Subgingival microbiota levels and their associations with periodontal status at the sampled sites in an adult Sudanese population using miswak or toothbrush regularly

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Little information is available on the effect of miswak use on gingival microbiota. We assessed levels of 28 oral bacteria in subgingival plaque of adult Sudanese miswak ($n = 38$) and toothbrush users ($n = 36$) age range 20–53 years (mean 34.6 years) to study associations between these bacteria, oral hygiene method, and periodontal status at the sampled sites. A pooled subgingival plaque sample from 6 probing sites of 1 selected tooth in each jaw was obtained from each subject. Whole genomic DNA probes and the checkerboard DNA-DNA hybridization were used in assessing 74 pooled samples. Using 10^5 bacterial cells threshold, between 2.6% and 47.4% of miswak users and between 2.8% and 36.1% of toothbrush users harbored the investigated species. The percentages of subjects with the investigated species at 10^6 bacterial cells varied between 2.6% and 39.5% in miswak and between 2.8% and 36.1% in toothbrush users. Miswak users harbored significantly higher *Streptococcus intermedius*, *Actinobacillus actinomycetemcomitans*, *Veillonella parvula*, *Actinomyces israelii*, and *Capnocytophaga gingivalis*, and significantly lower *Selenomonas sputigena*, *Streptococcus salivarius*, *Actinomyces naeslundii*, and *Streptococcus oralis* than did toothbrush users. Probing pocket depth $\geq$6 mm showed significantly ($P < 0.05$) higher levels of *Porphyromonas gingivalis*, *Treponema denticola*, *Bacteroides forsythus*, *Fusobacterium nucleatum*, and *V. parvula* than those 4–5 mm. Our results indicate that the type of oral hygiene had a significant effect on levels of 11 out of 28 bacterial species, and that the type of effect was also dependent on type of bacteria and probing pocket depth. □ *Chewing stick; miswak; oral hygiene; periodontal status; subgingival microbiota*

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Subgingival dental plaque is a biofilm comprising a heterogeneous and highly complex mixture of microorganisms. About 400 species have been cultivated from the gingival crevice (1). Recent data show that the microbial composition of subgingival plaque is different between periodontally healthy individuals and periodontitis patients (2). While levels of species in the genera *Actinomyces* and *Streptococcus* predominate in subgingival plaque of periodontally healthy individuals, it has been found that certain species, e.g. *Bacteroides forsythus, Porphyromonas gingivalis*, and *Treponema denticola*, often coexist and are more prevalent in periodontally diseased than in healthy subjects. However, several other species could be found in both healthy and diseased subjects (2).

In studies of the occurrence of bacterial species in subgingival plaque, conveniently selected groups of periodontitis patients and non-periodontitis subjects are often used. Hence, inference from these studies may not be pertinent to populations of developing countries. There is also considerable evidence that the occurrence of periodontal pathogens is markedly different among different ethnic groups (3–5). In addition, the finding that the distribution of *Actinobacillus actinomycetemcomitans* in adolescents diagnosed with early-onset aggressive periodontitis differs by geographical area and race is a further indication of the importance of reporting data from populations in developing countries (6–8). Oral hygiene is another factor that has a strong impact on the composition of supra and subgingival plaque microflora. For instance, in subjects with poor oral hygiene, *P. gingivalis* was detected in 79% of diseased sites and 36% of healthy sites in periodontitis patients, and in 35% of healthy sites in healthy subjects (9).

In some developing countries, certain plants are fashioned into chewing sticks that are thought to help maintain good oral hygiene (for references, see Wu et al. (10)). Extracts from these plants have various biological properties, including antibacterial and antifungal effects (11–13). Hence, the use of chewing sticks for oral hygiene may influence the occurrence of periodontal pathogens and other bacteria that are involved in the development of dental plaque. It has been shown, for instance, that black pigmented *Bacteroides* were not commonly found in the subgingival microflora of adult Nigerian chewing stick users (14). Homer et al. (15–16) showed in vitro that aqueous extracts of chewing sticks (Mswaki) used in Kenya inhibited peptidase and glycosidase activities of *P. gingivalis*, *Prevotella intermedia*, and *Treponema denticola*.

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In Sudan, chewing sticks (miswak) are prepared from the roots, twigs, or stems of the shrub *Salvadora persica*, and are used commonly as oral hygiene tools, mainly among males. There are reports demonstrating good periodontal health in miswak users (17–20). The Consensus Statement on Oral Hygiene (21) states that 'chewing sticks may have a role to play in the promotion of oral hygiene' and that 'evaluation of their effectiveness warrants further research'.

Little information is available in the literature on the effect of miswak use on the gingival microflora. The aims of this study were: 1) to assess the levels of 28 oral bacteria in subgingival sites of groups of adult Sudanese habitual miswak and toothbrush users, and 2) to study the associations between occurrence of bacteria, oral hygiene method, and periodontal status at the sampled sites.

Materials and Methods

Study subjects

A total of 74 individuals (age range 20–53 years; mean 34.6 years) were included in this study. The group consisted of 12 (16.2%) women (mean age 35.6 years) and 62 (83.8%) men (mean age 34.5 years) selected from a group of 213 individuals used in a previous study (20). The larger group comprised volunteer participants who were recruited for the study among employees and students at the Faculty of Medical Sciences, University of Khartoum, Sudan. The inclusion criteria of this study group were: good general health, ≥18 teeth present, at least 1 maxillary and 1 mandibular tooth with ≥4 mm probing pocket depth and gingival bleeding on probing, no present or past history of cigarette smoking or use of other tobacco products, regular use of miswak or toothbrush as the main oral hygiene tool for the previous year, and no history of using antibiotics in the past 3 months. Thirty-eight of the subjects (including 11 women) were regular miswak users and 36 subjects (including one woman) used a toothbrush habitually.

Clinical examination

A full-mouth clinical examination was performed for the study group. The teeth were dried in air, and the World Health Organization periodontal probe (TRS-621 C-version) (22) was used to measure the probing pocket depth (PPD) at 6 sites per tooth: the mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual. Sites exhibiting bleeding on probing were recorded. PPD was defined as the distance from the free gingival margin to the bottom of the pocket/sulcus. This distance was measured in millimeters and was rounded to the lowest whole millimeter. The clinical examinations were undertaken at the University of Khartoum Dental Center, Sudan. The measurements were performed by one examiner (I.A.D), who had been trained and calibrated at the Department of Periodontology, the Faculty of Dentistry, University of Bergen, Norway.

Microbiological sampling

Using the measurements of probing depth, 1 maxillary and 1 mandibular posterior tooth exhibiting 4–5 mm or ≥6 mm PPD and bleeding on probing were randomly selected for bacterial sampling. If posterior teeth were missing, anterior teeth were used instead. Teeth were isolated with cotton rolls, carefully scaled supragingivally with sterile Gracey curettes, cleaned with sterile cotton pellets, and dried in air. A sterile curette was then inserted into the pocket, and subgingival plaque was collected by multiple scaling strokes of the 6 sites per tooth. Effort was taken so that pressure against the root surfaces ceased when the curette tip reached the gingival margin, this in order to minimize contamination from the supragingival area. For each subject, dental plaque collected from the maxillary and mandibular sites was immediately transferred into one Eppendorf tube containing 150 µl of sterile TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 7.6). The plaque samples were then suspended in the buffer by gentle shaking. The 74 Eppendorf tubes containing the pooled subgingival plaque samples were stored at −20°C and later transported on dry ice in a polystyrene box to the Laboratory of Oral Microbiology, University of Bergen, Norway, where they were stored at −20°C until analyzed.

Probe bacteria

A total of 28 bacterial species were used as probe bacteria in this study. These species comprised 3 categories of reference strains: (i) Periodontitis-associated bacteria (23–24): *Porphyromonas gingivalis* (ATCC 33277), *Treponema denticola* (ATCC 36405), *Bacteriodes forsythus* (ATCC 43037), *Prevotella intermedia* (VPI 4196), *Fusobacterium nucleatum* (ATCC 10935), *Campylobacter rectus* (ATCC 33612), *Selenomonas sputigena* (ATCC 33150), *Streptococcus intermedius* (CCUG 27335), *Eikenella corrodens* (ATCC 23834), *Actinobacillus actinomycetemcomitans* (ATCC 43719), *Veillonella parvula* (ATCC 10790), *Peptostreptococcus micros* (CCUG 17638), and *Prevotella melaninogenica* (ATCC 33184); (ii) cariogenic bacteria (25): *Streptococcus mutans* (ATCC 25172), *Streptococcus sobrinus* (CCUG 25735), and *Lactobacillus acidophilus* (ATCC 4646); and (iii) bacteria associated with health (24, 26): *Streptococcus anginosus* (ATCC 10566), *Streptococcus salivarius* (CCUG 25736), *Actinomyces israelii* (ATCC 12103), *Actinomyces viscosus* (ATCC 15987), *Actinomyces naesludii* (ATCC 12104), *Streptococcus sanguis* (ATCC 10566), *Capnocytophaga sputigena* (ATCC 33612), *Capnocytophaga gingivalis* (ATCC 33624), *Leptotrichia buccalis* (ATCC 14201), *Streptococcus gordonii* (CCUG 33482), *Streptococcus oralis* (ATCC 35037), and *Streptococcus mitis* (CCUG 31611). With the exception of the *Treponema denticola* strain the bacterial strains were purchased from the American Type Culture Collection (ATCC, Rockville, Md, USA), Virginia Polytechnique Institute (VPI Virginia, USA) and the

Culture Collection, the University of Gothenburg (CCUG), Sweden. They were rehydrated and cultivated as recommended by the ATCC, CCUG, and/or VPI. The *T. denticola* DNA was a gift from Dr. Ulf Dahle, the School of Dentistry, University of Oslo, Norway.

Preparation of whole chromosomal DNA probes

The DNA probes used in the study were prepared from the 28 probe bacteria using the Puregene DNA Isolation Kit (Genera Systems, Inc., Minn., USA), and from the *T. denticola* DNA. Digoxigenin-labeling of the probes was done using the random primer technique (27) in accordance with the manufacturer's protocol. The prepared probes were standardized by spot assay of labeling efficiency as described in the labeling kit manual (Boehringer Mannheim, Mannheim, Germany). Each probe was adjusted to give a final concentration in hybridization solution of 6 ng/ml. All the probes were then tested against standardized reference samples of the homologous species and further adjusted if necessary to give the same sensitivity for each probe.

Microbiological assessment

The levels of bacterial species were determined in each of the 74 pooled subgingival plaque samples using whole genomic DNA probes and the checkerboard DNA-DNA hybridization technique (28). Briefly, the lysed plaque samples, along with 2 positive controls, each containing 10^5 or 10^6 cells of each probe species, and the probe DNA were placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, Mass., USA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Imunetics) with lanes of DNA perpendicular to the lines of the device. Digoxigenin-labeled whole genomic probes to the 28 probe bacteria were hybridized in individual lines of the Miniblotter. After hybridization, the membranes were washed at low and high stringency, incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase, washed, further incubated with chemiluminescent substrate (CSPD) (Boehringer Mannheim, Mannheim, Germany), and finally exposed to X-ray films to detect bound probes. The densities of chemiluminescent signals were assessed by visual inspection. Evaluation of numbers of bacteria in the test samples was made by comparing the signals generated by test samples with those generated by the controls. The sensitivity of the assay was adjusted to permit detection of 10^4 cells of a given species. The signals were coded on a scale scored from 1 to 5, where score 1 = signal density 0 or weaker than the one of the low standard (i.e. $<10^5$ cells); score 2 = signal density equal to the one of the low standard ($=10^5$ cells); score 3 = signal density higher than the one of the low standard, but lower than that of the high standard ($>10^5$ but $<10^6$ cells); score 4 = signal density equal to the one of the high standard (10^6 cells); and score 5 = signal density higher than the one of the high standard ($>10^6$ cells).

Validation of the whole genomic DNA probe specificity in the checkerboard assay has been described previously (29). Bacterial assessment was done by one examiner (I.A.D).

Data analysis

The mean values of pocket depths 4–5 mm and ≥6 mm were computed for each tooth and the teeth values were averaged for each subject. The numbers and percentages of subjects with different clinical parameters and bacterial levels were calculated. Contingency tables were compiled for the number and percentage of subjects by levels of various bacterial species in subgingvial plaque. The chi-square test was used to test the significance of the difference in frequencies between the 2 user groups and the pocket depths. The frequencies of miswak users and toothbrush users testing positive for each of the 28 DNA probe bacteria were assessed at 2 bacterial cell counts (10^5 and 10^6 cells). In this way, colonized (10^5 cells) versus non-colonized individuals ($<10^5$ cells), and heavily colonized ($\geq 10^6$ cells) versus non-colonized and less heavily colonized (10^5–10^6 cells) individuals, were compared. Comparisons by oral hygiene method were done by grouping the subjects into those showing 10^5 or 10^6 cells. Differences in detection frequencies of the probe bacteria at 10^5 cells threshold were assessed at PPD 4–5 mm and ≥6 mm. Differences with a $p < 0.05$ were considered statistically significant. Statistical analysis was performed by SAS statistical package (SAS version 8.0, SAS Institute Inc., Cary, NC, USA).

Results

Clinical findings

Table 1 summarizes the clinical characteristics of the study subjects and the teeth from which subgingival samples were selected. There were no significant differences between the proportion of subjects with PPD 4–5 mm and those with PPD ≥6 mm in the miswak and toothbrush groups.

Table 1. Clinical characteristics of the study subjects and the teeth from which subgingival plaque samples were obtained

Clinical variables	Miswak group ($n = 38$)	Toothbrush group ($n = 36$)
No. of sampled teeth	76	72
Mean pocket depth ± standard deviation of sampled teeth	4.06 ± 0.3 mm	4.09 ± 0.3 mm
Range pocket depth	4–7 mm	4–6 mm
No. and (%) of pockets 4–5 mm	32 (84.2%)	28 (77.8%)
No. and (%) of pockets ≥6 mm	6 (15.8%)	8 (22.2%)

Table 2. Numbers and percentages of subjects by 3 levels of the 28 bacterial species identified in subgingival plaque using whole genomic DNA probes and checkerboard DNA-DNA hybridization (33)

	Levels of the bacteria in subgingival plaque					
	$<10^5$		10^5		$\geq 10^6$	
Bacterial species*	No.	%	No.	%	No.	%
P. gingivalis (Pg)	34	45.9	19	25.7	21	28.4
T. denticola (Td)	57	77.1	7	9.5	10	13.5
B. forsythus (Bf)	64	86.5	5	6.8	5	6.8
P. intermedia (Pi)	50	67.6	9	12.2	15	20.3
F. nucleatum (Fn)	46	62.2	17	22.9	11	14.9
C. rectus (Cr)	52	70.3	15	20.3	7	9.5
S. sputigena (Ssp)	54	72.9	9	12.2	11	14.9
S. intermedius (Si)	46	62.2	20	27	8	10.8
E. corrodens (Ec)	56	75.7	10	13.5	8	10.8
A. actinomycetemcomitans (Aa)	42	56.8	20	27	12	16.2
V. parvula (Vp)	55	74.3	13	17.6	6	8.1
P. micros (Pm)	66	89.2	5	6.8	3	4.1
P. melaninogenica (Pme)	61	82.4	2	2.7	11	14.9
S. mutans (Smu)	73	98.7	1	1.4	0	0
S. sobrinus (Sb)	70	94.6	4	5.4	0	0
L. acidophilus (La)	74	100	0	0	0	0
S. anginosus (San)	73	98.7	0	0	1	1.4
S. salivarius (Sa)	67	90.5	7	9.5	0	0
A. israelii (Ai)	60	81.1	9	12.2	5	6.8
A. viscosus (Av)	67	90.5	4	5.4	3	4.1
A. naeslundii (An)	25	33.8	18	24.3	31	41.9
S. sanguis (Ss)	66	89.2	1	1.4	7	9.5
C. sputigena (Cs)	67	90.5	5	6.8	2	2.7
C. gingivalis (Cg)	38	51.4	28	37.8	8	10.8
L. buccalis (Lb)	73	98.7	1	1.4	0	0
S. gordonii (Sg)	72	97.3	2	2.7	0	0
S. oralis (So)	58	78.4	15	20.3	1	1.4
S. mitis (Smi)	64	86.5	6	8.1	4	5.4

* Subdivided into 3 categories of bacteria, starting from top: periodontitis-associated species ($n = 13$), cariogenic species ($n = 3$), and species associated with dental health ($n = 12$).

Microbiological findings

The numbers and percentages of subjects by 3 levels of 28 bacterial species in subgingival plaque are given in Table 2. The detection frequencies of the 28 investigated species at $<10^5$, 10^5, and $\geq 10^6$ bacterial cells varied between 33.8% and 100%, 1.4% and 37.8%, and 1.4% and 41.9%, respectively. Small percentages of subjects had 10^5 bacterial cells of *L. acidophilus* (0%), *S. mutans* (1.4%), *S. anginosus* (0%), *S. sangius* (1.4%), *L. buccalis* (1.4%), *S. gordonii* (2.7%), *P. melaninogenica* (2.7%), and *S. sobrinus* (5.4%).

The percentages of subjects with 10^5 bacterial cells by type of bacteria and oral hygiene group are shown in Fig. 1. At this threshold, the detection frequencies of the 28 investigated species varied between 2.6% and 47.4% in the miswak group and between 2.8% and 36.1% in the toothbrush group. Similarly, the prevalences of periodontopathic species, including *P. gingivalis*, *T. denticola*, *B. forsythus*, *P. intermedia*, *A. actinomycetemocmitans*, and *V. parvula*, were between 10.5% and 36.8%, and between 2.8% and 19.4% in the two groups.

At 10^5 bacterial cells, there were significantly more miswak users than toothbrush users harboring *S. intermedius* ($P = 0.05$), *A. actinomycetemocmitans* ($P = 0.03$), *V. parvula* ($P = 0.04$), *A. israelii* ($P = 0.02$), and *C. gingivalis* ($P = 0.03$), and significantly fewer of the former group harboring *S. sputigena* ($P = 0.01$), *S. salivarius* ($P = 0.04$), *A. naeslundii* ($P = 0.02$), and *S. oralis* ($P = 0.001$) (Fig. 1).

In the miswak group, the percentages of subjects harboring 10^6 bacterial cells in the subgingival plaque were 26.3% *P. gingivalis*, 7.9% *P. intermedia*, 5.3% *B. forsythus*, 15.8% *T. denticola*, and 15.8% *A. actinomycetemocmitans* (Fig. 2). The corresponding percentages in the toothbrush group were 22.2%, 25.0%, 8.3%, 11.1%, and 16.7%. Using this threshold level, significantly more toothbrush users than miswak users harbored *P. intermedia* ($P = 0.05$), *S. sangius* ($P = 0.04$), and *S. mitis* ($P = 0.03$) (Fig. 2).

The detection frequencies of the 28 investigated species at 10^5 bacterial cells varied between 1.7% and 28.3% for PPD 4–5 mm and between 7.1% and 71.4% for PPD ≥ 6 mm (Table 3). Significantly more subjects with PPD ≥ 6 mm harbored *P. gingivalis* ($P = 0.001$), *T. denticola* ($P = 0.002$), *B. forsythus* ($P = 0.04$), *F. nucleatum* ($P = 0.003$), and *V. parvula* ($P = 0.001$) than did the subjects with PPD 4–5 mm (Table 3).

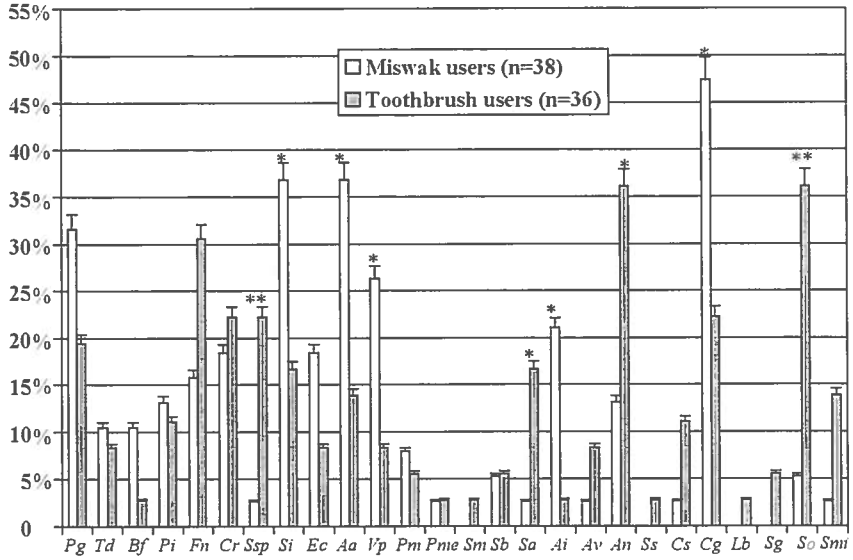

Fig. 1. Percentage of subjects with 10^5 bacterial cells in the subgingival plaque by type of bacteria and oral hygiene group. ** $P = 0.001$, * $P = 0.05$. (For abbreviations of bacterial names, see Table 2).

Discussion

The study was undertaken to investigate whether habitual mechanical tooth brushing using miswak or toothbrush influenced the detection frequencies and levels of subgingival bacteria and the relationships between these bacteria and periodontal status at the sampled sites in adult Sudanese. The bacterial composition of subgingval plaque in periodontal health and disease has been evaluated in several studies (e.g. 2, 30–31). It is notable that few of these studies examined the effect of daily supragingival plaque control by manual toothbrush on the composition of subgingival plaque bacteria (32–34), and none compared the effect of miswak chewing sticks and manual toothbrush. In this study, we investigated the bacterial composition of subgingival plaque from two groups of Sudanese adults performing habitual mechanical tooth cleaning using miswak or toothbrush. For this purpose we analysed pooled plaque collected from 12 subgingival sites in each test subject.

Our results showed that the type of oral hygiene had a significant effect on the subgingival plaque levels of 11 out

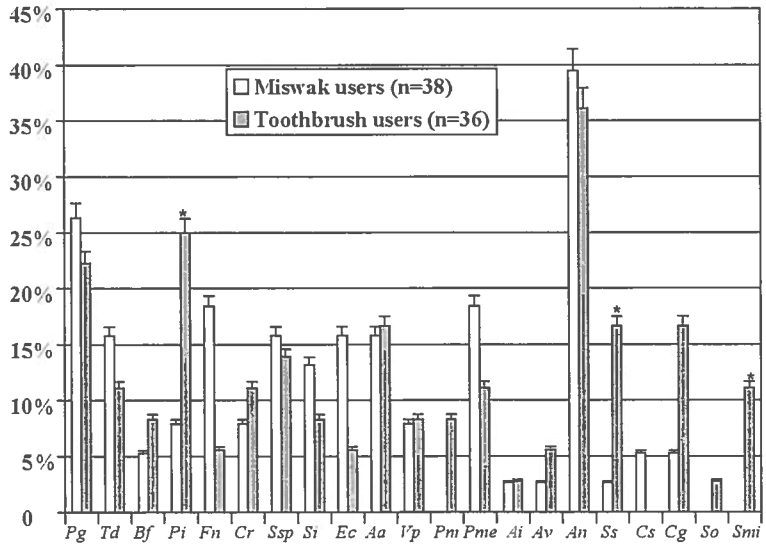

Fig. 2. Percentage of subjects with 10^6 bacterial cells in the subgingival plaque by type of bacteria and oral hygiene group. ** $P = 0.001$, * $P = 0.05$. (For abbreviations of bacterial names, see Table 2).

Table 3. Percentage of subjects showing the probe bacteria at 10^5 cells by probing pocket depth and type of bacterial species (statistically significant differences shown in boldface)

Bacterial species*	Pocket depths 4–5 mm ($n = 60$)	Pocket depths ≥6 mm ($n = 14$)	*P* value
P. gingivalis	15	71.4	**0.001**
T. denticola	3.3	35.7	**0.002**
B. forsythus	3.3	21.4	**0.04**
P. intermedia	10	21.4	0.22
F. nucleatum	15	57.1	**0.003**
C. rectus	16.7	35.7	0.11
S. sputigena	13.3	7.1	0.45
S. intermedius	28.3	21.4	0.43
E. corrodens	11.7	21.4	0.28
A. actinomycetemocmitans	25	35.7	0.31
V. parvula	10	50.0	**0.001**
P. micros	8.3	0	0.33
P. melaninogenica	1.7	7.1	0.34
S. mutans	1.7	0	0.81
S. sobrinus	6.7	0	0.42
S. salivarius	8.3	0	0.43
A. israelii	13.3	7.1	0.45
A. viscosus	6.7	0	0.43
A. naeslundii	26.7	14.3	0.25
S. sangius	1.7	0	0.81
C. sputigena	8.3	0	0.33
C. gingivalis	35	50.0	0.23
L. buccalis	0	7.1	0.18
S. gordonii	1.7	7.1	0.34
S. oralis	20	21.4	0.58
S. mitis	8.3	7.1	0.68

* See footnote to Table 2.

of 28 bacterial species investigated in this study, and that the type of effect was also dependent on the type of bacteria. At the 10^5 bacterial cells threshold, *S. intermedius, A. actinomycetemocmitans, V. parvula, A. israelii*, and *C. gingivalis* were present in significantly higher numbers in subgingival plaque of the miswak than of the toothbrush group. *S. sputigena, S. salivarius, A. naeslundii*, and *S. oralis* were found in significantly lower numbers in the miswak group. When we used the 10^6 bacterial cells threshold, *P. intermedia, S. sanguis*, and *S. mitis* were significantly higher in the toothbrush group than in the miswak group. This is in general agreement with our previous report comparing the levels of the 25 bacterial species in salivary samples of adult Sudanese habitual miswak or toothbrush users (29).

Interestingly, similar to our previous report (29), the present study showed that species including *P. gingivalis, T. denticola, C. rectus, F. nucleatum*, and *L. buccalis* did not seem to be influenced by the type of oral hygiene used. This may suggest that the 2 oral hygiene methods had similar effects on the levels of these species. This is consistent with the findings of studies showing that supragingival plaque control had little or no effect on the levels of subgingival species, not at least in sites with deeper probing depths (35–37). In addition, it has been suggested that the group of periodontal pathogens that includes *B. forsythus, Fusobacterium* spp., *P. gingivalis, P. intermedia*, and *P. micros* are considered a causal factor in therapy-resistant periodontitis (38).

Comparison of the subgingival plaque species at the 10^5 bacterial cells threshold by PPD showed significantly higher numbers of subjects with ≥6 mm PPD harboring *P. gingivalis, T. denticola, B. forsythus, F. nucleatum*, and *V. parvula* than did subjects with PPD 4–5 mm. This is consistent with the findings of Socransky et al. (39–40), who suggested that *F. nucleatum, B. forythus*, and *C. rectus* or *P. gingivalis, P. intermedia*, and *S. intermedius* were found in sites which on average had the most severe attachment loss and the deepest pockets (≥6 mm). A strong positive relationship was also found between high counts ($\geq 10^5$ cells) of *P. gingivalis, A. actinomycetemcomitans*, and *P. intermedia*, and increased probing pocket depths (23).

One (1.4%) and 4 (5.4%) of the study subjects had 10^5 cells of *S. mutans* or *S. sobrinus*, respectively, in their subgingival plaque. The subgingival sites have not been considered a normal habitat for mutans streptococci. However, a recent study (41) used a bacterial culture technique and demonstrated in a Dutch population that the prevalence of mutans streptococci varied from 82% in untreated periodontitis patients to 94% in maintenance patients. The lower detection frequencies reported in the present study may be attributed to difference in the threshold levels of detection frequencies used in the 2 studies as well as ethnic differences.

Whole genomic DNA probes and checkerboard DNA-DNA hybridization (28) have been utilized to analyze one pooled subgingival plaque sample from each test subject (42) or subgingival plaque collected from individual teeth in each subject (e.g. (31)). Increasing the numbers of sampled sites will diminish the numbers of false negatives, and the magnitude of this effect is related to the frequency with which a species is detected, being greater for infrequently detected species than for frequently detected ones (43). We used the subject as the unit of analysis and analyzed 1 pooled subgingival plaque sample, representing 12 subgingival probing sites, permitting the study of bacterial levels and their associations with the oral hygiene method used and the PPD. Pooling plaque samples has a diluting effect on the levels of microbes detectable in some but not all the pooled samples. The magnitude of this dilution on a given species will depend on the relative numbers of individual samples in which this microbe is present in low number or is missing compared to the number of samples harboring it in higher levels. This dilution effect may have impacted some of the bacterial levels reported in our study.

Bostrom et al. (44) also examined subgingival microflora using $<10^5$ bacterial cells as the cut-off level to compare colonized versus non-colonized periodontitis patients. In the present study, we used 10^5 bacterial cells for the same reason in order to reduce the number of possible false positives. Both studies used 10^6 bacterial cells to contrast heavily colonized versus less heavily colonized samples. This is also in line with Haffajee & Socransky (23), who suggested that increased levels of pathogens (i.e. 3×10^4 *A.*

actinomycetemcomitans and 6×10^5 *P. gingivalis*) in subgingival plaque are associated with a greater risk of periodontal disease progression. Darveau et al. (24) showed that the total microbial load in subgingival plaque of periodontitis patients was increased 10^5 to 10^8 bacterial cells and that elevated level of *P. gingivalis* differentiated periodontitis from gingivitis.

In addition to a mechanical effect, the enzyme inhibitory properties of miswak may be significant in deactivating the virulence effects of subgingival species that are associated with periodontal disease (19). This is in line with Homer et al. (15–16). Furthermore, we identified several anionic components, including thiocyanate from miswak (45). It has recently been shown that thiocyanate has potent promoter effects on the salivary peroxidase thiocyanate and hydrogen peroxide antimicrobial system and increased unspecific and specific resistance mechanisms of the tissues against the infection (46). In vitro studies have shown that extracts from miswak inhibited growth of various oral aerobic and anaerobic bacteria, and *Candida albicans* (11, 13, 47). The present study compared subgingival plaque levels of 28 bacterial species in miswak and/or toothbrush groups and detected significant differences in the levels of 11 out of 28 bacterial species. The results further indicate that the type of effect was also dependent on type of bacteria and PPD.

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