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Dental plaque pH and ureolytic activity in children and adults of a low caries population

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Abstract

Objectives. The aim of this study was to evaluate the plaque pH level and ureolytic activity among children and adults of Karen Hill tribes. ***Methods.*** Thirty-four children aged 6–10 years and 46 adults aged 20–38 years were interviewed regarding oral hygiene practices, sucrose intake and betel chewing. Caries experience (DMFT and DT), calculus, bleeding on probing (BoP) and Plaque index (PlI) were registered. Ureolytic activity in supragingival plaque was tested at two interproximal sites (11/12 and 41/42) with the rapid urease test (RUT). Registration of plaque pH was performed at two interproximal sites (15/16 and 31/41) before, during and 30 min after rinsing with an urea solution (0.25%). Four interproximal plaque samples (one from each quadrant) per individual were collected to test the bacterial composition using the checkerboard technique. ***Results.*** Children and adults had similarly low DMFT and DT values. Children had a higher baseline pH and a higher ureolytic activity in the maxilla ($p < 0.05$) compared with adults. A significant correlation ($r^2 = 0.63$) was found between baseline pH and urease activity in the mandibular anterior teeth. Caries-free individuals had a higher baseline pH compared with caries active individuals in the anterior mandibular region ($p < 0.01$). The microbiological composition was characterized by an anaerobic low acidiogenic microbiota. ***Conclusions.*** Dental plaque pH is related to the ureolytic activity, which explains the low acidogenic plaque microflora and the low caries levels in the Karen population.

Key Words: *calculus, caries, Karen population, oral microflora, pH, ureolytic activity*

Introduction

Dental caries is a multi-factorial and widespread disease caused by a prolonged plaque acidification. Many different bacterial species contribute to acid formation that cause tooth demineralization [1].

An ongoing project on the Karen Hill tribe population in Northern Thailand has the overall aim to explain the low caries prevalence in both children and adults. In previous studies [2], the low caries prevalence was confirmed despite the presence of risk factors such as bad oral hygiene and no fluoride exposure. Betel chewing has been claimed to be cariostatic, but both non-chewers and chewers showed a similarly low caries prevalence [2]. Another possible and probably very significant explanation is the low sucrose intake among the inhabitants of these very rural and isolated villages. It is also possible that this population has inherited caries preventive factors in saliva and plaque. The dental plaque flora is also very little studied and there may be certain traits that are specific for this population.

Urea, e.g. carbamide, is an alkaline product that exists naturally in saliva [3]. Some oral bacteria have the ability to convert carbamide into ammonia with the aid of bacterial ureases and a more alkaline environment is temporarily produced [4–6]. This counteracts plaque acidification caused by bacterial fermentation of carbohydrates and thereby contributes to a pH rise and a possible decrease in caries activity [7,8]. Formation of alkaline products may thus be as important in caries development as high levels of acid formation [3,6]. It has been shown that rinsing with sucrose solution results in a plaque pH drop. The reverse occurs when rinsing with an alkaline product, e.g. urea, when the pH may instead

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increase [9]. According to Kleinberg [6], urea is the only nitrogenous substrate that can produce alkali fast enough to buffer salivary acids and thereby contribute to a pH rise. Different locations in the dentition are unevenly exposed to saliva and have therefore shown different plaque pH values and thus variations in the bacterial flora [3].

Previous studies *in vitro* have shown that caries-free individuals have a higher pH and a higher ammonia ($NH4^+$) concentration in plaque fluid [7,10]. Moreover, if the ureolytic activity was high, the pH effect of the carbohydrate metabolism was negligible. The results of an *in vitro* study suggested that plaque cariogenicity might be inversely related to salivary urea concentrations [11]. Studies in rats suggest that even relatively small differences in urea concentrations and in the amount of urease enzyme may dramatically affect caries initiation and progression [12]. *In vivo*, the urease activity in plaque and saliva is an important factor in preventing caries over time [13]; however, the urease activity in plaque *in vivo* has not been well studied.

High ureolytic activity is one reason for a high pH [9]. An alkaline pH fosters the deposition and retention of calcium and phosphate in the plaque and thereby the formation of calculus [3,6]. Previous studies have shown that the inhabitants of Hill tribe villages in Northern Thailand have high amounts of calculus, which may be a general characteristic for East-Asian populations [2,14]. The low caries prevalence in Hill tribe populations makes it interesting to study the ureolytic activity in the plaque and pH changes after rinsing with an urea solution as well as the bacterial composition and especially the prevalence of bacterial species known to be ureolytic. Our hypothesis was that caries-free adults and children would have a more pronounced ureolytic activity in comparison with caries-experienced individuals.

Materials and methods

Subjects

Thirty-four children aged 6–10 years (mean 8.5 years) and 46 adults aged 20–38 years (mean 27.2 years) were recruited in the Omkoi District, Chiang Mai Province of Northern Thailand. They came from four rural villages (Mae-Hong-Thai Village, Mae-Hong-Klang Village, Hauy Klang Village and Hauy Bong Village) accessible only after a 3–4 h drive by 4-wheel drive vehicles. The visits were arranged through a mobile dental team organized by The Princess Mother Medical Voluntary Foundation, Bangkok, Thailand, which also ethically approved the study. The participating subjects were chosen randomly among those who attended the mobile dental team for screening of dental treatment need. The subjects were informed about the study and voluntarily agreed to participate and received necessary dental treatment afterwards. The only inclusion criterion was the age range of 6–10 years for children and 20–40 years for adults. We ended up with a gender distribution for children of 19 girls and 15 boys and for adults 25 females and 21 males.

Questionnaire

With the help of an interpreter, the subjects were interviewed to ascertain age, oral hygiene procedures (yes or no), betel chewing habits (yes or no) and frequency of sucrose-containing dietary intake (> 1 time/day, > 1 time/week, not so often, never).

Clinical examinations

Three calibrated examiners (L.A., A.D., C.E.) carried out clinical registrations in daylight, equipped with a mirror and a periodontal probe (Hu-Friedy, Chicago, IL). Decayed, missing and filled teeth (DMFT) were registered. Absent teeth were registered as missing, although it was not possible to establish why they were absent. The periodontal examination included plaque index (PlI) and bleeding on probing (BoP) for all teeth present.

Calculus was scored 0, 1 or 2 in six areas in the mouth, where 0 = free of calculus, 1 = calculus around the gingival margin and 2 = significant quantity of calculus. At the level of the individual, we scored calculus as 0 = up to grade 1 in one sextant, 1 = grade 1 in at least two sextants or grade 2 in one sextant, 2 = grade 2 in at least two sextants.

The urease activity was measured by the use of a modified Rapid Urease Test (RUT) [15] in interproximal plaque samples from two sites (11–21 and 41–42). The urea broth used consisted of urea (10%) in distilled water and 0.02 g/L of phenol red; the pH was adjusted to 6.8. The broth was used in 100 µl volumes to which a 'pinhead' amount of dental plaque was added. The reaction was read after 2 h at a temperature of 20–25°C. The color reaction was graded from 0–4, where 0 corresponded to no reaction (colorless) and 1–4 to gradually increased purple reaction, where grade 4 showed the deepest purple reaction. One examiner performed the color readings (GD).

Microbiological supragingival plaque samples were collected at four interproximal sites (11–12, 25–26, 31–32, 45–46) with a curette. The samples were transferred to Eppendorf tubes containing 100 µl of TE buffer (0.5 mM Tris-EDTA) and to which 100 µL 0.1 M NaOH was added. The analysis of the samples was undertaken in the Oral microbiological diagnostic service laboratory at the Oral Microbiology Department, Institute of Odontology of Gothenburg University, Sweden, within 6 months after sampling.

pH registrations

Plaque pH was measured at baseline at two interproximal sites (16–15 and 31–41) with the use of the pH-strip method [16]. The pH registrations where made with pH indicator strips, cut into an arrow shape to facilitate interproximal placement. The subjects were instructed to rinse with 0.25% urea in water solution for 1 min. Plaque pH was measured 2, 5, 10, 20 and 30 min after rinsing. When teeth at the selected interproximal sites were missing, the registration was done at the nearest approximal site.

Microbiological analysis

Whole genomic DNA probes were prepared from a panel of 14 bacterial species, consisting of common dental plaque bacteria and bacterial species with known ureolytic activity. The species used were: *Actinomyces oris*, *Fusobacterium nucleatum*, *Haemophilus parainfluenzae*, *Lactobacillus fermentum*, *Prevotella intermedia*, *Prevotella tannerae*, *Streptococcus salivarius*, *Streptococcus sanguinis* and *Streptococcus mutans* [3,10,17,18]. Subgingival *Campylobacter* species (*Campylobacter gracilis*, *Campylobacter rectus*, *Campylobacter ureolyticus*) and other anaerobic species (*Filifactor alocis*), were included because they have been found in our laboratory to be ureolytic. *Helicobacter pylori* is a well-known and strong ureolytic bacteria associated primarily with peptic ulcers, but may be present in the dental plaque.

DNA was extracted with mutanolysin and lysozyme as previously described [19] and the DNA quality was evaluated from the UV spectrum between 200–300 nm using a Gene Quant spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Probe DNA (1 μg) was labeled with deoxygenin using the DIG High Prime kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The analysis was made with the aid of the checkerboard DNA-DNA hybridization methodology according to Wall-Manning et al. [20]. A volume of 150 μl each of all plaque samples were boiled for 5 min, neutralized with 800 μl 5 M ammonium acetate, transferred onto nylon membranes (Minislot device, Immunetics, Cambridge, MA) and fixed by ultraviolet light (UV Stratalinker 1800, Stratagene, La Jolla, CA). After 2 h of pre-hybridization at 42°C, the DNA probes (1–10 ng) were allowed to hybridize overnight in lanes vertical to the plaque and saliva samples using a Miniblotter device (Immunetics) at 42°C. Buffer-set 2 [21] was used as buffers for pre-hybridization and hybridization. After a series of stringency washes at 70°C, hybrids were detected using phosphatase-conjugated anti-digoxygenin antibodies and the signals were visualized with a chemiluminescent substrate (CDP Star, Roche Diagnostics). Evaluation of the number of bacteria in the samples was performed by comparing the obtained signals with the ones generated by pooled standard samples containing 10^6 and 10^5 cells of each species. The signals were then coded on a scale of 0–5, where 0 indicated no signal, 1 indicated signal with a lower intensity than that gained from 10^5 cells; 2 indicated a signal equivalent to 10^5 cells (low standard); 3 indicated a signal stronger than 10^5 cells but lower than 10^6 cells (high standard); 4 was equivalent to 10^6 cells and 5 indicated a signal stronger than 10^6 cells.

Statistical analyses

Statistical analyses were performed using KaleidaGraph (Synergy Software, 2010) and Microsoft Excel for Mac (version 14.1.3, 2011). The mean plaque pH ± SD for the different time points and all subjects was calculated for (11–12, 25–26, 31–32, 45–46). The maximum pH increase and maximum pH after the 0.25% urea solution rinse were also calculated. Possible correlations between max pH and calculus was analyzed using Pearson's *r*. The student's two-sample, non-paired *t*-test was used to analyze the significance of differences between the CF and CE groups and paired *t*-test within the same group. Chi-square test was used for calculation of significant differences for frequences. $p < 0.05$ was considered to be statistically significant and $p < 0.01$ highly significant.

Results

Questionnaire

The majority of the adults reported that they performed some kind of oral hygiene regularly. More women performed oral hygiene (80%) compared with men (66.7%). Children performed oral hygiene to a lesser extent than adults (41.2%) ($p < 0.01$). Betel chewing was reported by 17.4% of the adults and by none of the children. The majority of the children (61.8%) consumed sucrose containing products sporadically but at least once a week. Only 2.9% of the children reported that they never ate sucrose, compared with 26% of the adults. More men (33%) than women (20%) responded that they never ate sugar. There was no difference in sugar consumption between caries experienced and caries-free individuals (data not shown).

Clinical registration

The caries experience was low and 61% of the adults and 50% of the children were caries-free (Table I). Children and adults had a mean DMFT of 1.9 ± 1.8 and 1.9 ± 2.7, respectively, and a mean DT of 1.4 ± 1.8 and 1.2 ± 2.4, respectively. Women had significantly more DT than men ($p < 0.05$). Fillings were uncommon and adults had on average 27.7 and the children 23.9 teeth present. The visible plaque index (PII%) was high in all subjects and plaque was

Table I. Clinical recordings in Karen adults and children.

Clinical variable	Adults (n = 46)	Children (n = 34)
Number of teeth present (mean)	27.7	23.9
DMFT (mean ± SD)	1.8 ± 2.7	1.9 ± 1.8
DT (mean ± SD)	1.2 ± 2.4	1.4 ± 1.8
Number of caries-free individuals (%)	28 (61)	17 (50)
BoP % (mean ± SD)	51.6 ± 21.5	49.0 ± 17.5
PI % (mean ± SD)	99.2 ± 3.8	99.1 ± 2.5
Calculus (mean ± SD)	1.2 ± 0.5	1.0 ± 0.5

present on almost 100% of the tooth surfaces. BoP levels were the same in children and adults (~ 50%). The calculus values were high and almost all subjects had calculus in the anterior mandibular region. In region 13–23, caries-experienced adults had a significantly higher amount of calculus (mean score 0.7) compared with the caries-experienced children (mean score 0.35) ($p < 0.03$) (data not shown).

pH registrations

Significantly higher pH ($p < 0.05$) was found at baseline in children compared with adults in the maxilla (Table II) . The interproximal pH at baseline was significantly higher ($p < 0.05$) at the mandibular incisors compared with the maxillary molars in the adults. The pH increase and maximal pH after 1 min of rinsing with 0.25% urea was slightly higher (although not significant) in site 15/16 compared with site 31/41 in both children and adults (Table II). Notably, the pH never exceeded 8.3 in any individuals and sites.

Caries-free individuals had a higher baseline pH in the anterior mandibular region than individuals with DT > 0 (mean pH 7.3 and 7.0, respectively) ($p < 0.01$). The difference was not significant in the maxillary molar region. Figure 1 shows that the longitudinal pH after an urea rinse was constantly higher in the caries-free individuals than in the caries-experienced ones at both sites 16/15 and 31/41. In fact, differences were statistically significant at site 15/16 after 5, 10 and 20 min and at site 31/41 after 2, 5 and 10 min ($p < 0.05$). The pH had almost returned to the baseline levels at both sites after 30 min.

There was a strong correlation ($r^2 = 0.998$) between high maximum pH and calculus levels in the first quadrant and in the anterior mandibular region (r^2 = 0.993) in adults, while this correlation was weaker in children (r^2 = 0.698). Caries-free individuals did not show more calculus than caries active individuals (both had a mean score of 1.1).

Table II. Interproximal pH for maxillary molars and mandibular insicors in 46 Karen adults and 34 children after 1 minute rinsing with 0.25% carbamide (urea).

	Maxillary molars		Mandibular incisors	
pH variable	Adults	Children	Adults	Children
Baseline pH (mean ± SD)	6.9 ± 0.4**	7.1 ± 0.6*	7.1 ± 0.4**	7.2 ± 0.4
pH increase (mean ± SD)	0.8 ± 0.41	0.7 ± 0.4	0.6 ± 0.4	0.6 ± 0.4
Max pH (mean ± SD)	7.7 ± 0.5	7.8 ± 0.4	7.7 ± 0.4	7.8 ± 0.43

* $p < 0.05$ for baseline pH in maxillary molars between children and adults.
** $p < 0.05$ for baseline pH between maxillary molars and mandibular incisors in adults.

Urease activity

There was a strong and significant difference in ureolytic activity obtained with the Rapid Urease Test

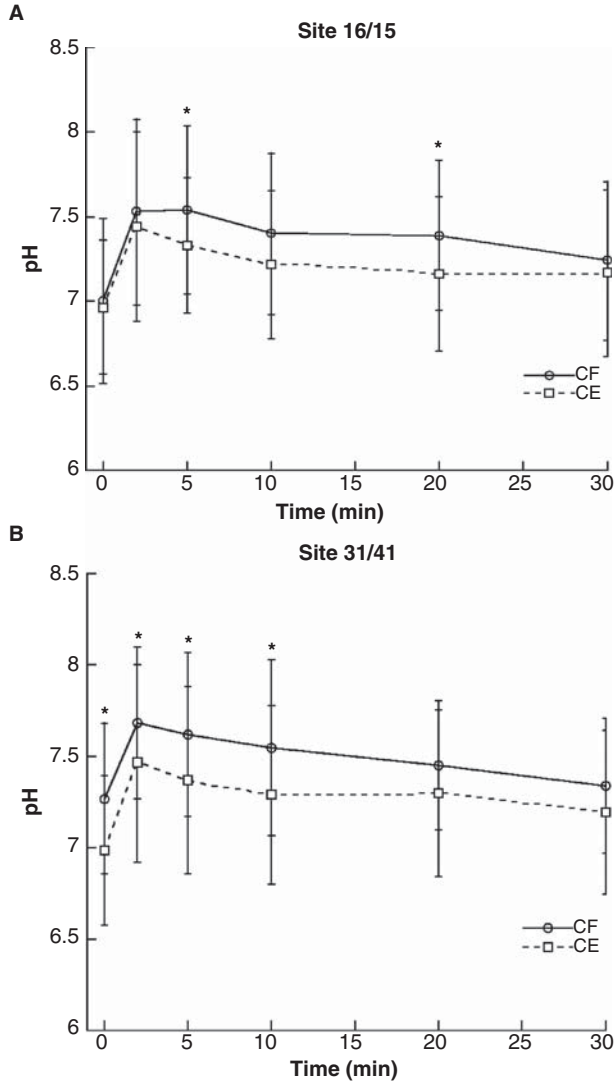

Figure 1. pH vs time (min) after 1 min rinsing with urea solution (0.25%) in (A) site 16/15 and (B) site 31/41 in 45 caries-free (CF) and 35 caries-experienced (CE) Karen individuals.

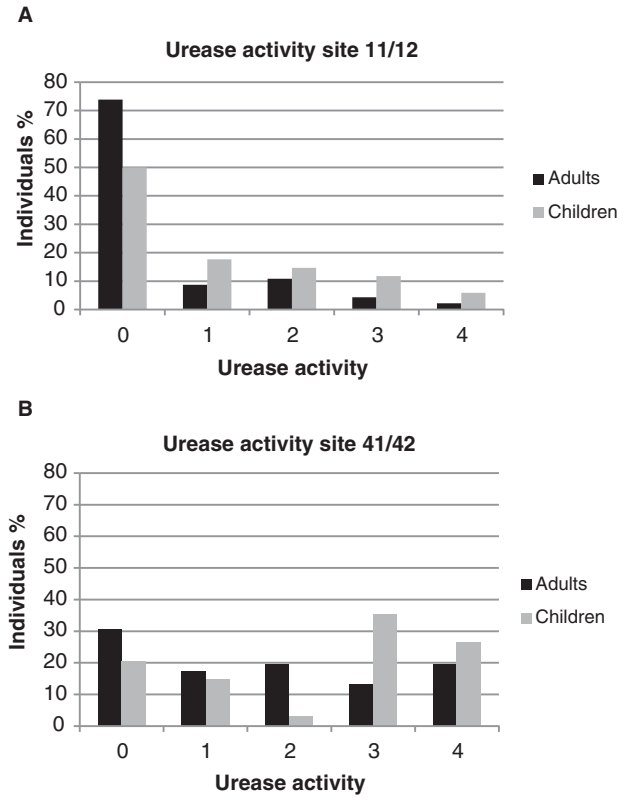

Figure 2. Frequency of individuals (adults and children) with different urease activity (score 0–4) in (A) site 11/12 and (B) site 41/42.

(RUT) in the anterior maxilla compared with the mandibular incisor area in both adults ($p < 0.01$) and children ($p < 0.01$) (Figures 2A and B) . There were also significantly ($p < 0.05$) more adults showing low urease activity (score 0) in site 11/12 than children, while children showed more frequently a high urease activity (Score 3 or 4) in site 41/42 than the adults.

Urease activity in caries-free individuals was not significantly higher than in those with caries (DT > 0) in both sites 11/12 and 41/42 (Figure 3) . Children with caries experience showed a higher ureolytic activity in the maxillary front region compared with caries-experienced adults ($p < 0.05$), although the urease activity was generally low at site 11/12.

A significant correlation was obtained ($r^2 = 0.63$) between the maximum pH and the ureolytic activity in the mandibular incisor region (Figure 4).

Microbiological analysis

The profile obtained by the microbiological analysis was similar in adults and children. The samples were predominated by *P. intermedia*, *F. nucleatum*, *A. oris* and *P. tannerae* (Figures 5A and B) . Strong ureolytic bacteria (e.g. *S. salivarius*, *Campylobacter* spp. and *H. pylori*) were found either not at all or in very small amounts. No statistically significant differences were found in the bacterial profiles between children and adults or between sites.

Discussion

This study describes sugar intake, oral hygiene practices, presence of calculus and caries experience as well as the pH level and ureolytic capacity of the dental plaque in a group of children and adults of the Karen Hill tribe of Northern Thailand. Ureolysis was measured *in vivo* as pH increase after a rinse with urea solution and *ex vivo* with the rapid urease test (RUT). In addition, the dental plaque flora was evaluated with a focus on the presence and level of some known ureolytic bacterial species.

Low caries experience in both children and adults of the Karen Hill tribe was demonstrated. In the same Hill tribe population, Reichart et al. [21] found in 1985 a mean DMFT values below 1 in subjects over the age of 10 years. Children under the age of 10 years had a significantly higher DMFT. Recently, low caries experience was confirmed in both adults and 13-year-old children in the Karen Hill tribes of the same district [2]. It was, thus, interesting to note that, among younger children, aged 6–10 years, 50% were caries-free and there were few open caries lesions (DT = 1.41), although they ate sucrose more

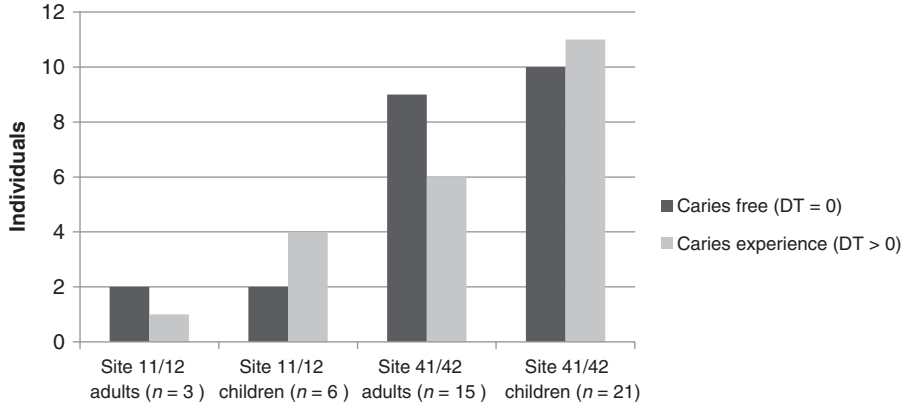

Figure 3. Number of caries-free and caries-experienced children and adults with a high urease activity (score 3 and 4) in sites 11/12 and 41/42.

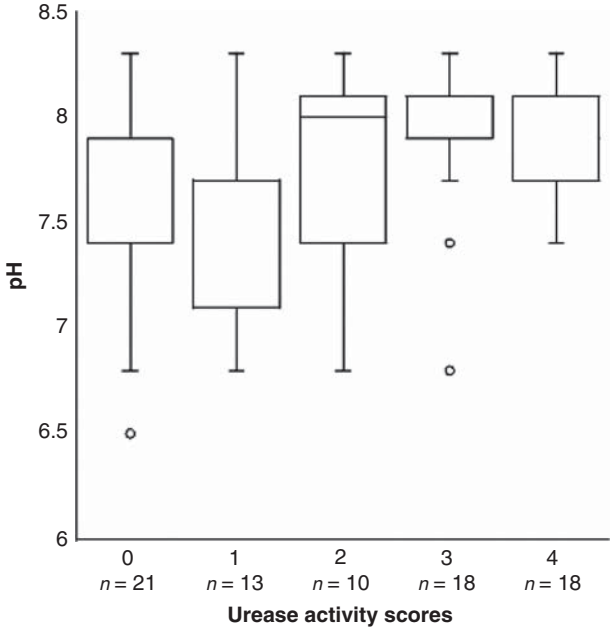

Figure 4. pH and urease activities (score 0–4) in the mandibular anterior teeth in Karen adults and children (n = 80). Correlation coefficient r^2 = 0.63 between caries-free and caries-experienced children and adults.

frequently and performed less oral hygiene than the adults.

In the comparison between caries experienced and caries-free individuals in the present study, DT measurements were used since it was not possible to establish why missing teeth had been lost (DMFT). The classification of caries-free individuals and those with caries was rough, classifying DT = 0 as caries-free and DT > 0 (1–13) as caries-experienced. Lesions were infrequent (most caries individuals had only 1–2 lesions), they progress slowly and many may have been arrested (especially in adults). It should, therefore, be noted that this Karen population is not ideal for comparing individuals with caries and those with no caries experience. Still, there was a significant difference between the resting pH in the anterior mandible in individuals with caries and caries-free individuals and the caries-free individuals had higher pH at all time points after an urea rinse. Our hypothesis implying a more pronounced ureolytic activity and higher pH among the caries-free compared with the caries-experienced was, thus, verified. In a previous study (unpublished) in the Karen Hills tribes, it was shown that 13-year old children and adults 20–40 years had a higher baseline pH and a

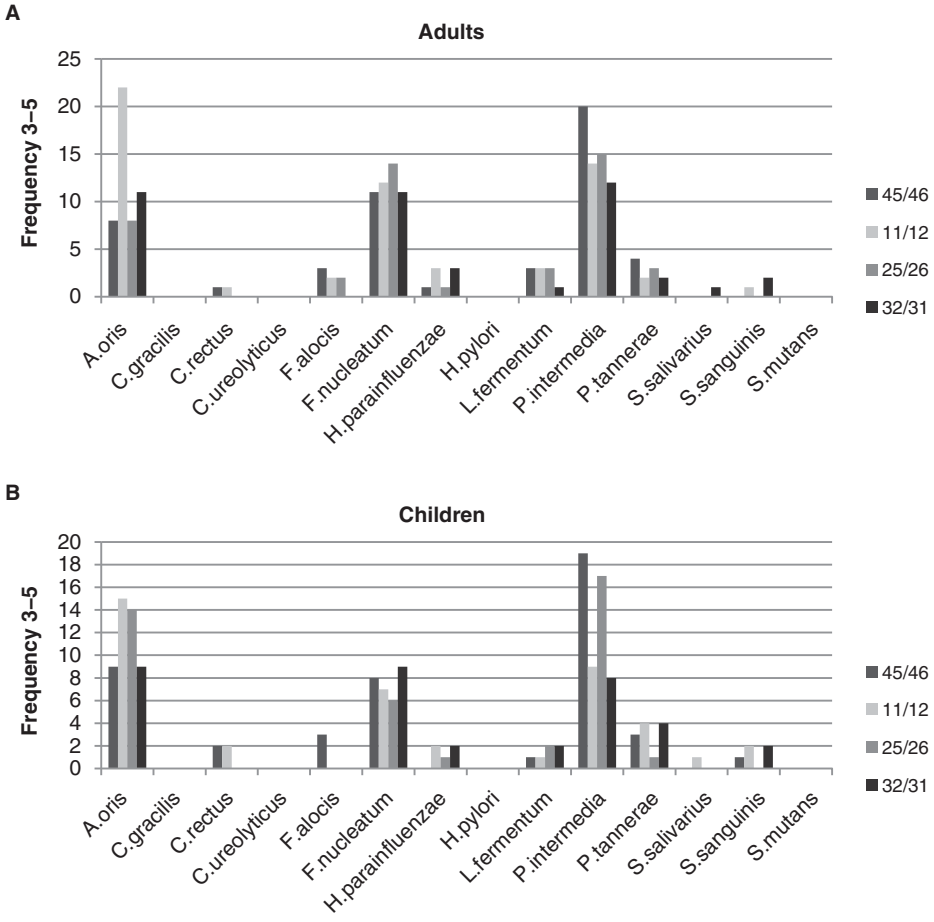

Figure 5. Frequency (%) of bacterial species found at a high score (score 3–5) in four interproximal dental sites in (A) Karen adults (n = 46) and (B) children (n = 34).

smaller pH drop than Swedish caries-free students after rinsing with sucrose. Further, Swedish individuals showed a deeper pH decrease after sucrose rinse than was disclosed using the same method for the Karen children and adults, as in the present study [16,22]. We therefore propose that the low caries activity of the Karen Hill tribe population is at least partly explained by a higher resting pH level and stronger ureolytic capacity of the plaque than have been reported from other populations such as the Swedish.

We found an overall correlation between baseline pH and urease activity in approximal plaque in the anterior mandible. Further, children showed a significantly higher ureolytic activity and a higher resting pH in the maxilla compared with adults. Ureolytic activity in plaque could compensate for the pH decrease caused by sucrose intake and, therefore, help to prevent the initiation of caries [3]. Children did not display a significantly greater increase in pH values after rinsing with a urea solution. This is not in accordance with the hypothesis that the pH increase would correlate strongly with the ureolytic activity as measured with RUT. It is possible that the urea content in saliva is higher in children than adults, which may explain the higher resting plaque pH in children. The pH is already higher (> 7.0) at baseline (by natural urea content in saliva/gingival exudate or by other NH_3 producing reactions) than the urea broth for RUT (pH 6.8) and there seems to be an upper limit for the pH maximum and pH increase *in vivo*, which never exceeded pH 8.3. It follows that the *ex vivo* RUT method may cover a broader pH interval than the pH measurements obtained *in vivo*. It should also be kept in mind that the pH scale in fact is logarithmic and an increase in color reaction in RUT may not fully correspond to a similar increase in pH. It should further be pointed out that the interpretation of the pH strips and the color of the RUT is partly subjective and may also entail some error. It is suggested that the simple RUT method clearly shows the immediate ureolytic capacity of the dental plaque, while the advantage of measuring pH with the pH strips *in vivo* is to follow the pH change longitudinally after the urea rinse under more realistic and dynamic conditions.

Children had less calculus compared with adults, even though the children had significantly higher urease activity and baseline pH. This may be explained by the fact that oral plaque accumulation has gone on for a longer time in adults and, therefore, calculus formation was more pronounced. Despite a correlation between the pH level and the quantity of calculus in adults, a weak correlation only between calculus levels and elevated ureolytic activity was found. Further studies are needed to find out what other factors are of importance in calculus formation.

Calculus was present in all adults and almost all children despite their low age and commonly included the entire dentition. This confirms that calculus is a significant finding in Karen populations and is consistent with East Asian populations in general [2,14]. It was most abundant in the mandibular incisor region, which is a common finding also in other populations and teeth in this dental region seldom have caries lesions. The strong correlation between calculus and pH level, found both in the anterior mandible and in the maxillary molar region, argues for the oral environment in this population as a whole having a generally high pH level and ureolytic activity, which buffers dramatic pH drops and caries development.

Detectable amounts of known obligate ureolytic bacteria (e.g. *S. salivarius*, *Campylobacter* spp. and *H. pylori*) were only infrequently found among the 12 bacterial species analyzed in the interproximal plaque samples. This should be interpreted with caution since these strong ureolytic species may be present in the individuals, but in undetectable amounts. The detection level with the checkerboard method is calculated to be 10^4 cells per sample [23]. The urease activity noted may also be derived from other bacterial species, not scanned for in this study. The predominating bacteria found in the dental plaque of both children and adults in this study were anaerobic species (*P. intermedia* and *F. nucleatum*) together with *A. oris*. This is strikingly different from the species commonly found in supragingival plaque in other populations, where streptococcal species usually predominate [24]. This could be explained by the fact that the Karen Hill population rarely practice oral hygiene and thereby have a more mature plaque which persists, involving more anaerobic micro-organisms. The low sugar intake does not favor strong sugar fermenters such as streptococci and lactobacilli and a suggested greater ureolytic activity buffers the plaque pH to a comparatively higher level. A low sugar intake (sucrose in particular) results in different plaque ecology than found in other populations exposed to more sugars in the diet, which promote an acidogenic and aciduric flora. Exposure of this population and especially the children, to more sugar, such as may occur when this population gets increased contact with the urban Thai community, will probably lead to a change of the oral plaque ecology and to increased susceptibility to caries.

In summary, this study found a correlation between the baseline ('resting') plaque pH and a high ureolytic activity, a high amount of calculus and a low caries experience/activity on the individual as well as site level, while the correlation with ureolytic plaque bacteria was low. The bacterial plaque profile in both children and adults was characterized by a high level of anaerobic bacteria and low levels of streptococci. It is suggested that the low caries prevalence in the Karen Hill tribe population of Northern Thailand is a result of low sucrose intake, a high resting plaque

pH, a strong ureolytic capacity of the dental plaque and a plaque microbiota low in sugar-fermenting bacteria.

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