Red fluorescent dental plaque: An indicator of oral disease?
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Red fluorescence of dental plaque in children – a cross-sectional study

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Submitted
Abstract

Aim
The relation between the presence of red fluorescent plaque and the caries status in children was studied. In addition, the microbial composition of dental plaque from sites with red fluorescent plaque (RFP) and from sites with no red fluorescent plaque (NFP) was assessed.

Methods
Fluorescence photographs were taken from fifty children (6 - 14 years old) with overnight plaque. Full-mouth caries scores (ICDAS II) were obtained. The composition of a saliva sample and two plaque samples (RFP and NFP) was assessed using 16S rDNA sequencing.

Results
At the site level, no clinically relevant correlations were found between the presence of RFP and the caries status. At the subject level, a weak correlation was found between RFP and the caries status when non-cavitated lesions were included ($r_s = 0.37, p = 0.007$). The microbial composition of RFP differed significantly from NFP. RFP had more anaerobes and more Gram-negative bacterial taxa. The most discriminative operational taxonomic units (OTUs) for RFP were Corynebacterium, Leptotrichia, Porphyromonas and Selenomonas, while the most discriminative OTUs for NFP were Neisseria, Actinomyces, Streptococcus and Rothia.

Conclusions
There were no clinical relevant correlations in this cross-sectional study between the presence of RFP and (early) caries lesions. There were differences in the composition of these phenotypically different plaque samples: RFP contained more Gram-negative, anaerobic taxa and was more diverse than NFP. The study outcomes provide more insight in the possibilities to use plaque fluorescence in oral health risk assessments.
Composition of fluorescent plaque

Introduction
Dental caries is a common oral disease (Petersen, 2009; WHO, 2012). Caries only progresses when dental plaque is present (Marsh, 1994). Therefore, frequent plaque removal is the most important preventive measure in maintaining oral health (Axelsson and Lindhe, 1978; Ekstrand and Christiansen, 2005). Another aspect involved in caries development and progression is the diet of the person (Selwitz et al., 2007). Especially a high frequency intake of carbohydrates in the diet can result in a change of the oral microbiome towards more aciduric species (Takahashi and Nyvad, 2011). The presence of a more aciduric dental plaque can disturb the demineralisation / remineralisation balance in a specific niche of the oral cavity, eventually resulting in caries.

To prevent caries from developing, it can be helpful to determine high-risk niches and high-risk patients (Zero et al., 2009). When patients are managed in time concerning their caries risk and caries progression, fewer operative dental procedures need to be performed (Thylstrup et al., 1997; Vermaire et al., 2014). So far, no specific tools are available to predict which niche is at high risk or which patient is at high-risk for developing tooth decay based on biological characteristics of a causal factor of the disease.

Some dental plaque fluoresces red when excited with 405 nm light (visible violet light) without the use of a disclosing solution (Heinrich-Weltzien et al., 2003). This red fluorescence has been observed from cavitated caries lesions (König et al., 1993; König et al., 1999; Lennon et al., 2002), non-cavitated caries lesions (Buchalla et al., 2004b; Buchalla, 2005; Felix Gomez et al., 2016) as well as from bacteria related to dental caries (Lennon et al., 2006b; Shigetani et al., 2008; Volgenant et al., 2013). Recent studies suggest that old and cariogenic plaque causes this red plaque to fluoresce (Lee et al., 2013; Kim et al., 2014; Volgenant et al., 2016a), while mature plaque is also related to caries progression (Marsh and Bradshaw, 1995). This red fluorescence can be observed and recorded using a dedicated fluorescence camera, which allows easy detection and recording of the fluorescence. With this camera, a tool to determine the caries risk of a patient on the basis of plaque characteristics may be within reach.

No clinical studies have been conducted so far that study the differences in the oral microbiome in relation to the presence or absence of red fluorescence of dental plaque or that link red fluorescent plaque to the presence of caries. Therefore, the main objective of this study was to study the relationship between the presence of red fluorescent plaque and the caries status in children. We also assessed if there was a difference in the bacterial composition of dental plaque from sites with and without red fluorescent plaque. The study results may provide an insight in the potential to use plaque fluorescence for caries risk assessment.
Chapter 8

Materials and Methods

Study design
The data for this cross-sectional study were collected in April and May 2014 at the Pediatric Dental Clinic of the Kornberg School of Dentistry, Temple University in Philadelphia, US. The Temple University Health Sciences Institutional Review Board approved the study protocol (protocol number 21916). The study was conducted in accordance with the ethical principles of the 64th WMA Declaration of Helsinki (October 2013, Brazil) and the Medical Research Involving Human Subjects Act (WMO), approximating Good Clinical Practice (CPMP/ICH/135/95) guidelines. Potential participants received oral and written information about the study and they could only join after signing an informed consent form. Informed consent was obtained from the parent or legal caregiver of the child, and a written assent was obtained from the child. Participation comprised a visit of approximately 60 minutes to the clinic, for which the parent received a money order (USD 25) as incentive and as a reimbursement for the costs of, for example, transportation, time off work.

Study population
Children between 6 and 14 years old in good general health were eligible to participate in the study. Volunteers for the study had to master the English language and were excluded if they had oral lesions, active orthodontic treatment or removable (partial) dentures, had used antibiotics during the last three months or had periodontal pockets with attachment loss. The caregiver was called by phone one day before the appointment to remind them of the appointment.

Clinical procedures
Participants were instructed not to brush their teeth or to perform any oral hygiene measures on the evening and morning before the appointment. A questionnaire was handed to the caregiver to collect data on oral health behaviours of the child, such as when the child last brushed his/her teeth. Thereafter, red fluorescent plaque (RFP) on the buccal surfaces of all teeth up to the first molars was recorded using a QLF-D camera (Figure 8.1, Inspektor Research Systems BV, Amsterdam, the Netherlands). The QLF-D biluminator system consists of an illumination tube (biluminator; Inspektor Research Systems) fitted onto an SLR-camera (Canon model 550D, fitted with a 60 mm macro lens; Canon, Tokyo, Japan). The illumination tube is composed of a ring, mounted with eight violet-blue-light-emitting diodes (LEDs, 405 ± 20 nm) and four white LEDs (broad spectrum, 6,500 K) with filtering optics in front of the camera lens. The camera system is controlled using dedicated software to capture both fluorescence photographs and white-light photographs (C3; Inspektor Research Systems BV).
Composition of fluorescent plaque

Systems). The photographs were named with a unique identifier to guarantee anonymity of the participants.

Saliva samples were taken using a sterile cotton roll (Salivette, Sarsted AG & Co KG, Numbrecht, Germany), which was held under the tongue of the participant with sterile tweezers to absorb the present saliva until the roll was saturated. Thereafter, two plaque samples per child were taken: one from a site with red RFP and one from a site without red fluorescent plaque (NFP). The plaque samples were preferably taken from the mid-buccal surfaces of the front teeth to avoid differences in composition due to variations in sample site. Plaque samples were collected using sterile regular sized microbrushes (Microbrush, Waterford, Ireland), by wiping the microbrush from the gingival margin two times towards the middle of the tooth, then turning the microbrush around and wiping two more times. The tip of the brush was cut off and stored in a sterile vial. The vials were immediately put at –20°C and stored at –80°C within 2 h.

Subsequently, professional cleaning was performed, after which caries was assessed (by C.M.C.V.) using the ICDAS II (Ismail et al., 2007), complemented with recent bitewings (less than two months old) of the right and left lateral teeth.

Figure 8.1 An example of fluorescence photographs of the buccal surfaces of the teeth of a child before (a) and after (b) professional cleaning, demonstrating that red fluorescence originates from removable plaque and calculus.
Chapter 8

Examiner training
The clinical examiner (C.M.C.V.) was trained and calibrated for clinical caries detection according to the criteria of the ICDAS coordinating committee (ICDAS-Committee, 2009) during several days of training and examination by experienced senior examiner (A.I.I.). The inter-examiner reliability (Cronbach’s alpha) was determined against a second senior examiner (G.M.) on 474 clinical surfaces. The intra-examiner reliability (Cronbach’s alpha) was determined on 312 clinical surfaces at least one week apart. The inter-examiner consistency (Cronbach’s alpha) for the ICDAS scoring was 0.92 and the intra-examiner consistency was 0.99. Two experienced dentists (I.F.P. and C.M.C.V.) assessed the bitewings (inter-examiner reliability 0.86 and intra-examiner reliability 0.92 / 0.92). The scoring system was used as described by Mejare et al. (1997).

Assessment of plaque fluorescence
The fluorescence-photographs were assessed by two experienced examiners (M.H.V. and C.M.C.V.) using a modified Quigley Hein Index (QLF-mQH) to determine the amount of RFP as described previously (Volgenant et al., 2016b). In brief, a six-point scale (0 - 5) was applied at three sites of the vestibular aspects of the teeth (mesio-buccal, mid-buccal and disto-buccal sites). All six scores of the QLF-mQH index (per site) were used, whereafter the average QLF-mQH score per child was calculated (per subject). From the QLF-mQH index, the scores 1 and 3 were used as cutoff values to obtain dichotomized data on red fluorescent plaque per site.

DNA isolation, sequencing and data processing
DNA isolation from saliva samples
The salivette tubes containing the cotton rolls were thawed and 500 µL TE sterile buffer was pipetted onto the cotton roll. The salivette tubes were centrifuged at 4500 g for 10 minutes. The effluent containing the TE and saliva was resuspended and transferred to sterile 1.5 mL Eppendorf tubes and subsequently centrifuged at 15,000 rpm for 15 minutes. The supernatants were removed and the pellets were resuspended in 150 µL sterile TE buffer. The resuspended pellets were transferred to a 1.1 mL deepwell plate (Axygen scientific inc., CA, USA), which contained 250 µL 0.1-mm Zirconia beads, 200 µL of phenol (Rotiphenol, Carl Roth GMBH & Co. KG, Germany) and 200 µL of lysisbuffer (MagMini DNA isolation kit, LGC Genomics ltd, UK). The deepwell plate was sealed with a silicone lid and placed in a Mini-BeadBeater-96 (BioSpec products, Bartlesville, OK, USA) for 2 minutes at 2,100 oscillations/min. DNA was extracted using the Mag Mini DNA Isolation Kit.
DNA isolation from plaque samples

The tubes containing microbrushes were thawed and the microbrushes were transferred, using sterile forceps to a 1.1 mL deepwell plate. The tubes were subsequently washed with 200 µl sterile TE buffer, which was also added to each microbrush containing well. The deepwell plate contained 250 µL of 0.1 mm Zirconia beads, 200 µl of phenol and 200 µL of lysisbuffer. The deepwell plate was sealed with a silicone lid (Axygen scientific inc., CA, USA) and placed in a Mini-BeadBeater-96 for 2 minutes at 2,100 oscillations/min. DNA was extracted using the Mag Mini DNA Isolation Kit.

Sequencing and data processing

Bacterial DNA concentration after purification was determined by quantitative PCR, with universal primers specific to the bacterial 16S rRNA gene (Ciric et al., 2010). The V4 hypervariable region of the 16S rRNA gene was amplified (Caporaso et al., 2011) using 1 ng DNA with 1 µM of each primer used and performing 33 amplification cycles. Paired-end sequencing (251 nucleotides) of the DNA was conducted on the MiSeq platform (Illumina, San Diego, CA, USA) at the VUmc Cancer Center Amsterdam (Amsterdam, the Netherlands). The flow cell was loaded with 4.5 pmol DNA containing 50% PhiX.

The paired-end sequencing reads were merged, processed and clustered using USEARCH version 8.0.1623 (Edgar, 2013). The sequences were quality filtered (maximum expected error rate 0.5, no ambiguous bases allowed) and clustered into operational taxonomic units (OTUs) using the following settings: uparse_maxdball 1,200, only de novo chimera checking, usearch_global with –maxaccepts 8 –maxrejects 64 –maxhits 1. QIIME version 1.8.0 (Caporaso et al., 2010) was used to select the most abundant sequence of each OTU as representative sequence and assign a taxonomy to it, using the RDP classifier (Cole et al., 2009) with a minimum confidence level of 0.8. The alignment of the 97 % representative 16S ribosomal DNA sequence set, provided by the QIIME developers, was first trimmed to the V4 region and the alignment was converted to a set of gap-free nonredundant sequences. This set was used to retrain the RDP classifier. The resulting OTU table was randomly subsampled to an equal depth per sample using QIIME. To further classify the relevant OTUs at species level, the OTU representative sequences were assigned a species name based on the Human Oral Microbiome Database (HOMD, Chen et al., 2010). First, BLAST was used to obtain the top 20 matches in the HOMD database (BLAST on homd.org; HOMD 16S rRNA RefSeq version 14.5 database). A HOMD taxonomy was assigned only if the similarity was at least 97% and if the entire query sequence was aligned. In case of tied top-hits (i.e., the same percentage of similarity), the species names were combined into the taxonomy assigned.
**Data analysis**

Ahead of the data analyses, all identifying information was removed and unique subject identification numbers were used. The deft/DMFT-scores per child were derived from the ICDAS II scores with ICDAS scores 2 and 3 as cutoff values and normalized for the number of teeth present. ICDAS score 3 as a cutoff value has been reported as comparable with the WHO criteria (Braga *et al.*, 2009). For data processing the ICDAS scores were divided in three groups: sound (score 0), no obvious decay (scores 1 and 2) and caries present (scores 3 - 6) as well as in four groups: sound (score 0), initial caries (scores 1 and 2), moderate caries (scores 3 and 4) and extensive caries (scores 5 and 6). For site-level comparisons between the QLF-mQH and the ICDAS score (in three or four groups), the partial correlation coefficient was calculated to correct for dependencies at subject level. Analyses were performed on just the buccal data, as well as on the buccal, mesial and distal data. A Spearman rank correlation was calculated to assess a possible relation between the average amount of RFP and the caries status of the child.

Plaque and saliva microbiome data were subsampled to an equal number of reads per sample to normalize for unequal sequencing depth. The OTU abundances were log2 transformed to normalize the data distribution for principal component analyses (PCA). This analysis takes the abundance of each OTU into account as well as the number of OTUs in the samples and ordinates it into dimensions or principal components.

PERMANOVA (permutational multivariate analysis of variance) -analyses were performed on the Bray-Curtis similarity indices to calculate the significance of the compositional differences between groups (with Bonferroni correction when applying to more than two groups). Compositional differences between sample-groups with significant differences were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata *et al.*, 2011) to determine the features (OTUs), which are most likely the ones that explain the differences between the groups. LEfSe was performed via the Galaxy framework onlineAn LDA value of 3.0 was used as threshold. OTUs, which were computed differentially abundant between the groups in LEfSe, were tested for differences in abundance with a paired samples t-test, using the false discovery rate (FDR) to correct for multiple testing (Benjamini and Hochberg, 1995).

Two important parameters of microbial communities are (1) the α diversity, which is the diversity within each sample, and (2) the β diversity, which is the partitioning of the biological diversity among environments or along a gradient, e.g., the number of species shared between two samples (Lozupone *et al.*, 2007). For the α diversity, the Shannon diversity index was used, for the β diversity the Bray-
Curtis similarity index was calculated. The Kruskal-Wallis test was used to assess
differences in saliva diversity in red plaque fluorescence groups (average QLF-mQH
divided into four groups) and the deft/DMFT score (four groups). The Wilcoxon
signed-rank test was used to test for differences in plaque composition between
RFP and NFP. The Kruskal-Wallis test was performed to test if two plaque samples
within the same individual correlate better with each other than independent
samples with the same phenotype (RFP / NFP), using the Mann-Whitney U test as
post-hoc comparison with Bonferroni correction (0.0167 as the level of significance).
Statistical analyses were performed using IBM SPSS Statistics (version 21, IBM
Inc., Chicago Ill., USA). The PCA, one-way PERMANOVA analyses, Bray-Curtis
similarity indices and Shannon Diversity indices were calculated using PAST
(PALEontological STatistics) version 3.11 (Hammer et al., 2001). P-values < 0.05 were
considered statistically significant unless stated otherwise.

Results
Participants
In total 50 children (28 females) attended a research appointment to participate in the
study with a mean age of 10 years (SD 2.3, range 6 - 14 years). The response rate of
this study was 35%, which was higher than the normal no-show rate in this pediatric
clinic. From three children no recent bitewings were available and from one child a
bitewing image was available only from the right side of the dentition. All children
had RFP, while in four of the 50 participants we were not able to detect NFP.

Caries scores
The normalized mean deft/DMFT score with ICDAS 3 as cutoff value was 4.6 (SD
3.6, median 4.2, range 0 - 15.4) and the mean deft/DMFT score with ICDAS 2 as
cutoff value was 14.3 (SD 5.3, median 14.0, range 4.7 - 25.7). In total 6,640 surfaces
were allocated an ICDAS score 0 - 6, with an average score of 0.47 (SD 1.01, median
0, range 0 - 6). For site-specific comparisons with presence of red fluorescent plaque,
the buccal surfaces (1,075 sites) as well as the buccal, mesial and distal surfaces were
used (3,109 sites).

RFP scores
On subject level, the mean QLF-mQH score for the buccal, mesial and distal surfaces
was 1.1 (SD 0.60; median 1.05, range 0.1 - 2.56). On site level, 2,618 buccal, mesial and
distal surfaces were allocated a QLF-mQH score 0 - 5, with an average score of 1.1
(SD 1.3, median 1.0, range 0 - 5). For site-specific comparisons of RFP with the caries
score, solely buccal surfaces were used as well (950 sites, with mean score 1.15, SD
1.25, median 1.0, range 0 - 5).
Chapter 8

Relationship between caries and RFP

Subject level

A weak though statistically significant correlation was found between the average amount of RFP on the buccal surfaces with the caries status of the child when using the deft/DMFT score with ICDAS 2 as cutoff value ($r_s = 0.37$, $p = 0.007$), but not when using the deft/DMFT score with ICDAS 3 as cutoff value ($p = 0.19$).

Site level

The correlations found between the presence of RFP and the caries status per site were significant ($p < 0.05$), but in all cases the correlation was below 0.2. These results applied to all cases, whether only the buccal data was used or the buccal and approximal data combined.

Microbial profile

The samples had on average 11,038 reads per sample after processing (SD 3,635). The data was subsampled at 7,850 reads per sample. After subsampling, 443 OTUs remained in the data set with an average of 98 OTUs per sample (range 33 - 161, supplementary Table 8.1). Seven samples were excluded, due to too few reads in these samples: three plaque samples and four saliva samples. Combined with the lack of NFP in four children, 43 matching plaque-pairs (a RFP and a NFP sample per child) and 46 individual saliva samples remained for analyses.

Figure 8.2 The microbial composition of red fluorescent plaque (RFP) and not red fluorescent plaque (NFP) represented in a two-dimensional ordination by principal component analysis (PCA). Samples from the same participant are connected with a line.
Composition of fluorescent plaque

Saliva samples
The relation between the microbial composition of saliva and the amount of RFP (mean QLF-mQH index, divided in four quartiles) as well as with the deft/DMFT score (cutoff values ICDAS 2 and 3, divided in four quartiles) was assessed using PCA and PERMANOVA. The PCA showed no clustering by the four RFP quartiles (QLF-mQH score, PERMANOVA, $p > 0.05$, data not shown). The PCA showed also no sample clustering based on the deft/DMFT scores (with ICDAS 2 and 3 as cutoff scores, PERMANOVA, $p > 0.05$, data not shown).

Plaque samples
Clear clustering of samples based on RFP / NFP was observed from the PCA (Figure 8.2). The difference between the microbial profiles of the RFP / NFP groups was statistically significant (PERMANOVA, $p < 0.0001$, $F = 5.518$). The RFP group consisted on average of 105 OTUs per sample (SD 29, range 44 - 161); the NFP consisted on average of 88 OTUs per sample (SD 26, range 33 - 130). The microbial profiles from the RFP were significantly more diverse, both by number of OTUs (species richness; Wilcoxon test, NFP: $n = 45$, median = 91.0; RFP: $n = 48$, median = 107.0, $z = -2.53$, $p = 0.011$, Figure 8.3a), as well as by the Shannon diversity index (Wilcoxon test, NFP: $n = 45$, median = 2.77; RFP: $n = 48$, median = 3.08, $z = -2.39$, $p = 0.017$, Figure 8.3b). No differences in microbial composition at the sampled sites were found based on the ICDAS score (PERMANOVA, $p = 0.04$; not significant after Bonferroni correction).

Figure 8.3 The diversity analyses of red fluorescent plaque (RFP) and not red fluorescent plaque (NFP) with (a) the observed species richness (number of OTUs / sample) and (b) the Shannon diversity index. The red fluorescent dental plaque samples were statistically significantly more diverse than the microbiomes without red fluorescence (Wilcoxon test).
Two corresponding plaque samples from the same individual (RFP / NFP pair) were more similar (Bray-Curtis similarity index) than two unrelated plaque samples of the same fluorescence phenotype (Figure 8.4, Kruskal-Wallis test; H (2) = 17.92, \( p < 0.001 \); RFP vs. paired samples: Mann-Whitney U test; U = 13,189, \( p < 0.001 \); NFP vs paired samples: U = 15,204, \( p < 0.001 \); RFP vs NFP: U = 557,497, \( p = 0.95 \)).

**Differences in composition between RFP and NFP**

The LDA effect size was used to determine which OTUs contributed to the observed differences between RFP and NFP. From the 443 OTUs, 30 OTUs statistically significantly discriminated between the two groups (\( p < 0.05 \), LDA > 3.0). Table 8.1 summarizes the output of the statistical analyses of the LDA effect size and the paired sample t-test. The corrected FDR-significance level was determined at 0.035. Figure 8.5 depicts the relative abundance of reads for the four most discriminative OTUs, which differed significantly between RFP and NFP (based on the LEfSe LDA scores and the paired samples t-test). The four most discriminative OTUs for RFP were *Corynebacterium*, *Leptotrichia*, *Porphyromonas* and *Selenomonas* and the four most discriminative OTUs for NFP were *Neisseria*, *Actinomyces*, *Streptococcus* and *Rothia* (Figure 8.5).
Figure 8.5 The relative abundance of the eight most discriminative OTUs between red fluorescent dental plaque (RFP) and not red fluorescent plaque (NFP) based on the LDA scores in LEfSe and a post-hoc paired t-test with FDR correction with 0.035 as corrected level of significance.

On the next page Table 8.1 A summary of the most significant OTUs that differentiate between red fluorescent dental plaque and not red fluorescent dental plaque. The taxonomy from the SILVA database (lowest level) and the HOMD database (lowest level) is given. The taxa are ranked by the LDA effect size in LEfSe (LDA scores above 3.0) for both groups; red fluorescent dental plaque (RFP) and not red fluorescent dental plaque (NFP). The corresponding p-values are given. The t-value and the p-value of the paired sample t-test are presented as well (FDR correction with 0.035 as corrected level of significance).
<table>
<thead>
<tr>
<th>OTU number</th>
<th>Taxonomy (SILVA)</th>
<th>Taxonomy (HOMD)</th>
<th>Group</th>
<th>LDA effect size</th>
<th>( p )-value (L.ESe)</th>
<th>( t )-value (t-test)</th>
<th>( p )-value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU 5</td>
<td>Corynebacterium</td>
<td><em>Corynebacterium matruchotii</em></td>
<td>RFP</td>
<td>4.12</td>
<td>0.002</td>
<td>-3.26</td>
<td>0.002</td>
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<tr>
<td>OTU 11</td>
<td>Fusobacterium</td>
<td><em>Fusobacterium nucleatum</em> subsp. <em>polymorphism</em> _HOT_202/ *Fusobacterium sp._HOT_203</td>
<td>RFP</td>
<td>3.98</td>
<td>0.022</td>
<td>-0.837</td>
<td>0.41</td>
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<td>OTU 12</td>
<td>Leptotrichia</td>
<td><em>Leptotrichia sp._HOT_417</em></td>
<td>RFP</td>
<td>3.98</td>
<td>0.003</td>
<td>-2.56</td>
<td>0.014</td>
</tr>
<tr>
<td>OTU 6</td>
<td>Porphyromonas</td>
<td><em>Porphyromonas sp._HOT_278</em> / <em>Porphyromonas sp._HOT_279</em></td>
<td>RFP</td>
<td>3.90</td>
<td>0.014</td>
<td>-3.13</td>
<td>0.003</td>
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<tr>
<td>OTU 18</td>
<td>Selenomonas</td>
<td><em>Selenomonas noxia</em></td>
<td>RFP</td>
<td>3.88</td>
<td>0.0003</td>
<td>-3.34</td>
<td>0.002</td>
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<tr>
<td>OTU 25</td>
<td>Prevotella</td>
<td><em>Prevotella melanogenica</em> / <em>Prevotella scopos</em> / <em>Prevotella sp._HOT_314</em></td>
<td>RFP</td>
<td>3.45</td>
<td>0.017</td>
<td>-2.08</td>
<td>0.044</td>
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<tr>
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<td>Prevotella</td>
<td><em>Prevotella sp._HOT_317</em></td>
<td>RFP</td>
<td>3.44</td>
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<td>0.007</td>
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<td>OTU 37</td>
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<td><em>Selenomonas sp._HOT_442</em> / <em>Selenomonas sp._HOT_446</em></td>
<td>RFP</td>
<td>3.41</td>
<td>0.002</td>
<td>-3.11</td>
<td>0.003</td>
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<tr>
<td>OTU 343</td>
<td>Fusobacterium</td>
<td><em>Fusobacterium periodonticum</em></td>
<td>RFP</td>
<td>3.36</td>
<td>0.026</td>
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<td>0.124</td>
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<td>OTU 312</td>
<td>Capnocytophaga</td>
<td><em>Capnocytophaga leadbetteri</em></td>
<td>RFP</td>
<td>3.35</td>
<td>0.019</td>
<td>-2.63</td>
<td>0.012</td>
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<tr>
<td>OTU 33</td>
<td>Campylobacter</td>
<td><em>Campylobacter rectus</em> / <em>Campylobacter showae</em></td>
<td>RFP</td>
<td>3.35</td>
<td>0.005</td>
<td>-3.10</td>
<td>0.003</td>
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<tr>
<td>OTU 178</td>
<td>Aggregatibacter</td>
<td><em>Aggregatibacter segnis</em> / <em>Aggregatibacter sp._HOT_458</em> / <em>Aggregatibacter sp._HOT_512</em></td>
<td>RFP</td>
<td>3.24</td>
<td>0.006</td>
<td>-3.02</td>
<td>0.004</td>
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<tr>
<td>OTU 44</td>
<td>Prevotella</td>
<td><em>Prevotella nigrescens</em></td>
<td>RFP</td>
<td>3.19</td>
<td>0.0007</td>
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<td>0.013</td>
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<tr>
<td>OTU 320</td>
<td>Leptotrichia</td>
<td><em>Leptotrichia buccalis</em></td>
<td>RFP</td>
<td>3.17</td>
<td>0.0003</td>
<td>-2.67</td>
<td>0.011</td>
</tr>
<tr>
<td>OTU 79</td>
<td>Capnocytophaga</td>
<td><em>Capnocytophaga sp._HOT_335</em> / <em>Capnocytophaga sp._HOT_336</em></td>
<td>RFP</td>
<td>3.15</td>
<td>0.0001</td>
<td>-2.85</td>
<td>0.007</td>
</tr>
<tr>
<td>OTU 169</td>
<td>Selenomonas</td>
<td><em>Selenomonas artemidis</em> / <em>Selenomonas sp._HOT_137</em></td>
<td>RFP</td>
<td>3.13</td>
<td>0.002</td>
<td>-3.85</td>
<td>0.0004</td>
</tr>
<tr>
<td>OTU 69</td>
<td>Prevotella</td>
<td><em>Prevotella oris</em></td>
<td>RFP</td>
<td>3.09</td>
<td>0.020</td>
<td>-1.87</td>
<td>0.07</td>
</tr>
<tr>
<td>OTU 58</td>
<td>Prevotella</td>
<td><em>Prevotella aurorum</em></td>
<td>RFP</td>
<td>3.05</td>
<td>0.008</td>
<td>-1.84</td>
<td>0.07</td>
</tr>
<tr>
<td>OTU 52</td>
<td>Leptotrichia</td>
<td><em>Leptotrichia hofstadii</em></td>
<td>RFP</td>
<td>3.03</td>
<td>0.006</td>
<td>-0.602</td>
<td>0.55</td>
</tr>
<tr>
<td>OTU 2</td>
<td>Neisseria</td>
<td><em>Neisseria flavia</em> / <em>Neisseria mucosa</em> / <em>Neisseria pharyngis</em> / <em>Neisseria sicca</em></td>
<td>NFP</td>
<td>4.35</td>
<td>0.045</td>
<td>2.41</td>
<td>0.02</td>
</tr>
<tr>
<td>OTU 7</td>
<td>Actinomyces</td>
<td><em>Actinomyces naeslundii</em> / <em>Actinomyces oris</em> / <em>Actinomyces sp.</em></td>
<td>NFP</td>
<td>4.32</td>
<td>0.002</td>
<td>3.38</td>
<td>0.002</td>
</tr>
<tr>
<td>OTU 1</td>
<td>Streptococcus</td>
<td><em>Streptococcus dentisani</em> / <em>Streptococcus infantis</em> / <em>Streptococcus mitis</em> / <em>Streptococcus oralis</em> / <em>Streptococcus sp.</em></td>
<td>NFP</td>
<td>4.23</td>
<td>0.035</td>
<td>2.86</td>
<td>0.007</td>
</tr>
<tr>
<td>OTU 9</td>
<td>Rothia</td>
<td><em>Rothia dentocariosa</em></td>
<td>NFP</td>
<td>4.12</td>
<td>0.001</td>
<td>2.80</td>
<td>0.008</td>
</tr>
<tr>
<td>OTU 97</td>
<td>Streptococcus</td>
<td><em>Streptococcus sanguinis</em></td>
<td>NFP</td>
<td>4.00</td>
<td>0.013</td>
<td>2.72</td>
<td>0.01</td>
</tr>
<tr>
<td>OTU 10</td>
<td>Lautropia</td>
<td><em>Lautropia mirabilis</em></td>
<td>NFP</td>
<td>3.39</td>
<td>0.011</td>
<td>1.52</td>
<td>0.14</td>
</tr>
<tr>
<td>OTU 75</td>
<td>Methylobacterium</td>
<td>no match</td>
<td>NFP</td>
<td>3.35</td>
<td>0.036</td>
<td>1.78</td>
<td>0.08</td>
</tr>
<tr>
<td>OTU 232</td>
<td>Haemophilus</td>
<td><em>Haemophilus haemolyticus</em> / <em>Haemophilus influenzae</em> / <em>Haemophilus sp._HOT_036</em> / <em>Haemophilus sp._HOT_908</em></td>
<td>NFP</td>
<td>3.29</td>
<td>0.003</td>
<td>2.85</td>
<td>0.007</td>
</tr>
<tr>
<td>OTU 141</td>
<td>Granulicatella</td>
<td><em>Granulicatella elegans</em></td>
<td>NFP</td>
<td>3.23</td>
<td>0.0003</td>
<td>2.74</td>
<td>0.009</td>
</tr>
<tr>
<td>OTU 21</td>
<td>Abiotrophia</td>
<td><em>Abiotrophia defectiva</em></td>
<td>NFP</td>
<td>3.22</td>
<td>0.016</td>
<td>2.86</td>
<td>0.007</td>
</tr>
<tr>
<td>OTU 30</td>
<td>Porphyromonas</td>
<td><em>Porphyromonas sp._HOT_930</em></td>
<td>NFP</td>
<td>3.13</td>
<td>0.006</td>
<td>1.69</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Discussion
The current study assessed the relation between red fluorescence of plaque and caries, as well as the composition of plaque. The presence of RFP correlated weakly with the caries status. However, clear differences in the microbial composition between RFP and NFP were found: the RFP contained taxa that are associated with more mature and more anaerobe plaque than the NFP.

This is the first study assessing the composition of dental plaque based on its red fluorescence characteristics. A remarkable difference in the bacterial composition was found between the two phenotypically different plaque types with the most discriminative OTUs of red fluorescent plaque being mainly Gram-negative anaerobes. An increase in the presence of anaerobe bacteria (Ritz, 1967) as well as the Gram-negative bacteria and Gram-positive rods is associated with older dental plaque (Syed and Loesche, 1978; Marsh and Bradshaw, 1995). *Corynebacterium matruchotti*, here found in red fluorescent plaque, is clinically related with dental calculus formation (Tsuzukibashi et al., 2014). In addition, the taxa of *Fusobacterium*, *Campylobacter*, *Leptotrichia*, *Selenomonas*, *Capnocytophaga*, *Campylobacter* and *Prevotella* were reported to be more prevalent after 1 or 2 weeks without oral hygiene (Kistler et al., 2013). These genera also represent the OTUs found in our study that differentiated most for RFP. Simultaneously, less *Actinomyces*, *Streptococcus* and *Rothia* were identified in samples after a period without oral hygiene (Kistler et al., 2013). This again is in agreement with the OTUs found in this study that differentiated most for NFP. Therefore, we hypothesize that the composition of RFP is related to poor oral hygiene.

Besides distinct differences in microbial profiles, we observed higher diversity of the microbiome of RFP compared to NFP. A higher microbiome diversity has been reported in periodontitis patients (Abusleme et al., 2013; Pozhitkov et al., 2015), while the opposite has been described for samples associated with caries (Gross et al., 2010). Higher microbial diversity, as found in RFP, suggests the presence of complex microbial interactions within the biofilm (Kuramitsu et al., 2007), resulting in the establishment of a greater variety of taxa than do not fluoresce red. Microbial interactions between *Parvimonas micra* and *Porphyromonas gingivalis* were reported to induce red fluorescence (Van der Veen et al., 2006; Volgenant et al., 2013). Higher similarity in the composition of plaque samples from the same person (RFP and NFP samples) was found compared to the non-matching pairs (irrespective of the fluorescence phenotype). This indicates that the inter-individual variation in this study population was higher than the expected differences in biofilm phenotype, which could be due to a relatively low sample size.
Red fluorescence is a characteristic of dental plaque. Therefore, it was not surprising to find that there was no correlation between the microbial composition of the saliva and the average amount of RFP. The microbial composition of saliva is more similar to the microbial composition of tongue and throat and less to the composition of dental plaque (Segata et al., 2012). This could explain the absence of a relation between the average amount of RFP and the saliva composition. The absence of a relation between the deft/DMFT and differences in saliva composition in our study contradicts a previous study where saliva microbiomes discriminated the caries-active individuals from healthy participants (Yang et al., 2012). The study of Yang et al. (2012) was performed in a different age-group (participants of 18 - 22 years), while it is known that the developmental stage of the dentition influences the microbial composition (Crielaard et al., 2011). Additionally, our study was not designed to compare two matched and distinct groups (caries versus caries-free). Moreover, we assessed RFP only on the buccal surfaces of teeth, which could be discrepant with the overall quantity of red fluorescent plaque in the oral cavity.

Taking the various results together, the hypothesis of RFP being mature, disease-related plaque was confirmed. Red fluorescence can be the result of specific microorganisms, present in an older biofilm, developing towards dysbiosis. Although the composition and relative diversity of RFP seems to be more related with plaque found after a period of refraining from oral health, we did find a weak correlation between RFP and the caries status on subject level when non-cavitated lesions were included.

Since dental plaque causes caries, the presence of plaque precedes the presence of caries (Axelsson and Lindhe, 1978). We found a high amount of sites with RFP with an ICDAS score of 0; these sites could (or could not) become caries active in time. The lack of a clinically relevant correlation between these two parameters could be due to the fact that we obtained ‘snap-shot’ information by a single examination and a single plaque sampling moment, while caries lesions are the result of events that progress over time (Fejerskov, 1997). A better way of assessing changes in plaque ecology and in caries activity would be by performing a longitudinal study. Finally, we found statistically significant though very weak correlations between the caries status on site level and the presence of RFP. The statistical significance of these very weak correlations on site level were most likely influenced by the large number of sites included in the calculation (Chavalarias et al., 2016).

In conclusion, red fluorescing plaque had a weak association with the caries status of the children, while its microbial composition was distinct from plaque that did not fluoresce red: it contained taxa that are associated with old, mature plaque. Since caries activity can only be determined over time, longitudinal studies are
Composition of fluorescent plaque

required to assess the actual potential of red fluorescent plaque to be used in oral health assessments.

Supplementary file

Table S8.1 The total OTU frequencies per sample are presented with the taxonomy as derived from SILVA and HOMD. Sample IDs with P0 are the NFP samples, sample IDs with P2 are the RFP samples and sample IDs with S9 are the saliva samples. The number after the letter c in the sample ID indicates the subject number.

This supplementary file is available at: http://hdl.handle.net/11245/1.538521