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ORIGINAL ARTICLE

Change in diet and oral hygiene over an 8-week period: effects on oral health and oral biofilm

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Abstract The aim of the study was to monitor changes in oral health and oral biofilm composition in vivo during an experiment simulating prehistoric lifestyle and diet and poor oral hygiene. Thirteen subjects lived for a period of 8 weeks under Neolithic conditions. The following clinical parameters were recorded before and after the project: gingival and plaque index (Löe and Silness, Acta Odontol Scand 21:533, 1963; Silness and Löe, Acta Odontol Scand 22:121–135, 1964), probing pocket depth, and bleeding upon probing. In addition, supragingival plaque samples were collected both before and after the project and were analysed quantitatively using multiplex fluorescence in situ hybridization and confocal laser scanning microscopy. The following plaque bacteria were evaluated: *Streptococcus* spp., *Veillonella* spp., *Fusobacterium nucleatum*, and *Actinomyces naeslundii*. The plaque index increased significantly from 1.12 up to 1.55 over the 8-week period (gingival index before, 0.46; after, 0.93; $p < 0.05$). A strong correlation of both indices was recorded before ($r = 0.77$) and after ($r = 0.83$) participation in the study. Each of the children in the study showed a progression of carious lesions and/or new areas of demineralisation. The probing pocket depth and bleeding upon probing were not affected. All subjects yielded an intra-individual shift in biofilm composition. The proportion of *F. nucleatum* decreased across all subjects. The proportion of *Veillonella* spp. increased among the children. Poor oral hygiene and change of diet lead to an increase in oral plaque and gingival inflammation. The inter-individual comparison indicated a shift in bacterial composition.

Keywords Nutrition · Oral hygiene · Dental biofilm · Gingivitis · Multiplex FISH

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Introduction

Dental plaque biofilm is the trigger for the dental diseases caries, gingivitis, and periodontitis. Mature dental plaque is made up of a multispecies biofilm which contains more than 500 different species of bacteria [1]. Its formation is the result of complex bioadhesive processes within the oral cavity [1-3]. The amount of supragingival plaque has been shown previously to be correlated with gingivitis over study periods of 10 to 20 days [4]. With respect to plaque and caries formation in man, a number of archaeological studies have indicated an increase in the amount of caries corresponding with the end of nomadism and the beginning of agriculture [5-9]. The increasing consumption of carbohydrates during the Neolithic period led to an increase in caries [5-8]. Carbohydrates such as starch accumulate in

dental plaque and are degraded by amylase, where the starch break down products in turn become substrates for cariogenic and glycolytic oral bacterial [10].

Several approaches have been adapted in the past for the microbial investigation of oral biofilms. In contrast to methods which depend on culturing of the biofilm bacteria, fluorescence in situ hybridization (FISH) is a useful and well-established method which allows the detection and quantification of specific bacteria without disrupting their natural environment [11-14]. Furthermore, viable plate count and culture-dependent techniques automatically involve selection for certain bacteria present in the dental plaque. This could lead to false results with regard to the actual bacterial structure.

The combination of FISH with confocal laser scanning microscopy has already been used to obtain images of three-dimensional reconstructions of supragingival dental plaque [11]. However, there have been no FISH studies to date which have looked at the bacterial composition of mature plaque, meaning plaque established in vivo over a period of several weeks in a defined setup without oral hygiene.

An anthropological project simulating the nutrition and living conditions of the Neolithic epoch over an 8-week period offered an opportunity to monitor the long-term effects of poor oral hygiene and changed diet on clinical dental parameters, as well as on the microbial composition of dental supragingival dental plaque in 13 volunteers. Microbiological and clinical data at the beginning and end of the test period were compared.

The hypothesis to be tested was the change of oral hygiene and Neolithic diet leads to an increase in plaque and gingivitis and to a formation of new initial caries lesions along with a shift in the composition of supragingival plaque.

Materials and methods

General setup

The University of Freiburg conducted an anthropological experiment simulating the living conditions of an early agrarian community of around 3,000 BC. A group of 13 subjects, six of them children, lived under these conditions (Neolithic period) for 8 weeks in August and September of 2006 in dwellings near Lake Constance in Germany. The participants were extensively trained in Neolithic agriculture and craft techniques. This study was reviewed and approved by the ethics committee of the Medical Faculty of the University of Freiburg. Informed written consent was given by the subjects for participation in the project and for medical and dental examinations, respectively. Oral examination was carried out by three experienced dentists directly before participation in the project and immediately afterwards.

Nutrition before and during the project was recorded. The conventional diet of the subjects before the project was rich in potatoes, bread, and noodles. Sausages and sweets as well as dairy products were frequently consumed. In addition, fruits, particularly bananas, were part of the diet.

During the project, the primary source of food was a type of porridge made from self-ground prehistoric grain (wild emmer *Triticum dicoccon* and prehistoric wheat *Triticum turgidum* L.). In addition, nuts and meat and some fruits, primarily berries and apples, were consumed. Dairy products from goat's milk also formed a part of the diet. The participants were extensively informed about this diet before the start of the project.

The subjects used neither toothbrushes nor other standard oral hygiene devices; instead, only twigs were used sporadically.

Subjects and samples

The group of participants included six children (aged 3–11) and seven adults (aged 31–64). A detailed dental examination was performed recording dental restorations as well as initial and open carious lesions and the DMF-t/dmf-t (decayed, missing, filled teeth) was calculated. When required, therapy of open carious lesions and endodontic treatment were carried out before the project.

Measurement of the plaque index was performed according to Silness and Löe [15], and the gingival index was calculated according to Löe and Silness [16] at buccal and oral sites for all teeth. A total of six measurements was carried out per tooth for each index.

Additionally, detailed periodontal examinations of the adult subjects were performed. At six sites per tooth, bleeding upon probing (yes/no) and probing depth were recorded. The gingival index was measured first. The probing of the periodontal pockets was carried out 30 min after the gingival index in order to avoid false positive results. During this time period, the subjects rinsed their mouths several times with water.

Plaque samples

The subjects stopped their oral hygiene for 24 h before the preliminary examination, in order to gain enough plaque for the laboratory experiments.

Pooled plaque samples for multiplex FISH analysis were collected at lingual sites on the mandibular second molars and at buccal and lingual sites on the maxillary first and second molars. Samples were taken before and immediately after participation in the project with sterile curettes and

transferred in sterile tubes containing phosphate-buffered saline. Dental prophylaxis was carried out at the end of the preliminary examination.

Caries risk assessment with commercial test kits

Caries risk was evaluated via measurement of lactate release (grade 0–9, Cario L-Pop, 3 M Espe®, Seefeld, Germany). Furthermore, the amount of streptococci and lactobacilli was determined using the CRT® bacteria kit (Ivoclar Vivadent, Schaan, Liechtenstein). Grade 1 means that there were less than 10^5 CFU/ml, while grades 2 and 3 had more than 10^5 CFU/ml.

Fluorescence in situ hybridization (FISH) and confocal microscopy

FISH was conducted according to Amann [17] but was modified slightly as previously described [11, 14]. In order to minimise cell loss during the following hybridization and washing steps, the plaque samples were coated with agarose [18]. For this purpose, the fixed plaque material was spotted onto microscope slides (Erie scientific company, Portsmouth, UK). The spotted samples were allowed to dry at 46°C. Afterwards, the slides were immersed in molten 0.5% agarose (PeQLab Biotechnologie GmbH, Munich, Germany) at 37°C for 3 s. The slides were then placed on ice until the agarose had solidified. The oligonucleotide probes used in this study, as well as the method used for image analysis, were described earlier in an in situ study on dental supragingival plaque [11].

Statistics

An aggregate statistical analysis was conducted by averaging all of the measured values for each subject at each time point. For each of the four bacterial targets, we fitted a linear mixed model [19]. Each continuous response variable is modelled as a linear function of time (end versus beginning), group (adults versus children), and the time-group interaction as explanatory variables. To take the dependency within each proband into account, we fitted probands as random. Variance components were used as a covariant structure. Model assumptions were graphically checked by residuals and other regression diagnostics (including Cook's distance). The normality of the error terms can be assumed. Least-square means with 95% confidence intervals were calculated and displayed graphically.

Changes in the plaque and gingivitis indices were evaluated using the paired t test ($p < 0.05$); Pearson correlations for these two indices were also calculated.

All calculations were carried out using the PROC MIXED option from the SAS 9.1.2 statistical software.

Results

Clinical parameters

The plaque-index level was 1.12 ± 0.50 before modern oral hygiene was stopped and then increased significantly to reach 1.55 ± 0.31 after 8 weeks ($p = 0.017$, t test). Additionally, the gingival index increased significantly from 0.46 ± 0.41 to 0.93 ± 0.38 at the end of the experiment ($p = 0.0001$). A strong correlation of both indices was recorded before ($r = 0.77$) and after ($r = 0.83$) participation in the study (Fig. 1).

The DMF-t was 15.9 ± 6.3 for the adults (D, 2.7 ± 2.4; M, 2.7 ± 3.1; F, 10.4 ± 7.5). It did not change throughout the course of the project. For the children, DMF-t and dmf-t were calculated separately. The DMF-t ($n = 3$ children) amounted to 2.7 ± 2.3 and was unchanged. The dmf-t ($n = 6$ children) was 3.5 ± 2.6 in the beginning (d, 1.2 ± 1.5; m, 0.2 ± 0.3; f, 2.3 ± 2.3) and increased to 4.2 ± 2.3 at the end of the study (d, 1.8 ± 1.5; m, 0.2 ± 0.3; f, 2.3 ± 2.3). Four of the children developed up to two new cavities which arose from previous initial lesions; nearly all children showed new initial lesions after participating in the project. The new demineralization was observed buccaly and at the occlusal sites.

A detailed periodontal examination was carried out only in the adult subjects ($n = 7$). One of the subjects showed no attachment loss, but five of them had an attachment loss of up to 40% with probing depths up to 6 mm. One subject had severe periodontitis with an attachment loss of more than 70% and a probing depth of up to 12 mm. The periodontal parameters for the adults remained unchanged. The probing pocket depth was 3.17 ± 1.36 mm at the beginning of the study and 3.01 ± 1.26 mm after living under conditions of poor oral hygiene for 8 weeks. On

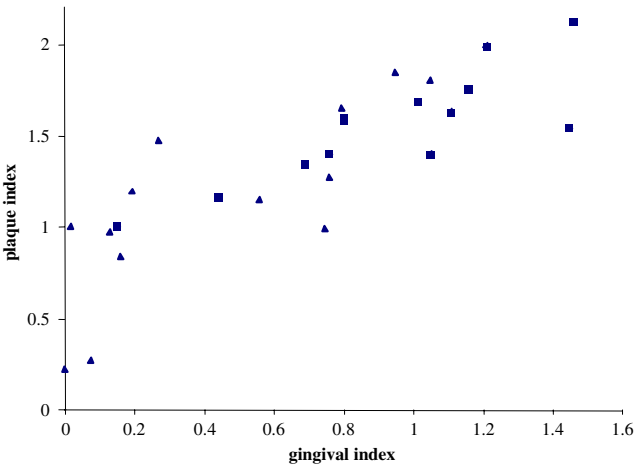

Fig. 1 Correlation of gingival index and plaque index, $n = 13$ subjects, data recorded before (*triangle*) and 8 weeks after (*square*) change of oral hygiene and diet

average, 55 ± 46% sites bled on probing before and 66 ± 45% at the end of the study.

Commercial test kits

High lactate release (grade 7.92 ± 0.95) was observed before and after (grade 7.15 ± 1.86) participation in the project, indicating a high caries risk (Cario L-Pop, 3 M Espe®). This was confirmed by determination of streptococci and lactobacilli (CRT® bacteria). Before the project, the score for streptococci had a grade of 2.92 ± 0. 85, while afterwards, it amounted to 3.00 ± 0.91 (lactobacilli: before, 2.69 ± 0.75; afterwards, 1.77 ± 0.71).

Multiplex FISH

Each of the bacterial targets was detected both before and after 8 weeks of the project. The predicted means with 95% confidence intervals for all adults and all children are shown separately in Fig. 2. The predicted means for *Streptococcus* spp. content for all adults were 14.4% in the beginning and 13.7% at the end. The corresponding values for *Streptococcus* spp. for the children were 14.1% and 12.7%. For the adult subjects, the values of *Fusobacterium nucleatum* were 26.3% before the project and 21.7% after 8 weeks, respectively; whereas, for the children, predicted means of 34.8% (begin) and 20.7% (end) were recorded. *Actinomyces naeslundii* was detected as a minor component with proportions between 2.5% before and 1.8% after the experiment in the adults (children: before, 2.7%; after, 5.6%). The *Veillonella* spp. data were 9.5% (begin) and 9.4% (end) for adults and 5.1% and 14.3% for children, respectively. When considering all subjects as a single group, significant changes in detected bacterial targets were observed over the 8-week period for *F. nucleatum* ($p = 0.0168$) and *Veillonella* spp. ($p = 0.0351$). A significant time-group interaction was only detected for *Veillonella* spp. ($p = 0.0342$), meaning that there was a time effect only among the children but not for the adults as can be seen in Fig. 2. A considerable intra-individual shift in bacterial composition was recorded within each subject. Representative stack of confocal images of dental plaque is shown in (Fig. 3).

Discussion

The present study is the first in which the influence of a simulated Neolithic lifestyle and diet on oral health was scientifically examined.

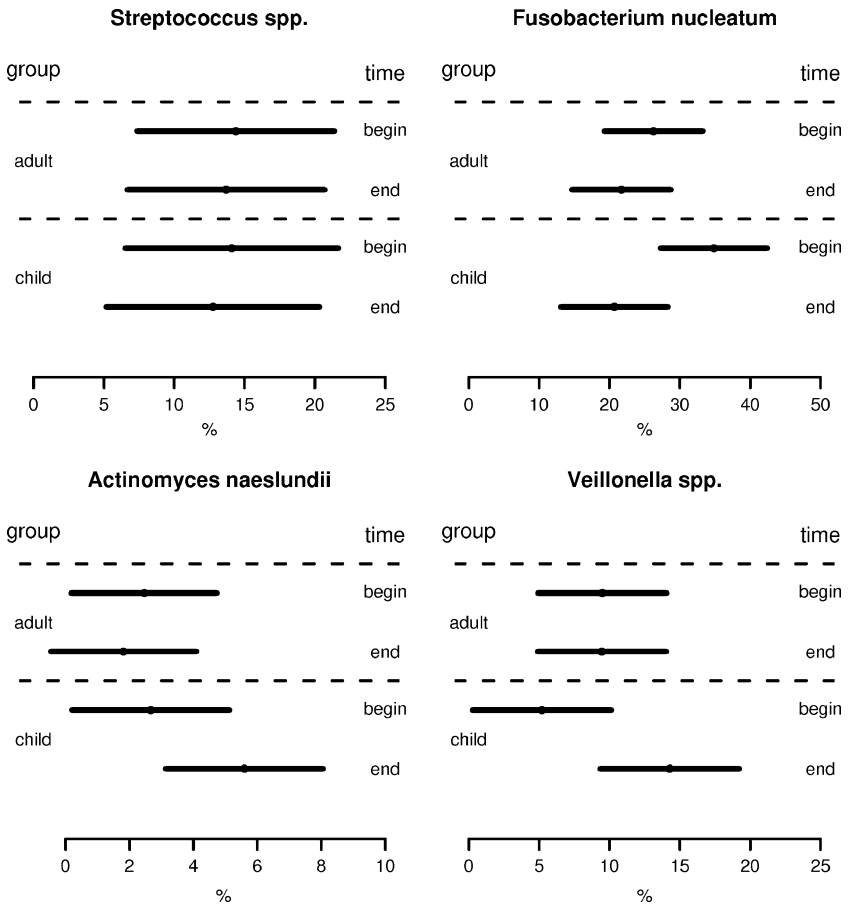

Fig. 2 Predicted means estimated from the linear mixed model with 95% confidence limits for each parameter, respectively. The proportions of *Streptococcus* spp., *Fusobacterium nucleatum*, *Actinomyces naeslundii*, and *Veillonella* spp. both before and after the project are shown. Data for adults ($n = 7$) and children ($n = 6$) are given separately

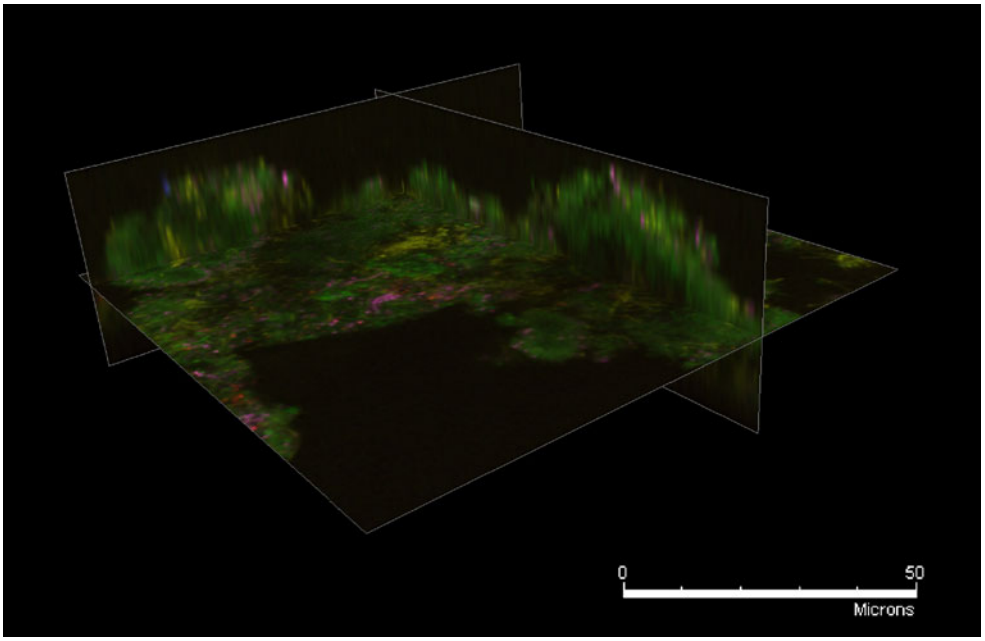

Fig. 3 Representative orthogonal slices through a stack of confocal images of mature dental plaque after multiplex fluorescence in situ hybridization. Eubacteria (*green*), *Streptococcus* spp. (*magenta*), *Veillonella* spp. (*red*), *Fusobacterium nucleatum* (*yellow*), and *Actinomyces naeslundii* (*blue*). The bar represents 50 μm

Due to its basic setup, the study was limited to a total of 13 subjects and to a duration of 8 weeks. Furthermore, the subjects were inhomogeneous as a group, as was their general oral health. This is because the selection of the participants was based on a large variety of criteria including the intention of recruiting families rather than matched single subjects. Accordingly, the data have to be interpreted with respect to these limitations, and it is not possible to draw general conclusions on oral health in the Stone Age. Furthermore, the modern lifestyle of the subjects before participation in the study has to be considered with respect to dental restorations, diet, oral hygiene, and therefore oral microbiology. Nonetheless, valuable information could be gained on several dental parameters with respect to the Neolithic lifestyle.

Previous studies on the correlation of plaque and gingivitis usually monitored periods of 10 to 20 days [4]. As was expected, a significant increase of plaque and gingivitis could be observed after 8 weeks. This corresponds to the observations of a previous long-term study on experimental gingivitis [20]. The well-known correlation of gingivitis and plaque could be clearly confirmed. The periodontal parameters were not affected, although presumably, during a longer period of poor oral hygiene, not only gingivitis but also progredient periodontal destruction would be conceivable. In agreement with this consideration, periodontal defects have been observed in prehistoric skulls from all periods [21, 22]. However, it could be expected that the participants developed their own devices for improvisational oral hygiene. Indeed, small pieces of twigs were used sporadically as toothpicks by the participants. Nonetheless, this cannot be compared with the well-proven effect of a miswaak, which releases fluoride [23].

Furthermore, an increase in initial carious lesions and a progression of existing carious lesions were observed in the children. In a previous study, it was shown that visible initial demineralisations may occur within the course of 23 days after cessation of oral hygiene [24]. The generally high caries risk of the subjects, as assessed by commercial test kits, offers an additional explanation for this finding. Furthermore, the main food of the subjects was derived from grain and composed of carbohydrates. With respect to starch-metabolism in humans, it has been shown recently that the amount of salivary amylase gene copy numbers increased in populations with diets rich in carbohydrates [25]. Due to the fact that amylase promotes caries progredience and bacterial adhesion, the increase of caries in agricultural populations after the onset of sedentism is feasible [10, 26].

The recently established FISH method was used to characterise the bacterial composition of the plaque. The advantages of using multiplex FISH to study the microbial composition of supragingival dental plaque have been discussed elsewhere in detail [11]. The present data confirmed the microbial distribution observed previously for 1–7-day-old biofilm for the bacterial targets analysed and offer more information on the composition of mature supragingival dental plaque. Due to that fact that FISH targets the ribosomes in bacteria, it should be emphasised that it more primarily detects bacteria which were active at the time of fixation [12], and that the bacteria detected in this study could make up the main portion of viable bacteria present in dental plaque [27]. All in all, this study showed that the mean composition of mature plaque was affected by an 8-week period of poor oral hygiene combined with a prehistoric diet with regard to *F. nucleatum* and *Veillonella*

spp. levels. Furthermore, all subjects yielded an intra-individual shift in bacterial composition with respect to the four species tested. The failure to detect a change in the proportions of oral streptococci and *A. naeslundii* could be caused by the limited number of plaque samples studied. Additionally, a more exhaustive analysis of the genus *Streptococcus* would have offered more details on the distribution of this predominant bacterial group.

Conclusions

The well-known non-specific correlation of supragingival plaque and gingivitis was confirmed. The current study tried to simulate Neolithic lifestyle, but no valid conclusion on the increase of caries in man after sedentism and the beginning of agriculture can be drawn due to the low number of participants. The data on bacterial targets offer information on their distribution in mature plaque. General changes in plaque composition were observed only for *F. nucleatum* and *Veillonella* spp., though a considerable intra-individual bacterial shift was observed for each subject.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Foster JS, Kolenbrander PE (2004) Development of a multispecies oral bacterial community in a saliva-conditiones flow cell. Appl Environ Microbiol 70:4340–4348
2. Moore WEC, Moore LVH (1994) The bacteria of periodontal diseases. Periodontol 2000 5:66–77
3. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE (2001) Bacterial diversity in human subgingival plaque. J Bacteriol 183:3770–3783
4. Löe H, Theilade E, Jensen SB (1965) Experimental gingivitis in man. J Periodontol 36:177–187
5. Lunt DA (1974) The prevalence of dental caries in the permanent dentition of Scottish prehistoric and mediaeval populations. Arch Oral Biol 19:431–437
6. O'Sullivan EA, Williams SA, Wakefield RC, Cape JE, Curzon ME (1993) Prevalence and site characteristics of dental caries in primary molar teeth from prehistoric times to the 18th century in England. Caries Res 27:147–153
7. Sobkowiak EM, Berg P, Held M, Schumacher GH (1978) Comparative studies of caries epidemiology using skulls from the Iron, Slavic, and Middle Ages as well as modern subjects (author's transl). Zahn- Mund- Kieferheilkd Zent.bl 66:359–366 German
8. Tayles N, Domett K, Nelsen K (2000) Agriculture and dental caries? The case of rice in prehistoric Southeast Asia. World Archeol 32:68–83
9. Temple DH, Larsen CS (2007) Dental caries prevalence as evidence for agriculture and subsistence variation during the Yayoi period in prehistoric Japan: biocultural interpretations of an economy in transition. Am J Phys Anthropol 134:501–512
10. Douglas CW (1994) Bacterial-protein interactions in the oral cavity. Adv Dent Res 8:254–262
11. Al-Ahmad A, Wunder A, Auschill TM, Follo M, Braun G, Hellwig E, Arweiler NB (2007) The in vivo dynamics of *Streptococcus* spp., *Actinomyces naeslundii*, *Fusobacterium nucleatum* and *Veillonella* spp. In dental plaque biofilm as analysed by five colour multiplex-FISH. J Med Microbiol 56:681–687
12. Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169
13. Dige I, Nilsson H, Kilian M, Nyvad B (2007) In situ identification of streptococci and other bacteria in initial dental biofilm by confocal laser scanning microscopy and fluorescence in situ hybridization. Eur J Oral Sci 115:459–467
14. Hannig C, Hannig M, Rehmer O, Braun G, Hellwig E, Al-Ahmad A (2007) Fluorescence microscopic visualization and quantification of initial bacterial colonization on enamel in situ. Arch Oral Biol 52:1048–1056
15. Silness J, Löe H (1964) Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. Acta Odontol Scand 22:121–135
16. Löe H, Silness J (1963) Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol Scand 21:533
17. Amann RI (1995) In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Akkermans ADL, van Elsas JD, de Bruijin FJ (eds) Molecular microbial ecology manual. Kluwer Publishers, Dordrecht, pp 1–15
18. Daims H, Ramsing NB, Schleifer KH, Wagner M (2001) Cultivation-independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence in situ hybridization. Appl Environ Microbiol 67:5810–5818
19. Brown H, Prescott R (1999) Normal mixed models. In: Barnett V (ed) Applied mixed models in medicine. Wiley, Hoboken, pp 33–83
20. Brecx MC, Fröhlicher I, Gehr P, Lang NP (1988) Stereological observations on long-term experimental gingivitis in man. J Clin Periodontol 15:621–627
21. Lavelle CLB (1973) Alveolar bone loss and tooth attrition in skulls from different population samples. J Periodontal Res 8:395–399
22. Molnar S, Molnar I (1985) Observations of dental diseases among prehistoric populations of Hungary. Am J Phys Anthropol 67:51–63
23. Wu CD, Darout IA, Skaug N (2001) Chewing sticks: timeless natural toothbrushes for oral cleansing. J Periodontal Res 36:275–284
24. Von der Fehr FR, Löe H, Theilade E (1970) Experimental caries in man. Caries Res 4:131–148
25. Perry GH, Dominy NJ, Claw KG, Lee AS, Fiegler H, Redon R, Werner J, Villanea FA, Mountain JL, Misra R, Carter NP, Lee C, Stone AC (2007) Diet and the evolution of human amylase gene copy number variation. Nat Genet 39:1256–1260
26. Douglas CW (1983) The binding of human salivary alpha-amylase by oral strains of streptococcal bacteria. Arch Oral Biol 28:567–573
27. Arweiler N, Hellwig E, Sculean A, Hein N, Auschill TM (2004) Individual vitality pattern of in situ dental Biofilms at different locations in the oral cavity. Caries Res 38:442–447