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Microbiological profile of untreated subjects with localized aggressive periodontitis

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Abstract

Aim: The microbial profile of localized aggressive periodontitis (LAgP) has not yet been determined. Therefore, the aim of this study was to evaluate the subgingival microbial composition of LAgP.

Material and Methods: One hundred and twenty subjects with LAgP ($n = 15$), generalized aggressive periodontitis (GAgP, $n = 25$), chronic periodontitis (ChP, $n = 30$) or periodontal health (PH, $n = 50$) underwent clinical and microbiological assessment. Nine subgingival plaque samples were collected from each subject and analysed for their content of 38 bacterial species using checkerboard DNA–DNA hybridization.

Results: Red complex and some orange complex species are the most numerous and prevalent periodontal pathogens in LAgP. The proportions of *Aggregatibacter actinomycetemcomitans* were elevated in shallow and intermediate pockets of LAgP subjects in comparison with those with GAgP or ChP, but not in deep sites. This species also showed a negative correlation with age and with the proportions of red complex pathogens. The host-compatible *Actinomyces* species were reduced in LAgP.

Conclusion: *A. actinomycetemcomitans* seems to be associated with the onset of LAgP, and *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Campylobacter gracilis*, *Eubacterium nodatum* and *Prevotella intermedia* play an important role in disease progression. Successful treatment of LAgP would involve a reduction in these pathogens and an increase in the *Actinomyces* species.

Key words: DNA probe; localized aggressive periodontitis; microbiology; periodontal disease; subgingival microbiota

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The accurate microbial diagnosis of an infectious disease is a critical step in order to establish effective therapeutic strategies. In the case of periodontal infections, this is not a simple task, because disease onset and progression is not related merely to the presence of a specific microorganism, but most importantly, to the imbalance between levels and proportions of periodontal pathogens and beneficial species in different sites of the mouth (Socransky & Haffajee 2005). The development of new molecular techniques that allow a large number of microorganisms and samples to be identified and quantified was very important in establishing the microbial profiles associated with periodontal health (PH) or disease (Socransky et al. 1994, Paster et al. 2001). Results from investigations using these diagnostic methods have greatly improved our knowledge of the microbiology of certain periodontal conditions, such as chronic periodontitis (ChP) (Socransky et al. 1998, Colombo et al. 2002, Haffajee et al. 2004, López et al. 2004, Ximenez-Fyvie et al. 2006, Fritschi et al. 2008), and were crucial for defining microbial endpoints for therapy. Nonetheless, at present, little information is available on the microbial profile of aggressive periodontitis (AgP). Few studies to date have comprehensively evaluated the microbiota

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.

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associated with generalized aggressive periodontitis (GAgP) (Kamma et al. 2004, Ximenez-Fyvie et al. 2006, Botero et al. 2007, Fritschi et al. 2008, Faveri et al. 2008).

The mean microbial profile of localized aggressive periodontitis (LAgP) is less well known. For a considerable period of time, this condition was thought to be microbiologically distinct. Studies of the predominant cultivable microorganisms in LAgP showed that the microbial composition of the healthy sites differed significantly from that of diseased sites, which were heavily colonized by a Gram-negative capnophilic rod, previously classified as *Actinobacillus actinomycetemcomitans* (currently *Aggregatibacter actinomycetemcomitans*) (Newman et al. 1976, Slots 1976, Newman & Socransky 1977). Subsequently, various investigations conducted in the 1980s and 1990s primarily focused on the detection of *A. actinomycetemcomitans* and showed its close association with the aetiology of LAgP (for a review, see Loesche & Grossman 2001). This bacterial species was found in higher proportions and prevalence in the subgingival microbiota of subjects with LAgP compared with those with ChP or PH (Slots et al. 1980, Mandell & Socransky 1981, Zambon et al. 1983, Bueno et al. 1998). Recent studies using polymerase chain reaction (PCR) have confirmed these findings (Haraszthy et al. 2000, Mullally et al. 2000, Haubek et al. 2002, 2008, Yang et al. 2004, Fine et al. 2007). Conversely, other authors were unable to detect higher prevalence or proportions of *A. actinomycetemcomitans* in LAgP in comparison with ChP subjects (Moore et al. 1985, Han et al. 1991, López et al. 1996, Doğan et al. 2003, Takeuchi et al. 2003, Gajardo et al. 2005).

The role of other bacterial species such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella* species and *Campylobacter rectus* in the aetiology of LAgP has also been suggested. However, most of these studies appraised only a few samples and/or bacterial species by PCR (Mullally et al. 2000, Lee et al. 2003, Takeuchi et al. 2003, Gajardo et al. 2005, Thiha et al. 2007), culture techniques (Moore et al. 1985, Han et al. 1991, López et al. 1995, Nonnenmacher et al. 2001) or DNA probes (López et al. 1996, Lee et al. 2003). Because there are few data analysing a comprehensive range of species in LAgP, and this knowledge would help to elaborate more specific treatments for this infection, the purpose of the present study was to describe the mean microbial profile of the subgingival microbiota of subjects with LAgP and to compare that directly with individuals who had GAgP, ChP and PH. The hypothesis tested was that species other than *A. actinomycetemcomitans* may play a role in the aetiology of LAgP.

Material and Methods

Subject population

One hundred and twenty systemically healthy subjects were selected from the population referred to the periodontal clinic of Guarulhos University (Guarulhos, SP, Brazil). Their medical and dental histories were obtained and a full-mouth periodontal examination was performed. Based on these data, the periodontal diagnosis was made, and subjects who fulfilled the inclusion/exclusion criteria were invited to participate in the study. The study protocol was explained to each subject, and a signed informed consent was obtained. This study protocol was approved previously by Guarulhos University's Ethics Committee in Clinical Research.

Clinical examination

Visible plaque (0/1), gingival bleeding (0/1), bleeding on probing (BOP, 0/1), suppuration (0/1), probing depth (PD, mm) and clinical attachment level (CAL, mm) were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) in all teeth excluding third molars at the baseline visit. PD and CAL measurements were recorded to the nearest millimetre using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA).

Table 1. Demographic characteristics and mean (± SD) full-mouth clinical parameters for the five clinical groups

	Clinical groups				
Variable	Localized aggressive periodontitis (LAgP) ($n = 15$)	Generalized aggressive periodontitis (GAgP) ($n = 25$)	Chronic periodontitis (ChP) ($n = 30$)	Healthy (PH) ($n = 30$)	Healthy young (PHy) ($n = 20$)
Gender (M/F)^{NS}	6:9	10:15	8:22	6:24	10:10
Age (years)*	15.2 ± 2.8^{c}	25.2 ± 3.2^{b}	42.0 ± 6.2^{a}	26.1 ± 8.5^{b}	17.1 ± 1.9^{c}
Range	12–20	20–29	30–49	18–40	13–19
Probing depth (mm)*	2.9 ± 0.3^{b}	4.8 ± 0.8^{a}	3.8 ± 0.7^{a}	2.2 ± 0.2^{c}	2.2 ± 0.3^{c}
Attachment level (mm)*	3.0 ± 0.4^{b}	4.7 ± 1.2^{a}	4.3 ± 1.0^{a}	2.2 ± 0.2^{c}	2.0 ± 0.2^{c}
Percentage of sites with					
Plaque accumulation*	41.7 ± 8.1^{b}	49.2 ± 12.9^{b}	84.7 ± 10.7^{a}	43.7 ± 17.8^{b}	38.5 ± 9.9^{b}
Gingival bleeding*	8.5 ± 5.4^{c}	12.1 ± 10.9^{b}	40.7 ± 22.4^{a}	6.5 ± 5.5^{c}	4.7 ± 4.4^{c}
Bleeding on probing *	35.5 ± 4.8^{b}	68.7 ± 15.8^{a}	63.6 ± 20.2^{a}	17.7 ± 6.5^{c}	15.2 ± 5.4^{c}
Suppuration *	1.0 ± 2.5^{b}	4.3 ± 3.5^{b}	3.0 ± 3.7^{b}	0.0^{a}	0.0^{a}
PD ⩽ 3 mm	78.0 ± 5.1^{b}	39.4 ± 21.2^{c}	55.8 ± 20.4^{c}	100^{a}	100^{a}
PD 4–6 mm	20.5 ± 5.4^{b}	40.9 ± 30.4^{a}	36.8 ± 34.4^{a}	–	–
PD ⩾ 7 mm	1.5 ± 0.9^{c}	19.7 ± 15.7^{a}	7.4 ± 4.4^{b}	–	–

*$p < 0.05$; Kruskal–Wallis test.

Different letters in the columns indicate statistically significant differences between groups; Mann–Whitney *U*-test.

NS, not significant, $p > 0.05$; χ^2 test; PD, probing depth; SD, standard deviation.

Investigator calibration

The clinical examination was performed by one trained and calibrated examiner. A total of 10 non-study subjects with ChP were recruited and used for the calibration exercise. The examiner (M. Fa.) measured one quadrant per subject. The choice of quadrant was based on the number of teeth present. For better standardization, quadrant 1 was the first choice, followed by 2, 3 and 4. The quadrant chosen should have at least six teeth. If a quadrant presented $<$six teeth, the next quadrant was chosen. Initially, the examiner measured PD and CAL in a given quadrant and 60 min. later this same protocol was repeated. Therefore, all 10 subjects were probed twice in the same visit by the examiner. Upon completion of all measurements, the intra-examiner variabilities for PD and CAL measurements were assessed. The calibration was performed according to Araujo et al. (2003), and the standard error of measurement (SE) was calculated. Intra-examiner variability was 0.13 mm for PD and 0.29 mm for CAL. The examiner was able to provide reproducible measures under 0.5 mm.

Inclusion criteria

LAgP, GAgP, ChP or PH was diagnosed based on the periodontal classification of the American Academy of Periodontology (Armitage 1999). Subjects had at least 20 teeth and were required to meet the following criteria in order to be included in this study:

LAgP

- $\leqslant$35 years of age;
- Minimum of six permanent incisors and/or first molars with at least one site each with PD and CAL$\geqslant$5 mm;
- No more than two teeth other than first molars and incisors with PD and/or CAL$>$3 mm; and
- Familial aggregation (at least one other member of the family present-

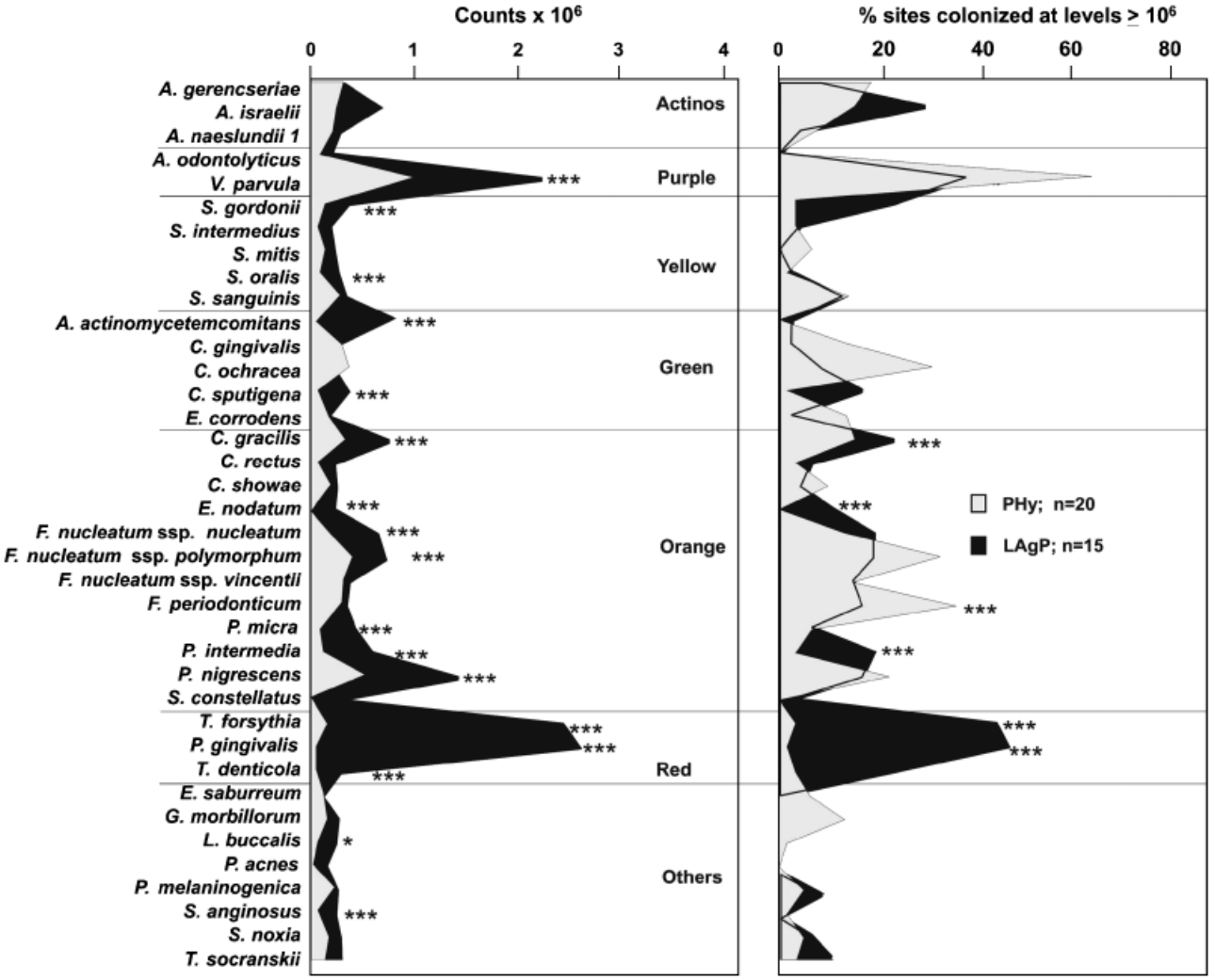

Fig. 1. Mean counts (left panel) and percentage of sites colonized by 38 bacterial species at levels $\geqslant 10^6$ (right panel) in subgingival plaque samples taken from 15 subjects with localized aggressive periodontitis (LAgP) and 20 young subjects with periodontal health (PHy). The species were ordered and grouped according to the microbial complexes described by Socransky et al. (1998). Mean values for each species were averaged within a subject and then across subjects in the two clinical groups. The significance of differences between groups was assessed using the general linear model (GLM); $^{*}p<0.05$ and $^{***}p<0.001$ after adjusting for multiple comparisons (Socransky et al. 1991).

ing or with a history of periodontal disease).

GAgP

- ⩽35 years of age;
- Minimum of six permanent incisors and/or first molars with at least one site each with PD and CAL⩾5 mm;
- Minimum of six teeth other than first molars and incisors with at least one site each with PD and CAL⩾5 mm; and
- Familial aggregation (at least one other member of the family presenting or with a history of periodontal disease).

Generalized chP

- >35 years of age;
- Minimum of six teeth with at least one site each with PD and CAL⩾5 mm; and
- At least 30% of the sites with PD and CAL⩾4 mm and presence of BOP.

PH

- >25 years of age; and
- No sites with PD and CAL measurements >3 mm and <20% of sites exhibiting gingival bleeding and/or bleeding on probing.

Young Subjects with Periodontal Health (PHy)

- <20 years of age; and
- No sites with PD and CAL measurements >3 mm and <20% of sites exhibiting gingival bleeding and/or bleeding on probing.

Exclusion criteria

Exclusion criteria were pregnancy, lactation, smoking, previous subgingival periodontal therapy, any systemic condition that could affect the progression of periodontal disease (e.g. diabetes and immunological disorders), long-term administration of anti-inflammatory medication, need for antibiotic coverage for routine dental treatment and antibiotic therapy in the previous 6 months.

Microbiological examination

Sample collection

Individual subgingival plaque samples were collected from nine non-contiguous interproximal sites per subject. For LAgP, GAgP and ChP groups, three sites at each of the following PD categories were sampled: ⩽3 mm, between 4 and 6 mm, and ⩾7 mm. Sites with PD⩽3 mm were collected from PH and PHy groups. The selected sites were randomized in different quadrants. After the clinical parameters had been recorded, the supragingival plaque was removed and the samples were taken with individual sterile Gracey curettes and immediately placed in separate polypropylene tubes containing 150 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). One hundred microlitres of 0.5 M NaOH was added to each tube and the samples were dispersed using a vortex mixer.

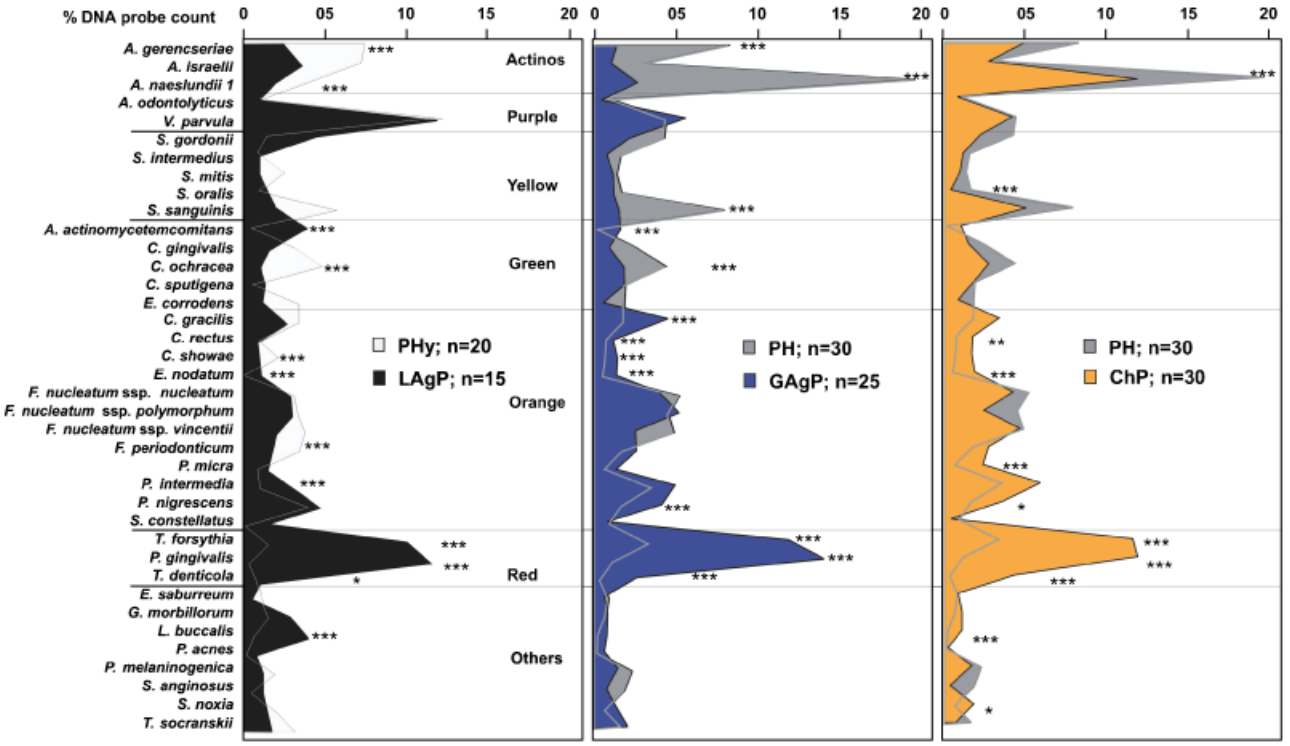

Fig. 2. Mean percentage of the total DNA probe count in subgingival plaque samples taken from 15 subjects with localized aggressive periodontitis (LAgP, left panel), 25 subjects with generalized aggressive periodontitis (GAgP, middle panel) and 30 subjects with chronic periodontitis (ChP, right panel). LAgP was compared with 20 young subjects with periodontal health (PHy), while ChP and GAgP were compared with another group of 30 periodontally healthy subjects (PH). The species were ordered and grouped according to the microbial complexes described by Socransky et al. (1998). The significance of differences between groups was assessed using the general linear model (GLM) adjusted for age between PH and CHP groups; $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$ after adjusting for multiple comparisons (Socransky et al. 1991).

Checkerboard DNA–DNA hybridization

Counts of the 38 bacterial species (Matarazzo et al. 2008) were determined in each sample, using the Checkerboard DNA–DNA hybridization technique (Socransky et al. 1994, 1998). The microbiological analysis was entirely performed at the Laboratory of Microbiology of Guarulhos University. The samples were boiled for 10 min. and neutralized using 0.8 ml of 5 M ammonium acetate. The released DNA was then placed into the extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA), concentrated on a 15×15 cm positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN, USA) and fixed to the membrane by baking it at 120°C for 20 min. The membrane was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labelled whole genomic DNA probes for 38 bacterial species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using the antibody to digoxigenin conjugated with alkaline phosphatase and chemiluminescence detection. The last two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. Signals were converted to absolute counts by comparison with the standards lanes on the membrane. The sensitivity of the assay was adjusted to allow detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe.

Statistical analysis

Clinical examination

The percentage of sites with visible plaque, gingival bleeding, BOP and suppuration, as well as mean PD, CAL and age were computed for each subject and then averaged across subjects in the different groups. The significance of differences among the five groups was determined using the Kruskal–Wallis test. If significance was achieved, the Mann–Whitney *U*-test was used to assess differences between two groups. The χ^2 test was used to compare differences in the frequency of gender.

Microbiological examination

The mean counts ($\times 10^6$ cells) of individual bacterial species were averaged within each subject and then across subjects in the different clinical groups. Similarly, the percentage of the total DNA probe counts was determined initially in each site, then per subject and averaged across subjects in the five groups. Prevalence was computed by determining the percentage of sites per subject colonized by each species at counts $\geqslant 10^6$ cells and then averaging across subjects in the five groups. The significance of differences between two groups or among the five groups was determined using the general linear model (GLM), adjusted for age. Adjustments were also made for multiple comparisons as described by Socransky et al. (1991). The level of significance

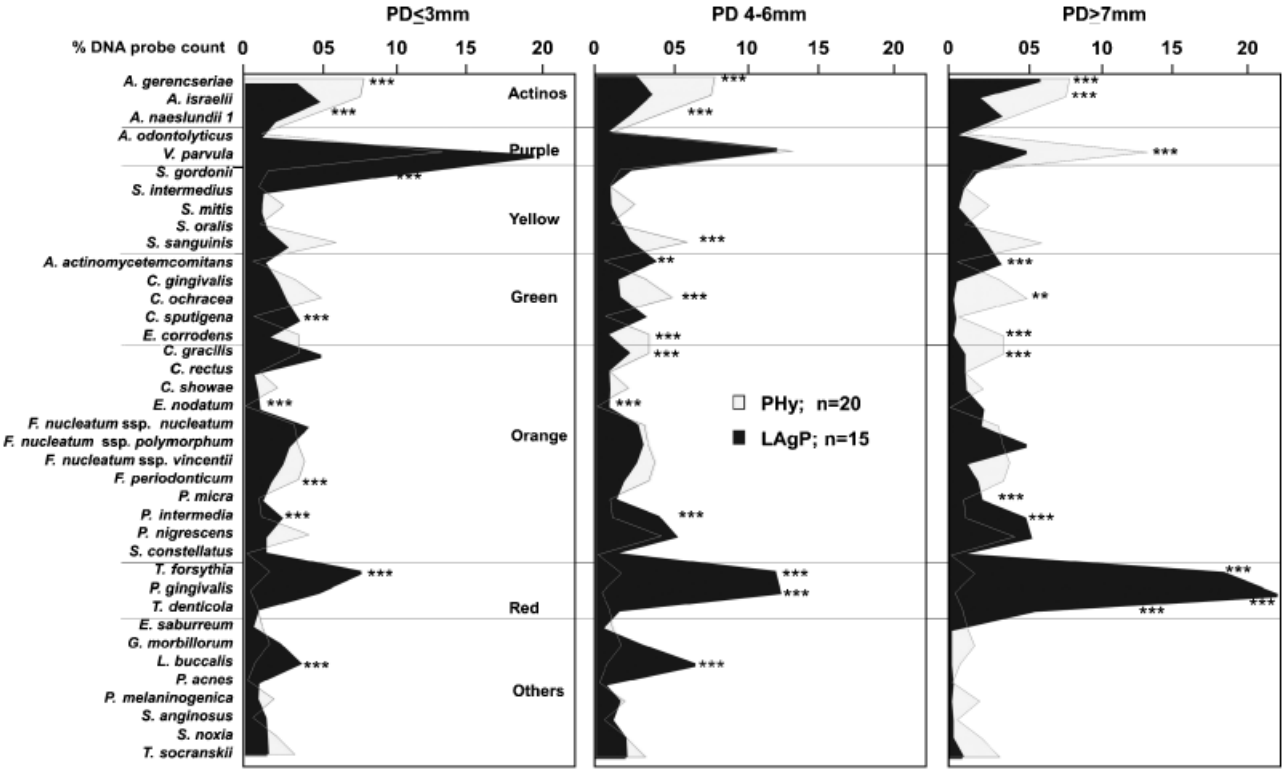

Fig. 3. Mean percentage of the total DNA probe count in subgingival plaque samples taken from 15 subjects with localized aggressive periodontitis (LAgP) in different probing depth (PD) categories ($\leqslant 3$, 4–6, $\geqslant 7$ mm) and from 20 young subjects with periodontal health (PHy). For the LAgP group, the mean values for each species were computed by averaging all samples within the three PD categories in each subject and then averaging across subjects. The species were ordered and grouped according to the microbial complexes described by Socransky et al. (1998). The significance of differences between groups was assessed using the general linear model (GLM) $^{**}p < 0.01$ and $^{***}p < 0.001$ after adjusting for multiple comparisons (Socransky et al. 1991).

was set at 5% for all the analyses and the data were analysed using the software SPSS 13.0. Pearson's rank correlation was used to assess possible associations between the mean proportion of *A. actinomycetemcomitans*, red complex species and age.

Results

Clinical data

The demographic characteristics and clinical parameters of the studied population are presented in Table 1. A total of one hundred and twenty subjects with LAgP ($n = 15$), GAgP ($n = 25$), ChP ($n = 30$), PH ($n = 30$) or PHy ($n = 20$) participated in this investigation.

Differences among groups were observed for all clinical and demographic parameters evaluated, except for gender. The mean age was similar between subjects with LAgP and PHy subjects, as well as between those with GAgP and PH. The mean PD and CAL measurements and the percentage of sites exhibiting gingival bleeding or BOP were significantly lower in the LAgP in comparison with ChP and GAgP groups. LAgP subjects also had fewer sites with PD $\geqslant$ 7 mm and more sites with PD $\leqslant$ 3 mm than the other diseased groups.

Microbiological data

Figure 1 presents the mean counts ($\times 10^6$ cells) and the mean percentage of sites colonized (at levels $\geqslant 10^6$ cells) by the 38 species evaluated in the subgingival plaque samples from the LAgP and PHy groups. The most numerous and prevalent species in LAgP were *T. forsythia* and *P. gingivalis*. In addition, these two pathogens as well as *Campylobacter gracilis*, *Eubacterium nodatum* and *Prevotella intermedia* were present in higher mean counts and prevalence in LAgP in comparison with PHy.

The proportions (mean percentage of the total DNA probe count) of the 38 bacterial species evaluated were compared between the periodontitis and the healthy groups and the data are presented in Fig. 2. The three pathogens of the red complex (*T. forsythia, P. gingivalis* and *T. denticola*), and the putative periodontal pathogen from the orange complex, *E. nodatum*, were elevated in LAgP, GAgP and ChP in comparison with the periodontally healthy subjects. Conversely, the host-compatible *Actinomyces naeslundii* 1 was elevated in the two periodontally healthy groups *A. actinomycetemcomitans* was observed in higher adjusted mean proportions in LAgP and GAgP in comparison with PHy and PH groups, respectively. Overall, most of the species considered to be host-compatible, from the purple, yellow and green complexes (except for *A. actinomycetemcomitans*) as well the *Actinomyces* species, were elevated in the healthy in comparison with periodontally diseased subjects.

Figure 3 presents the proportions of the bacterial species in the different baseline PD categories (shallow: $\leqslant$ 3 mm, intermediate: 4–6 mm and deep: $\geqslant$ 7 mm) in subjects with LAgP, compared with PHy. As one goes from shallow to deep sites, there is an increase in the proportions of the pathogens from the red complex and a decrease in the proportions of the host-compatible species from the purple, yellow and green complexes, as well as *Actinomyces* species. *A. actinomycetemcomitans* was found in higher proportions in LAgP in comparison with PHy at intermediate and deep site categories.

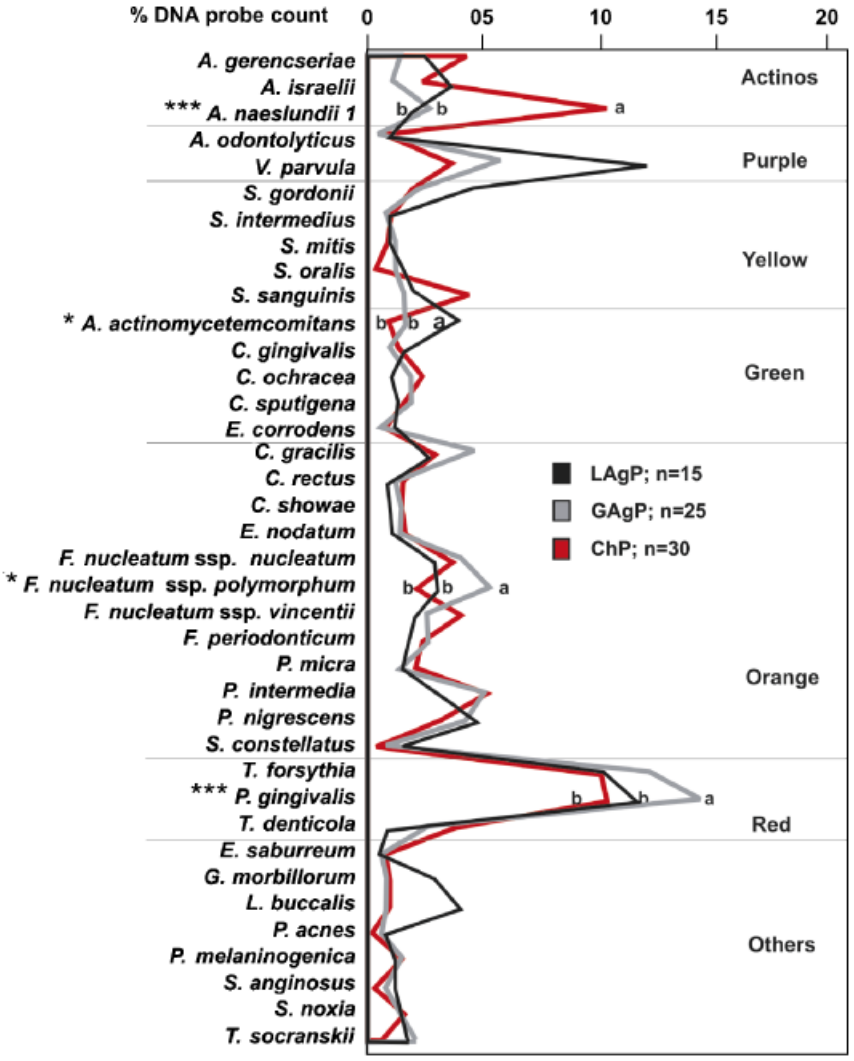

Fig. 4. Mean percentage of the total DNA probe count in subgingival plaque samples taken from 15 subjects with localized aggressive periodontitis (LAgP); 25 subjects with generalized aggressive periodontitis (GAgP); and 30 subjects with chronic periodontitis (ChP). The species were ordered and grouped according to the microbial complexes described by Socransky et al. (1998). The significance of differences among groups was assessed using the general linear model (GLM) adjusted for age. Different small letters indicate statistically significant differences between pairs groups ($p < 0.05$) after adjusting for multiple comparisons (Socransky et al. 1991).

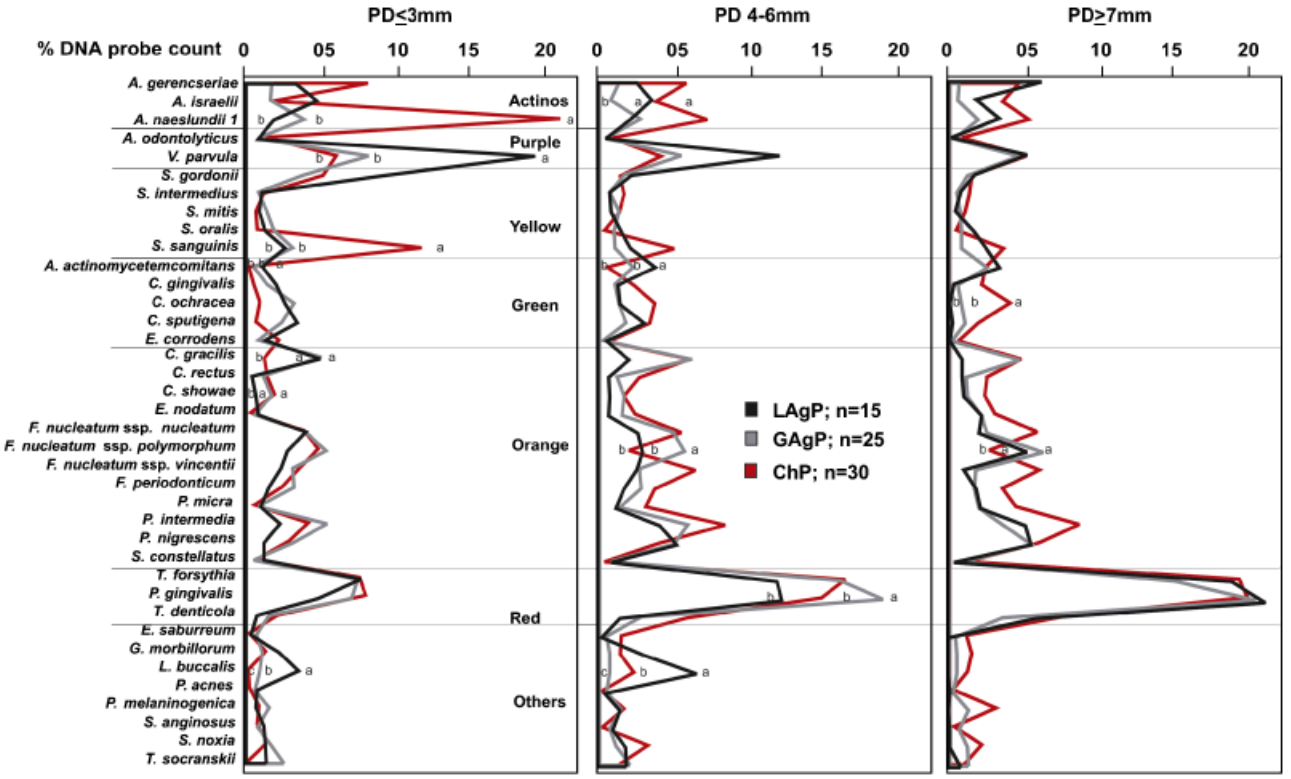

Fig. 5. Mean percentage of the total DNA probe count in subgingival plaque samples taken from 15 subjects with localized aggressive periodontitis (LAgP), 25 subjects with generalized aggressive periodontitis (GAgP) and 30 subjects with chronic periodontitis (ChP) in different probing depths (PD) categories ($\leqslant 3$, 4–6, $\geqslant 7$ mm). The mean values for each species were computed by averaging all samples within the three PD categories in each subject and then averaging across subjects. The species were ordered and grouped according to the microbial complexes described by Socransky et al. (1998). The significance of differences among groups was assessed using the general linear model (GLM) adjusted for age. Different small letters indicate statistically significant differences between pairs of groups ($p < 0.05$) after adjusting for multiple comparisons (Socransky et al. 1991).

The comparison among the individual mean proportions of the species evaluated in the three diseased groups is presented in Fig. 4. Four species differed significantly among groups: *A. actinomycetemcomitans* was elevated in LAgP, *Fusobacterium nucleatum* ssp. *polymorphum* and *P. gingivalis* in GAgP and *A. naeslundii 1* in ChP.

Figure 5 presents the comparisons among the groups for the mean proportions of the bacterial species evaluated in the three PD categories. Interestingly, the greatest differences among the groups were observed at the shallow pockets, followed by the intermediate sites. The subgingival microbial profiles of the deep sites were very similar among the three groups. The proportions of the pathogens of the red complex were similar among the three groups in all PD categories, except for *P. gingivalis*, which was significantly elevated in the intermediate sites of GAgP subjects. *A. actinomycetemcomitans* was in significantly higher proportions in shallow and intermediate sites of LAgP in comparison with GAgP and ChP, but not in the deep sites. The proportions of *C. gracilis* were significantly elevated in the shallow sites of LAgP and GAgP, in comparison with ChP.

The mean proportions of the microbial complexes of the diseased and PHy groups are described in Fig. 6. *A. actinomycetemcomitans* is presented separately (light green) from the other species of the green complex. *A. actinomycetemcomitans* was found in higher proportions in LAgP subjects in comparison with PHy, GAgP and ChP. The red complex pathogens were elevated in LAgP in comparison with PHy. However, the proportions of red complex species did not differ significantly among the three diseased groups. The *Actinomyces* species were significantly reduced in the LAgP group in comparison with PHy and ChP. The putative species from the orange complex were in lower proportions in the LAgP subjects in comparison with the GAgP and ChP groups. The comparison between GAgP or ChP and the PH subjects showed a higher proportion of red and orange complex species in both diseased groups, as well as of *A. actinomycetemcomitans* in GAgP. Conversely, the *Actinomyces* species were in lower proportions in the GAgP subjects.

Pearson' correlations between age, proportions of *A. actinomycetemcomitans* and red complex in subjects with LAgP are presented in Fig. 7. Age was negatively correlated with proportions of *A. actinomycetemcomitans* ($r = -0.74$, $p = 0.001$) and positively correlated with proportions of the red complex ($r = 0.51$, $p = 0.049$). In addition, a negative correlation was observed between proportions of *A. actinomycetemcomitans* and red complex ($r = -0.65$; $p = 0.02$). No significant correlations were observed between *A. actinomycetemcomitans*, red complex and age in subjects with ChP, GAgP, PH and PHy (data not shown).

Discussion

It is well recognized that periodontal destruction is associated with the imbal-

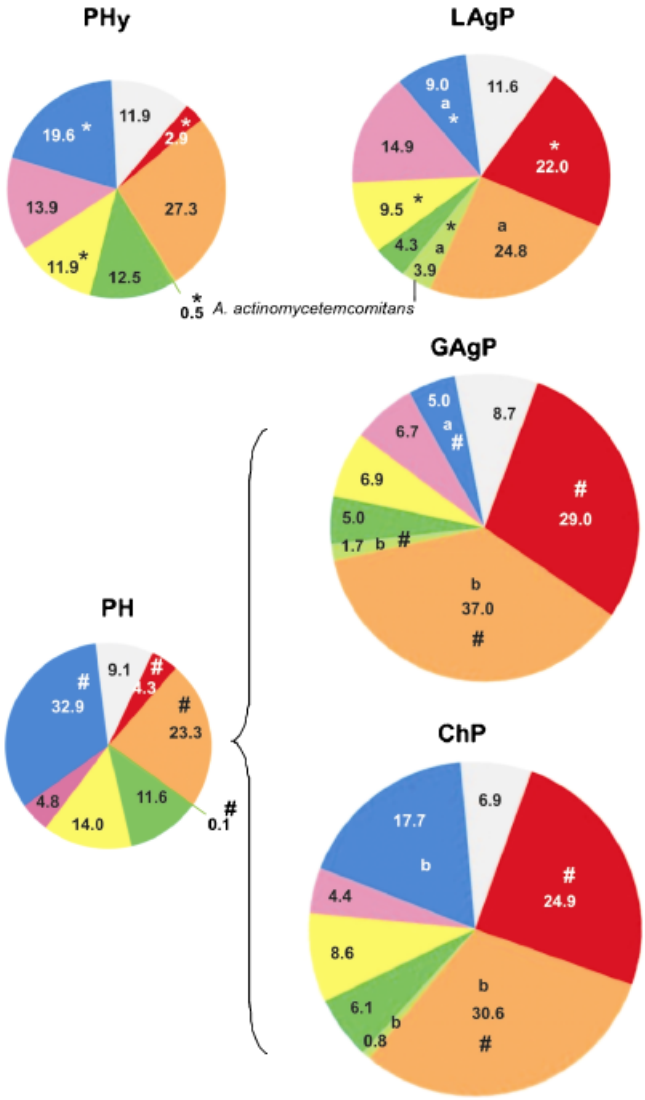

Fig. 6. Pie charts describing the mean proportion of microbial complexes from 20 younger subjects with periodontal health (PHy), 15 subjects with localized aggressive periodontitis (LAgP), 25 subjects with generalized aggressive periodontitis (GAgP), 30 subjects with chronic periodontitis (ChP) and 30 periodontally healthy subjects (PH). Species in the complexes were summed and the proportions that each complex comprised were determined. The areas of the pies were adjusted to reflect the mean total counts in each clinical group. The colours represent the different complexes described by Socransky et al. (1998). The blue colour represents three species of *Actinomyces* (*Actinomyces gerencseriae, Actinomyces israelii* and *Actinomyces naeslundii* 1), and the grey colour represents species that do not fall into any complex (''Others'' group described in Figs 1–5). The significance of differences among groups was determined by the general linear model (GLM) and was represented as follows: $^{*}p<0.05$ between PHy and GAgP groups; $^{\#}p<0.05$ between PH and LAgP or ChP groups; and small letters – $p<0.05$, adjusted for age, among the three diseased groups.

ance between periodontal pathogens and host-compatible species in the oral cavity. Therefore, defining the microbial profile of different periodontal conditions is an important step in delineating more specific treatments. To our knowledge, this is the first report in which the composition of the subgingival microbiota of subjects with LAgP has been extensively evaluated and comprehensively compared with that of GAgP, ChP and periodontally healthy subjects. However, the data of the present study should be considered with caution, because they are derived from a relatively small sample size of 15 subjects in the LAgP group. The levels, prevalence and proportions of 38 bacterial species in 1080 subgingival plaque samples from a total of 120 subjects were analysed.

Subjects with LAgP, GAgP and ChP were selected according to the parameters of PD and CAL, as well as age. The three common features of AgP according to the Consensus Report of the American Academy of Periodontology (Armitage 1999, Lang et al. 1999) are: otherwise clinically healthy subjects, familial aggregation and rapid attachment loss and bone destruction. The first two features were used as inclusion criteria in the present study. However, there are no consistent means of determining the rate of attachment loss while selecting subjects for cross-sectional studies. This situation was well elucidated by Picolos et al. (2005) and Ximenez-Fyvie et al. (2006) in recent reports. Alternatively, as done in the present study, one would include younger subjects with severe disease, and estimate rapid destruction by setting the earliest starting point of disease around puberty. Therefore, although it is understood that AgP may affect individuals at any age; the present study used age-related definitions for LAgP, GAgP and ChP to provide the best possible diagnosis of AgP.

In agreement with previous studies (Zambon et al. 1983, Haraszthy et al. 2000, Haubek et al. 2002, Yang et al. 2004) the data support the notion that *A. actinomycetemcomitans* is an important pathogen in the aetiology of LAgP. This species was more prevalent and present at higher levels and proportions in subjects with LAgP in comparison with periodontally healthy subjects. However, this was not the only known or suspected periodontal pathogen to be elevated in lesions of LAgP. Species from the red and orange complexes, such as *P. gingivalis*, *T. forsythia*, *T. denticola*, *C. gracilis*, *E. nodatum* and *P. intermedia*, were also elevated in these subjects. In fact, *A. actinomycetemcomitans* was present at much lower levels (0.89×10^6 cells) and proportions (3.9% of the species evaluated) in subjects with LAgP than some of these other pathogens, especially *P. gingivalis* (2.8×10^6 cells; 11.5% of the species evaluated) and *T. forsythia* (2.5×10^6 cells; 10% of the species evaluated). The low proportions of A. *actinomycetemcomitans* in subjects with LAgP have been described previously (Moore et al., 1985, Nonnenmacher et al. 2001, Doğan et al. 2003, Gajardo et al. 2005). Nonetheless, it has been suggested that the threshold number of *A. actinomycetemcomitans*

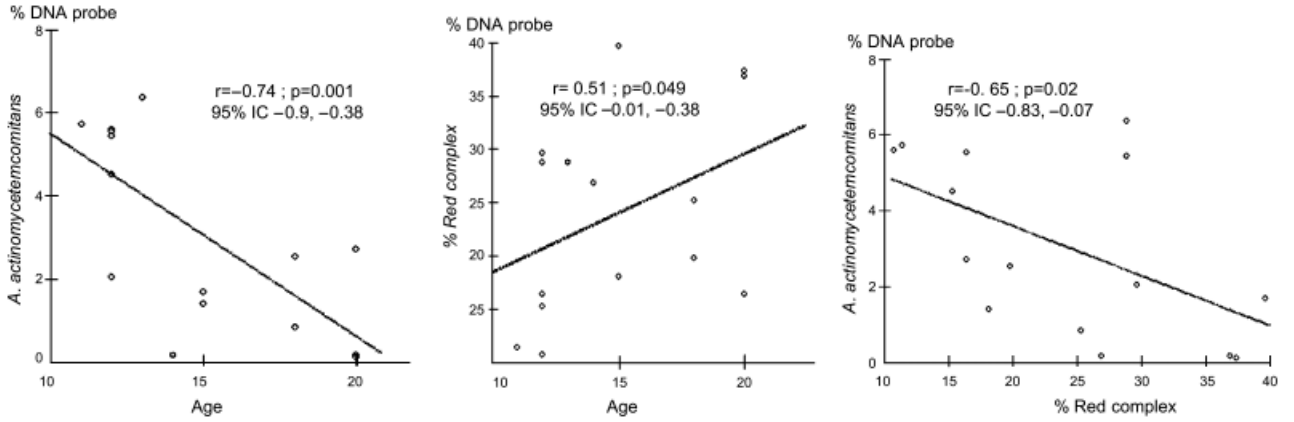

Fig. 7. Pearson' correlation between proportions of *Aggregatibacter actinomycetemcomitans* and age (left panel); proportions of red complex and age (middle panel); and proportions of *A. actinomycetemcomitans* and red complex (right panel) in subgingival plaque samples taken from 15 localized aggressive periodontitis (LAgP) subjects.

for the onset or the progression of periodontal disease may be lower than that needed for species such as *P. gingivalis* (Haffajee & Socransky 1994). This finding may be explained by the fact that *A. actinomycetemcomitans* is highly virulent and even low levels of this species, especially in the non-mature oral biofilm of young subjects, may trigger periodontal destruction (Bragd et al. 1987, Rams et al. 1997).

Several species from the red and orange complexes have been implicated previously in the aetiology of ChP (for a review, see Socransky & Haffajee 2005), and more recently AgP (Takeuchi et al. 2003, Kamma et al. 2004, Gajardo et al. 2005, Ximenez-Fyvie et al. 2006, Botero et al. 2007, Fritschi et al. 2008). However, few studies to date have suggested that pathogens other than *A. actinomycetemcomitans* could also be associated with the aetiology of LAgP, particularly in very young individuals (Moore et al. 1985, López et al. 1996, Gajardo et al. 2005). Therefore, the heavy colonization of some of these pathogens in LAgP subjects observed in the present study was rather unexpected.

Interestingly, *A. actinomycetemcomitans* was significantly increased in shallow and intermediate but not in deep periodontal pockets of subjects with LAgP in comparison with those with GAgP and ChP (Fig. 5), and the proportion of this pathogen showed a negative correlation with age as well as with the proportions of red complex species (Fig. 7). A recent study by Fine et al. (2007) reinforces the importance of the early colonization by *A. actinomycetemcomitans* for the onset of LAgP in young individuals. The authors followed 98 periodontally healthy children from 11 to 17 years of age for 2–3 years, and observed that 21% of the children harbouring *A. actinomycetemcomitans* ($n = 38$) developed LAgP over time, whereas none of the *A. actinomycetemcomitans*-negative children ($n = 58$) developed periodontal disease during this period of observation. Rodenburg et al. (1990) also suggested that the prevalence of severe periodontitis subjects colonized with *A. actinomycetemcomitans* may decrease with increasing age. Taken together, these data support the notion that *A. actinomycetemcomitans* is implicated in the onset of the disease; however, the deepening of the pockets and the resultant increase in the anaerobic environment may favour the growth of other pathogens, such as the strict anaerobic species from the red complex. It could also be speculated that because *A. actinomycetemcomitans* may not be particularly resistant to bacterial competition (Teughels et al. 2007), the presence of a more complex microbiota may lead to a reduction in its counts and proportions as the pockets deepen.

The comparison of the microbial profiles of LAgP, GAgP and ChP subjects revealed certain specific differences among the three groups. The proportions of *A. actinomycetemcomitans* were elevated and those of *A. naeslundii* 1 were reduced in LAgP subjects. This fact could be further investigated given that these species are not considered to be antagonists (Van Hoogmoed et al. 2008). The levels of *F. nucleatum* ssp *polymorphum* and *P. gingivalis* were the highest in GAgP subjects and the lowest in ChP (Fig. 4). The increased proportion of *P. gingivalis* may have contributed to the widespread destruction in the GAgP subjects. Of importance were the lower proportions of *Actinomyces* species observed in LAgP and GAgP subjects (Fig. 6). This information might explain the lower degree of plaque accumulation observed in AgP, because the *Actinomyces* are important plaque formers. Furthermore, these bacterial species have been strongly associated with PH (Colombo et al. 2002, López et al. 2004, Socransky & Haffajee 2005) and their increased proportions may be necessary for a successful therapeutic outcome.

In conclusion, the results of the present study suggest that *A. actinomycetemcomitans* seems to be associated with the onset of periodontal disease in LAgP, and other bacterial species, such as *P. gingivalis*, *T. forsythia*, *T. denticola*, *C. gracilis*, *E. nodatum and P. intermedia*, play an important role in disease progression. These results have direct clinical implications as they describe the microbial profile of LAgP and provide microbiological endpoints for therapy. Successful treatment of LAgP would involve a reduction in the levels and proportions of red complex pathogens, *A. actinomycetemcomitans* and some orange complex species and an increase in the *Actinomyces* species.

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Clinical Relevance

Scientific rationale for the study: Subjects with periodontal diseases present with different clinical conditions. It is not clear why these differences occur, but a likely explanation is that the composition of the subgingival microbiota differs among clinical groups.

Principal findings: Red complex species are closely associated with the aetiology of LAgP, as well as *A. actinomycetemcomitans* and some orange complex species. Specific differences were observed among the microbiota of LAgP, GAgP and ChP.

Practical implications: While *A. actinomycetemcomitans* was present in significantly higher levels in the LAgP, it was not the dominant pathogen, rather the red complex species. This finding may have implications regarding therapeutic approaches for this condition.