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Oral microbiota analyses of Saudi sickle cell anemics with dental caries

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ABSTRACT

Objectives: The objectives of this study were to identify the composition of oral microbiota in a cohort of patients with sickle cell anemia (SCA) and a high mean number of decayed, missing, and filled permanent teeth (DMFT) and compare it to a cohort of patients with SCA and a low number of DMFT and elucidate the effect of fetal haemoglobin levels on the oral microbiota composition.

Methods: Patients who had been diagnosed with SCA, who were homozygous for sickling β-globin mutation (β^S/β^S), who had Arab-Indian haplotype, and who ranged in age from 5 to 12 years were included in this study. Oral saliva from each participant (n = 100) was collected in GeneFiX™ Saliva DNA Microbiome Collection tube and DNA was extracted using GeneFiX™ DNA Isolation Kits. The composition of oral 16S rRNA from patients with SCA and high dental caries (n = 27, DMFT ≥5) and low dental caries (n = 73, DMFT ≤4) was analysed. Sequencing was performed on an Ion Personal Genome Machine using, Ion PGM Hi-Q view Sequencing 400-bp kit.

Results: We observed an overall increase in abundance of Proteobacteria, Chloroflexi, and Bacteroidetes in the high DMFT index group compared to those with a low DMFT index. In addition, there was an overall increased abundance of microbiota from Proteobacteria, Fusobacteria, Firmicutes, and Bacteroidetes in the patients with SCA with low fetal haemoglobin compared to those with high fetal haemoglobin ($P < .05$). *Enterobacteriaceae* species were the most significant abundant species of bacteria found in both the high DMFT index group and low fetal haemoglobin cohort ($P < .05$).

Conclusions: Our data indicate that SCA in Saudi patients with high DMFT have a higher predominance of pathogenic bacteria compared to those with low DMFT. Furthermore, SCA in Saudi patients with low fetal haemoglobin have a higher predominance of pathogenic bacteria compared to those with higher fetal haemoglobin.

Introduction

Sickle cell anemia (SCA) is a common hereditary monogenic disease prevalent in many parts of the world and, as such, represents a worldwide health burden. Reports have estimated that more than 10 million individuals in Africa, Arab countries, and India live with the disease.[1,2] SCA is prevalent in 2 major areas of Saudi Arabia: the Eastern and Southwestern provinces.[3] SCA is predominantly associated with the Arab-Indian (AI) haplotype of the β-globin gene cluster in the Eastern Province and with the Benin haplotype in the Southwestern Province.[3] Patients with SCA and the AI haplotype maintain a high level of fetal haemoglobin (HbF, $\alpha_2\gamma_2$) when compared to other haplotypes of African origin. HbF is the major modulator of SCA severity because of its ability to

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inhibit deoxygenated sickle haemoglobin polymerisation, the main cause of vaso-occlusion. However, the maintenance of high HbF is limited to the childhood period and as these children age, the HbF level declines and consequently the disease phenotype evolves into that of the African haplotypes.[3]

Oral manifestations are well recognised in SCA. Several reports have indicated an association between SCA and jaw changes.[4,5] This association has been attributed to SCA being a multisystem disease, including changes in the teeth that are caused by deficiency in the formation of dental and bone tissues with an associated prevalence of opacities in the teeth.[4-6] Additionally, several reports have also shown an association between SCA and dental caries.[7-12] Dental caries is a multifactorial disease process promoted by several factors, including the formation of microbial biofilm, diffusion process, saliva, and sugar that cause an imbalance between disease-causing and protective factors that influence the dynamic of demineralisation and remineralisation. Subsequently, the progressive dysbiosis is thought to cause demineralisation of hard dental tissue.[13,14]

Normal oral bacterial colonisation changes as a newborn ages.[15,16] These changes can be classified into 3 phases of bacterial colonisation.[15] The early colonisers that begin to inhabit the oral cavity in the first 3 to 6 months of a newborn's life include *Streptococcus, Veillonella,* and *Lactobacillus* spp. The abundance of constant colonisers, bacteria that are already present during the first 3 to 6 months of life (less than 1%), increases with time. These bacteria include the bacterial genera *Gemella, Granulicatella, Haemophilus,* and *Rothia*.[15] The late colonisers start colonising the newborn's oral cavity after the first year of life, and these include *Actinomyces, Porphyromonas, Abiotrophia,* and *Neisseria*.[15]

A healthy mouth has a balance of bacteria, but inadequate oral health narrows the range of bacteria, resulting in oral dysbiosis, a state in which beneficial bacteria decrease and potentially pathogenic bacteria increase. Oral dysbiosis results in the onset and development of dental caries. The normal oral microbiome that colonises teeth is responsible for neutralising acid production from the intake of sugars through the process of ammonia production.[17] However, the dysbiosis of the microbial community is thought to be due to overproduction of acid which favours the increases in acidogenic, acid-tolerant bacteria such as *Streptococcus mutans, Streptococcus mitis, Streptococcus sanguinis, Streptococcus oralis, Lactobacilli, Actinomyces*, and *Bifidobacteria*, which have been identified in healthy patients with a high number of dental caries.[18-20] Cohorts with low numbers of dental caries were found to harbour lower levels or none of these pathogens.[21] However, next-generation sequencing has revealed that there is a microbial diversity amongst different populations due to varying cultural habits.[22]

Diet, especially sugar intake, has a tremendous influence on the process of dental caries, which can impact the ecology of this community by biasing acidogenic and acid-resistant bacteria that are thought to drive the development and progression of dental caries.[13] The polymicrobial synergy and dysbiosis (PSD) hypothesis proposes that a synergistic polymicrobial community is necessary to fulfill the distinct roles that congregate to manipulate and alleviate dysbiosis microbiota, which disturbs host immune homeostasis.[23] The other part of the PSD hypothesis is that it focusses on the continuous process in which dysbiosis and host inflammation synergistically drive the disease, rather than answering the question "which comes first, the inflammation or the dysbiosis?"[23]

Although ample evidence indicates a causative correlation between the disruption of the oral microbiome and dental caries,[7-10] the effect in SCA has not been investigated. The main aim of this study was to identify the composition of oral microbiota in a cohort of patients with SCA with high numbers of decayed, missing, and filled permanent teeth (DMFT) and compare it to a cohort of patients with SCA with low numbers of DMFT. Furthermore, we aimed to elucidate the effect of HbF levels on the oral microbiota composition by comparing patients with SCA with low vs high HbF.

Methods

Patients

Between 2019 and 2020, oral samples and clinical data were collected from 100 patients with SCA (homozygous, β^S/β^S genotype) attending Qatif Central Hospital, Al Qatif, Saudi Arabia. Participants ranged in age from 5 to 12 years and had been clinically diagnosed with SCA. Baseline measurements were obtained from each patient's hospital records, with DMFT index measurements and general oral health being determined by a qualified dentist. The patients included in the study were subdivided according to low and high DMFT (low DMFT index = 0–4: high DMFT index = ≥5) and low and high HbF (low HbF <10%; high HbF ≥20%), as described previously.[21]

Written informed consent was obtained from the parent/guardian of all participants. The study received ethical approval from the Imam Abdulrahman Bin Faisal University Institutional Review Board (IRB) committees (Reference IRB-2013–01–113) and was conducted according to the ethical principles of the Declaration of Helsinki and Good Clinical Practice guidelines.

DNA extraction

All participants were given a GeneFiX™ Saliva DNA Microbiome Collection tube containing a stabilising solution and, prior to ingesting any food or liquid or cleaning their teeth in the morning, the subjects were requested to spit into the funnel of the tube until the 2-mL line was reached, excluding any bubbles. The tube was then shaken several times by each participant's parent/guardian in order to mix the sample with the solution in the tube. The tube, with its contents, was then stored at room temperature until collection by the research group for analysis. The abundance of microbial communities was determined by analysing the composition of oral 16S rRNA in the saliva of paediatric patients with SCA. These patients had both low and high DMFT, as determined by the DMFT index. Microbiome DNA extraction was caried out using Genefix Saliva DNA Isolation Kit (GSPN-50, Isohelix) according to the manufacturer's protocol. DNA concentrations and purity were estimated by fluorometry using a NanoDrop 2000 Spectrophotometer (Thermo Fisher).

Methods for DNA library preparation and sequencing

Amplification of the bacterial 16S rRNA genes was carried out by polymerase chain reaction (PCR) using 16S Ion Metagenomic Kit™ (Thermo Fisher). Primer sets V2, V4, V8 and V3, V6–7, and V9 were used in 2 separate PCR reactions to increase the resolving power of 16S rRNA profiling, and the reaction yielded amplicon fragments of ~250 bp, ~288 bp, ~295 bp, ~215 bp, ~260 bp, and ~209 bp, respectively. The PCR cycling conditions were denaturing at 95 °C for 10 minutes, then 20–25 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 20 seconds followed by a final extension step at 72 °C for 7 minutes. All PCR products were checked for size and specificity by agarose gel (2%) electrophoresis. After that, equal volumes of amplification product from both V2, V4, V8 and V3, V6–7, and V9 were pooled equally for subsequent sequencing. A set of 100 samples was processed for the DNA library preparation and sequencing. Purification of the pooled PCR product was done using Agencourt AMPure beads (Beckman Coulter) on a magnet support (DynaMag-2 magnet). Then, quantification of the purified product was done using Qubit high-sensitivity DNA kit (Invitrogen). End repair and adapter ligation was done to 40 nanograms of combined amplicons using Ion Plus Fragment Library Kit ™ and Ion Xpress Barcodes Adapters, 1–16 ™ (Thermo Fisher) to make the DNA library. Both these steps were followed by purification using Agencourt AMPure beads and eluted in a low Tris-EDTA buffer. Evaluation of the size and concentration of the prepared DNA libraries was done with Ion universal library quantitation kit (Thermo Fisher). Each sample was adjusted to a 10-picomolar concentration prior to pooling all 100 samples and subsequent processing with Emulsion PCR and enrichment of template-positive particles with One-Touch 2 using an Ion PGM Hi-Q view OT2 Kit (Thermo Fisher) and One-Touch ES systems (Thermo Fisher) according to the manufacturer's instructions. The enriched template-positive ion sphere particles were loaded onto an Ion 316 chip v2 (Thermo Fisher) and sequenced on an Ion Personal Genome Machine (PGM) using Ion PGM Hi-Q view Sequencing 400-bp kit (Thermo Fisher) according to the manufacturer's instructions.

Data processing

The sequence data were analysed using automated and streamlined Torrent Suite Software v5.6. Analysis was performed using QIIME2 (Quantitative Insights Into Microbial Ecology) 16S Metagenomics Analysis tool. Paired-end reads were joined using VSEARCH. Deblur was used to process Chimera amplicon removal and abundance filtering.[24] Amplicon sequences were clustered and assembled into Operational Taxonomical Units (OTUs) using closed reference clustering against the Greengenes 13_8 database via VESEARCH. A pretrained Naÿve Bayes classifier with Greengenes OTU database was used to perform taxonomic assignment. The abundance tables and data obtained from QIIME2 were put together into a Phyloseq object, and further analysis in R with custom scripts was carried out.[25] The authors are in the process of uploading the data sets generated during the current study in the European Repository.

Results

We identified a total of 416 bacterial species (the top 20 species are shown in Figure 1). The percentage of microbiota at

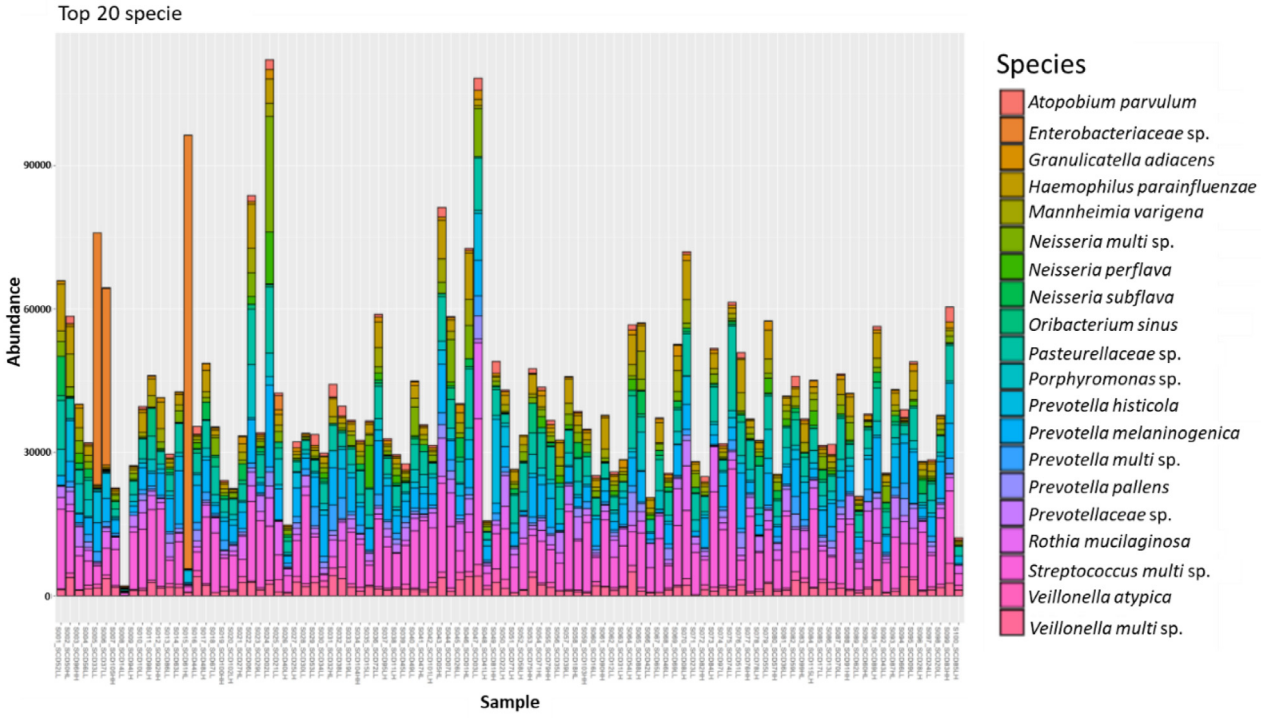

Fig. 1 – Taxonomic profiling of top 20 species in 100 samples. The stacked barplots show the Operational Taxonomical Units abundance of species as assigned by different colours.

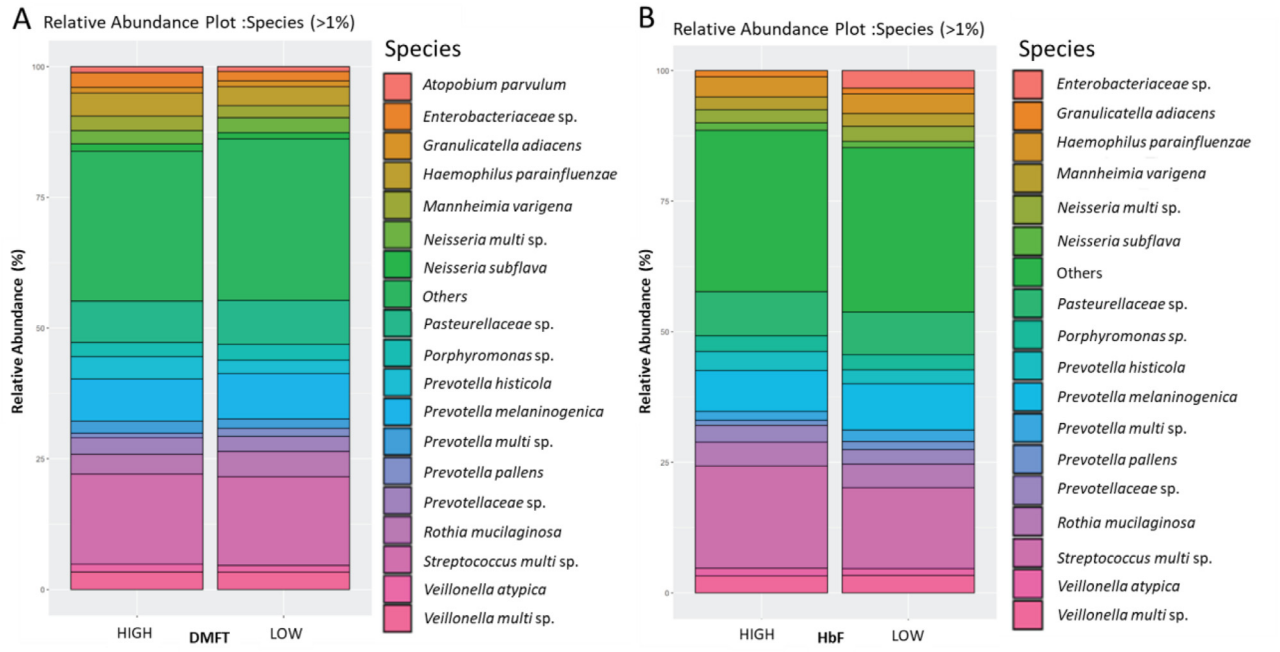

Fig. 2 – Histogram of relative abundance of microbiota at species level. A, Relative abundance between high and low decayed, missing, and filled permanent teeth at species level. B, Relative abundance between high and low fetal haemoglobin at species level.

species level is shown in Figure 2. *Enterobacteriaceae* species were significantly more abundant in the high DMFT index group compared to the low DMFT index group (Figure 2A). Table 1 shows the species of bacteria that were found to be significantly more abundant in the high DMFT index group compared to the low DMFT index group. Additionally, *Enterobacteriaceae* species were found to be significantly more abundant in the low HbF group compared to the high HbF cohort (Figure 2B). Table 2 shows the significantly more abundant bacteria species in the low HbF group compared to the high HbF cohort.

The difference in abundance of oral microbiota (phylum/genus) between high vs low DMFT index and high vs low HbF in patients with SCA was assessed using DEseq2 analysis (Supplementary Figure S1). We observed an overall increase in abundance for Proteobacteria, Chloroflexi, and Bacteroidetes in the high DMFT index group compared to the low DMFT index group (Supplementary Figure S1A). In addition, there was an overall increased abundance of microbiota from Proteobacteria, Fusobacteria, Firmicutes, and Bacteroidetes in the patients with SCA who had low HbF compared to those with high HbF (Supplementary Figure S1A). An increase in alpha diversity in patients with SCA with low HbF as compared to those with high HbF was detected using the Shannon diversity index and the raw number of observed taxa (Supplementary Figure S3B). This comparison did not show a difference between SCA cases with a high DMFT index vs those with a low DMFT index (Supplementary Figure S3A). Similarly, comparison of alpha diversity in males vs females showed no differences in the raw number of observed taxa and in Shannon indices (Supplementary Figure S2).

Discussion

In this study, we performed the largest microbiome study ever conducted in Saudi Arabia and the first-ever characterisation of oral microbiota in patients with SCA. SCA is highly

Table 1 – Species that show significantly higher abundance in the high DMFT group compared to low DMFT group.

Bacterial species	Phylum	baseMean*	Log2 fold change	lfcSE**	*P* value
Lautropia spp.§	Proteobacteria	7.01	5.66	1.55	.00027
Enterobacteriaceae spp.§	Proteobacteria	119.84	3.14	0.91	.00058
Prevotella shahii	Bacteroidetes	51.33	2.32	0.90	.00974
Ardenticatenaceae spp.§	Chloroflexi	7.85	4.51	1.86	.01559
Prevotella tannerae	Bacteroidetes	15.04	3.09	1.30	.01774
Lautropia multi spp.§	Proteobacteria	260.70	1.52	0.64	.01813
Capnocytophaga gingivalis	Bacteroidetes	4.08	3.21	1.61	.04605

§ Species could not be identified.
* BaseMean is the average of the normalised count values, taken over all samples with division by size factors.
** lfcSE is log2 fold change standard error.DMFT, decayed, missing, and filled permanent teeth.

Table 2 – Species that show significantly higher abundance in the low HbF group compared to high HbF group.

Bacterial species	Phylum	baseMean*	Log2 fold change	lfcSE**	*P* value
Enterobacteriaceae spp.§	Proteobacteria	119.13	4.67	0.79	3.23×10^{-9}
Prevotella aurantiaca	Bacteroidetes	62.84	3.13	0.96	.0011
Leptotrichia spp.§	Fusobacteria	3.41	3.92	1.46	.0074
Leptotrichia genomosp	Fusobacteria	17.66	2.76	1.11	.0130
Morococcus cerebrosus	Proteobacteria	12.04	6.58	2.99	.0277
Actinobacillus parahaemolyticus	Proteobacteria	28.57	3.97	1.82	.0288
Erysipelotrichaceae spp.§	Firmicutes	14.66	2.13	1.00	.0331
Atopobium spp.§	Actinobacteria	12.72	1.90	0.96	.0470

§ Species could not be identified.
* baseMean is the average of the normalised count values, taken over all samples with division by size factors.
** lfcSE is log2 fold change standard error.HbF, fetal haemoglobin.

prevalent in the Eastern Province of Saudi Arabia, where this study was conducted. The AI haplotype is the most prevalent in the area and characterised by some children having high HbF.[3,26] Given this observation and the possible correlation between dental caries and SCA, we assessed the microbiota abundance based on high DMFT index vs low DMFT index and high HbF vs low HbF.[7-12] Our results provide a valuable addition to the global microbiome reference data set in an underexamined community. These efforts are essential and warranted given the scarcity of microbiome data in Middle Eastern populations.

Dental caries is a multifactorial disease process that results from an imbalance between disease-causing and protective factors that influence demineralisation and remineralisation processes. Complications of dental caries seen in patients with SCA often include pulpitis, periapical periodontitis, and acute pain and are often associated with a poor quality of life.

Although a metanalysis report has indicated that there is no increased level of dental caries in patients with SCA in comparison to children without SCA,[27] other reports have shown that there is an association.[7-9] The discrepancy between studies in finding the association between SCA and high DMFT can be due to confounding factors, such as sample size, different countries with different dietary habits, different socioeconomic status, different haplotypes, and different genotypes (homozygous β^S/β^S or heterozygous β^S/β^C). In our study, we included only patients who are homozygous for sickling β-globin mutation (β^S/β^S) with AI haplotype. Several reports have indicated that there is an association between SCA and periodontal diseases and dental caries.[7-9] This increase has been attributed to the SCA-mediated impairment of blood flow to dental tissue that induces the complications of pulpitis, periapical periodontitis, with associated deficiency in the formation of dental and bone tissues and, consequently, an increased risk of infection.[7,28]

These conditions of insufficient blood flow favour the colonisation of facultative anaerobic bacteria such as *Streptococci* and *Actinomycin* species.[23] *Streptococci sanguinis*, which is a normal inhabitant of the oral cavity in humans, was present in our patients with SCA and could be the prime source of the cariogenic biofilm, but their presence alone is not sufficient for the formation of the cariogenic biofilm. *Streptococci sanguinis* is known to produce H_2O_2, which inhibits the growth of many other microorganisms.[29]

Patients with SCA and a high DMFT index have more abundant bacteria from the Proteobacteria phylum than their counterparts who have SCA and a low DMFT index. These results are similar to a study conducted in children from China in which the percentage of Proteobacteria was increased in children with dental caries compared to children with no dental caries and in children from Myanmar where Proteobacteria bacteria were the most abundant oral microbiota in children with dental caries.[30,31]

Additionally, the spontaneous periodontal inflammation murine model (Gas6−/− mice) has shown a microbial dysbiosis with a selective expansion of nitrate reductase expression Proteobacteria.[23] In our study, the *Lautropia* and *Enterobacteriaceae* species were the most significant abundant species of bacteria found in the high DMFT index group. Both species belong to the phylum of Proteobacteria that are nitrate reductase positive and a known pathogenic species.

Patients with SCA have a chronic inflammation status, especially patients with low HbF.[32,33] The insufficient blood flow and the high inflammation status in patients with SCA will favour the growth of more facultative anaerobic bacteria. Accordingly, our data show that patients with low HbF have a significant abundance of *Enterobacteriaceae* species, a facultative anaerobic bacterium. Multiple studies have isolated *Enterobacteriaceae* species bacteria from samples taken from patients with dental caries.[34,35] It has been suggested that the presence of the *Enterobacteriaceae* species in the oral cavity is favoured when an individual's immunity is compromised.[34,35] This suggestion is corroborated by studies of oral bacterial cultures from hospitalised patients undergoing chemotherapy that show a great number of *Enterobacteriaceae* species bacteria.[36,37]

Patients with SCA are immunocompromised due to the dysfunction of the innate and adaptive immune system.[38] The source of these oral *Enterobacteriaceae* species in our low HbF group with SCA could also be mediated through the nosocomial environment of the hospital since patients with low HbF are very likely to be hospitalised for some days during their SCA complications. Despite this limitation of our study, our data further emphasise the importance of routine oral hygiene visits for patients with SCA. This is especially important for patients with SCA and low HbF, who have a higher probability of hospitalisation and clinical complications compared to patients with SCA and high HbF; as we show here, patients with SCA with low HbF have a greater abundance of

oral microbiota species that favour dental caries. Nevertheless, a well-designed longitudinal study with a large sample size evaluating the oral microbiota association with dental caries in patients with SCA is warranted.

Author contributions

YMA, FAA, AAA, AAA, NNA, and MIA were involved in the design of the work, critically revising protocol, patient recruitment, data acquisition, analysis, interpretation of data, and drafting of the manuscript. CBV, CCY, AHH, and BPK were involved in the design of the work, laboratory work, analysis, critically revising protocol, interpretation of data, and drafting the manuscript. These authors have contributed equally to this work.

Conflict of interest

None disclosed.

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Supplementary materials

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Corrigendum

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The authors regret that one of Authors, Dr. Abukabbos, who contributed to the paper is missing from the main Author list. The Author list has been updated above.

The author contributions list should also be updated to reflect this change. The new Author contributions list is as below:

Author contributions

YMA, FAA, AAA, AAA, NNA, HSA and MIA were involved in the design of the work, critically revising protocol, patient recruitment, data acquisition, analysis, interpretation of data, and drafting of the manuscript. CBV, CCY, AHH, and BPK were involved in the design of the work, laboratory work, analysis, critically revising protocol, interpretation of data, and drafting the manuscript. These authors have contributed equally to this work.

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