



Research article

Temporal oral microbiome changes with brushing in children with cleft lip and palate



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ABSTRACT

This cohort study aimed to characterize the oral microbiome of children with CLP, from two different age groups, and evaluate the effect of supervised or unsupervised toothbrushing on the microbiome of the cleft over time. Swab samples were collected from the cleft area at three different time points (A; no brushing, B; after 15 days and C; after 30 days) and were analyzed using next-generation sequencing to determine the microbial composition and diversity in these time points. Overall, brushing significantly decreased the abundance of the genera *Alloprevotella* and *Leptotrichia* in the two age groups examined, and for *Alloprevotella* this decrease was more evident for children (2–6 years old). In the preteen group (7–12 years old), a significant relative increase of the genus *Rothia* was observed after brushing. In this study, the systematic brushing over a period of thirty days also resulted in differences at the intra-individual bacterial richness.

1. Introduction

The human oral cavity contains more than 600 different bacterial taxa, most of them are commensal, but a small number are opportunistic pathogens that can cause various oral diseases, as well as systemic diseases (de Castilho et al., 2006; Parapanisiou et al., 2009; Perdikogianni et al., 2009; Edlund et al., 2018). Several factors influence the oral microbiome, including diet, genetics and life style (Lif-Holgerson et al., 2011). Cleft lip and palate (CLP) is the most common congenital orofacial malformation, (prevalence close to 1:700) (Worth et al., 2017) and significantly affects the structure and functions of the oral cavity (Ahluwalia et al., 2004; Gopakumar and Hegde, 2010). Children with CLP suffer from skeletal discrepancies, causing respiratory problems and malocclusion that hinders the removal of bacterial plaque (Shashni et al.,

2015; Hazza'a et al., 2011; Rivkin et al., 2000; Bolor and Thomas, 2010). Treatment consists of corrective surgery and the use of palatal obturator plates or orthodontic appliances, which may increase microbial colonization due to accumulation of bacterial plaque (Freitas et al., 2013). Furthermore, fear of sanitizing the cleft area (Sundell et al., 2015) leads to a higher plaque index in these children (Parapanisiou et al., 2009; Pisek et al., 2014). Previous studies have shown that children, adolescents, and young adults with CLP have increased risk of developing dental caries and periodontal diseases compared to non-CLP counterparts (Perdikogianni et al., 2009; Rivkin et al., 2000; Howe et al., 2017; Mutarai et al., 2008; Plakwicz et al., 2017). Together this indicates that children with such malformations can be expected to have an unusual oral microbiome. The colonization of the oral cavity commences within the first hours (8–16 h) after birth and this oral microbiome forms the

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foundation upon which newer communities develop as new and more niches appear (Mason et al., 2018; Wade, 2013). Moreover, a 24-hour analysis, using metagenomic and metatranscriptomic approaches, of an *in vitro* oral biofilm assembly and maturation, revealed that the taxonomic composition and functional capacity of mature biofilm is gradually shaped through processes of co-aggregation, competition and production of reactive agents within the microbial community (Edlund et al., 2018). Variations in the microbial composition may be explained by the dynamic development of the oral microbiome that matures and evolves, following the modifications of the dentition, throughout childhood and adolescence (Kilian et al., 2016; Xu et al., 2015; Crielaard et al., 2011).

Oral health problems may be related to the presence of specific putative periodontal pathogens such as Gram-positive facultative anaerobic bacteria, like *Streptococcus mutans* and *Lactobacillus* spp. (Machorowska-Pieniążek et al., 2017; Marsh, 2003), or Gram-negative proteolytic species, from the genera *Prevotella*, *Porphyromonas*, *Fusobacterium* and *Treponema* (Marsh, 2003). The cleft area is vulnerable to this since is a site for dental plaque accumulation (Wyrebek et al., 2017; Perdikogianni et al., 2009; Gaggl et al., 1999; Bragger et al., 1985). In dental caries, there is a shift towards community dominance of acidogenic and acid-tolerant bacteria (*S. mutans* and *Lactobacillus* spp.) that demineralize enamel (Marsh, 2003; Wade 2013; Rosier et al., 2018). These bacteria metabolize dietary sugars to acid, creating locally a low pH (5.5); if these acidic conditions persist without sufficient time for remineralization, caries lesions will develop (Rosier et al., 2018). Periodontitis-associated bacteria (e.g. the Gram negative genera previously mentioned) cause tissue damage directly, by the production of proteases such as collagenase or hyaluronidase, however, much of the tissue damage can be caused by activation of the pro-inflammatory pathways and the inflammatory response (Marsh, 2003; Wade, 2013).

In children with CLP many factors can inhibit optimal toothbrushing, whether autonomous or parent-assisted. These factors include reticence, due to fear of traumatizing soft tissues or concern with bleeding from inflamed gums, and physical hindrances, such as malocclusion or lip fibrosis resulting from cheiloplasty, making it difficult to thoroughly clean teeth and the deepest area of the cleft (Ahluwalia et al., 2004; Gopakumar and Hegde, 2010; Crerand et al., 2020). These constraints impede tooth removal of the bacterial plaque and favor microbial colonization, consequently leading to an increased risk of developing caries and periodontal diseases (Kolawole et al., 2010; Kolawole and Folayan, 2019). Several studies have highlighted the relevance of cleaning and brushing the cleft area in order to maintain good oral health and avoid oral diseases (Ahluwalia et al., 2004; Hazza'a et al., 2011; Rivkin et al., 2000; Lin et al., 2017). This study characterizes the oral microbiome of children with CLP, from different ages before and after toothbrushing, and evaluated the importance of direct parent involvement in the toothbrushing. Next generation sequencing of the 16S rRNA gene from oral swabs was used to: *i*) determine the microbiome of the children with CLP before or after tooth brushing; *ii*) compare the impact supervised vs unsupervised toothbrushing; *iii*) compare the combined effects of toothbrushing and supervision on the alteration of the microbiome of CLP children with different ages; *iv*) correlate the plaque index with the microbiome.

2. Materials and methods

2.1. Ethics

The study follows the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Faculdade de Medicina Dentária da Universidade do Porto (FMDUP/July/2016; Ref. n.º. 406). All legal guardians of the subjects enrolled in the study provided informed written consent of their participation.

2.2. Study design and experimental protocol

The cohort was designed to follow the oral health of children and preteens with CLP and how it was affected by tooth brushing. A multi-disciplinary group including dentists for the dental care followed all subjects at the Compor Clinic in Porto, Portugal. All the children and preteen have had their lips and palates repaired and did not have any clinically observed oronasal fistula or alveolar bone graft surgery performed at this age.

The inclusion criteria were: 1) age within 2–6 years (children) or 7–12 years (preteen); 2) CLP type I complete unilateral or bilateral and type II of Spina Classification (Spina, 1973) (type I – cleft lip and type II – cleft lip and palate); 3) children without motor development problems of the upper limbs or cognitive problems. Any child needing an orthodontic appliance or a CLP surgical intervention, which could significantly change the oral conditions, were excluded from the study.

The children (2–6 years) received parent supervision during oral hygiene, while the preteens (7–12 years), performed their oral hygiene autonomously. Parents of both groups were instructed to maintain the routine oral hygiene. Oral swabs were collected, evaluated and measured at baseline (time point A), without prior brushing, at day 15 (time point B), and day 30 (time point C), after toothbrushing. Of the initial 28 subjects, one child and three preteens were lost to follow-up, which meant that 72 samples were collected, from 13 children and 11 preteens.

2.3. Sample collection

The cleft area was swabbed 3 times, using sterile FLOQSwabs™ (COPAN, USA), spinning the swab along the deepest area of the cleft at a perimeter of 0.5 cm around it. The samples were stored at -20 °C until DNA extraction. One investigator collected all samples, thereby ensuring uniform sample collection. After performing the swab, an oral plaque index was evaluated. The adapted plaque index (aPI) was calculated at time point A as described in a different study by Rodrigues et al. (2018).

2.4. DNA extraction

The swab tips were submerged in 1 mL of molecular grade water (W4502, Sigma, UK), and bacteria released by 10s manual agitation. DNA extraction from 200 µl of the water was performed using NucleoSpin® 96 soil kit (740787, Macherey-Nagel, Germany) using an EpMotion 5075vt (Eppendorf, USA), following manufacturer instructions, using lysis buffer SL1 with 150 µl Enhancer SX and eluted in 70 µl elution buffer.

2.5. 16S amplicon sequencing and bioinformatics pipeline

The 16S rRNA gene amplification procedure was performed using a two-step procedure to amplify the hypervariable V3–V4 region of the 16S rRNA gene, using PCR BIO HiFi Polymerase (PB10.41, PCRBIOSYSTEMS, UK) in 25 µL reactions (2 µL template, 5 µL reaction buffer, 1 µL forward primer, 1 µL reverse primer, 0.12 µL Polymerase, 15.88 µL molecular grade water (W4502, Sigma, UK). Amplification was performed in 96-well microtiter plates, in a 2720 thermal cycler (Applied Biosystems®, Life Technologies, CA, US) according to the following cycling program: 1 min of denaturation at 95 °C, followed by 30 cycles of 15 s at 95 °C (denaturing), 15 s at 56 °C (annealing) and 30 s at 72 °C (elongation), final extension at 72 °C for 5 min, and storage at 10 °C thereafter. The PCR products from both steps were purified using High Prep™ PCR (AC-60500, MagBio Genomics Inc., USA) PCR Clean Up System, using 0.65:1 beads to amplicon ratio (vol/vol). The first step used 30 amplification cycles and the modified broad range primers Uni341F (5'-CCTAYGGGRBGCASCAG-3') and Uni806R (5'-GGACTACHVGGGTWTCTAAT-3') (Klindworth et al.,

2013; Takai and Horikoshi, 2000). The second step used 15 amplification cycles and primers developed in-house, which contain sequencing adaptors and unique combinations of forward and reverse indices (Nunes et al., 2016). A negative template-free control and a positive control containing 2.0 μ l DNA from a known bacterial mock community (1.0 ng/ μ l; HM-782D, BEI Resources, VA, US) were included.

Samples were normalized using SequalPrep™ Normalization Plate (96) Kit (Invitrogen, MD, USA), pooled, and concentrated using the DNA Clean and Concentrator™-5 kit (Zymo Research, Irvine, CA, USA). The concentration of the pooled libraries was determined using the QuantiT™ High-Sensitivity dsDNA Assay Kit (Q33120, Invitrogen, MD, USA) and adjusted to 1.65 ng/ μ l (4 nM). Amplicon sequencing was performed on the Illumina MiSeq Desktop Sequencer (Illumina Inc., CA, US), with the denatured libraries adjusted to a final concentration of 16 pM. For each run, a 5.0% PhiX internal control was included. All reagents used were from the MiSeq Reagent Kits v2 (Illumina Inc., CA, US). Automated cluster generation and 250 paired-end sequencing with dual-index reads was performed. The sequencing output was as the demultiplexed fastQ-files generated directly on the MiSeq instrument. Up to 192 samples, including controls were sequenced per run.

Sequencing data was analyzed using the Qiime2 (Bolyen et al., 2018) implementation of DADA2 using default parameters (Callahan et al., 2016) producing 2,682,145 high quality merged sequencing reads, representing 3,251 amplicon sequence variants (ASV). Taxonomical classification was performed against SILVA database version 132 (Yilmaz et al., 2014).

2.6. Statistical analysis

Statistical analyses and data treatment were performed using the R software platform (R Core Team, 2018). The R package phyloseq was used for data handling (McMurdie and Holmes, 2013), and all

plots were created using the ggplot2 package (Wickham, 2016). Based on the rarefaction curves (Figure 1), the single samples with less than 5000 reads were removed from the data set.

The alpha diversity measures, observed richness and Shannon-diversity index (H), were calculated as the mean of 100 separate rarefactions to 6,688 reads per sample (90% of minimum sample depth). Between groups, comparisons of alpha diversity were performed using analysis of variance (anova) (function: ANOVA, package: stats), and correlation with aPI was tested using Spearman, Kendall, and Pearson r correlations (function: ggscatter, package (Kassambara, 2018)). Beta-diversity was calculated using Bray-Curtis dissimilarity index (function: distance, package: phyloseq), and analyzed using Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA, function: adonis, package (Oksanen et al., 2019)), for aPI we further calculated a PCoA (function: ordinate, package: phyloseq) and correlated aPI with the first 10 axes. To investigate differential abundance of bacteria we used the package DAtest to determine which statistical method to employ (function: testDA, package (Russel et al., 2018)) and then to perform the optimal tests. The relative abundance change (RAC) was calculated for each genus with mean abundance of at least 0.2%, using the formula: $RAC = \ln(A_1)/\ln(A_0)$, where A_0 is the abundance at one time point and A_1 is the abundance at a later time point. The significance of between group variance of the RAC were evaluate using anova and Wilcoxon signed rank test, followed by correction for false discovery rates (fdr) ($q = \text{fdr adjusted p-values}$), to identify genera that changed significantly. To test for correlations with aPI we calculated Spearman, Pearson r, and Kendall correlations with the 10 most abundant ASV, species, and genera, based on relative abundance and log transformed relative abundances.

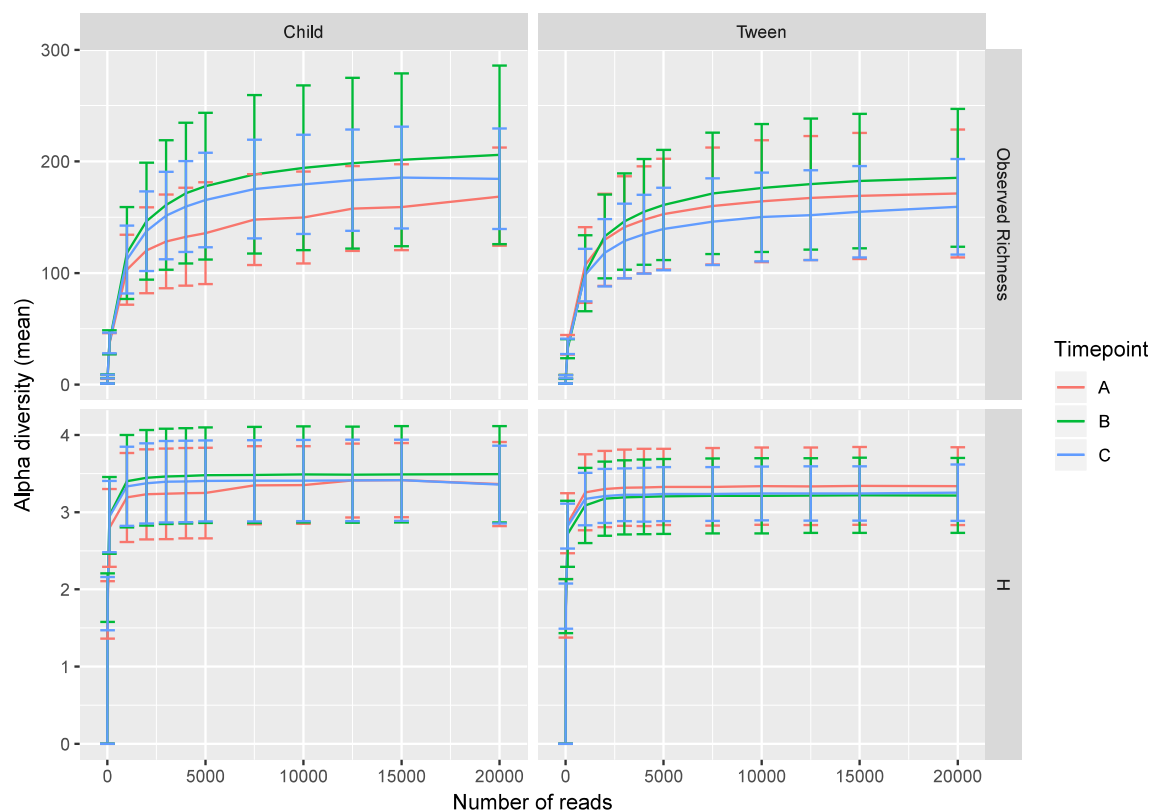


Figure 1. Rarefaction curves. Rarefaction curves of calculated for observed richness (top) and H (bottom) at 1 to 20000 sequencing reads, separated for child (left) and Tween (right), with samples grouped by timepoint with bars indicating standard deviations.

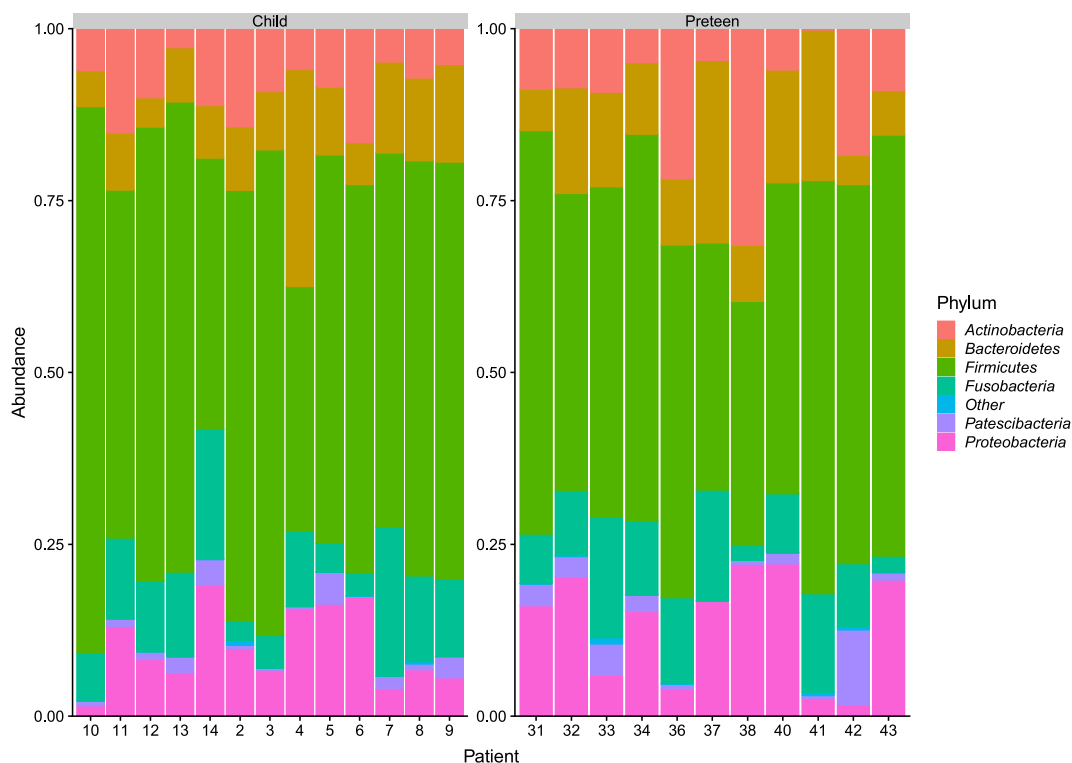


Figure 2. Phylum level microbiome composition at timepoint A, per patient. Bar plot showing the relative abundance of all phyla with a mean abundance above 1% and remaining phyla group as Other.

3. Results

3.1. Characterization of the oral hygiene status

The aPI of both groups of children showed overall insufficient oral hygiene. The aPI were not significantly different between age groups

(Mann-Whitney *t*, *p* = 0.057), although the children showed a slightly higher median values than the preteens (Me (Q1-Q3): 2.4 (1.8–2.6) vs 1.9 (0.2–2.2), respectively). Moreover, based on the information regarding their brushing habits, their brushing frequencies did not significant differ between groups (Mean (Q1-Q3): 2 (1–2) for children and 1 (1–2) for preteen; *p* = 0.207).

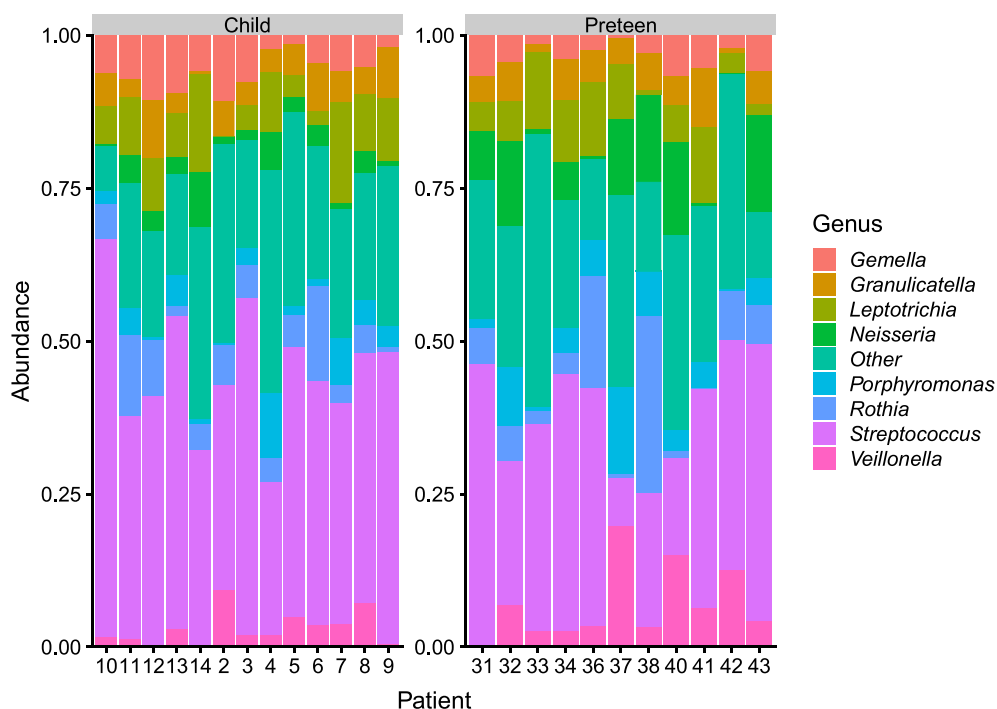


Figure 3. Genus level microbiome composition at time point A, per patient. Bar plot showing the relative abundance of all genera with a mean abundance above 4% and remaining genera group as “Other”.

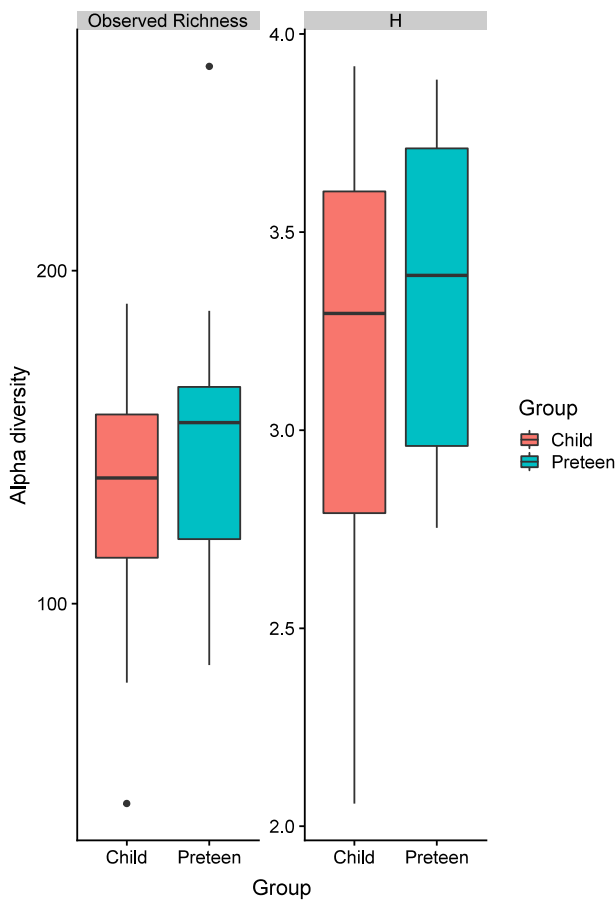


Figure 4. Boxplot of alpha diversity at time point A for children and preteen determined by observed richness and Shannon Diversity Index (H). Line in box indicates median value, box covers 95% confidence intervals, vertical lines reaches to furthest samples within 1.5 x box height and any outliers are indicated as points.

3.2. The microbiome at baseline (time point A)

At baseline (time point A), in the 24 samples, from 13 children and 11 preteen, the major bacterial phyla present were *Firmicutes* (54.7%), *Proteobacteria* (11.6%), *Bacteroidetes* (11.5%) and *Actinobacteria* (10.0%) (Figure 2). The five most abundant genera were *Streptococcus* (37.4%), *Leptotrichia* (7.6%), *Rothia* (6.6%), *Neisseria* (5.3%), and *Granulicatella* (5.0%) (Figure 3). There were no statistically significant differential abundant phyla or genera between the two groups.

When comparing baseline alpha diversity in the two groups, no significant differences were observed in either observed richness (132 ± 40.6 and 151 ± 48.0 , respectively, $p = 0.31$) or H (3.2 ± 0.54 and 3.3 ± 0.42 , respectively, $p = 0.47$) (Figure 4). The variation in baseline microbiome composition between children and preteen was measured by Bray-Curtis dissimilarity and showed that the age groups significantly explain 6.4% (PERMANOVA, $p = 0.04$) of the overall variation.

When testing for correlations between the microbiome and aPI at baseline, independent of age group, alpha diversity was not correlated with aPI ($p = 0.123-0.99$). Bray-Curtis distances were not correlated with aPI (PERMANOVA, $R^2 = 0.04$, $p = 0.61$), and when correlating the first 10 axis of a PCoA with aPI only axis 5 showed a significant Kendall correlation with aPI ($r^2 = -0.35$, $p = 0.028$), but no other axis or type of correlation were significant. Neither of the 10 most abundant ASVs, species, or genera was significantly correlated with aPI.

3.3. Microbiome per group age (child and preteen) after brushing (time point B + C)

The CLP microbiome, after toothbrushing was dominated by the phyla *Firmicutes* (51.7–52.5%), *Actinobacteria* (16.4–19.2%), *Proteobacteria* (12.1–15.1%), *Bacteroides* (8.0–8.4%) and *Fusobacteria* (6.0–7.2%). At genus level, the dominant bacteria were *Streptococcus* (32.9–33.6%), *Rothia* (9.6–12.3%), *Gemella* (6.0–7.5%), *Veillonella* (5.3–5.4%), and *Haemophilus* (4.5–5.4%). *Rothia* was a major constituent of the oral microbiome in seven preteen and four children, while having a small relative abundance in the other patients. *Gemella* was a major genus in only one child and one preteen. *Neisseria* and *Veillonella* were major genera in two groups of two preteen's microbiome each. *Haemophilus* was a major constituent in only one child's oral microbiome (Figure 5). No

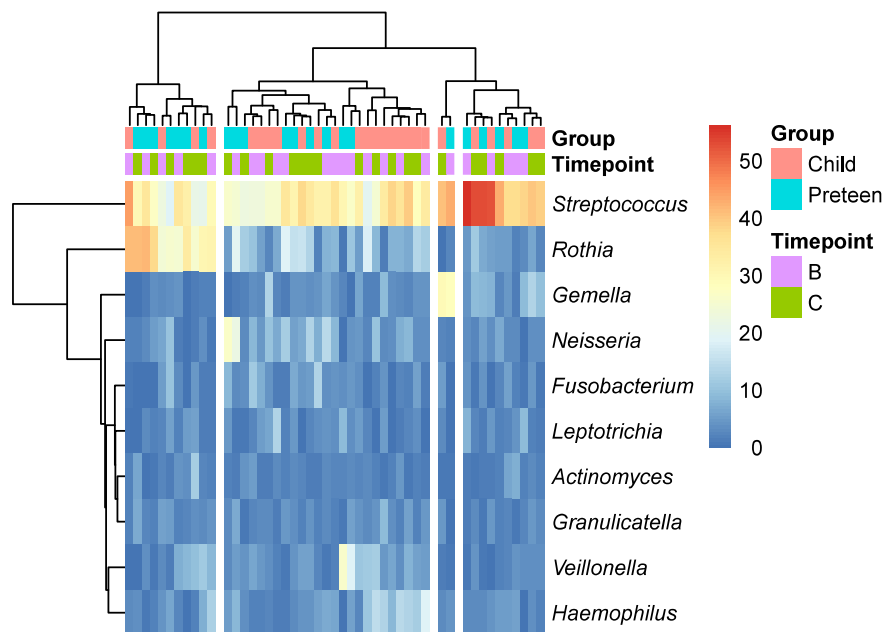


Figure 5. Heatmap of 10 most abundant genera at time point B and C. The two top rows indicate the age group and time point of each sample. The abundances are shown as percentages with the blue-yellow-red color scale.

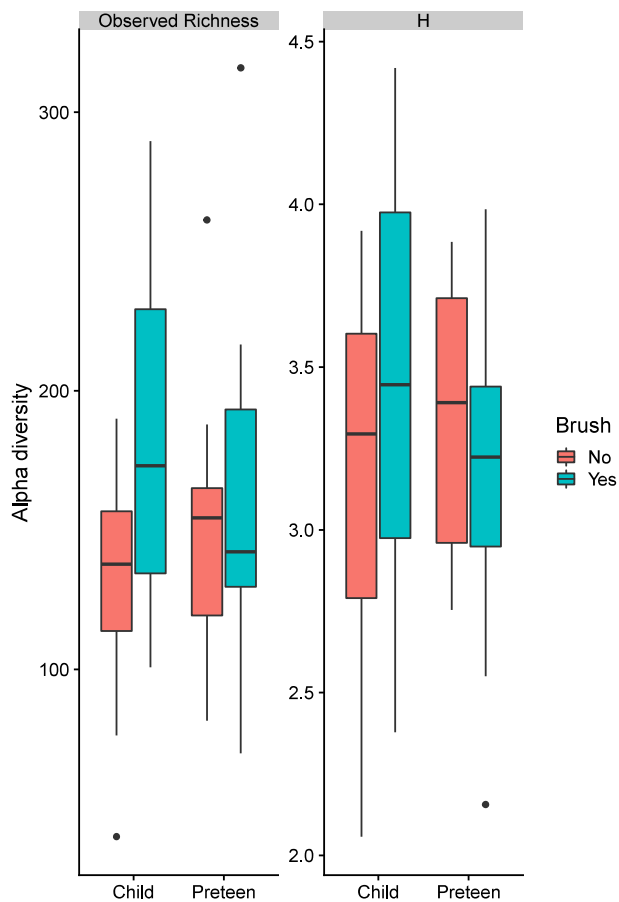


Figure 6. Boxplot of the alpha diversity for children and preteen. Boxplots are colored according to brushing (Baseline: red, Brushing: turquoise), measured as both observed richness (left) and Shannon Diversity Index (H) (right). Line in box indicates median value, box covers 95% confidence intervals, vertical lines reaches to furthest samples within 1.5 x box height and any outliers are indicated as points.

genera were significantly different between the two age groups at either time point (Quasi-Poisson GLM, predictor = group, covariable = Time point) or between time points in either group (DESeq2, predictor = Time point, covariable = Group).

3.4. Effect of toothbrushing supervision after 30 days (B + C) compared to the baseline microbiome

To evaluate the impact of the different oral hygiene strategies, the initial microbiome diversity (time point A) was compared with the microbiome diversity of the samples taken after brushing, at day 15 and 30 (time point B and C). Children showed higher observed richness after brushing (183 ± 60.8) compared to their initial microbiome (132 ± 40.6 , $p = 0.001$), while Shannon-diversity index (H) did not show a significant change (Figure 6). For preteens the observed richness was so similar after brushing that there was a higher mean value (151 ± 48.0 and 159 ± 51.2 , respectively), but lower median values (154 vs 142).

The effects of toothbrushing supervision and difference from baseline were analyzed using Bray-Curtis dissimilarities showing that the supervision significantly explains 2.7% of the overall variation ($p = 0.012$), while toothbrushing explains further 4.2% by itself ($p < 0.001$).

To further understand the changes in composition, the relative abundance change (RAC) of each genus (with mean abundance $>0.5\%$) was calculate within the individual patients. This was done for the three timespans: initiation (A to B), continuation (B to C) and overall (A to C).

Of the 29 genera tested, four genera changed (RAC different from 0) significantly during initiation (Wilcoxon, $q < 0.1$ (Figure 6), and three had significantly changed abundance overall.

When comparing between groups, eight genera had a significantly different RAC between groups at one or more of the timespans (ANOVA, $q < 0.05$) (Figure 7). *Actinobacillus* had significantly positive RAC overall (1.37, $q = 0.041$), driven by increased abundance during initiation, and no RAC differences between group. *Alloprevotella* were significantly decreased during initiation ($q = 0.042$) and especially in the child group, which were significantly lower than the preteen group ($q = 0.030$), and significantly decreased when seen over the entire period ($q = 0.041$). *Gemella* tended to have negative RAC overall, with a significant difference between group during continuation, where RAC were positive for the child group (0.46) and negative for the preteen group (-0.70). *Granulicatella* did not show any differences between groups, but were found to have significantly negative RAC during initiation (-0.49, $q = 0.093$). *Haemophilus* showed a general tendency towards positive RAC over time, with significant differences between group at initiation, which were driven by a significant higher RAC in the child group (1.06 and -0.13, for children and preteen, respectively, $q = 0.024$). *Leptotrichia* had a significantly negative RAC during initiation (-0.83, $q = 0.004$) and overall (-0.93, $q = 0.041$), with no significant differences during continuation or between groups. *Porphyromonas* showed a significant lower RAC in the preteen group, when compared overall ($q = 0.016$) that were driven by a tendency to decreased RAC in the preteen group during initiation and slightly increased RAC in the child group during continuation. *Prevotella* were significantly different between groups during initiation ($q = 0.015$) and overall ($q = 0.006$), with positive RAC in the child group and negative RAC in the preteen group. There was a significant increase of *Rothia* during initiation (0.76, $q = 0.090$), with significant differences at continuation ($q = 0.025$) driving an overall difference between the groups ($q = 0.021$). Furthermore, in the child group *Rothia* had a negative RAC (-0.54) during continuation, whereas the preteen group had a steady positive RAC during both in initiation and continuation (0.81 and 0.82, respectively). *Streptococcus*, the most abundant genus, did not show any great variation in general, but were found to differ significantly between groups during initiation (RAC: -0.27 and 0.21, for child and preteen, respectively, $q = 0.014$). Lastly, *Veillonella* had positive RAC in the child group and negative RAC in the preteen group, resulting in significant differences between group seen over the entire project (RAC: 1.08 and -0.73, respectively, $q = 0.011$).

4. Discussion

In this study, we evaluated the oral microbiome of children (2–6 years old) and preteens (7–12 years old) with CLP, using massive high-throughput amplicon sequencing. The data on oral microbiome of CLP patients are scarce and, to our knowledge, this is the first report on the oral microbiome of the cleft area in CLP children and preteens assessed in depth using next generation sequencing. This approach showed that *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were the most abundant phyla in CPL oral microbiome. Additionally, *Streptococcus*, *Leptotrichia*, *Rothia*, *Neisseria*, and *Granulicatella* were the dominant genera in the two age groups. This resembles the oral microbiome in non-CLP children, where these taxa have been reported as abundant members, supporting the idea that there is a core oral microbiome (Xu et al., 2015; Grielaard et al., 2011).

Other than CLP, all children and preteens included in this study did not have any health problem and presented a health status suitable for their ages. The child group consisted of preschool children with primary dentition and children with mixed dentition (primary and permanent), while the preteen group consisted of children with mixed dentition and pre-adolescents with permanent dentition. The replacement of the primary teeth with a permanent dentition is a striking biological change that would significantly explain the observed age-dependent changes in the oral microbiome. The expansion of biodiversity may be attributable to

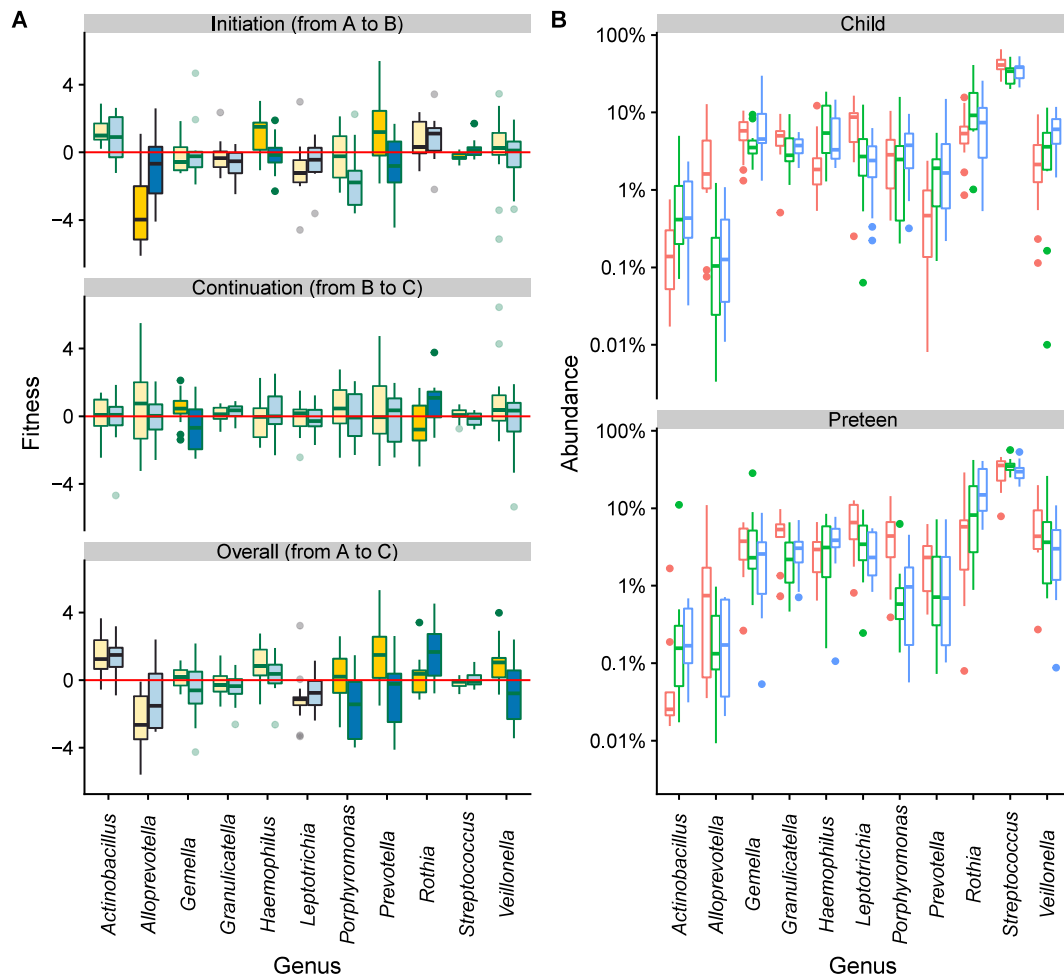


Figure 7. Boxplots of the relative abundance change (RAC) and abundance of significantly changing genera. A. Boxplot of RAC, per group (child: yellow, preteen blue), for any genus with RAC significantly different from 0 in any of the 3 timespans (Significant change, yes: Black edge, no: green edge), or which differs significantly between groups in any of the timespans (Significant difference, yes: solid fill, no: pale fill). B. Boxplot of the genus abundance of the relevant genera, edge color indicates time point (A (day 0): red, B (day 15): green, C (day 30): blue). Line in box indicates median value, box covers 95% confidence intervals, vertical lines reaches to furthest samples within 1.5 x box height and any outliers are indicated as points.

both introductions of new species and increase in abundance of the already present organisms. Further explanation includes oral hygiene behavior (Lewis et al., 2017), exposure to external bacteria (Könönen, 2000) and the development of the immune system (Costello et al., 2012).

We did not observe any correlations between the cleft scar microbiome and the aPI at baseline. Studies have shown that the functional potential in plaque biofilm is more important than the abundance of specific species (Espinoza et al., 2018) and that spatial variation is expected in the oral microbiome (Proctor et al., 2018); it is not surprising that we did not find any correlation between the microbiome of a soft tissue site and the dental surface.

Introduction of the supervised toothbrushing resulted in inter-individual differences in the bacterial composition, without presenting group specific patterns of change, since no genera were significantly different between the two age groups at either time point B or C, or between the two time points in either age group. However, a closer look to the dynamics of the oral microbiome in the cleft area showed relative abundance changes (RAC) of different genera within individual patients. From the beginning to the end of the trial, in which both age groups were requested to brush their teeth, the relative abundances of members of the genera *Alloprevotella* and *Leptotrichia* significantly decreased, and the relative abundance of members of the genus *Actinobacillus* significantly increased. These three genera are naturally occurring members of the

microbiome in healthy individuals, but the overabundances of members of the genera *Alloprevotella* and *Leptotrichia* have been correlated with oral cancer and periodontitis disease (Zhao et al., 2017; Chen et al., 2018; Li et al., 2014). The RAC of genera *Leptotrichia*, *Granulicatella*, *Alloprevotella* (RAC<0), and *Rothia* (RAC>0) were significant in the first 15 days of the trial and this change was observed in both groups, with exception of the genus *Alloprevotella*, where the change occurred mainly in the child group. In addition, the positive RAC of *Rothia* was evident for the group preteen during the entire trial, while for the child group, the relative abundance of this genus showed a tendency to decrease from time point B to time point C. This group of microorganisms is commonly found associated with both health (core microbiome of children with primary dentition but not in preteens) and oral disease, demonstrating a distinct pathogenic potential of members of this genus (Mason et al., 2018; Crielaard et al., 2011; Zhao et al., 2017). Our data does not enable us to draw any conclusions about the pathogenicity or about the specific species of *Rothia* present. Members of *Streptococcus*, *Gemella*, *Haemophilus*, *Porphyromonas*, *Prevotella* and *Veillonella* also showed significantly different RAC for the two groups between two or more time points, during the trial. These significant differences between age groups, supports the idea that dynamic changes of the oral microbiome can potentially respond to host development and maturation. Oral bacteria and their metabolites interact with human host cells, both as protective

barriers against pathogen invasion and as causative agents of oral diseases (e.g. caries and periodontal disease). Many oral diseases are being viewed as a consequence of a deleterious shift in the balance of the resident oral microbiome (Willis and Gabaldón, 2020). In dental caries there are changes in the oral microbiome that favors acid-producing (acidogenic) species, from genera such as *Streptococcus* and *Veillonella* (Rosier et al., 2018). Members of the genera *Porphyromonas* and *Prevotella* have also been strongly associated with caries (Hurley et al., 2019). Periodontal disease, results from an inappropriate inflammatory reaction to the normal microbiome, exacerbated by the presence of some disease-associated bacterial species. Increased abundance of Gram-negative proteolytic species, such *Porphyromonas gingivalis* and *Prevotella* spp. have been associated with periodontal diseases (Rosier et al., 2018; Willis and Gabaldón, 2020). However, it is not always clear, whether changes in microbial compositions causes disease or if they are caused by the disease. Some bacterial genera that have been associated with oral disease are commonly found as parts of a healthy oral microbiome, these include *Neisseria*, *Haemophilus*, *Prevotella*, *Veillonella*, *Gemella*, *Streptococcus*, and *Porphyromonas* (Willis and Gabaldón, 2020). Moreover members of the genus *Streptococcus* are also well known for their production and secretion of antimicrobial compounds (e.g. hydrogen peroxide, bacteriocins), which can act as defenses against oral pathogens (Edlund et al., 2018).

In the child group, the observed richness was higher after supervised brushing when compared to the initial diversity. An increased diversity of the oral microbiome is generally associated with a healthier microbiome; caries-free children have a higher alpha diversity than children with caries (Li et al., 2014; Gross et al., 2010). This indicates that the supervision and guidance by parents promoted a better overall oral hygiene in this group. The supervised toothbrushing affected the composition of the oral microbiome of each individual in this study. Although, these effects were specific to each individual and could not be predicted for the groups in general. The action of toothbrushing, independently of the period of toothbrushing, significantly explained the differences observed in the microbiome of CLP individuals (4.2%). Therefore, preventive intervention (adequate toothbrushing) is of paramount importance to promote a good oral hygiene of CLP children. While the increased risk of dental caries, in individuals with CLP, is still inadequately understood, many of the potential risks factors are related to insufficient cleaning of areas with impeded access.

The consequences of poor oral health are considerable for children and preteens with CLP, since it may affect their candidacy for orthodontic treatment. Furthermore, if good oral hygiene is not maintained during the orthodontic treatment, it can impair future oral health and cleft-related outcomes (Lewis et al., 2017).

This is the first longitudinal study of the oral microbiome of children with CLP. By comparing the oral microbiome over time, we have identified inter-individual changes in the oral microbiome composition, although no general inter group differences were not observed. Nevertheless, we did observe that brushing increased the richness and evenness in the child group and not in the preteen group.

5. Limitations of the study

The strength of this study was limited by the impossibility to ensure a strict and continuous monitoring of the children and their families during the entire study period. The participants were given guidelines for good brushing efficiency (frequency or time of brushing), but the daily brushing was not observed by the study staff and adherence to the guidelines were followed up through a study questionnaire.

We also acknowledge that the NGS data lacked the resolution to identify all bacteria at species level, limiting the transferability of our conclusions to a clinical setting. More research will be needed to clarify the major cause of variation in the oral microbiome of CLP patients.

Declarations

Author contribution statement

Rita Rodrigues: Conceived and designed the experiments; Performed the experiments.

Ana P. Chung: Analyzed and interpreted the data; Wrote the paper.

Martin S. Mortensen: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Maria H. Fernandes, António B. Monteiro, Rowney Furfuro, Cátia C. Silva: Performed the experiments.

Maria C. Manso: Conceived and designed the experiments.

Søren J. Sørensen: Contributed reagents, materials, analysis tools or data.

Paula V. Morais: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

The sequence data from this study is available in the Sequence Read Archive (SRA) under BioProject ID PRJNA512456.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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