Clustering of subgingival microbial species in adolescents with periodontitis

Rodrigo López[1], Gunnar Dahlén[2], Carolina Retamales[3], Vibeke Baelum[4]

[1]Department of Periodontology, Faculty of Health Sciences, Aarhus University, Aarhus, Denmark; [2]Department of Oral Microbiology, University of Gothenburg, Gothenburg, Sweden; [3]Faculty of Dentistry, University of Chile, Santiago, Chile; [4]School of Dentistry and Department of Epidemiology, Institute of Public Health, Faculty of Health Sciences, Aarhus University, Aarhus, Denmark

López R, Dahlén G, Retamales C, Baelum V. Clustering of subgingival microbial species in adolescents with periodontitis.
Eur J Oral Sci 2011; 119: 141–150. © 2011 Eur J Oral Sci

It has become increasingly recognized that groups of microorganisms interact within the subgingival plaque of adult subjects with periodontitis. It is much less clear, however, whether the consortia of microorganisms associated with periodontitis are different in early and more advanced cases of periodontitis. To investigate this point further, subgingival plaque was collected from six sites in 87 adolescents with periodontitis and 73 controls and the samples were analyzed for the detection of 18 microbial species using the DNA–DNA hybridization technique. *Actinomyces oris* accounted for the highest proportion of the flora and was more predominant among controls. *Prevotella nigrescens*, *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Tannerella forsythia* were present at higher levels among the subjects with periodontitis. Factor analyses identified one factor characterized by highly positive loadings for *T. forsythia*, *Campylobacter rectus*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *Parvimonas micra*, and *Treponema denticola*, and another factor characterized by highly positive loadings of *A. oris*, *Capnocytophaga ochracea*, *Eikenella corrodens*, *Streptococcus intermedius*, *Selenomonas noxia*, *Streptococcus oralis*, *Streptococcus sanguinis*, and *Veillonella parvula*. *Aggregatibacter actinomycetemcomitans* and *Streptococcus mutans* did not load on any of the two factors, while *Fusobacterium nucleatum* loaded on both. These findings confirm the occurrence of clustering of subgingival bacteria according to case status also among young individuals.

Rodrigo López, Department of Periodontology, Aarhus University, Vennelyst Boulevard 9, Aarhus C, 8000, Denmark

Telefax: +45-86-198122
E-mail: rlopez@odont.au.dk

Interest in the microbiological features of periodontitis among adolescents is reflected in numerous case reports and case series (1–13), in studies contrasting different patient groups (14–16), and in studies contrasting periodontitis patients with control groups selected by convenience (17–21). Valid inference from such studies is hampered by either the omission of comparison groups or by the lack of appropriate reference groups, as valid inference necessitates the comparison groups to represent the non-case population. Only two studies have, to the best of our knowledge, used a study design involving non-cases originating from the same population that gave rise to the cases (22, 23). ALBANDAR *et al.* (22) contrasted a spectrum of periodontitis manifestations among adolescents with respect to the occurrence of seven putative periodontal pathogens [*Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Campylobacter rectus*, *Aggregatibacter actinomycetemcomitans* (at the time: *Actinobacillus actinomycetemcomitans*), *Eikenella corrodens*, and *Treponema denticola*], and found that detectable levels of all species, except for *A. actinomycetemcomitans* and *E. corrodens*, were statistically significantly more frequent among cases of early-onset periodontitis than among non-cases. Moreover, except for *A. actinomycetemcomitans*, which was relatively rare, statistically significantly higher levels of bacteria were found among the cases of early-onset periodontitis. HAUBEK *et al.* (23) studied *A. actinomycetemcomitans* among Moroccan adolescents and observed a statistically significant positive association between the presence of the highly leucotoxic clone of *A. actinomycetemcomitans* and early-onset periodontitis (categorized as localized juvenile periodontitis, generalized juvenile periodontitis, and incidental attachment loss); other types of *A. actinomycetemcomitans* were not associated with early-onset periodontitis. It should be noted, however, that the highly leucotoxic clone of *A. actinomycetemcomitans* seems to be specific to subjects whose descent can be traced back to the Mediterranean or western parts of Africa (24).

Adolescence represents the transition from childhood to adulthood, and from a microbiological viewpoint it is interesting that the colonization patterns of putative periodontal pathogens observed among adults (25) are strikingly similar to those observed among even young children (26, 27). Moreover, several studies have shown that many putative periodontal pathogens can be found among children who present no signs of periodontitis (27–37). These findings suggest that the main differences between subjects with periodontitis and subjects without

periodontitis are found in the amount and proportions of the individual microbial species present in the lesions (25), indicating that putative periodontal pathogens belong to the commensal microbiota and that their presence at high levels represents an endogenous infection (26, 27, 36). This view is further supported by the results of studies of periodontitis among young adults, as evidenced by the clinical detection of periodontal attachment loss of ≥ 2 mm (38), which suggests that the microbial characteristics of these young adults with periodontitis are similar to those commonly found for more advanced cases of periodontitis (38).

It has become increasingly recognized that putative periodontal bacteria interact in the subgingival plaque of subjects with periodontitis (25, 39, 40). This observation is particularly interesting because it has been found that particular categories of periodontitis cannot be discriminated through the occurrence of putative periodontal pathogens such as *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *Bacteroides forsythus*, and *C. rectus* (41).

These observations might suggest that rather than focusing on the presence and counts of particular putative periodontal pathogens, more useful information may be gathered by using an ecological approach to investigate the distribution of microbial consortia in the subgingival environment. It is unknown whether the constellations of microorganisms associated with periodontitis are different in subjects with no periodontitis, with incipient periodontitis or with advanced periodontitis.

To the best of our knowledge, no study has investigated the formation of microbial consortia in the subgingival microbiota of adolescents with periodontitis using a study design with appropriate comparability of study groups. Therefore, the aim of the present study was to describe, using a case–control design, the occurrence of, and constellations among, key putative periodontal pathogens and species generally considered to be beneficial, in adolescents with and without periodontitis, nested in an adolescent population (42).

Material and methods

Study population

Cases and non-cases were nested in a fully enumerated adolescent population that had been screened for signs of periodontitis (42). The target population for the screening examination comprised all adolescent students in the high schools of the Province of Santiago, Chile. A screening examination for the occurrence of clinical attachment loss (CAL) was performed on 9,163 adolescents, identified using multistage cluster random sampling, from 310 high school classes in 98 high schools (42). A student was considered as a case (i.e. to have periodontitis) when a CAL of ≥ 3 mm was found in at least two of the 16 teeth examined during the screening examination. A total of 148 students were found to fulfil the inclusion criterion for being a case. Non-cases did not fulfil the case-defining criterion and were identified by drawing a simple random sample of 250 students from the screened population representing potentially

eligible non-cases. Seven of these potentially eligible non-cases were excluded because they were already in the case group, leaving 243 eligible non-cases.

A standard invitation letter, without information on the student's status as a case or a control, was sent to all eligible subjects. A total of 160 participants [73 non-cases (response rate 30%) and 87 cases (response rate 59%)] were recruited for the present non-matched case–control study. Cases presented with a wide spectrum of severity and extent of periodontitis. Further details on the sampling and recruitment strategies, sample size estimations, participation rates, and intra-examiner and interexaminer reliability of the clinical recordings used to identify the cases, as well as a comprehensive description of the socio-demographic profile of the underlying population and a detailed description of the clinical periodontal features of cases and non-cases, have previously been published (42–46). The periodontist (R.L.) who conducted the clinical examinations in the case–control study was also the gold standard for the training and calibration of the examiners who conducted the screening examinations. Calibration was achieved using test–retest exercises in groups of patients before the screening examination study and repeated test–retest exercises in large groups of adolescents at different intervals during the screening examination (45). The overall percentage agreement for intra-examiner reliability when assessing CAL ≥ 3 mm in the screening examinations was 99% and the corresponding overall kappa value was 0.85 (45).

All participants (72 male subjects and 88 female subjects; age range 13–20 yr) answered a questionnaire on general health status, current and past use of medication, and health-related behaviours (44). None of the participants had previously received periodontal treatment or had received antibiotic treatment within the last 3 months. No attempt was made to identify subjects with 'aggressive periodontitis' as there is no agreement regarding the defining criteria for this disease entity. Ethical approval for the study was obtained from the local Committee of Ethics, and informed written consent was provided by each participant/guardian.

The clinical examinations comprised direct recordings of CAL, probing depth (PD), bleeding on probing (BOP), supragingival plaque, and calculus at six sites per tooth (mesio-buccal, midbuccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual) for all teeth present, excluding third molars. A detailed description of the distribution of all the clinical parameters investigated has been recently published (44).

Samples of subgingival plaque were obtained during the same visit from six fixed sites in each subject, immediately before the clinical examinations were conducted. The sites selected for sampling included the mesio-palatal site of the upper first molars, the mesio-buccal sites of the upper left and lower right central incisors, and the mesio-buccal site of the lower first molars. However, in some participants, one or more of the designated teeth could not be sampled owing to missing teeth or the presence of orthodontic appliances, and replacements were therefore made. Replacements included the substitution of 16 upper first molars with upper second molars in the same side of the mouth, and the substitution of 32 lower first molars with lower second molars in the same side of the mouth. Three upper central incisors were substituted by the contralateral tooth. In one subject, only four of the six designated teeth could be sampled and suitable replacements could not be made.

Subgingival plaque samples were obtained using sterile Gracey curettes (Hu-Friedy, Chicago, IL, USA). After removing gross supragingival deposits, the sterile curette was inserted into the pocket and the subgingival plaque was collected from the bottom of the pocket by a single scaling stroke. Caution was exercised to ensure that all pressure on the root surface ceased when the curette tip reached the gingival margin, in order to minimize contamination with supragingival plaque. Each sample of plaque was immediately transferred into an Eppendorf tube (Eppendorf, Westbury, NY, USA) containing 150 μl of sterile TE buffer (10 mM Tris–HCl, 1.0 mM EDTA, pH 7.6). The tube was vortexed, and 150 μl of 0.5 M NaOH was added. Each tube was then closed and kept at −20°C until processed at the Laboratory of Oral Microbiology, University of Gothenburg, Sweden. In total, 958 bacterial samples from 160 subjects were available for analysis.

Checkerboard DNA–DNA hybridization

Digoxigenin-labelled, whole genomic probes were prepared by random priming using the High-Prime labelling kit (Boehringer-Mannheim, Mannheim, Germany) from the following 18 strains of bacteria: *A. actinomycetemcomitans* strain FDC Y4 (OMGS 76; originally obtained from Dr Socransky, Forsyth Dental Center, Boston, MA, USA); *Actinomyces oris* (formerly *Actinomyces naeslundii* genospecies 2), ATCC 27044 (CCUG 34288); *Capnocytophaga ochracea*, ATCC 33624 (OMGS 2844); *C. rectus*, ATCC 33238 (OMGS 1236); *E. corrodens*, ATCC 23834 (OMGS 2844); *F. nucleatum*, ATCC 10953 (OMGS 2865); *P. gingivalis*, FDC381 (OMGS 2860; originally obtained from Dr Socransky, Forsyth Dental Center); *P. intermedia*, ATCC 25611 (OMGS 2514); *Parvimonas micra*, ATCC 33270 (OMGS 2852); *Prevotella nigrescens*, ATCC 33563 (OMGS 2513); *Streptococcus intermedius*, ATCC 27335 (OMGS 3177); *Streptococcus mutans*, ATCC 25175 (OMGS 2483); *Selenomonas noxia* (OMGS 3118; originally obtained from Dr Socransky, Forsyth Dental Center); *Streptococcus oralis*, ATCC 33624 (OMGS 2470); *Streptococcus sanguinis*, ATCC 10556 (OMGS 2482); *T. denticola*, (OMGS 3271) (originally obtained from Dr R. Ellen at the University of Toronto, Toronto, ON, Canada); *T. forsythia*, ATCC 43037 (OMGS 3072), and *Veillonella parvula*, ATCC 10790 (OMGS G186). The target species were selected to facilitate comparisons from previous studies in other populations and age groups (47, 48).

The analysis was performed using the checkerboard DNA–DNA hybridization method (49) as modified by Papapanou *et al.* (47). The samples were boiled for 5 min, neutralized, transferred onto nylon membranes using a Minislot device (Immunetics, Cambridge, MA, USA), and immobilized by ultraviolet light and baking at 120°C. Immobilization of bacterial samples on the membranes was completed briefly after sample collection. After 2 h of prehybridization, the DNA probes were allowed to hybridize overnight with the sample DNA in a Miniblotter device (Immunetics) at 42°C. After a series of stringency washes, hybrids were detected by application of an anti-digoxigenin immunoglobulin conjugated with alkaline phosphatase and incubated with an appropriate chemiluminescent substrate (CSPD; Boehringer-Mannheim). The chemiluminescence signals were read using a LumiImager Workstation (Boehringer-Mannheim) and evaluated by comparing the signals obtained with those of pooled standard samples containing 10^6 (high-standard) or 10^5 (low standard) bacteria of each of the 18 species. The probes were cross-tested for specificity against the 18 species of the two panels in order to distinguish cross-hybridizations, as described by Socransky *et al.* (50).

Statistical analysis

Graphical plots of the chemiluminescence values for the two standards containing 10^6 and 10^5 bacteria of each species were made for each of the 37 membrane gels used as well as for each of the 18 bacterial species investigated. These plots clearly indicated that a logarithmic transformation of the data would support the use of an additive logarithmic model. The log-transformed chemiluminescence values for each species in each sample were converted to logarithmic bacterial counts by means of a linear transformation of the logarithmic chemiluminescence values for the two standards represented in each membrane gel. This procedure adjusts for the membrane gel effect and ensures comparability of findings across different membrane gels.

The data set analyzed consisted of the log bacterial counts for each of the 18 bacterial species from each of the six sampled teeth from each of the 160 subjects (87 cases and 73 non-cases). In order to produce subject-level plots illustrating the occurrence and counts of each species, a subject-level log bacterial count was generated as the average log count over the six teeth sampled. Based on these subject-level data, cumulative frequency-distribution graphs for the log counts of each species were generated as well as of the proportion of the 'total' flora accounted for by each of the 18 species.

In order to investigate the possible effect of case status on the prevalence of bacterial counts in excess of 10^5 cells, we used multilevel logistic regression analysis (procedure xtlogit of STATA 11.0) (StataCorp, College Station, TX, USA) to account for the clustering of the 958 sample sites in the 160 subjects. The effect of case status on the log bacterial counts was explored using a multilevel mixed linear regression model (procedure xtmixed of STATA 11.0) (51) with case status and sample tooth as fixed effects and subject as random effect. These analyses allowed us to estimate the 'effects' of case status while accounting for the fact that the six sample teeth are nested within subjects because the multilevel analysis takes into account the dependency of plaque samples from different teeth in the same mouth.

Finally, factor analysis was used to explore the patterns of co-occurrence of the 18 microbial species. A separate analysis was carried out for each of the six sample teeth in order to explore the consistency of the results. Factor analysis is a statistical method that describes the variability among observed variables (in this study, the log counts of the 18 species of bacteria) in terms of a lower number of unobserved (latent) variables called factors, which represent the microbial constellations. Factor analysis searches for joint variations in the observed variables in response to unobserved latent variables and models the observed variables as linear combinations of the factors. The information gained on the co-variation of the observed variables can later be used to reduce the original set of variables into a smaller number of variables. Factor analysis is closely related to principal components analysis but differs from this technique by accounting for the co-variation among variables, where principal components analysis accounts for the total variation among variables. The factor analysis was carried out using the STATA (51) procedure 'factor' and two factors were extracted for each of the six teeth sampled. The factors were rotated using Varimax rotation to obtain an orthogonal factor solution.

Results

Table 1 summarizes the clinical findings among cases and non-cases.

Table 1

Clinical findings among cases and non-cases

	Cases (n = 87)	Non-cases (n = 73)
Sites with BOP (mean % (SD))	44.6 (22.9)	13.6 (16.0)
Sites with CAL ≥ 3 mm (mean % (SD))	4.1 (6.4)	0.2 (0.5)
Sites with PD ≥ 4 mm (mean % (SD))	5.3 (6.7)	1.2 (2.6)
Persons with BOP (%)	100	97.3
Persons with CAL ≥ 3 mm (%)	97.7	11.0
Persons with PD ≥ 4 mm (%)	88.5	45.2
CAL of sampled sites (mean (SD))	0.63 (1.03)	0.08 (0.35)
Sampled sites with CAL ≥ 1 mm (%)	37.0	5.7
Sampled sites with CAL ≥ 3 mm (%)	6.7	0.2
Sampled sites with PD ≥ 4 mm (%)	8.8	1.6
PD of sampled sites (mean (SD))	2.31 (1.00)	1.75 (0.75)
Sampled sites with BOP (%)	64	23

BOP, bleeding on probing; CAL, clinical attachment level; PD, probing depth.

Irrespective of case status, all 18 species of bacteria were present at detectable levels in virtually all sites sampled (Fig. 1). However, the counts of *A. actinomycetemcomitans, C. ochracea, E. corrodens, S. noxia*, and *T. denticola* less frequently exceeded 10^5 cells. Cases had statistically significantly higher levels (i.e. > 10^5 cells) of *T. forsythia, C. rectus, F. nucleatum, P. gingivalis, P. intermedia, P. micra, P. nigrescens*, and *S. intermedius* than did non-cases.

The subject-level logarithmic counts of *A. oris, T. forsythia, P. gingivalis, P. intermedia*, and *P. nigrescens* provided a clear distinction between cases and non-cases, while a small distinction between cases and non-cases was seen for *C. rectus, F. nucleatum*, and *P. micra* (Fig. 1). The log count distribution for all other bacterial species was similar in cases and in non-cases. Except for *A. oris*, all bacteria for which a distinction between cases and non-cases was observed occurred in higher numbers among the cases than among the non-cases.

Upon considering the subject level proportion accounted for by each species, it was clear that the dominant species was *A. oris*, followed by *P. nigrescens, P. intermedia, S. mutans, S. oralis, P. gingivalis*, and *T. forsythia* (Fig. 2). Again, *A. oris* was more dominant among non-cases than among cases, whereas *P. nigrescens, P. intermedia, P. gingivalis*, and *T. forsythia* were
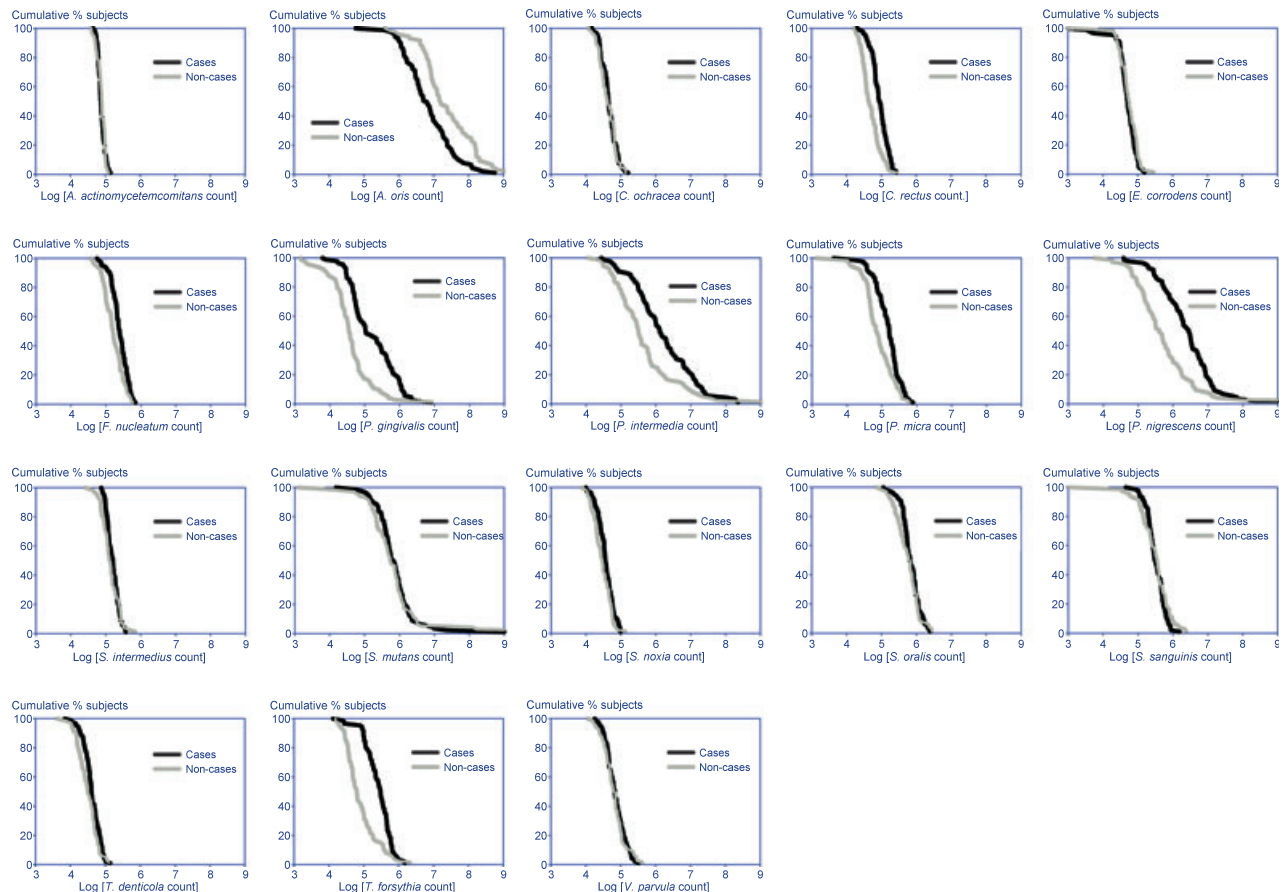

Fig. 1. Cumulative frequency distribution of subjects according to the logarithmic bacterial counts of each of the 18 species of bacteria investigated (representing the average log count over the six teeth sampled).
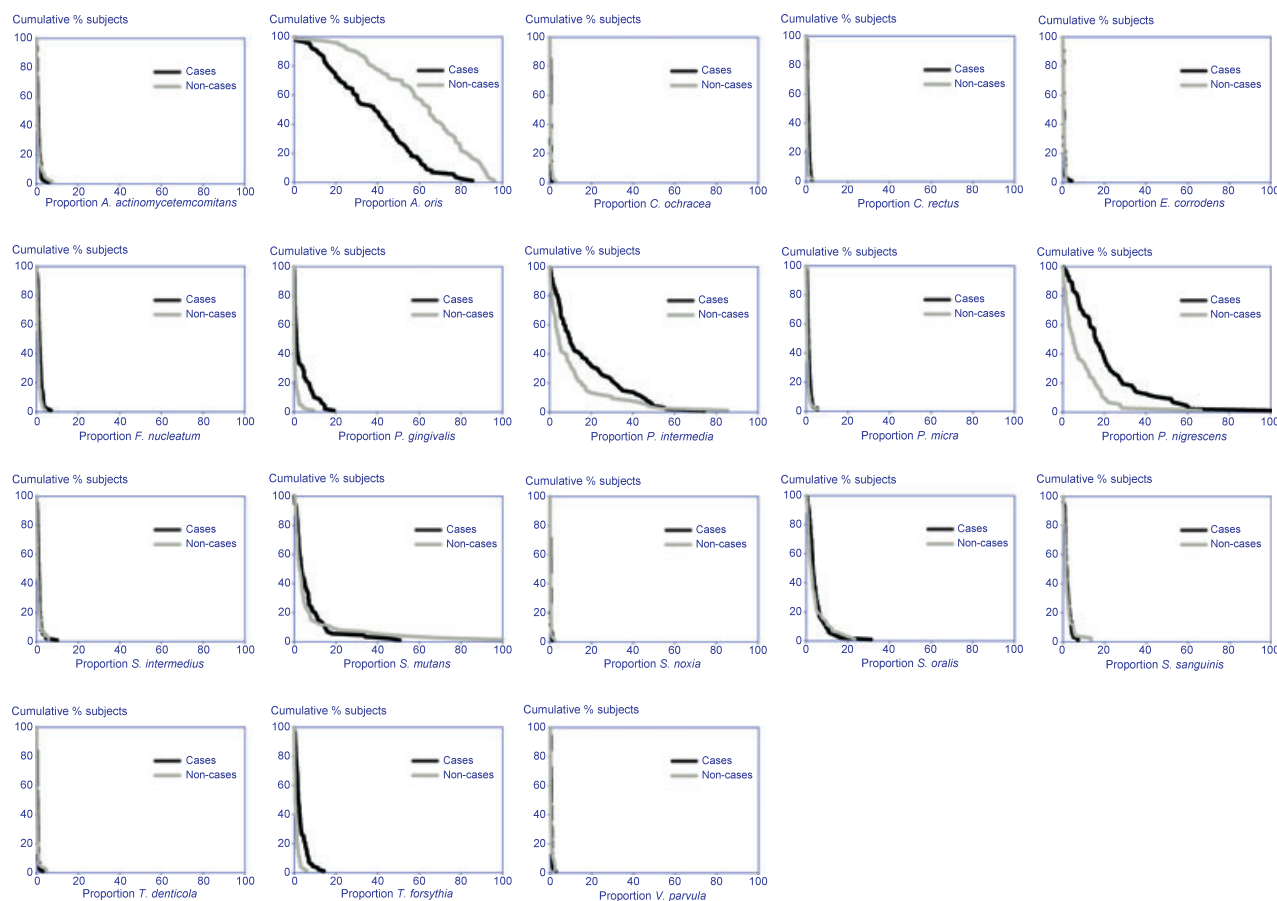
Fig. 2. Cumulative frequency distribution of subjects according to the proportion of the 18 species combined, accounted for by each species investigated.

more dominant among the cases. All other species investigated constituted only negligible fractions of the flora investigated.

Table 2 shows the results of mixed-effects linear-regression analyses of the effect of case status on the log bacterial counts, adjusted for the effects of the sample tooth. Non-cases had a statistically significant average of 0.55 higher log counts of *A. oris* than did cases, whereas cases had significantly higher log counts (in particular of *P. nigrescens*, *P. gingivalis*, *P. intermedia*, and *T. forsythia*) than did non-cases (Table 2). The average log count difference between cases and non-cases for these species ranged from 0.51 to 0.74. The sample tooth also influenced the log bacterial counts for most species in such a way that the reference tooth (the upper central incisor) had lower counts than did the other sample teeth. The exception to this general rule was seen for *A. oris,* where the reference tooth had significantly higher counts than did the upper right first molar, the lower left first molar, and the lower central incisor. Considerable and statistically significant between-subject variation not attributable to case status was observed for all species, and was most pronounced for *P. gingivalis*, *P. nigrescens*, *S. mutans*, and *P. intermedia* (Table 2).

The results of the factor analyses for each of the six sample teeth (Table 3) showed that the analyses consis-

tently identified one factor in which *T. forsythia*, *C. rectus*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *P. micra*, and *T. denticola* loaded highly positively, and another factor in which *A. oris, C. ochracea, E. corrodens, S. intermedius, S. noxia, S. oralis, S. sanguinis,* and *V. parvula* loaded highly positively. *A. actinomycetemcomitans* and *S. mutans* did not load on any of the two factors identified, whereas *F. nucleatum* loaded similarly on both (Table 3). Depending on the sample tooth, the two factors identified explained between 86.0% and 90.7% of the common variance among the 18 microbial variables. Except for tooth 21, the factor explaining most of the variation was the factor with high loadings for *T. forsythia*, *C. rectus*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *P. micra*, and *T. denticola*.

Discussion

The present study is, as far as we are aware, the first to explore the occurrence of constellations of subgingival microbial species among adolescents. The use of six fixed sample sites allowed us to carry out factor analyses separately for each of the sample sites to check consistency of results, and the results of these analyses corroborated each other in identifying the same two

Table 2

Mixed-effects linear regression of the influence of case status and tooth sampled on the bacterial counts for each of 18 selected species

Species	Fixed effects						Random effect
	Case status* β (95% CI)	# 16[†] β	# 26[†] β	# 46[†] β	# 41[†] β	# 36[†] β	Subject variance
Aggregatibacter actinomycetemcomitans	0.00 (−0.03 to 0.04)	−0.01	−0.00	−0.02	−0.02	−0.01	**0.09 (0.08–0.11)**
Actinomyces oris	**−0.55 (−0.78 to −0.32)**	**−0.33**	−0.13	**−0.48**	**−0.67**	**−0.22**	**0.59 (0.49–0.70)**
Capnocytophaga ochracea	0.03 (−0.04 to 0.10)	**0.20**	**0.21**	0.04	**−0.10**	0.04	**0.19 (0.16–0.22)**
Campylobacter rectus	**0.26 (0.18 to 0.34)**	**0.28**	**0.17**	**0.21**	**0.11**	**0.13**	**0.22 (0.19–0.26)**
Eikenella corrodens	−0.04 (−0.15 to 0.07)	0.01	0.04	−0.06	−0.02	**−0.12**	**0.32 (0.28–0.36)**
Fusobacterium nucleatum	**0.18 (0.10 to 0.26)**	**0.34**	**0.32**	**0.19**	**0.14**	**0.20**	**0.22 (0.19–0.26)**
Porphyromonas gingivalis	**0.62 (0.43 to 0.82)**	**0.55**	**0.44**	**0.42**	**0.34**	**0.28**	**0.58 (0.51–0.67)**
Prevotella intermedia	**0.56 (0.28 to 0.83)**	**0.83**	**0.66**	**0.52**	**0.34**	**0.67**	**0.78 (0.68–0.90)**
Parvimonas micra	**0.28 (0.15 to 0.41)**	**0.32**	**0.20**	**0.16**	**0.13**	**0.16**	**0.38 (0.33–0.44)**
Prevotella nigrescens	**0.74 (0.34 to 1.13)**	**0.87**	**0.80**	**0.83**	**0.58**	**0.71**	**1.12 (0.97–1.30)**
Streptococcus intermedius	**0.07 (0.00 to 0.13)**	**0.20**	**0.18**	**0.09**	0.04	**0.09**	**0.18 (0.15–0.21)**
Streptococcus mutans	0.01 (−0.26 to 0.28)	**0.41**	**0.29**	0.13	0.15	**0.25**	**0.81 (0.72–0.92)**
Selenomonas noxia	**0.09 (0.01 to 0.17)**	**0.19**	**0.14**	−0.00	**−0.12**	−0.03	**0.20 (0.17–0.24)**
Streptococcus oralis	0.09 (−0.01 to 0.18)	**0.29**	**0.21**	**0.10**	0.04	0.08	**0.23 (0.19–0.28)**
Streptococcus sanguinis	0.04 (−0.08 to 0.16)	**0.22**	**0.17**	0.04	0.05	0.02	**0.32 (0.28–0.38)**
Treponema denticola	**0.11 (0.03 to 0.20)**	**0.15**	**0.13**	**0.11**	**0.06**	**0.08**	**0.25 (0.22–0.29)**
Tannerella forsythia	**0.51 (0.38 to 0.64)**	**0.50**	**0.37**	**0.41**	**0.30**	**0.34**	**0.37 (0.32–0.43)**
Veillonella parvula	0.03 (−0.06 to 0.12)	**0.21**	**0.18**	0.07	**−0.19**	**0.11**	**0.24 (0.20–0.28)**

Statistically significant estimates are given in bold.
*Reference = non-cases.
[†]Reference = tooth # 21 (upper central incisor).

microbial factors or constellations, one dominated by putative periodontopathogens (*T. forsythia, C. rectus, F. nucleatum, P. gingivalis, P. intermedia, P. nigrescens, P. micra,* and *T. denticola*) and one dominated by *A. oris, C. ochracea, E. corrodens, S. intermedius, S. noxia, S. oralis, S. sanguinis,* and *V. parvula.* Interestingly, the first of these factors comprised microorganisms identified by SOCRANSKY *et al.* (39, 52) as 'red' (*T. forsythia, P. gingivalis,* and *T. denticola*) or 'orange' (*P. intermedia, P. nigrescens, P. micra, F. nucleatum,* and *C. rectus*) complex bacteria. The other factor comprised microorganisms classified by SOCRANSKY & HAFFAJEE (52) as 'yellow' (*S. oralis, S. sanguinis,* and *S. intermedius*), 'green' (*E. corrodens* and *C. ochracea*), 'purple' (*V. parvula*), and 'blue' (*A. oris*). Moreover, *S. noxia,* which, according to SOCRANSKY & HAFFAJEE (52), does not belong to any particular colour complex, also loaded on this factor, as did the 'orange' complex species *F. nucleatum,* which thereby loaded on both factors. SOCRANSKY & HAFFAJEE (52) described the bacterial species belonging to the 'yellow', 'green', 'purple', and 'blue' complexes as early colonizers, which lead to autogenic succession whereby members of the 'red' and 'orange' complexes are allowed to proliferate. This change in habitat will, it has been hypothesized, result in gingivitis whereby a feedback mechanism is activated, leading to further proliferation, not only of the 'red' and 'orange' bacteria, but probably also of the early colonizer complexes (52). However, our observation of a negative association between *A. oris* and case status, which corroborates previous reports among young Chinese adults (20), is apparently in contrast to this proposition, at least as far as this 'blue' complex species is concerned. Not-

withstanding, our finding supports the earlier proposal of antagonistic relationships between certain species and the members of the red and orange complexes (39) by also demonstrating it among adolescents. In this context, it was intriguing to observe how the sample tooth effect, mainly reflected in lower counts for 'red' and 'orange' complex species and high counts of *A. oris* in the upper central incisor, corroborated this by reflecting the generally lower disease experience of this tooth relative to the other sampled teeth (44). However, a full account of the relationship between the occurrence of bacteria from different colour complexes and the clinical features of the sample teeth warrants a more detailed analysis. Specifically, the present study is cross-sectional in nature and we may therefore only speculate on the reasons for the observed associations.

The advantage of the checkerboard analysis in this type of study is the detection and semiquantification of a large number of species in many samples using a simple and rapid method. However, the disadvantage is low specificity, owing to the use of whole-genomic probes, as a result potential cross-hybridization between related species (50). Cross-reactivity occurred between *P. intermedia* and *P. nigrescens* and between the streptococcal species. The prevalence of low levels of bacteria (e.g. 10^4–10^5 cells) should therefore be interpreted with caution.

It may be argued that the results presented here are chiefly confirmatory, and that the target species selected are those also studied by SOCRANSKY & HAFFAJEE (52) using essentially the same technique. However, whereas previous results typically pertain to adult periodontal patients, the findings of the present study may indeed be considered novel because they were obtained from very

Table 3

Results of factor analysis of the log bacterial counts for each of the six teeth sampled

Species	Tooth 16		Tooth 21		Tooth 26		Tooth 46		Tooth 31		Tooth 36	
	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2
Aggregatibacter actinomycetemcomitans	0.15	0.23	0.17	0.27	0.18	0.20	0.21	0.14	0.16	0.30	0.04	0.39
Actinomyces oris	−0.30	**0.52**	−0.12	**0.51**	−0.36	**0.50**	−0.21	**0.57**	−0.19	**0.63**	−0.23	0.45
Tannerella forsythia	**0.89**	−0.07	**0.88**	0.12	**0.90**	0.16	**0.91**	0.07	**0.89**	−0.00	**0.89**	0.08
Capnocytophaga ochracea	0.24	**0.67**	0.35	**0.76**	0.31	**0.70**	0.32	**0.65**	0.34	**0.58**	0.34	**0.70**
Campylobacter rectus	**0.90**	0.09	**0.85**	0.27	**0.88**	0.25	**0.88**	0.14	**0.88**	0.16	**0.87**	0.17
Eikenella corrodens	0.24	0.42	0.09	**0.73**	0.30	**0.53**	0.09	**0.54**	0.05	**0.65**	0.11	0.49
Fusobacterium nucleatum	**0.61**	**0.56**	**0.65**	**0.62**	**0.66**	**0.58**	**0.68**	**0.56**	**0.68**	**0.51**	**0.65**	**0.63**
Porphyromonas gingivalis	**0.68**	0.03	**0.65**	0.24	**0.73**	0.13	**0.71**	0.22	**0.70**	0.13	**0.67**	0.16
Prevotella intermedia	**0.79**	0.13	**0.72**	0.40	**0.80**	0.23	**0.73**	0.32	**0.72**	0.38	**0.68**	0.41
Prevotella nigrescens	**0.63**	0.10	**0.57**	0.23	**0.58**	0.13	**0.56**	0.12	**0.55**	0.28	**0.65**	0.40
Parvimonas micra	**0.52**	0.42	**0.60**	0.44	**0.60**	0.34	**0.56**	**0.56**	**0.61**	0.42	**0.58**	0.48
Streptococcus intermedius	0.21	**0.64**	0.34	**0.79**	0.39	**0.53**	0.42	**0.63**	0.39	**0.71**	0.46	**0.63**
Streptococcus mutans	−0.01	0.35	0.18	0.41	0.19	0.45	0.17	0.44	0.20	0.25	0.07	0.02
Selenomonas noxia	0.45	**0.57**	0.39	**0.67**	0.46	**0.56**	0.44	**0.53**	0.43	**0.50**	0.49	**0.58**
Streptococcus oralis	0.11	**0.73**	0.24	**0.83**	0.28	**0.73**	0.30	**0.79**	0.19	**0.82**	0.31	**0.69**
Streptococcus sanguinis	0.02	**0.74**	0.14	**0.85**	0.20	**0.74**	0.17	**0.79**	0.08	**0.78**	0.22	**0.68**
Treponema denticola	**0.58**	0.13	**0.57**	0.20	**0.60**	0.12	**0.63**	0.11	**0.66**	0.03	**0.58**	0.10
Veillonella parvula	−0.13	**0.72**	0.33	**0.81**	0.03	**0.78**	0.10	**0.80**	0.34	**0.75**	0.08	**0.72**
Cumulative percentage variance	46.3	86.0	40.1	90.7	49.0	87.1	44.5	87.2	44.6	86.9	45.8	87.1

Factor analysis was restricted to provide two factors.
The factors presented are orthogonal (Varimax rotation).
Factor loadings ≥ 0.50 are given in bold.

young and periodontally untreated subjects. The number of bacterial species studied here was somewhat smaller than the typical 27–40 species considered in studies among adults (39, 47, 53, 54). However, the ecological analyses performed by Socransky *et al.* (39), which led to the description of the microbial complexes, were based on no more than 32 species of bacteria because eight species occurred too infrequently to justify their inclusion in the analyses. Given our expectation of a less complex subgingival microbiota in this very young population, we suggest that the 18 species selected for the present study cover the key bacteria previously investigated (39, 47, 53, 54), just as they outnumber the quantity of species hitherto investigated among adolescents (14, 15, 17, 22, 23).

We are indeed aware that the current periodontal microbiological paradigm suggests that further studies should be based on open-ended molecular techniques, allowing for the identification of a much broader spectrum of species (55–57). While it might be ideal if all the estimated 500–600 species currently thought to be constituents of the subgingival microbiota (58) could be included in ecological analyses, it should also be acknowledged that relatively few may be disease-related. As an example, Paster *et al.* (58) suggested that only 29 species and phylotypes of the total of 347 identified were 'often-identified disease-associated species and phylotypes [… that] are clearly candidates for further study'. Moreover, one should also bear in mind that interpretable and statistically valid analyses of data on a broad range of species require inclusion of a considerably larger number of subjects than is commonplace.

A variety of methods have been used to detect bacteria (52), ranging from culture techniques (2) that necessarily select for less fastidious species, to molecular techniques, such as the use of amplified 16S ribosomal RNA (57), that typically result in considerably higher prevalence estimates (59, 60). The results of the present study among Chilean adolescents confirmed the observations from previous studies (47, 48, 52) that the investigated putative periodontal pathogens occur ubiquitously in a number of diverse populations. Moreover, our results are in agreement with previous studies reporting that *P. gingivalis* (17, 18, 22, 38), *T. forsythia* (17, 18, 38, 61), *P. intermedia* (18, 22), *C. rectus* (17, 22), *T. denticola* (17, 22), and *F. nucleatum* (22) are more common among adolescents and young adults presenting with periodontitis than among healthy subjects. Our findings are also compatible with findings among adults that *P. gingivalis* (47, 48, 53, 54, 62), *T. forsythia* (47, 48, 53, 54, 62), *T. denticola* (47, 48, 54, 62), *P. nigrescens* (47, 48, 53), *C. rectus* (48), and *P. intermedia* (48) are associated with periodontitis.

Although the detection methods used and the specific subset of species investigated may affect the relative contribution of each species to the total bacterial count of the investigated species, our findings regarding the more dominant bacteria among cases corroborate previous findings among young adults and adults with periodontitis in that *P. gingivalis* (18, 50, 53, 54, 63), *T. forsythia* (18, 53, 54, 63), *P. nigrescens* (50, 64), and *P. intermedia* (50, 64) are important species of the

microbiota investigated. Similarly, the finding that *A. oris* was a dominant species in the whole study population, and particularly preponderant among the non-cases, corroborate the results reported previously for adults (50, 53, 54, 64) [for review see (52)].

Particularly interesting in the context of an adolescent population are our findings of no association between case status and *A. actinomycetemcomitans* in this Chilean adolescent population, which corroborate previous findings reported for US American adolescents (22), and Chinese (20) and Japanese (17) adolescents and young adults. Haubek *et al.* (23) found that the highly leucotoxic strain of *A. actinomycetemcomitans* [the JP2 clone (65)] was significantly associated with early-onset periodontitis among Moroccan adolescents, whereas this was not the case for *A. actinomycetemcomitans* without the 530-bp deletion (23). Our results thereby corroborate the observation that the mere presence of *A. actinomycetemcomitans* may not differentiate cases of periodontitis from non-cases in adolescent populations where the JP2 clone of *A. actinomycetemcomitans* has not been identified (24, 66). A significant association between the presence of *A. actinomycetemcomitans* and aggressive periodontitis among adolescents and young adults was reported in a study from Brazil (18), but because the JP2 clone has been identified among Brazilians (66, 67) it is possible that the positive association could be attributed to the presence of this clone. Moreover, the Brazilian study included somewhat older periodontitis cases recruited among periodontal referral patients (18).

One may speculate whether participation rates of 59% and 30% for cases and non-cases, respectively, provide study groups that are representative of the eligible underlying study populations and whether this differential participation may have been a source of selection bias in the study. However, the comparability of study groups depends on the influence of the determinants of differential participation on the parameters investigated. While we have identified putative determinants of differential participation in this study (43), we have no reason to suspect that these determinants, which mainly reflect social parameters, would differentially affect the distribution and composition of the subgingival microbiota in cases and non-cases.

Taken together, our findings demonstrate that species considered to belong to the 'red' (*P. gingivalis* and *T. forsythia*) and 'orange' (*P. intermedia*, *P. nigrescens*, *P. micra*, and *F. nucleatum*) complexes (52) are significantly associated with periodontitis among adolescents in this Chilean population and these species load highly positively on the same factor. This confirms previous findings on studies among adults that 'members of the red complex are rarely found in the absence of members of the orange complex' (39). Moreover, a factor describing the early colonizers was also identified and, taken together, these two factors explained 86–91% of the common variance among the 18 microbial species investigated.

clinical settings for this case–control study. The authors are also grateful to Associate Professor Morten Frydenberg for inspiring discussions on the analytical aspects of the study and for his critical comments to a previous version of the manuscript. The technical expertise provided by Mrs Susanne Blomqvist and Mrs. Lisbeth Bengtsson is greatly appreciated. Colgate-Palmolive, Chile, is kindly thanked for the donation of toothbrushes to be given to the participating students. The study was partially supported by a grant from the Danish Medical Research Council.

Conflicts of interest – The authors report no conflicts of interest related to this study.

References

1. WILSON ME, ZAMBON JJ, SUZUKI JB, GENCO RJ. Generalized juvenile periodontitis, defective neutrophil chemotaxis and *Bacteroides gingivalis* in a 13-year-old female. A case report. *J Periodontol* 1985; **56**: 457–463.
2. VAN STEENBERGEN TJM, VAN DER VELDEN U, ABBAS F, DE GRAAFF J. Microbiological and clinical monitoring of non-localized juvenile periodontitis in young adults: a report of 11 cases. *J Periodontol* 1993; **64**: 40–47.
3. LOPEZ NJ, MELLADO JC, GIGLIO MS, LEIGHTON GX. Occurrence of certain bacterial species and morphotypes in juvenile periodontitis in Chile. *J Periodontol* 1995; **66**: 559–567.
4. NONNENMACHER C, MUTTERS R, DE JACOBY LF. Microbiological characteristics of subgingival microbiota in adult periodontitis, localized juvenile periodontitis and rapidly progressive periodontitis subjects. *Clin Microbiol Infect* 2001; **7**: 213–217.
5. MANDELL RL, EBERSOLE JL, SOCRANSKY SS. Clinical immunologic and microbiologic features of active disease sites in juvenile periodontitis. *J Clin Periodontol* 1987; **14**: 534–540.
6. SLOTS J. The predominant cultivable organisms in juvenile periodontitis. *Scand J Dent Res* 1976; **84**: 1–10.
7. MULLALLY BH, DACE B, SHELBURNE CE, WOLFF LF, COULTER WA. Prevalence of periodontal pathogens in localized and generalized forms of early-onset periodontitis. *J Periodont Res* 2000; **35**: 232–241.
8. VANDESTEEN GE, WILLIAMS BL, EBERSOLE JL, ALTMAN LC, PAGE RC. Clinical, microbiological and immunological studies of a family with a high prevalence of early-onset periodontitis. *J Periodontol* 1984; **55**: 159–169.
9. LOPEZ NJ, MELLADO JC, LEIGHTON GX. Occurrence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* in juvenile periodontitis. *J Clin Periodontol* 1996; **23**: 101–105.
10. LOPEZ NJ, GIGLIO MS, MELLADO JC, LEIGHTON GX. Microbiological study of juvenile periodontitis in Chile. *Clin Infect Dis* 1995; **20**: s308–s310.
11. ASIKAINEN S, JOUSIMIES-SOMER H, KANERVO A, SAXÉN L. *Actinobacillus actinomycetemcomitans* and clinical periodontal status in Finnish juvenile periodontitis patients. *J Periodontol* 1986; **57**: 91–93.
12. KAMMA JJ, NAKOU M, PERSSON RG. Association of early onset periodontitis microbiota with aspartate aminotrasferase activity in gingival crevicular fluid. *J Clin Periodontol* 2001; **28**: 1096–1105.
13. DELCOURT-DEBRUYNE EMC, BOUTIGNY HRA, HILDEBRAND HF. Features of severe periodontal disease in a teenager with Chédiak-Higashi syndrome. *J Periodontol* 2000; **71**: 816–824.
14. KAMMA JJ, NAKOU M, GMÜR R, BAEHNI PC. Microbiological profile of early onset/aggressive periodontitis patients. *Oral Microbiol Immunol* 2004; **19**: 314–321.
15. CORTELLI JR, CORTELLI SC, JORDAN S, HARASZTHY VI, ZAMBON JJ. Prevalence of periodontal pathogens in Brazilians with aggressive or chronic periodontitis. *J Clin Periodontol* 2005; **32**: 860–866.
16. KAMMA J, NAKOU M. Subgingival microflora in smokers with early onset periodontitis. *Anaerobe* 1997; **3**: 153–157.
17. TAKEUCHI Y, UMEDA M, ISHIZUKA M, HUANG Y, ISHIKAWA I. Prevalence of periodontopathic bacteria in aggressive periodontitis patients in a Japanese population. *J Periodontol* 2003; **74**: 1460–1469.
18. FAVERI M, FIGUEIREDO LC, DUARTE PM, MESTNIK MJ, MAYER MPA, FERES M. Microbiological profile of untreated subjects with localized aggressive periodontitis. *J Clin Periodontol* 2009; **36**: 739–749.
19. SLOTS J, REYNOLDS HS, GENCO RJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. *Infect Immun* 1980; **29**: 1013–1020.
20. HAN NM, XIAO XR, ZHANG LS, RI XQ, ZHANG JZ, TONG YH, TANJG MR, XIAO ZR. Bacteriological study of juvenile periodontitis in China. *J Periodont Res* 1991; **26**: 409–414.
21. ASIKAINEN S, JOUSIMIES-SOMER H, KANERVO A, SUMMANEN P. Certain bacterial species and morphotypes in localized juvenile periodontitis and in matched controls. *J Periodontol* 1987; **58**: 224–230.
22. ALBANDAR JM, BROWN LJ, LÖE H. Putative periodontal pathogens in subgingival plaque of young adults with and without early-onset periodontitis. *J Periodontol* 1997; **68**: 973–981.
23. HAUBEK D, ENNIBI O-K, POULSEN K, POULSEN S, BENZARTI N, KILIAN M. Early-onset periodontitis in Morocco is associated with the highly leucotoxic clone of *Actinobacillus actinomycetemcomitans*. *J Dent Res* 2001; **80**: 1580–1583.
24. RYLEV M, KILIAN M. Prevalence and distribution of principal peirodontal pathogens worldwide. *J Clin Periodontol* 2008; **35**(Suppl 8): 346–361.
25. XIMENEZ-FYVIE LA, HAFFAJEE AD, SOCRANSKY SS. Comparison of the microbiota of supra-and subgingival plaque in health and periodontitis. *J Clin Periodontol* 2000; **27**: 648–657.
26. TANNER ACR, MILGROM PM, KENT RJR, MOKEEM SA, PAGE RC, RIEDY CA, WEINSTEIN P, BRUSS J. The microbiota of young children from tooth and tongue samples. *J Dent Res* 2002; **81**: 53–57.
27. PAPAIOANOU W, GIZANI S, HAFFAJEE AD, QUIRYNEN M, MAMAI-HOMATA E, PAPAGIANNOULIS L. The microbiota on different oral surfaces in healthy children. *Oral Microbiol Immunol* 2009; **24**: 183–189.
28. ALALUUSUA S, ASIKAINEN S. Detection and distribution of *Actinobacillus actinomycetemcomitans* in the primary dentition. *J Periodontol* 1988; **59**: 504–507.
29. LAMELL CW, GRIFFEN AL, MCCLELLAN DL, LEYS EJ. Acquisition and colonization stability of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in children. *J Clin Microbiol* 2000; **38**: 1196–1199.
30. OOSHIMA T, NISHIYAMA M, HOU B, TAMURA K, AMANO A, KUSUMOTO A, KIMURA S. Occurrence of periodontal bacteria in healthy children: a 2-year longitudinal study. *Community Dent Oral Epidemiol* 2003; **31**: 417–425.
31. KÖNÖNEN E, ASIKAINEN S, SAARELA M, KARJALAINEN J, JOUSIMIES-SOMER H. The oral gram-negative anaerobic microflora in young children: longitudinal changes from edentulous to dentate mouth. *Oral Microbiol Immunol* 1994; **9**: 136–141.
32. SUDA R, KURIHARA C, KURIHARA M, SATO T, LAI C-H, HASEGAWA H. Determination of eight selected periodontal pathogens in the subgingival plaque of maxillary first molars in Japanese school children aged 8–11 years. *J Periodont Res* 2003; **38**: 28–35.
33. OKADA M, HAYASHI F, NAGASAKA N. PCR detection of 5 putative periodontal pathogens in dental plaque samples from children 2 to 12 years of age. *J Clin Periodontol* 2001; **28**: 576–582.
34. YANG EY, TANNER ACR, MILGROM P, MOKEEM SA, RIEDY CA, SPADAFORA AT, PAGE RC, BRUSS JON. Periodontal pathogen detection in gingiva/tooth and tongue flora samples from 18- to 48- month- old children and periodontal status of their mothers. *Oral Microbiol Immunol* 2002; **17**: 55–59.
35. UMEDA M, MIWA Z, TAKEUCHI Y, ISHIZUKA M, HUANG Y, NOGUCHI K, TANAKA M, TAKAGI Y, ISHIKAWA I. The

distribution of periodontopathic bacteria among Japanese children and their parents. *J Periodont Res* 2004; **39**: 398–404.

36. TANNER ACR, MILGROM PM, KENT RJR, MOKEEM SA, PAGE RC, LIAO SIA, RIEDY CA, BRUSS J. Similarity of the oral microbiota of pre-school children with that of their caregivers in a population-based study. *Oral Microbiol Immunol* 2002; **18**: 379–387.

37. KIMURA S, OOSHIMA T, TAKIGUCHI M, SASAKI Y, AMANO A, MORISAKI I, HAMADA S. Periodontopathic bacterial infection in childhood. *J Periodontol* 2002; **73**: 20–26.

38. TANNER ACR, PASTER BJ, LU SC, KANASI E, KENT R, VAN DYKE TE, SONIS ST. Subgingival and tongue microbiota during early periodontitis. *J Dent Res* 2006; **85**: 318–323.

39. SOCRANSKY SS, HAFFAJEE AD, CUGINI MA, SMITH C. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998; **25**: 134–144.

40. MOORE WEC, HOLDEMAN LV, CATO EP, SMIBERT RM, BURMEISTER JA, PALCANIS KG, RANNEY RR. Comparative bacteriology of juvenile periodontitis. *Infect Immun* 1985; **48**: 507–519.

41. MOMBELLI A, CASAGNI F, MADIANOS PN. Can presence or absence of periodontal pathogens distinguish between subjects with chronic and aggressive periodontitis? A systematic review. *J Clin Periodontol* 2002; **29**(Suppl 3): 10–21.

42. LOPEZ R, FERNÁNDEZ O, JARA G, BAELUM V. Epidemiology of clinical attachment loss in adolescents. *J Periodontol* 2001; **72**: 1666–1674.

43. LOPEZ R, FRYDENBERG M, BAELUM V. Non-participation and adjustment for bias in case–control studies of periodontitis. *Eur J Oral Sci* 2008; **116**: 405–411.

44. LOPEZ R, FRYDENBERG M, BAELUM V. Clinical features of early periodontitis. *J Periodontol* 2009; **80**: 749–758.

45. LOPEZ R, RETAMALES C, CONTRERAS C, MONTES JL, MARIN A, VÆTH M, BAELUM V. Reliability of clinical attachment level recordings: effects on prevalence, extent, and severity estimates. *J Periodontol* 2003; **74**: 512–520.

46. LOPEZ R, FERNÁNDEZ O, BAELUM V. Social gradients in periodontal disease among adolescents. *Community Dent Oral Epidemiol* 2006; **34**: 184–196.

47. PAPAPANOU PN, TEANPAISAN R, OBIECHINA NS, PITHPORNCHAIYAKUL W, PONGPAISAL S, PISUITHANAKAN S, BAELUM V, FEJERSKOV O, DAHLÉN G. Periodontal microbiota and clinical periodontal status in a rural sample in southern Thailand. *Eur J Oral Sci* 2002; **110**: 345–352.

48. PAPAPANOU PN, BAELUM V, LUAN WM, MADIANOS PN, CHEN X, FEJERSKOV O, DAHLÉN G. Subgingival microbiota in adult Chinese: prevalence and relation to periodontal disease progression. *J Periodontol* 1997; **68**: 651–666.

49. SOCRANSKY SS, SMITH C, MARTIN L, PASTER FE, DEWHIRST FE, LEVIN AE. "Checkerboard" DNA–DNA hybridization. *BioTechniques* 1994; **17**: 788–792.

50. SOCRANSKY SS, HAFFAJEE AD, SMITH C, MARTIN L, HAFFAJEE JA, UZEL NG, GOODSON JM. Use of checkerboard DNA–DNA hybridization to study complex microbial ecosystems. *Oral Microbiol Immunol* 2004; **19**: 352–362.

51. STATACORP. StataCorp. Statistical Software: Release 11.0. 2009.

52. SOCRANSKY SS, HAFFAJEE AD. Periodontal microbial ecology. *Periodontol 2000* 2005; **38**: 135–187.

53. XIMENEZ-FYVIE LA, ALMAGUER-FLORES A, JACOBO-SOTO V, LARA-CORDOBA M, MORENO-BORJAS J-Y, ALCANTARA-MARURI E. Subgingival microbiota of periodontally untreated Mexican subjects with generalized aggressive periodontitis. *J Clin Periodontol* 2006; **33**: 869–877.

54. XIMENEZ-FYVIE LA, ALMANGUER-FLORES A, JACOBO-SOTO V, LARA-CORDOVA M, SANCHEZ-VARGAS LO, ALCANTARA-MARURI E. Description of the subgingival microbiota of periodontally untreated Mexican subjects: chronic periodontitis and periodontal health. *J Periodontol* 2006; **77**: 460–471.

55. KEIJSER BJ, ZAURA E, HUSE SM, VAN DER VOSSEN D, SCHUREN FH, MONTIJN RC, TEN CATE JM, CRIELAARD W. Pyrosequencing analysis of the oral microflora of healthy adults. *J Dent Res* 2008; **87**: 1016–1020.

56. DEWHIRST FE, CHEN T, IZARD J, PASTER BJ, TANNER AC, YU WH, LAKSHMANAN A, WADE WG. The human oral microbiome. *J Bacteriol* 2010; **192**: 5002–5017.

57. CLARRIDGE JE III. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev* 2004; **17**: 840–862.

58. PASTER BJ, BOCHES SK, GALVIN JL, ERICSON RE, LAU CN, LEVANOS VA, SAHASRABUDHE A, DEWHIRST FE. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001; **183**: 3770–3783.

59. FAVERI M, MAYER MP, FERES M, DE FIGUEIREDO LC, DEWHIRST FE, PASTER BJ. Microbiological diversity of generalized aggressive periodontitis by 16S rRNA clonal analysis. *Oral Microbiol Immunol* 2008; **23**: 112–118.

60. ASIKAINEN S, DOGAN B, TURGUT Z, PASTER BJ, BODUR A, OSCARSSON J. Specified species in gingival crevicular fluid predict bacterial diversity. *PLoS ONE* 2010; **5**: e13589.

61. HAMLET S, ELLWOOD R, CULLINAN M, WORTHINGTON H, PALMER J, BIRD P, NARAYANAN D, DAVIES R, SEYMOUR G. Persistent colonization with *Tannerella forsythensis* and loss of attachment in adolescents. *J Dent Res* 2004; **83**: 232–235.

62. WARA-ASWAPATI N, PITIPHAT W, CHANCHAIMONGKON L, TAWEECHAISUPAPONG S, BOCH JA, ISHIKAWA I. Red bacterial complex is associated with the severity of chronic periodontitis in a Thai population. *Oral Dis* 2009; **15**: 354–359.

63. LOPEZ NJ, SOCRANSKY SS, DA SILVA I, JAPLIT MR, HAFFAJEE AD. Subgingival microbiota of Chilean patients with chronic periodontitis. *J Periodontol* 2004; **75**: 717–725.

64. XIMENEZ-FYVIE LA, HAFFAJEE AD, SOCRANSKY SS. Microbial composition of supra- and subgingival plaque in subjects with periodontitis. *J Clin Periodontol* 2000; **27**: 722–732.

65. BROGAN JM, LALLY ET, POULSEN K, KILIAN M, DEMUTH DR. Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. *Infect Immun* 1994; **62**: 501–508.

66. HAUBEK D. Racial tropism of a highly toxic clone of *Actinobacillus actinomycetemcomitans* associated with Juvenile Periodontitis. *J Clin Microbiol* 1997; **35**: 3037–3042.

67. CORTELLI SC, COSTA FO, KAWAI T, AQUINO DR, FRANCO GC, OHARA K, ROMAN-TORRES CV, CORTELLI JR. Diminished treatment response of periodontally diseased patients infected with the JP2 clone of *Aggregatibacter (Actinobacillus) actinomycetemcomitans*. *J Clin Microbiol* 2009; **47**: 2018–2025.