Dental caries and microbiota in children with black stain and non-discoloured dental plaque

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Abstract

Aim: Assessment of caries experience and microbiota in systemically healthy children with black stain (BS) and non-discoloured plaque. ***Methods:*** 46 children with BS and 47 counterparts with non-discoloured plaque aged 7.9 ± 1.3 years were clinically examined. Dental caries was scored using WHO criteria. Samples of BS and non-discoloured dental plaque were collected from tooth surfaces. The DNA of the samples was extracted and real-time PCR was performed to determine the total number of bacteria and the species *Streptococcus mutans, S. sobrinus*, *Lactobacillus* sp., *Actinomyces naeslundii*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *and Fusobacterium nucleatum*. ***Results:*** Children with BS had lower dmft (p=0.013), dt values (p=0.005) and a tendency to lower caries prevalence (p=0.061) than children with non-discoloured plaque. Plaque samples of the BS group contained higher numbers of *A. naeslundii* (p=0.005) and lower numbers of *F. nucleatum* (p=0.001) and *Lactobacillus* sp. (p=0.001) compared to non-discoloured plaque samples of the control group. Comparing caries-free and caries-affected children with BS and non-discoloured plaque higher counts for *A. naeslundii* (p=0.013) were observed in caries-free children with BS while in caries-affected children with BS higher counts of *F. nucleatum* (p=0.007) were found. Counts of *Lactobacillus* sp. were higher in non-discoloured plaque samples than in BS of caries-free and caries-affected children.

Conclusion: Results suggest that the different microbial composition of BS might be associated with the lower caries experience in affected subjects. The role of black pigmented bacteria associated with periodontitis needs further studies.

The interest in genesis and nature of extrinsic black tooth stain in dentistry has now continued for nearly a century [Pickerill, 1923; Ronay and Attin, 2011; França-Pinto et al., 2012]. Black stain (BS) may be clinically diagnosed as pigmented dark lines parallel to the gingival margin [Pedersen, 1946; Shourie, 1947; Leimgruber, 1950; Commerell, 1955; Gülzow, 1963; Koch et al., 2001] or as incomplete coalescence of dark dots rarely extending beyond the cervical third of the crown. It belongs to the group of extrinsic tooth discolouration of non-metallic origin [Watts and Addy, 2001]. Dietary chromogens found in tea, coffee and other beverages (tannins) are possible aetiological agents such as tobacco and chlorhexidine mouthrinses resulting in extrinsic staining by adsorption onto the tooth surface via plaque or the acquired pellicle [Manuel et al., 2010]. Metallic extrinsic discolouration may be associated with metal salts containing medicaments and with industrial exposure to iron, manganese and silver [Ronay and Attin, 2011; Watts and Addy, 2001]. In people using iron supplements, especially iron containing oral solutions, characteristic black staining of teeth was reported [Nordbo et al., 1982; Manuel et al., 2010; Kumar et al., 2012]. A recent study among 5-year-old Spanish children with BS found a significant higher consumption of iron supplements in the children (11%) and their mothers during pregnancy (not specified) compared to counterparts without BS (2.7%) and their mothers (not specified) [Garcia Martin et al., 2013]. The authors observed also higher consumption rates of iron containing foods in children with than without BS. Furthermore, a positive correlation between black extrinsic stains and the concentration of iron in water sources was documented [Pushpanjali et al., 2004]. In contrast to BS, these forms of black extrinsic staining are commonly covering the entire tooth surface as thin film and are not limited to the cervical third of the crown. In addition to the history of subjects affected this feature should be used as differential diagnostic criterion. Intrinsic discolourations, resulting from incorporation of pigments into the dental tissues during tooth development (genetic and metabolic disorders, medication), have a completely different appearance and can hardly be mistaken for BS [Manuel et al., 2010; Ronay and Attin, 2011; Kumar et al., 2012].

Black stain as particular type of pigmentation has been considered to be a special form of dental plaque because it contains an insoluble ferric salt, probably ferrous sulfide, and a high content of calcium and phosphate [Reid and Beeley, 1976]. The chemical composition of saliva in children with BS indicated a significantly higher content of calcium, inorganic phosphates, copper, sodium and total protein but less glucose compared to children without staining [Surdacka, 1989b]. The assessment of the amount and pH of saliva in children with staining revealed a significantly higher pH but an equal amount of saliva in these children compared to a control group [Surdacka, 1989a].

Early ultrastructural examinations of BS demonstrated that this deposit consists of microorganisms embedded in an intermicrobial matrix [Theilade et al., 1973], characterised by its simple bacteriological composition of gram-positive rods and its tendency to calcify [Reid et al., 1977; Theilade and Pang, 1987]. Actinomycetes have been reported as the predominant microorganisms in BS [Slots 1974, Saba et al., 2006]. Rarely, pigmented gram-negative rods were detectable by using cultivation [Slots, 1974]. A possible interaction between the microbiota related to extrinsic pigmentation, cariogenic pathogens and caries remains obscure.

There is no consensus in the literature concerning prevalence of BS among age groups [Commerell, 1955; Gülzow, 1963; Surdacka, 1987; Koch et al., 2001; Gasparetto et al., 2003; Paredes Gallardo and Paredes Cencillo, 2005; Heinrich-Weltzien et al., 2009; Bhat, 2010; Bartsch and Heinrich-Weltzien, 2011; França-Pinto et al., 2012, Garcia Martin et al., 2013], but the presence of these stains has been commonly associated with a lower caries prevalence and/or experience [Shourie, 1947; Commerell ,1955; Koch et al., 2001; Gasparetto et al., 2003; Heinrich-Weltzien et al., 2009; Bhat, 2010; Bartsch and Heinrich-Weltzien, 2011]. The recent study of França-Pinto et al. [2012] investigated the presence of BS and its potential caries protective effect considering an adjustment for demographic, social and behavioral factors that may influence both, dental caries and BS. According to their findings the authors suggested that BS is a protective factor for dental caries development.

Up to now, no study has addressed the investigation of caries experience and microbiota in children with BS and non-discoloured plaque. Therefore, this study tests the hypotheses: 1) caries prevalence and experience differ in children with and without BS, and 2) the microbiota differs between BS and non-discoloured plaque.

Subjects and Methods

Subject Selection

During the annual dental examination of 3- to 10-year-old Westphalian children attending kindergartens and elementary schools in the city of Dormagen, located in the Rhein-District Neuss, Germany, the occurrence of BS was recorded. Black stain was found in 113 children (1.5%) among the total population of 7.624 children [Bartsch and Heinrich-Weltzien, 2011]. Parents and/or caregivers of elementary school children with BS were asked for participation of their children in a clinical and microbiological study to determine the microbiota in BS samples. Goal and procedure of the study were detailed explained in writing. To create a control group to that BS group these children were asked to invite their school friends attending the same grade to participate in this study. Parents and/or caregivers of these recruited children with non-discoloured plaque were informed just as children with BS. Parents and/or caregivers of 47 children with BS and 46 children with non-discoloured plaque signed the informed consent forms. According to their interviewed parents all children involved were systemically healthy subjects. None of the children had undergone treatment with antibiotics within the past 6 month before the study. Children taking drugs incl. iron supplementation for longer than 6 months were excluded. None of the children preferred a special diet. The study was approved by the ethics committee of the University Hospital Jena under the registration number 1921-12/06.

Clinical Examination

The caries status of the children was assessed by one experienced and calibrated paediatric dentist (BB) on tooth level according to WHO criteria [WHO, 1997]. Presence of BS was recorded as present or absent independently from the number of teeth affected. The criterion for scoring BS was the presence of firmly adherent black dots generally forming linear discolouration parallel to the gingival margin and occasionally covering up to one third or more of the clinical tooth crown [Koch et al., 2001]. The examination was performed in classrooms using an intra-oral mouth mirror, a CPI ball end probe and a LED headlight (Waldmann, HX 35 N, Germany) after plaque sampling and tooth brushing supervised by the dental nurse.

A 1-day calibration training of the examiner (BB) was performed by an epidemiological experienced dentist (RHW). Calibration of caries scoring on dentin level was carried out at a local school that was not included in the survey sample. Calibration of scoring BS was restricted to training with typical images.

Plaque Sampling Procedure

Approximately 1 mg BS or non-discoloured plaque was collected from buccal and/or lingual surfaces of the upper first and second primary molar (preferred tooth: 64 and 65) by scraping with sterile new metal scalers (Hu-Friedy Mfg. Co., LLC., Tuttlingen-Moehringen, Germany). The samples were transferred on endodontic sterile paper points (ISO 55, Roeko GmbH, Langenau, Germany) stored in DNA and RNA free Eppendorf tubes (Save lock tubes Biopur, Eppendorf, Wesseling, Germany) at -60˚ C until laboratory processing. Samples were taken always in the morning (from 9.00 to 11.00 am). Children were advised to refrain from brushing their teeth in the morning.

Microbiological Analysis of Plaque Samples

Total bacterial counts as well as the counts of *Streptococcus mutans*, *S. sobrinus*, *Lactobacillus* sp., *Actinomyces naeslundii*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum* were determined using real-time polymerase chain reaction (PCR). The DNA extraction of the plaque samples was made using the

Genomic Mini Kit (A & A Biotechnology, Gdynia, Poland) according to the manufacturer's recommendation. Real-time PCR was carried out using a real-time rotary analyzer (RotorGene 2000; Corbett Research, Sydney, Australia). The primers for *P. gingivalis*, *A. actinomycetemcomitans, P. intermedia,* and the total counts were designed as described before [Ashimoto et al., 1996]. Primers for *S. mutans* and *S. sobrinus* were previously described by Yoshida et al. [2003], for *A. naeslundii* by Xia and Baumgartner [2003], for *F. nucleatum* by Fouad et al. [2002] and for *Lactobacillus* sp. by Byun et al. [2004]. PCR amplification was carried out in a reaction volume of 20 µl consisting of 2 µl template DNA and 18 µl of reaction mixture containing 2 µl 10 x PCR buffer, 2.75 mM $MgCl_2$, 0.2 mM nucleotides, 0.5 µM primer each, 10^{-4} SybrGreen, 1 U taq polymerase (Fermentas Life Science, St. Leon-Rot, Germany). Negative and positive controls were included in each batch of specimens. The positive control consisted of 2 µl genomic DNA in concentrations in a range from 10^2 to 10^7 bacteria of the reference strains, the negative control was 2 µl of sterile water, each added to 18 µl reaction mixture. The cycling conditions comprised an initial denaturation step at 95°C for 5 min, followed by 45 cycles at 95°C for 15 s, at 65°C (exception *A. actinomycetemcomitans*, *P. intermedia, S. mutans*, *S. sobrinus* 62°C) for 20 s using a touch-down for five cycles, at 72°C for 20 s. The sensitivity and specificity of the method was checked by well characterized bacterial strains and plaque samples. Furthermore, the specificity of the amplification was always assayed with the use of melting curves. For quantification, the results from unknown plaque specimens were projected on the counted pure culture standard curves of the target bacteria. The numbers of bacteria were classified using log-stages. For adjustment of the methods (sensitivity) and as references the following species were used: *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* ATCC 33384, *F. nucleatum* ATCC 25586, *P. intermedia* ATCC 25611, *S. mutans* ATCC 25175, *S. sobrinus* ATCC 33478, *A. naeslundii* ATCC 43013 and *Lactobacillus acidophilus* ATCC 11975. The cut-off was set to 10^2 bacterial counts per sample.

Statistical methods

The collected data were entered in Microsoft Excel worksheets and analysed using statistical software (SPSS, version 19.0). Cohen's kappa (κ) used for measuring the intra- and inter-examiner reliability for caries scoring was 0.97 for intra-examiner reliability (BB, RHW) and ranged from 0.96 (BB) to 0.98 (RHW) for inter-examiner reliability. The κ-values calculated for BS scoring was 1.00 for intra- and inter-examiner reliability for both dentists (BB, RHW). For statistical comparisons of differences between the ordinal scaled data of caries experience (dmft/DMFT/Dt/dt/ft/mt) in children with and without BS the Mann-Withney U test was used. Caries prevalence in the mixed dentition and primary teeth and the proportion of caries-affected subjects (dmft+DMFT >0 and dmft >0) with and without BS was compared by the contingency table test of independence (Chi-square test). Odds ratio (OR) and 95% confidence interval (95% CI) was calculated to assess the influence of BS on caries experience (dmft). Quantitative microbiological data were compared by the Mann-Whitney U test; for qualitative data the Chi-square test was used. The level of significance was set at 5%.

Results

The mean age of German children with BS and non-discoloured plaque involved in this study was equal (7.9 $\pm$ 1.3 years, table 1). Children with BS revealed a lower overall caries prevalence in the mixed dentition and in primary teeth than children with non-discoloured plaque, but these differences were not significant (p=0.061, p=0.071). While the caries experience in primary teeth among children with BS was significantly lower expressed as dmft and dt values compared to children of the non-discoloured plaque group (p=0.013 and 0.005, respectively), no differences were observed for caries experience in permanent teeth. Moreover, these children had by trend a higher (not statistically significant) risk for caries experience than their counterparts with BS (OR 2.29, 95% CI: 0.88-6.06, p=0.061).

The total number of bacterial counts was equal in plaque samples of children with BS and those with non-discoloured plaque (table 2, fig. 1).

However, plaque samples of the BS group contained a significantly higher number of *A. naeslundii* (p=0.005) and a tendency to more *P. gingivalis* compared to non-discoloured plaque samples. Contrary, there were significantly lower numbers of *F. nucleatum* (p=0.001) and *Lactobacillus* sp. (p=0.001) in BS samples compared to non-discoloured plaque samples. No significant differences between both groups were observed for the species *S. mutans*, *S. sobrinus*, *A. actinomycetemcomitans* and *P. intermedia*.

Comparing caries-free children with BS and non-discoloured plaque higher counts for *A. naeslundii* (p=0.013) were observed in children with BS (table 3, fig. 2). In both, caries-free and caries-affected children groups *Lactobacillus* sp. counts were higher in non-discoloured plaque samples than in BS (p=0.003; p=0.024). In caries-affected children with BS lower quantities of *F. nucleatum* (p=0.007) were found; more samples showed a trend for higher *P. gingivalis* (p=0.085) and for *A. actinomycetemcomitans* (p=0.061).

Discussion

This is the first study that addressed the investigation of caries experience and microbiota in children with BS and non-discoloured plaque. As these children were attending same elementary schools in the Westphalian Rhein-District Neuss, Germany, the socio-economic background of children involved was comparable. The influence of age on caries prevalence and experience could be excluded as the mean age of children in both groups was equal. The significantly lower caries experience in primary teeth (dmft, dt) found in our children with BS compared to their counterparts with non-discoloured plaque confirmed data observed in the population of 3- to 10-year-old Westphalians [Bartsch and Heinrich-Weltzien, 2011]. Other studies agree with this finding, demonstrating also lower caries experience in children with BS [Shourie, 1947; Commerell, 1955; Koch et al., 2001; Gasparetto et al., 2003; Heinrich-Weltzien et al., 2009; Bhat, 2010; França-Pinto et al., 2012]. Furthermore, a tendency to a higher odds ratio for caries experience was found in children with non-discoloured plaque. Therefore, the first hypothesis of this

study that caries prevalence and experience differ in children with BS and non-discoloured plaque could be confirmed for caries experience in primary but not in permanent teeth. The low caries experience of permanent teeth may be due to their short exposure time to the oral environment or the time from eruption to the onset of caries. In general the period of peak caries susceptibility occurs at 4 years after eruption of the tooth [Carlos and Gittelsohn, 1965]. Therefore, we assume that differences in caries experience of permanent teeth in children with and without BS will be observed in children older than 10 years as reported previously [Bhat, 2010; Heinrich-Weltzien et al., 2009; Koch et al., 2001].

The microbiological findings demonstrate an equal total number of bacterial counts in BS and non-discolored plaque samples. Therefore, significant differences of individual species of bacteria between plaque samples both reflect a different microbial composition. Considering the cariogenic microbiota a significantly higher number of *A. naeslundii* and a significantly lower number of *Lactobacillus* sp. was found in BS samples, while the number of *S. mutans* tends to be lower, and the numbers of *S. sobrinus* were not different between BS and non-discoloured plaque samples. These findings seem to be in good agreement with previous microbiological studies reporting a high proportion of gram-positive rods, especially of *Actinomyces* sp., and low numbers of streptococci [Slots 1974; Saba et al., 2006]. The morphological studies of BS confirmed that this kind of stain is a special type of dental plaque by its microbiota and with a tendency to calcify [Theilade et al., 1973; Reid et al., 1977]. Analysis of the chemical composition of BS [Reid and Beeley, 1976] and saliva of affected children [Surdacka, 1998] indicate high levels of calcium and phosphate within the black material and saliva that may contribute to the lower caries experience. *A. naeslundii* may play a special role in development of BS. It has been shown that *A. naeslundii* and *S. mutans* adhesion correlated with low and high caries experiences, respectively [Stenuddd et al., 2001]. The significantly higher level of *A. naeslundii* and the tendency to lower numbers of *S. mutans* detected in BS samples might be associated with the lower caries experience in these children. This suggestion is supported by the reported increased *Actinomyces* antibody level associated with increased *Actinomyces*

sp.colonization and lower caries experience [Levine et al., 2005]. Thus, bacterial composition of the microbiota may modulate susceptibility and resistance to dental caries [Stenuddd et al., 2001].

F. nucleatum well known as a bridging species between early and late colonizers in the dental plaque [Kolenbrander and London 1993] was found more often in non-discoloured plaque. Its role in caries development is not clearly defined. This species was detected in carious dentine lesions in association with moderate counts of *Lactobacillus* sp. [Chhour et al., 2005].

Actinomyces sp. support the adhesion of the periodontopathogenic bacterium *P. gingivalis* by co-aggregation and foster its growth by releasing succinates [Sanderink et al., 2004]. Therefore, with a higher prevalence of *Actinomyces* sp. increased growth of *P. gingivalis* could be assumed. In the present BS samples a significantly higher number of *Actinomyces* sp.and a tendency to more *P. gingivalis* were detected. This black pigmented gram-negative rod was found in 34% of BS but in 17% of non-discolored plaque samples only. The counts determined by real-time PCR were low in general. By using a less sensitive PCR method Saba et al. [2006] did not detect *P. gingivalis* and *P. melanonigenica* in children with and without BS. *P. gingivalis* is considered to be a major pathogen in chronic periodontitis, but reports on its occurrence are inconsistent in children. Some authors did not find this bacterium independently of oral sample sites collected in periodontal healthy children with mixed dentition [Cortelli et al., 2012; Kimura et al., 2002] and others found *P. gingivalis* as well as *P. intermedia* and *A. actinomycetemcomitans* in supragingival plaque samples in 3- to 17-year-olds [Tanaka et al., 2006]. In the present study no significant differences were observed between the prevalence of the periodontopathogenic species *A. actinomycetemcomitans* and *P. intermedia* in BS and non-discoloured plaque samples. Contrary to this finding a higher frequency of the periodontal pathogen *A. actinomycetemcomitans* was detected in BS samples (70%) versus non-discoloured plaque samples (20%) by PCR which could be involved in the emergence of pigmentation [Saba et al., 2006]. Furthermore, the authors discussed a potential risk of development forms of local and/or generalized

aggressive periodontitis in children harbouring this bacterium. On the other hand, Sakai et al. [2007] found that a high percentage of children with mixed dentition harboured at least one of the four putative periodontal pathogens (*A. actinomycetemcomitans*, *P. gingivalis*, *P. nigrescens* and *T. denticola*) in saliva without clinical signs of periodontal disease. Other previous studies reported that *P. intermedia* was associated with gingival bleeding [Kamma et al., 2000] and periodontal disease [Okada et al., 2001]. The children examined in the present study were not scored for gingival and periodontal health, which might be a limitation afterwards as the association between periodontopathogenic bacteria and periodontal health in children with BS could not be assessed. Until now *Gülzow* [1965] and *Surdacka* [1987] scored gingival health related to BS in children, but both did not find any association between gingival inflammation and BS. The recent study of França-Pinto et al. [2012] considered several demographic, social and behavioral factors that may have influence on the presence of BS and its caries protective effect, but unfortunately neither a plaque score nor a gingival screening index in the statistical approach with multivariable analysis was included.

In conclusion, the bacterial composition of BS with significantly increased numbers of *A. naeslundii* and significantly decreased numbers of *Lactobacillus* sp. and *F. nucleatum* might be associated with lower caries experience in children with BS. The hypothesis of this study that caries prevalence and experience differ in children with BS and non-discoloured plaque was confirmed for caries experience in primary but not in permanent teeth. To elucidate the role of periodontopathogenic bacteria in BS in development of periodontal disease in children longitudinal studies are necessary. Recently methods analyzing in one assay hundreds of taxa were introduced for determining oral microbiota in children [Crielaard et al., 2011; Tanner et al., 2011]. Data are still rare and real-time PCR is still used in addition for selected species. Nevertheless, applying these techniques to BS samples as well as the suspicion that the intake of iron supplements and the regular consumption of iron rich foods could favour the development of a chromogenic microbiota [Garcia Martin et al., 2013] should be a topic in future studies. As the

phenomenon of BS may be linked to differences in the microbiota and its metabolism, or composition of saliva further studies should investigate simultaneously these different aspects to understand its influence on dental and periodontal health more detailed.

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Legends

Table 1. Caries prevalence and experience (DMFT, dmft, single components) of children with black stain and non-discoloured plaque

Table 2. Prevalence of samples tested positively for selected bacterial species (≥10/sample) incl. statistics (Chi^2 test) detected in children with black stain and non-discoloured plaque samples

Table 3. Prevalence of samples tested positively for selected bacterial species (≥10/sample) incl. statistics for (Chi^2 test) detected in caries-free and caries-affected children in mixed dentition with black stain and non-discoloured plaque samples

Figure 1

Counts (median, 10% and 90% percentiles and outliers) of total numbers of bacteria as well as of *Actinomyces naeslundii*, *Lactobacillus* sp. and *Fusobacterium nucleatum* in black stain (BS, n=47) and non-discoloured plaque (no BS; n=46) incl. statistically significant differences (determined by Mann Whitney test) in all children (A), in caries-free children (n=33; BS=21, no BS=12 (B)) and caries-affected children (n=60; BS=26, no BS=34 (C))

Table 1

Subjects	N	Age (yrs) (x ± SD)	% Caries mixed dentition [95% CI]	% Caries primary teeth [95% CI]	DMFT (x ± SD)	DT (x ± SD)	dmft (x ± SD)	dt (x ± SD)	mt (x ± SD)	ft (x ± SD)
with black stain	47	7.9 ± 1.3	55.3[a] [40.6-70.1]	48.9[b] [34.1-63.8]	0.3 ± 0.7	0.1 ± 0.3	1.6 ± 2.1[c]	0.6 ± 1.0[d]	0.2 ± 0.4	0.8 ± 1.7
with non-discoloured plaque	46	7.9 ± 1.3	73.9 [60.7-87.1]	67.4 [53.3-81.5]	0.3 ± 0.7	0.1 ± 0.3	3.0 ± 3.2	1.7 ± 2.3	0.2 ± 0.5	1.2 ± 1.2
Total	93	8.0 ± 1.3	64.5 [54.6-74.4]	58.1 [47.9-68.3]	0.3 ± 0.7	0.1 ± 0.3	2.3 ± 2.8	1.2 ± 1.9	0.2 ± 0.5	1.0 ± 1.5

Mann-Witney U test: [a]p = 0.061, [b]p = 0.071; Chi-square test: [c]p = 0.013, [d]p = 0.005

Table 2

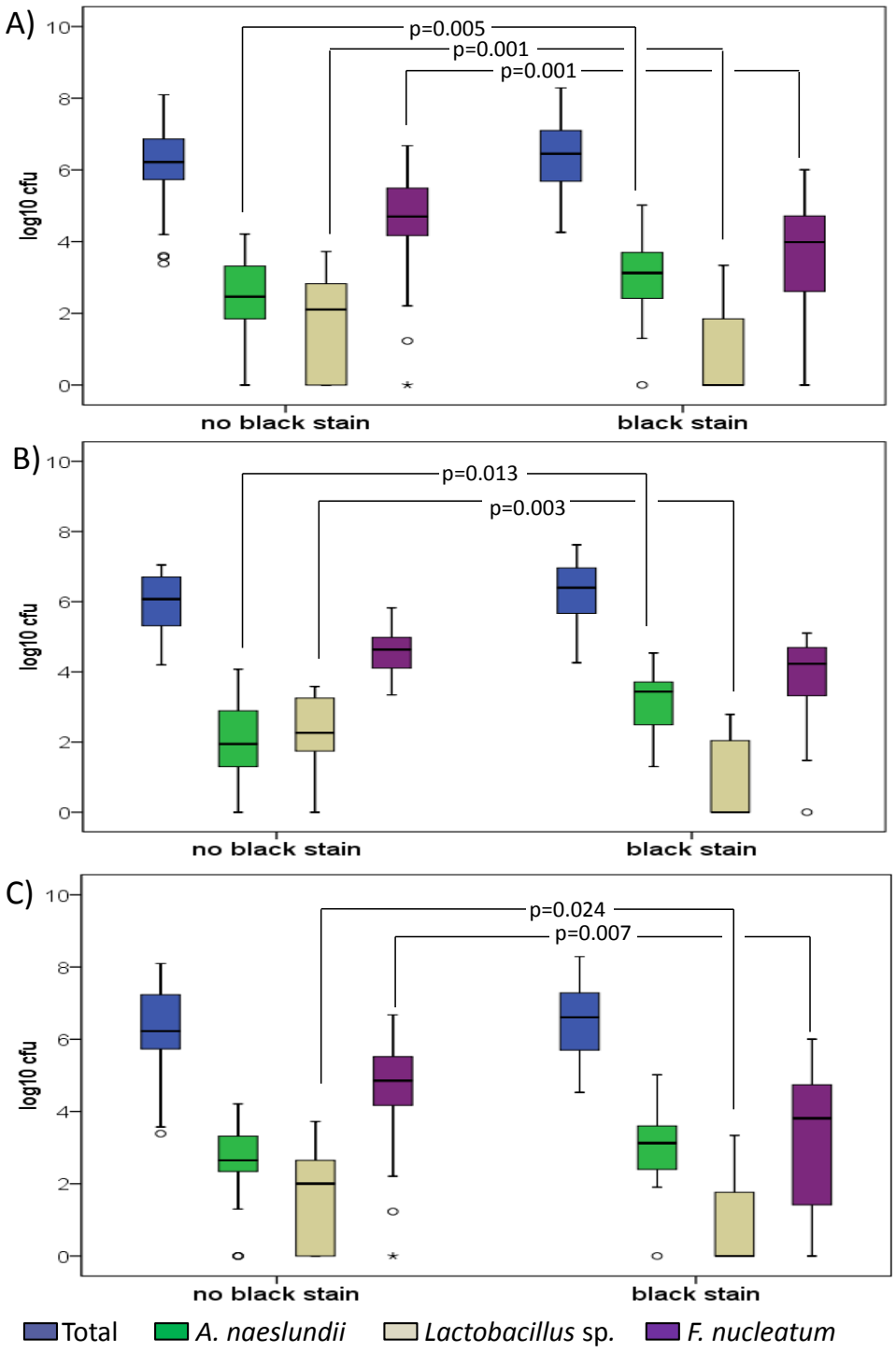
Species	Black stain samples (n=47)		Non-discoloured plaque (n=46)		p Chi^2 test
	n	%	n	%	
Total counts (≥10^7)	14	30	10	22	0.357
S. mutans	19	40	22	48	0.472
S. sobrinus	25	53	23	50	0.758
Lactobacillus sp.	20	43	32	70	0.012
A. naeslundii	46	98	42	91	0.160
P. gingivalis	16	34	8	17	0.067
A. actinomycetem.	10	21	7	15	0.450
P. intermedia	4	9	7	15	0.317
F. nucleatum	41	87	45	98	0.053

Table 3

	Caries-free children (dmft+DMFT = 0)				
Species	Black stain samples (n=21)		Non-discoloured plaque (n=12)		p Chi^2 test
	positive n	%	positive n	%	
Total counts (≥10^7)	5	24	1	8	0.268
S. mutans	6	29	2	17	0.443
S. sobrinus	13	62	9	75	0.443
Lactobacillus sp.	10	48	11	92	0.011
A. naeslundii	21	100	11	92	0.179
P. gingivalis	6	39	2	17	0.443
A. actinomycetem.	3	14	4	33	0.198
P. intermedia	2	10	1	8	0.909
F. nucleatum	20	95	12	100	0.443
	Caries-affected children (dmft+DMFT >0)				
Species	Black stain samples (n=26)		Non-discoloured plaque (n=34)		p Chi^2 test
Total counts (≥10^7)	9 (35)	35	9	27	0.444
S. mutans	13 (50)	50	20	69	0.463
S. sobrinus	12 (46)	46	14	41	0.700
Lactobacillus sp.	10 (39)	39	21	62	0.073
A. naeslundii	25 (96)	96	31	91	0.444
P. gingivalis	10 (38)	38	6	18	0.085

A. actinomycetem.	7 (27)	27	3	9	0.062
P. intermedia	2 (8)	8	6	18	0.261
F. nucleatum	21 (81)	81	33	97	0.037

Figure 1


A)
p=0.005
p=0.001
p=0.001
log10 cfu
no black stain
black stain
B)
p=0.013
p=0.003
log10 cfu
no black stain
black stain
C)
p=0.024
p=0.007
log10 cfu
no black stain
black stain
Total
A. naeslundii
Lactobacillus sp.
F. nucleatum