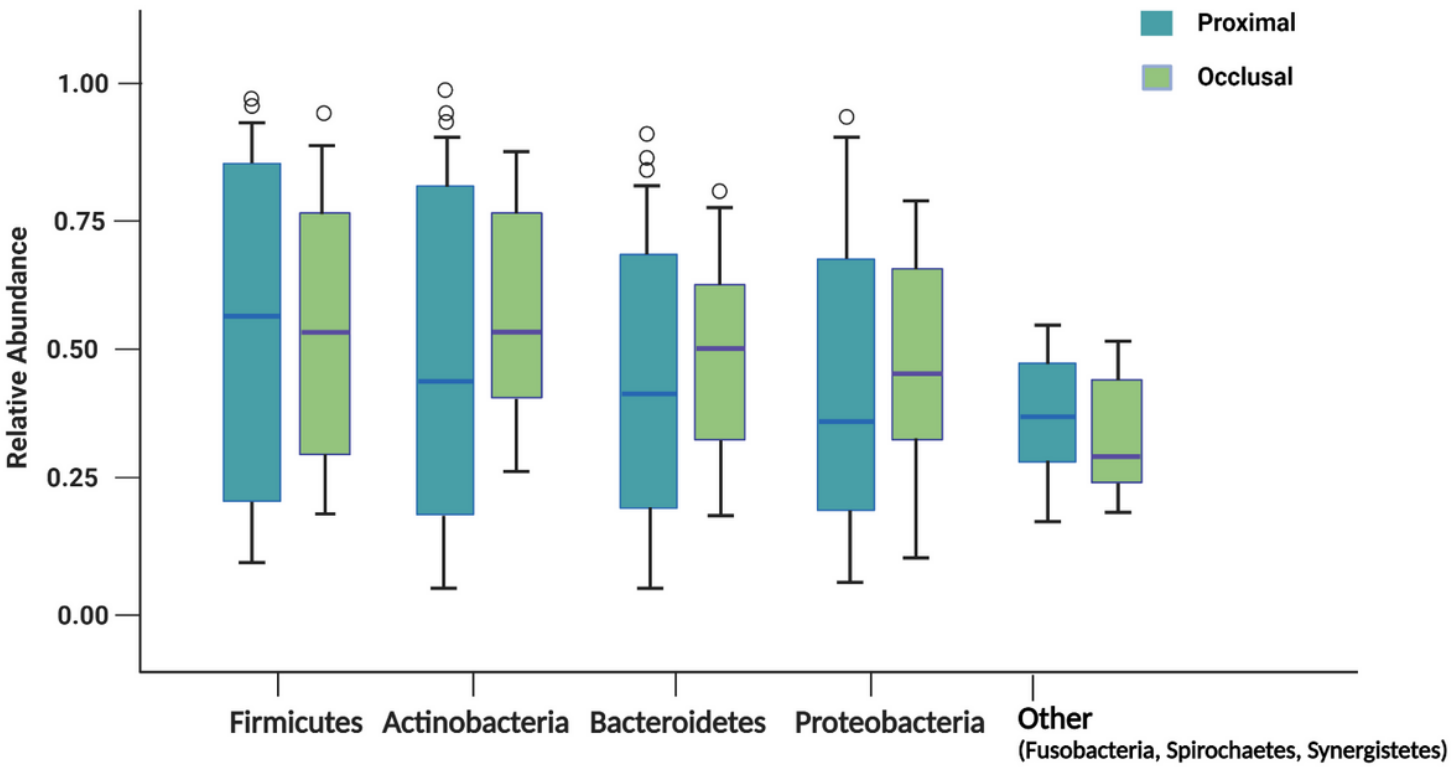


Figure 1

Box and whiskers plot representing the relative abundance of dominant phyla (Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria) and minor occurrence phyla including Fusobacteria, spirochetes, and Synergistetes by occlusal (green) and proximal (blue) caries samples. The upper and lower end of the box representing the first (Q1) and third (Q3) quartiles. The horizontal lines in the box represent the median. The whiskers representing 1.5 x interquartile range, and the circles represent outliers.

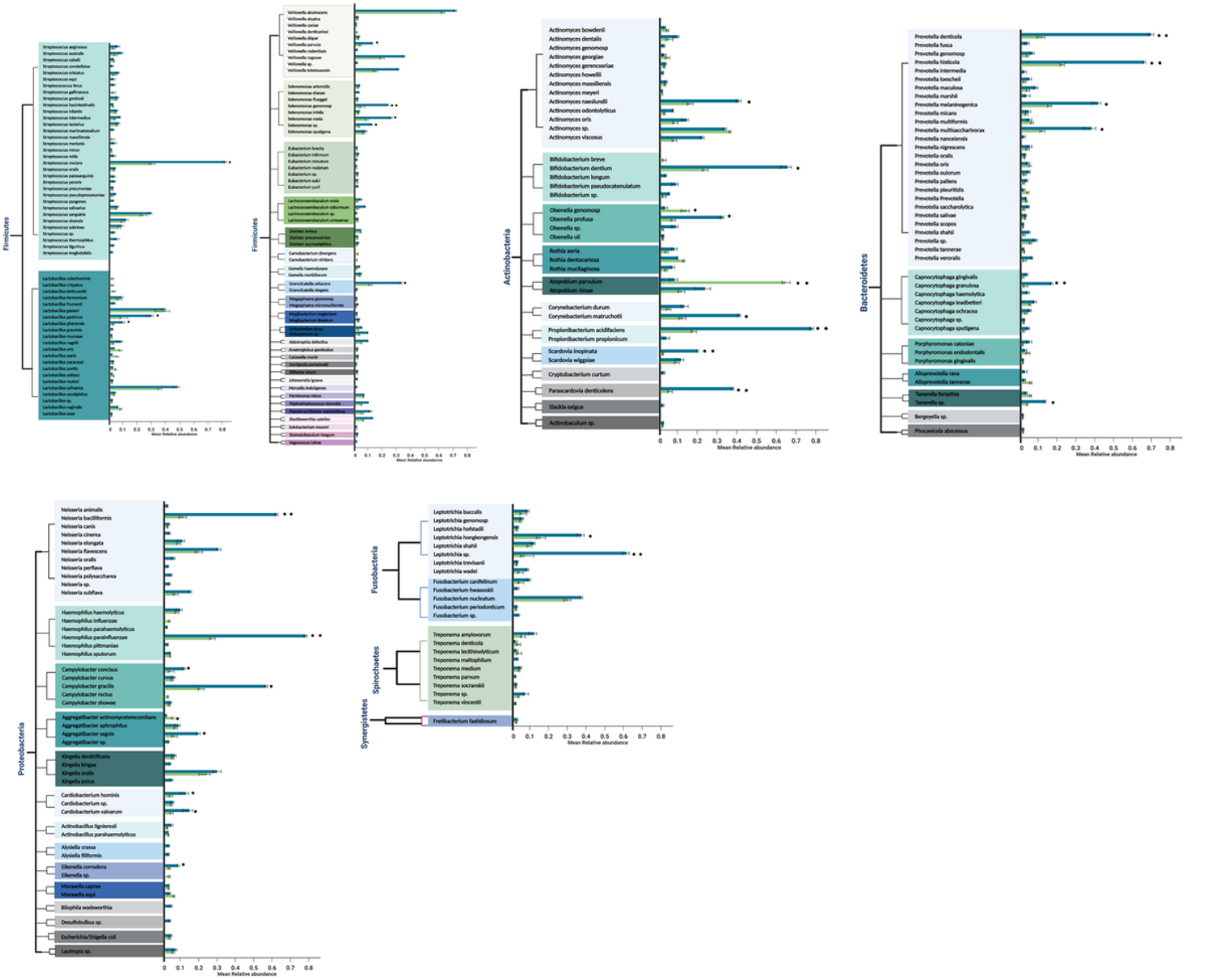


Figure 2

a: Mean relative abundances of differentially prevalent genera belonging to most dominant phylum Firmicutes in the occlusal and proximal caries samples. Error bars represent standard deviation, indicative of the variation between occlusal samples or proximal samples in relation to the frequency of occurrence of the species. Taxon showing a significant difference in abundance between proximal and occlusal samples is marked by ($p \leq 0.05$* and $p = 0.01$**). A p-value was obtained using t- test. A different color indicates each genus in the left column; occlusal caries samples are green, and proximal caries samples are blue on the right column. b: Mean relative abundances of differentially prevalent genera

belonging to phylum Actinobacteria in the occlusal and proximal caries samples. Error bars represent standard deviation, indicative of the variation between occlusal samples or proximal samples in relation to the frequency of occurrence of the species. Taxon showing a significant difference in abundance between proximal and occlusal samples is marked by asterisks ($p \leq 0.05$* and p=0.01**). A p-value was obtained using t-test. A different color indicates each genus in the left column; occlusal caries samples are in green, and proximal caries samples are in blue on the right column. c: Mean relative abundances of differentially prevalent genera belonging to phylum Bacteroidetes in the occlusal and proximal caries samples. Error bars represent standard deviation, indicative of the variation between occlusal samples or proximal samples in relation to the frequency of occurrence of the species. Taxon showing a significant difference in abundance between proximal and occlusal samples is marked by asterisks ($p \leq 0.05$* and p=0.01**). A p-value was obtained using t-test. A different color indicates each genus in the left column; occlusal caries samples are in green, and proximal caries samples are in blue on the right column. d: Mean relative abundances of differentially prevalent genera belonging to phylum Proteobacteria in the occlusal and proximal caries samples. Error bars represent standard deviation, indicative of the variation between occlusal samples or proximal samples in relation to the frequency of occurrence of the species. Taxon showing a significant difference in abundance between proximal and occlusal samples is marked by asterisks ($p \leq 0.05$* and p=0.01**). A p-value was obtained using t-test. Different color indicates each genus in the left column; occlusal caries samples are in green, and proximal caries samples are in blue on the right column. e: Mean relative abundances of differentially prevalent genera belonging to minor phyla Fusobacteria, Spirochaetes, and Synergistetes in the occlusal and proximal caries samples. Error bars represent standard deviation, indicative of the variation between occlusal samples or proximal samples in relation to the frequency of occurrence of the species. Taxon showing a significant difference in abundance between proximal and occlusal samples is marked by asterisks ($p \leq 0.05$* and p=0.01**). A p-value was obtained using t-test. Different color indicates each genus in the left column; occlusal caries samples are in green, and proximal caries samples are in blue on the right column.

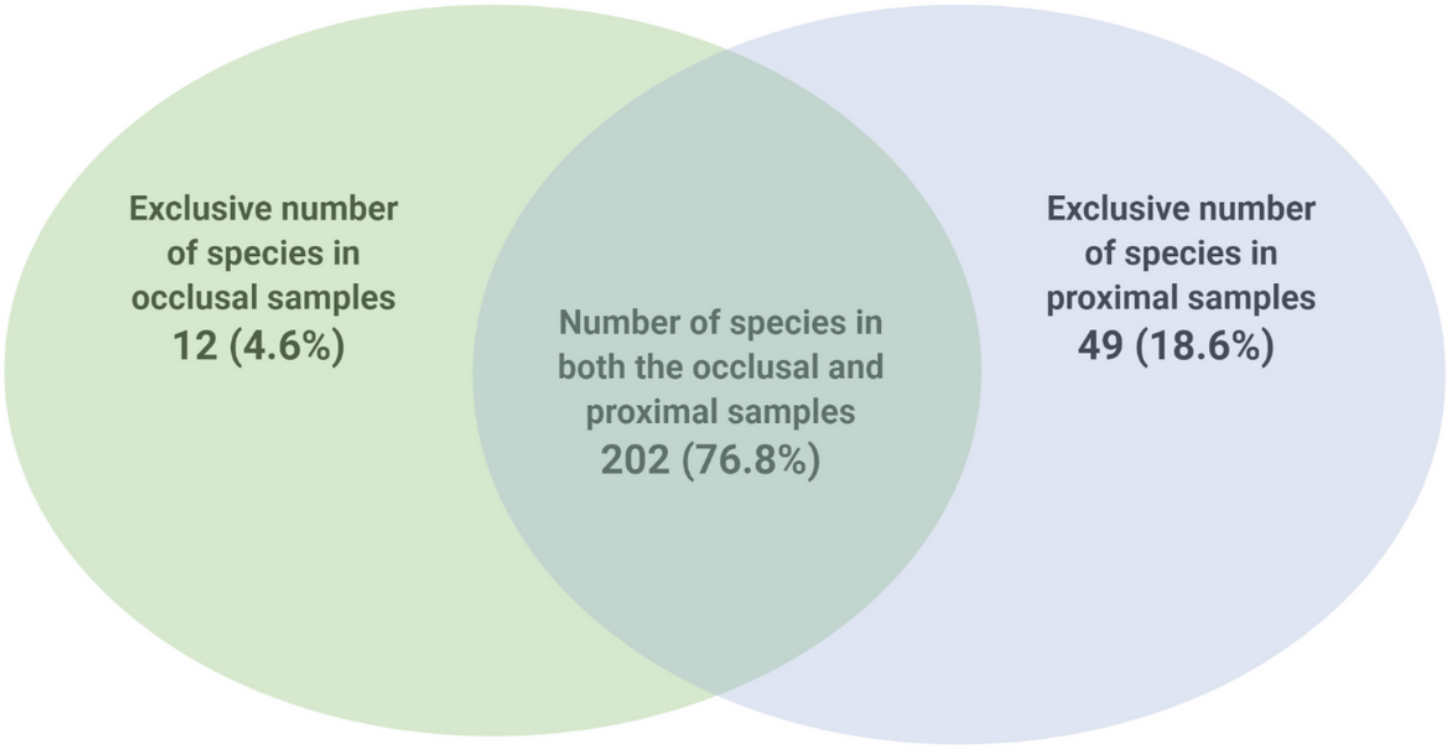
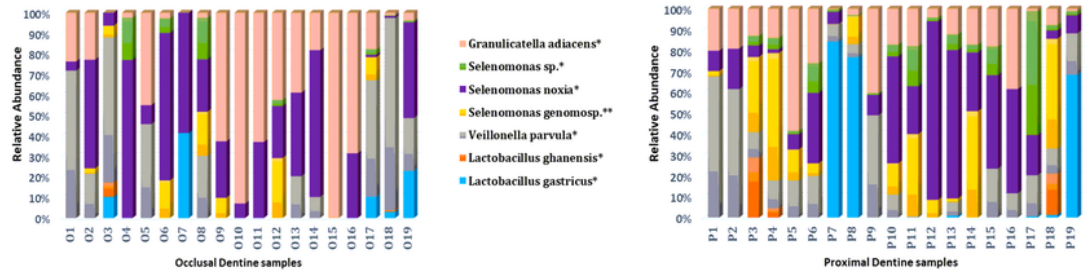
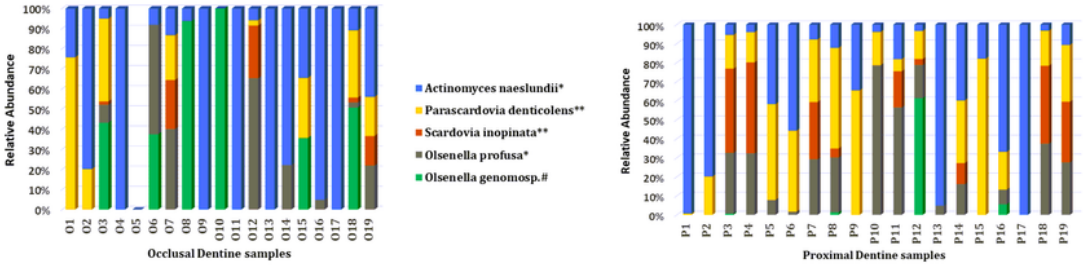


Figure 3

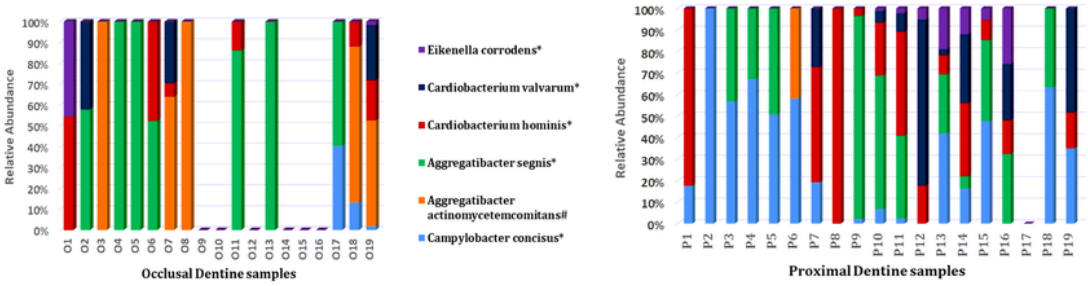
Species occurrence in 19 occlusal and 19 proximal caries samples in a cohort of 19 children with S-ECC.



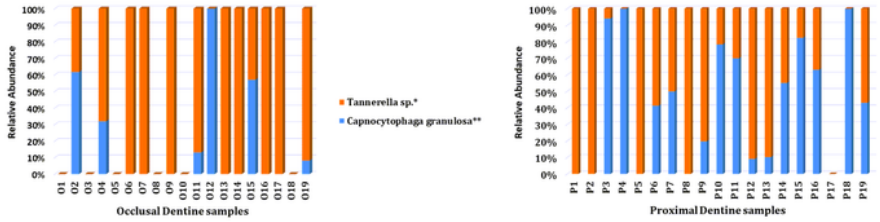
4a. Phylum Firmicutes



4b. Phylum Actinobacteria



4c. Phylum Proteobacteria



4d. Phylum Bacteroidetes

Figure 4 (a-d): Relative abundance of low occurrence taxa across both occlusal and proximal caries samples of phlya Firmicutes (4a), Actinobacteria (4b), Proteobacteria (4c), and Bacteroidetes (4d). Percentages for each taxon represent the median abundance values for each sample. Taxon showing a significant difference in abundance between proximal or occlusal dentine samples are marked $p \leq 0.05$* and $p = 0.01$** and $p \leq 0.05$#, respectively. p-value was obtained using t-test.

Figure 4

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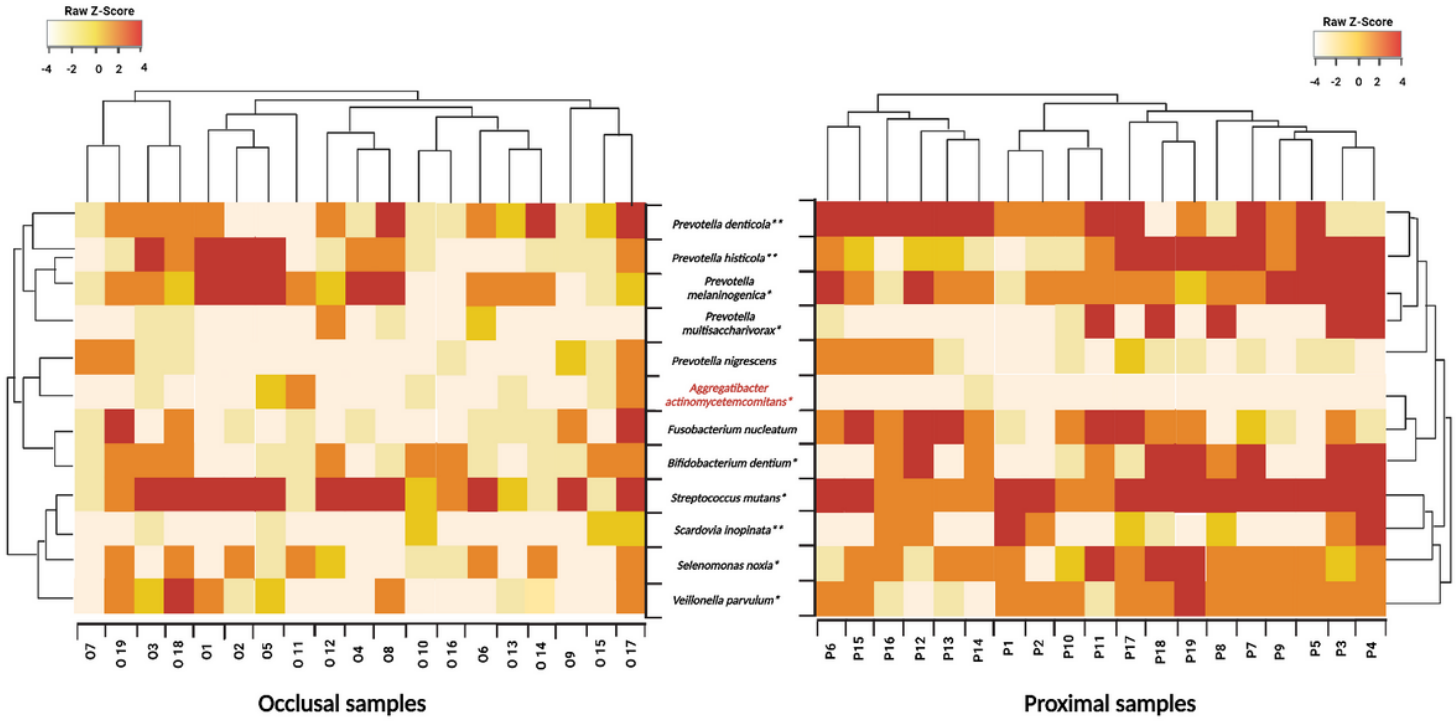

Figure 5

Heatmap showing 12 species belonging to 8 genera with known collagenolytic attributes present in the proximal and occlusal caries samples of children with S-ECC. The heat map was generated using the g-plots package by clustering proximal and occlusal infected dentin samples based on the distribution and relative abundance of the microbial species with proteolytic potential. Clustering shows the similarity of samples. The heat map scale displays the row Z score (Z score = [actual relative abundances of a species in each sample – mean relative abundance of the same species in the proximal/occlusal samples/standard deviation). The proteolytic species belonging to the eight genera are shown in the Y-axis, and the individual sample numbers are shown on the x-axis.. On the color scale, tan indicates low relative abundance, and dark brown, a high relative abundance of the given species. The gradient from tan to bright red indicates the z-score of the abundance from low to high Legends showed the Z-scores, demonstrating the relative abundance levels. Microbial taxon showing a significant difference in abundance between proximal or occlusal niches, are asterisked ($p \leq 0.05$* and $p=0.01$**). All species except A. actinomycetemcomitans had significantly higher abundance in the proximal rather than the occlusal samples. The latter (marked in red) was the only species with a high abundance in the proximal samples. p-value (≤ 0.05) was obtained using t-test.

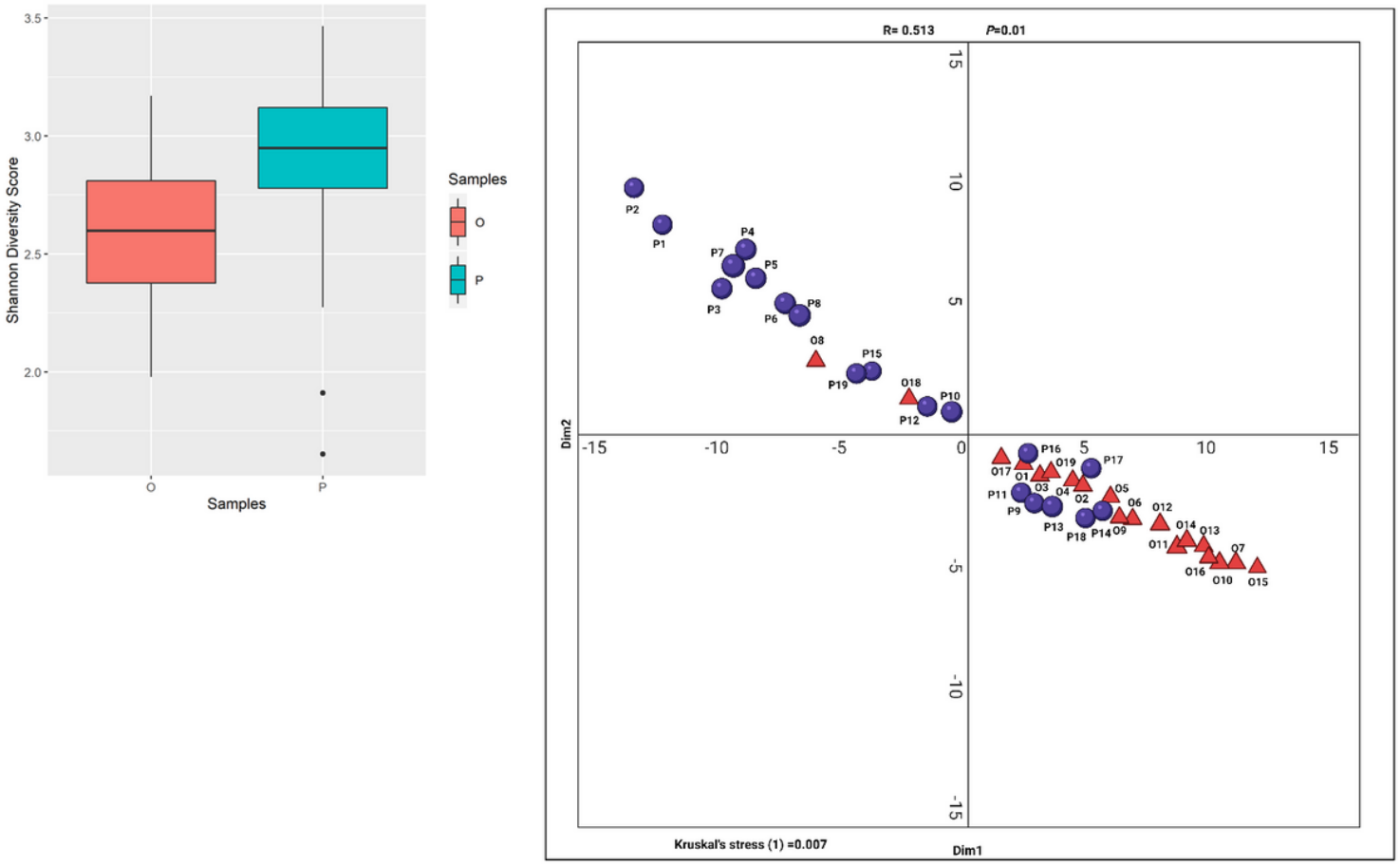

Figure 6

a: Alpha diversity (Shannon Index) of caries-dentin microbial communities. Boxplots of Shannon alpha diversity metrics grouped by sampling site (O: occlusal vs. P: proximal). Box represents the median and interquartile range. Student's t-test identified significant alpha diversity with a p-value <0.05. b: Bray-Curtis Multidimensional scaling (MDS) analysis of occlusal and proximal caries microbiota of S-ECC. The plot shows the wider spread of microbiota in proximal caries samples (purple circles) in comparison to the more concentrated clustering of occlusal caries samples in the lower right sector of the plot (red triangles). Analysis of Similarity (ANOSIM) = R= 0.513; p=0.01.