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Diversity and quantitative analysis of *Archaea* in aggressive periodontitis and periodontally healthy subjects

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Abstract

Aim: To investigate the diversity, levels and proportions of *Archaea* in the subgingival biofilm of generalized aggressive periodontitis (GAgP; $n = 30$) and periodontally healthy (PH; $n = 30$) subjects.

Materials and methods: Diversity was determined by sequencing archaeal *16S rRNA* gene libraries from 20 samples (10/group). The levels and proportions of *Archaea* were analysed by quantitative PCR (qPCR) in four and two samples/subject in GAgP and PH groups, respectively.

Results: *Archaea* were detected in 27/28 subjects and 68% of the sites of the GAgP group, and in 26/30 subjects and 58.3% sites of the PH group. *Methanobrevibacter oralis* was found in all 20 samples studied, *Methanobacterium curvum/congolense* in three GAgP and six PH samples, and *Methanosarcina mazeii* in four samples from each group. The levels and proportions of *Archaea* were higher in GAgP than in PH, whereas no differences were observed between the two probing depth category sites from the GAgP group.

Conclusion: *Archaea* were frequently found in subjects with periodontal health and GAgP, especially *M. oralis*. However, the higher levels and proportions (*Archaea*/total prokaryotes) of this domain observed in GAgP in comparison with PH subjects indicate a possible role of some of these microorganisms as an environmental modifier in GAgP.

Key words: *16S rRNA* gene; aggressive periodontitis; *Archaea*; microbial diversity; periodontal health; subgingival biofilm

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The microbiota of humans is not only comprised by *Bacteria* and *Eukarya*, but members of the third domain of life, *Archaea*, may also colonize human mucosa surfaces (Miller et al. 1982, Belay et al. 1990), including the oral cavity (Belay et al. 1988, Ferrari et al. 1994). *Archaea* are prokaryotes, evolutionary closely more linked to *Eukarya* than to *Bacteria*, and are distinguished from the other domains based on differences in ribosomal RNA (rRNA) subunits and other features such as distinct macromolecules (Woese et al. 1990, Gribaldo et al. 2010). The *Archaea* usually found in the human and animal microbiota are members of the methanogens (Conway de Macario & Macario 2009), which produce methane from various substrates, such as H_2 and CO_2, methylated C_1 compounds (methanol, methylamines, methylthiols) or acetate (Deppenmeier 2002). Although these organisms have been linked to human diseases in a few studies, they have not been conclusively shown to be the causative agent of any pathology (Cavicchioli et al. 2003, Eckburg et al. 2003, Conway de Macario & Macario 2009).

Archaea can be found in the oral cavity of humans, in the saliva, oral biofilms and endodontic lesions (Belay et al. 1988, Brusa et al. 1993, Vianna et al. 2006). PCR assays targeting the archaeal small subunit of ribosomal RNA gene (SSU *rRNA*) have been able to detect this domain in subgingival

Conflict of interest and source of funding statement

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samples from subjects with periodontal disease (Kulik et al. 2001), particularly in sites with severe periodontal destruction (Lepp et al. 2004), in a prevalence ranging from 22% to over 70% (Yamabe et al. 2008, Li et al. 2009). Nevertheless, *Archaea* was not detected in subgingival samples from periodontally healthy (PH) subjects (Lepp et al. 2004, Yamabe et al. 2008, Li et al. 2009), suggesting that these organisms may play a role in the pathogenesis of periodontitis.

Periodontitis is a polymicrobial infection in which the sequence of microbial colonization is fundamental to the onset and progression of the disease (Socransky & Haffajee 2005). The possible contribution of methanogens to pathogenesis may be explained by their ability to provide conditions for the growth of non-archaeal organisms, which are either pathogens themselves or promote the growth of real pathogens (Lepp et al. 2004, Vianna et al. 2006, Conway de Macario & Macario 2009).

The recent implication of indirect association of *Archaea* with periodontal disease prompted us to perform a cross-sectional examination of the ubiquity of methanogens in subgingival biofilms of generalized aggressive periodontitis (GAgP) and PH subjects. Such knowledge could provide information about the diagnostic and prognostic value of *Archaea* evaluation in periodontitis subjects, and, consequently, increase our understanding on the whole microbiota colonizing diseased and healthy periodontal sites.

This study aimed to investigate the diversity, levels and proportions of *Archaea* in the subgingival biofilm of GAgP and PH subjects. We tested the hypothesis that the diversity of this domain would indicate differences in the pathogenic potential of different genus and species, similar to what occurs in *Bacteria*. In addition, analyses of levels and proportions of *Archaea* in relation to the total prokaryotes in GAgP and healthy subjects and in deep and shallow sites of diseased subjects would indicate whether *Archaea* play a role in the ecological shift of the subgingival microbiota towards disease.

Material and Methods

Subject population

Thirty GAgP and 30 PH subjects were selected from a population referred to the Dental Clinic of Guarulhos University (Guarulhos, SP, Brazil). A complete clinical examination was performed, including medical and dental histories, intra-oral examination and full-mouth periodontal probing. The protocol was previously submitted and approved by the Human Research Ethical Committees of University of São Paulo and University of Guarulhos. All subjects signed a committee-approved informed consent.

The parameters for clinical examinations, as well as the inclusion and exclusion criteria have been previously published elsewhere (Faveri et al. 2008). Briefly, subjects diagnosed with GAgP were $\leqslant 30$ years of age; presented a minimum of six permanent incisors and/or first molars with at least one site each with probing depth (PD) and clinical attachment level (CAL) $\geqslant 5$ mm; had a minimum of six teeth, other than first molars and incisors, with at least one site each with PD and CAL $\geqslant 5$ mm; and had a family history (at least one other member of the family presently showing, or with history of, periodontal disease) (Armitage 1999). On the other hand, PH subjects were >20 years of age; exhibited PD and CAL <4 mm; had no clinical signs of generalized gingivitis, and had no more than 15% of sites presenting bleeding on probing (BOP).

The clinical monitoring was performed by one examiner, calibrated according to the method described by Araujo et al. (2003). Visible plaque (presence or absence), gingival bleeding (presence or absence), BOP (presence or absence), suppuration (presence or absence), PD (mm) and CAL (mm) were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) in all teeth, excluding the third molars. PD and CAL measurements were recorded to the nearest millimetre using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA). The intra-examiner variability was 0.14 mm for PD and 0.31 mm for CAL. This trained examiner was able to provide reproducible measurements of under 0.5 mm.

Microbiological evaluation

Sample collection

Four subgingival samples were taken from each subject in the GAgP group, two from each of the following PD categories: shallow (PD $\leqslant 3$ mm) and deep (PD $\geqslant 5$ mm). Two subgingival plaque samples were collected from each PH individual from two sites with PD $\leqslant 3$ mm without BOP. All samples were randomized in different quadrants. After the clinical parameters had been recorded, supragingival plaque was removed and subgingival samples were collected with individual sterile mini-Gracey curettes (#11–12) and placed into TE buffer [10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6]. The samples were stored at -20°C until the DNA extraction.

Diversity of Archaea

Diversity was determined by sequencing archaeal *16S rRNA* amplified DNA obtained from a single sample per subject from 10 GAgP (PD $\geqslant 7$ mm) and 10 PH subjects. DNA of subgingival samples was obtained according to Dewhirst et al. (2000).

A primers-pair specific for the *16S rRNA* gene of *Euryarchaea* was used (Kulik et al. 2001) (300fEyAr 5′-GC (A/G)(A/G)GAGCCCGGAGATGG-3′ and 954rEyAr 5′-CGGCGTTGA(A/G) TCCZZTTAAAC-3′) (Invitrogen Life Technologies, Carlsbad, CA, USA). PCR was performed in a total volume of 50 μl, containing 1 μl of template DNA, 2 mM of $MgCl_2$, 25 pmol of each primer, 0.2 mM of deoxynucleoside triphosphates and 2 U of Platinum® Taq DNA polymerase (Invitrogen). The negative control consisted of the same reaction with no added template DNA, whereas positive control reactions were added with 1 μl of a pool of methanogenic *Archaea* DNA from an environmental source, consisting of a mixture of *Methanosaeta concilli*, *Methanosaeta lacustris, Methanobrevibacter arboriphilus* and *Methanobrevibacter smithii*. The samples were preincubated at 94°C for 4 min., followed by amplification for 35 cycles of 94°C for 15 s, 64°C for 30 s and 72°C for 15 s, and a final extension step at 72°C for 7 min. PCR products were subjected to electrophoresis in a 1.5% agarose gel (Gibco, Invitrogen) in Tris acetate EDTA buffer (Tris acetate 40 mM, pH 8.5; EDTA 2 mM), stained with ethidium bromide (Sigma, St. Louis, MO, USA) and visualized under short-wavelength UV light (Pharmacia Biotech, UV20, San Francisco, CA, USA).

The amplicons were cloned using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions and sequenced. Sequencing was performed with ABI Prism fluorescent bases (BigDye Terminator Cycle

Sequencing kit with AmpliTaq DNA Polymerase FS; Perkin-Elmer, Foster City, CA, USA) using 3.2 μM of reverse primer (Kulik et al. 2001), with 35 cycles of denaturation at 96°C (45 s), annealing at 64°C (30 s) and extension at 60°C (4 min.) (ABI Prism 3100 Genetic Analyzer, Hitachi, San Mateo, CA, USA) at the Sequencing Facility at the Chemistry Institute, University of São Paulo.

For identification of the closest relatives, the sequences of approximately 500 bases of unrecognized inserts were compared with the *16S rRNA* gene sequences of Genbank database. A level of 99% sequence identity was used as the cut-off point for the identification of a specific taxon. Similarity matrices were constructed from the aligned sequences corrected for multiple base changes at a single position using Jukes & Cantor's method (1969).

Quantification of Archaea

Total genomic DNA from subgingival samples was extracted and purified using the Qiamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration (A_{260}) and purity ($A_{260/280}$) were estimated using a spectrophotometer (NanoDrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA).

The amplification and detection of *Archaea* and *Bacteria* DNA by quantitative PCR (qPCR) were performed in four samples per subject in the GAgP group and in two samples per subject in the PH group, randomly selected, on a thermocycler iQ 5 Bio-Rad (Bio-Rad, Rio de Janeiro, Brazil) using the QuantiMix Easy SYG kit (Biotools, Madrid, Spain). PCR reactions were comprised of 50–75 ηg of extracted DNA in a final volume of 25 μl. Quantification of the *Archaea 16S rRNA* gene was determined using 0.5 μM of the primers 931f and m1100r (Ovreas & Torsvik 1998; Table 1), with the following thermal program: preincubation at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 15 s, annealing at 64°C for 30 s, extension at 72°C for 30 s and plate read at 72°C for 10 s. The cycling was followed by a final extension of 7 min., and melting curve analysis was performed at 65–95°C with a plate read every 0.5°C.

Amounts of *Bacteria 16S rRNA* gene were estimated using 0.5 μM of the primers 338f and 518r (Einen et al. 2008; Table 1). The following thermal program was used: 95°C for 15 min., followed by 45 cycles of denaturation at 94°C for 15 s, annealing at 61°C for 30 s, extension at 72°C for 30 s and plate read at 72°C for 10 s. The cycling was followed by a final extension of 7 min., and a melting curve analysis was performed at 65–95°C with a plate read every 0.5°C.

Table 1. Sequence of the primers used for quantitative PCR analysis

Primer	Nucleotide sequence (5′ → 3′)	Product size (bp)	References
931f	AGGAATTGGCGGGGGAGCA	169	Ovreas & Torsvik (1998)
M1100r	BGGGTCTCGCTCGTTRCC		
338f	ACTCCTACGGGAGGCAGCAG	180	Einen et al. (2008)
518r	ATTACCGCGGCT GCTGG		

Calibration standards comprising serially diluted SSU *rRNA* genes cloned DNA obtained from *Methanobrevibacter oralis* (for *Archaea*) or *Porphyromonas gingivalis* W83 (for *Bacteria*) were used to establish the standard curves and allow conversion from data of threshold cycles to gene copy numbers. The standard curve for *Archaea* and *Bacteria* qPCR had an R^2 value of 0.999 minimum, and the amplification efficiency estimated ranged from 95% to 105%. All samples were run in duplicate and the mean values of target molecule numbers were used for analysis.

The levels and proportions of *Archaea* and *Bacteria* were expressed as the number of copies of the *16S rRNA* gene in the initial sample, calculated after normalization to the total DNA in each reaction.

Statistical analysis

A χ^2-test was used to test differences in the gender distribution between groups. The percentages of sites with visible plaque, gingival bleeding, BOP and suppuration, as well as mean age, PD and CAL were compiled for each subject and then averaged across subjects in both groups. The mean counts for *Archaea* ($\times 10^4$) and *Bacteria* ($\times 10^7$) were averaged within each subject and then across subjects in both groups. To calculate the proportion of the *Archaea* domain in subgingival biofilm, the number of *16S rRNA* gene copies of *Archaea* in initial sample was divided by the total amount of prokaryotes (*Archaea*+*Bacteria*) in the respective sample. The proportions were determined for sites and subjects and averaged across subjects in the two groups. The significance of differences between groups for clinical and microbiological parameters was assessed using the Mann–Whitney *U*-test. The χ^2-test was used to test differences in the prevalence rates of *Archaea* between GAgP and PH subjects, as well as among the PD categories. The level of significance was set at 5%.

Results

Clinical findings

Data of the clinical findings of the population studied are summarized in Table 2. No statistically significant differences were observed between groups in relation to age, gender and gingival bleeding ($p > 0.05$). GAgP subjects showed higher PD and CAL mean values, as well a higher percentage of sites with BOP, suppuration and visible plaque, when compared with PH subjects.

Microbiological findings

Diversity of Archaea

A total of 720 clones with a *16S rRNA* gene fragment of correct size were sent to sequencing. The phylogenetic identities of the 629 clones were determined, whereas 91 clones could not be identified due to technical reasons. The number of *16S rRNA* gene clones per library that were sequenced ranged from 33 to 47 in samples of the GAgP group and from 15 to 23 in samples of the PH group (mean 42.8 ± 3.9 and 20.1 ± 2.2 for GAgP and PH, respectively).

Overall, three different genera of methanogenic *Archaea* were identified in both clinical groups: *Methanobrevibacter*, *Methanobacterium* and *Methanosarcina*. *M. oralis* was detected in all the samples studied, representing 82% of the clones identified in the samples from the GAgP group and 70.1% from the PH group. Species identity could not be determined for the phylotypes *Methanobacterium curvum* and *Methanobacterium congolense*, as the homology in the *16S rRNA* gene shared by these species is over 99%. Therefore,

they were classified as *M. curvum/congolense* phylotype, which comprised 7.2% and 17.9% of the clones analysed for GAgP and PH group samples, respectively. *Methanosarcina mazeii* represented 10.8% and 12% of the sequenced clones, for samples of the GAgP and PH groups, respectively.

M. oralis was detected in a high proportion (>50% of the sequenced clones) in most of the samples analysed, and it was the unique *Archaea* specie identified in 5/10 GAgP and 3/10 PH samples. An exception was found in two samples of the GAgP group and in three of the PH group, where *M. curvum/congolense* and/or *M. mazeii* were found in higher proportions (Fig. 1).

Quantification of Archaea

The quantitative data were obtained for 28 subjects/103 sites of the GAgP group and 30 subjects/60 sites of the PH. Two subjects/17 sites of the GAgP group were excluded, due to no detection of bacterial DNA.

Archaea was detected in 27 of 28 subjects in the GAgP group, and in 26 of 30 subjects of the PH group. This domain was detected in 68% of analysed sites in the GAgP group. *Archaea* was detected in a similar prevalence in shallow (72.5%) and in deep sites (63.5%) of the GAgP subjects (χ^2-test, $p>0.05$). The frequency of detection of *Archaea* in sites of the PH group reached 58.3% of the sites, with no difference in the prevalence of this domain when compared with sites of subjects of the GAgP group, regardless of the PD category of the sampled sites (χ^2-test, $p>0.05$).

Samples of the GAgP group presented $11.2 \times 10^4 \pm 6.6 \times 10^4$ copies of the *16S rRNA* gene of *Archaea*/sample, whereas samples of the PH group presented significantly lower levels ($0.6 \times 10^4 \pm 0.2 \times 10^4$) (Mann–Whitney *U*-test, $p<0.05$). As expected, the levels of *Bacteria* were also increased in the GAgP group ($11.5 \times 10^7 \pm 2.3 \times 10^7$ copies of *16S rRNA* gene of *Bacteria*/sample) when compared with PH ($6.1 \times 10^7 \pm 1.7 \times 10^7$) (Mann–Whitney *U*-test, $p<0.05$).

There were no differences in the levels of *Archaea* when shallow and deep sites of the GAgP groups were compared ($10.7 \times 10^4 \pm 9.5 \times 10^4$ and $12.5 \times 10^4 \pm 5.1 \times 10^4$ *16S rRNA* gene copies of *Archaea*/sample) but differed from the levels of samples of the PH group ($p>0.05$, Fig. 2a). However, the levels of *Bacteria* differed in samples of deep ($16 \times 10^7 \pm 3.5 \times 10^7$ copies *16S rRNA Bacteria*/sample) and shallow sites ($7.8 \times 10^7 \pm 1.8 \times 10^7$), as well as between samples of the GAgP and PH subjects ($6.1 \times 10^7 \pm 1.8 \times 10^7$) ($p>0.05$, Fig. 2b).

The proportions of *Archaea* in relation to the total prokaryotes were significantly lower (0.02%) in PH samples

Table 2. Demographic characteristics and the mean (± SD) full-mouth clinical parameters of generalized aggressive periodontitis (GAgP) and periodontally healthy (PH) subjects

Variables	GAgP $n=30$	PH $n=30$
Gender (female/male)	16/14	17/13
Age (years)	26.2 ± 4.1 (20–29)	24.5 ± 5.1 (20–28)
Probing depth*	4.36 ± 0.88	2.28 ± 0.59
Clinical attachment level*	4.41 ± 1.27	2.39 ± 0.35
% sites		
Visible plaque*	48.79 ± 11.91	26.91 ± 10.49
Gingival bleeding	11.49 ± 11.22	7.91 ± 5.48
Bleeding on probing*	72.30 ± 23.05	10.31 ± 9.25
Suppuration*	3.57 ± 3.78	0.0

*Statistically significant difference ($p<0.05$).
Mann–Whitney *U*-test.

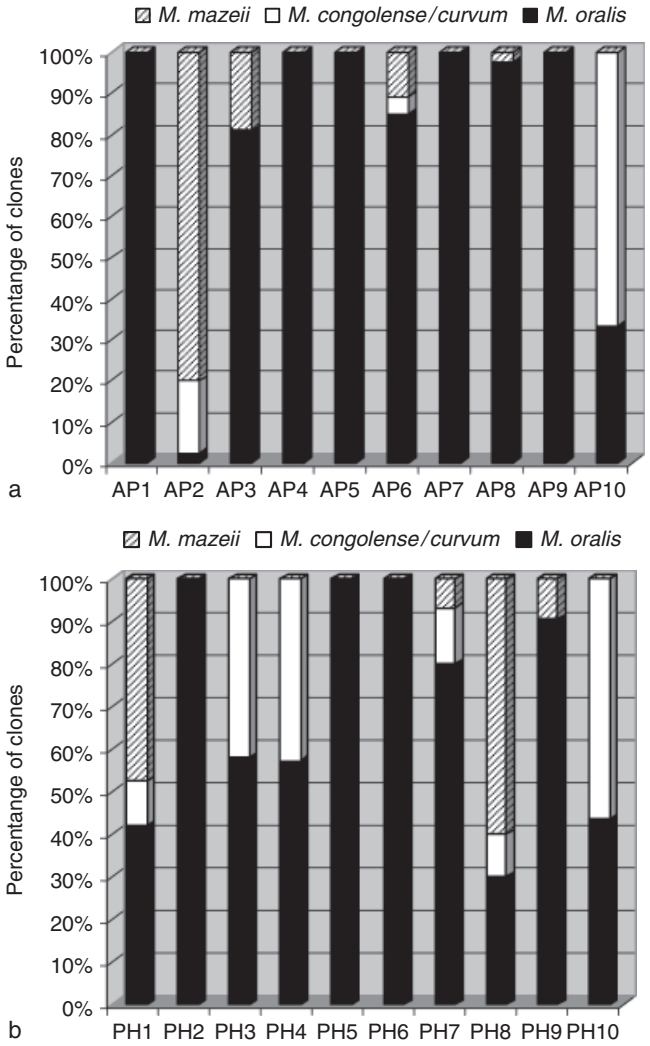

Fig. 1. Percentage of identified clones as *Methanobrevibacter oralis*, *Methanobacterium curvum/congolense* and *Methanosarcina mazeii* in samples of subgingival biofilm in generalized aggressive periodontitis (a) and periodontally healthy subjects (b).

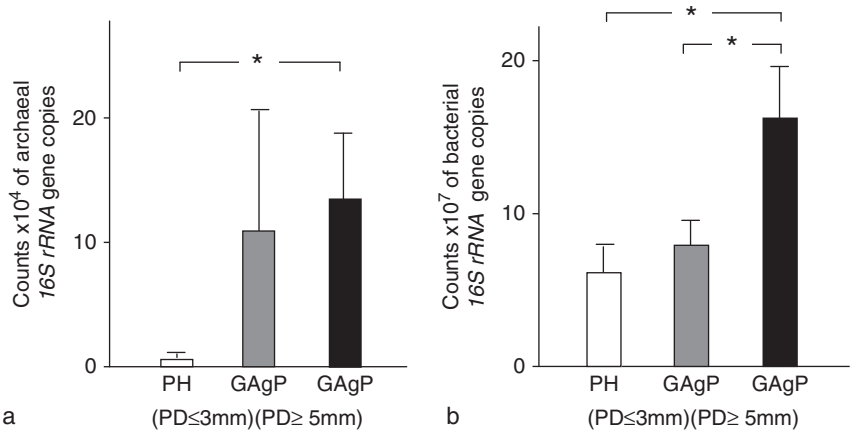

Fig. 2. Mean counts of *16S rRNA* gene copies of *Archaea* ($\times 10^4 \pm$ SEM, a) and *Bacteria* ($\times 10^7 \pm$ SEM, b) in samples obtained from periodontally healthy subjects and from shallow and deep sites of generalized aggressive periodontitis individuals. Mann–Whitney *U*-test, $^*p < 0.05$.

when compared with the GAgP group (0.08%) (Mann–Whitney *U*-test, $p < 0.05$). However, there were no differences in the proportions of the *Archaea* domain in shallow sites (0.12%) when compared with sites with PD $\geqslant$ 5 mm (0.08%) in the GAgP group.

Discussion

The role of *Archaea* in subgingival environment is still not established, although previous data suggested that members of this domain could be indirectly associated with the pathogenesis of periodontitis (Lepp et al. 2004, Vianna et al. 2008, Conway de Macario & Macario 2009).

Our data indicated that the diversity of *Archaea* in subgingival sites is very low, and that *M. oralis* is found frequently. Previous studies have also reported a restricted diversity of *Archaea* in human ecosystems, including the oral cavity (Kulik et al. 2001, Lepp et al. 2004, Vianna et al. 2006, Vickerman et al. 2007, Li et al. 2009). *M. oralis* has been detected previously in subgingival biofilms (Ferrari et al. 1994, Kulik et al. 2001, Lepp et al. 2004, Li et al. 2009) and in dental root canals (Vianna et al. 2006, Vickerman et al. 2007). However, *M. mazzei* and *M. curvum/congolense* had not been previously reported conclusively in humans. A microorganism with physiological, morphological and Gram-staining reaction closely related to *Methanosarcina* sp. has been reported in subgingival samples (Robichaux et al. 2003). The phylotype *M. curvum/congolense* was detected on the cassava root peel on the sludge of a beer factory floor (Cuzin et al. 2001), but not in humans.

The origin of *Archaea* in the oral cavity is still not elucidated. Food such as cheese, fish and meat may harbour methanogens of the genera *Methanogenium*, *Methanobacterium* and *Methanosarcina* but not of the genus *Methanobrevibacter* (Brusa et al. 1998). Hence, the phylotypes *M. mazeii* and *M. curvum/congolense* detected in the present study may have originated from the ingestion of contaminated food. On the other hand, the predominance of *M. oralis* in both healthy and periodontitis subjects, in addition to the high levels of copies of *16S rRNA* gene of *Archaea* in samples of which this phylotype was the only identified *Archaea* (data not shown), support the hypothesis that this species should be considered a resident organism of the oral cavity.

The low diversity of *Archaea* observed in subgingival sites could indicate that, unlike *Bacteria*, members of the *Archaea* domain, except for *M. oralis*, are slightly adapted to this particular niche (Lepp et al. 2004), as well as to other environments with a competitive microbiota (Aller & Kemp 2008). Then, the data suggested that *Archaea* may perceive and make use of the environment in ways that are more restrictive than for *Bacteria*, presenting a lower physiologic flexibility in non-extreme environments (Aller & Kemp 2008), and being numerically dominant only under extreme conditions, due to natural selection.

The diversity analysis indicated that *M. oralis* is the main species of *Archaea* in subgingival sites, and no certain archaeal species is associated with either periodontal health or disease. Thus, the hypothesis that the amount and proportions of *Archaea* would increase in diseased subgingival sites of GAgP subjects when compared with healthy sites either in diseased or in healthy subjects was accepted.

Differing from previous data (Belay et al. 1988, Kulik et al. 2001, Vianna et al. 2008, Yamabe et al. 2008, Li et al. 2009), similar prevalence rates of *Archaea* were demonstrated in both GAgP subjects and PH individuals.

Archaea was detected in 96.4% of subjects and 68% of sites from the GAgP group. This frequency of detection is much higher than that reported among chronic periodontitis individuals (36%) using another pair of primers also targeting the SSU *rRNA* of *Archaea* (Lepp et al. 2004). However, the results were similar when the prevalence per site was considered, indicating that the analysis of multiple sites per subject may have enabled us to detect *Archaea* in a higher proportion of subjects.

Our data indicated a high prevalence of *Archaea* in subgingival sites of PH subjects (86.7% of individuals and 58.3% of sites). To our knowledge, *Archaea* has not been detected in samples from PH subjects (Yamabe et al. 2008, Li et al. 2009). These differences may not be attributed only to methodological discrepancies but also quantitative and qualitative variability of the microbiota according to the geographical location, as already shown for some periodontopathogenic *Bacteria* (Haffajee et al. 2004, 2005). Likewise, evidence that the levels of methanogens differ among populations came from studies on methane levels in breath, showing that the proportion of methane producers among the adult human population in the United States or Great Britain was higher than that in Japan (Morii et al. 2003). Moreover, differences in the microbiota associated with aggressive and chronic periodontitis might explain the distinct results between studies.

The subgingival samples from subjects with GAgP presented higher counts of *Bacteria* than those from subjects with a healthy periodontium, and a positive association among *Bacteria* levels and PD was also observed in diseased subjects. These findings are in accordance with other studies (Ximenez-Fyvie et al. 2000, 2006, Faveri et al. 2009). The proportions of *Archaea* in subgingival sites were very low ($< 0.1\%$

of the prokaryotes), much lower than the 18.5% reported in chronic periodontitis subjects (Lepp et al. 2004). However, they were more similar to data obtained in chronic periodontitis subjects in Germany, for whom *Archaea* comprised only 0.26% of microbiota (Vianna et al. 2008).

The levels and proportions of *Archaea* in GAgP subjects were significantly higher than those in PH individuals. The percentage of copies of the archaeal *16S rRNA* gene was four times higher in samples from subjects with GAgP than in those with a healthy periodontium. However, the proportion of *Archaea* did not differ in diseased and healthy sites of aggressive periodontitis subjects, as the levels of *Bacteria* increased proportionally with increased PD.

Periodontitis is not a disease attributed to a single aetiological agent, and the different microbial groups, even at low proportions, able to use H_2, such as *Archaea*, might alter the ecosystem and could contribute to shift the microbiota associated with periodontal health to one associated with periodontal disease. Hydrogen is a fundamental by-product in anoxic environments (Deppenmeier 2002), and the consumption of H_2 and CO_2 by methanogens would probably favour the proliferation of fermentatives (Chassard et al. 2008), whereas high partial pressures of H_2 would reduce fermentation efficiency (Persson et al. 1990). On the other hand, sulphate-reducing bacteria, such as *Treponema*, may compete with methanogens for H_2 (Lepp et al. 2004), although both populations can coexist at comparable levels of abundance in the gut (Conway de Macario & Macario 2009) and in the subgingival environment (Vianna et al. 2008).

Furthermore, the production of other bacterial by-products present in the dental biofilm such as sulphide and nitrites may be toxic to methanogens, under certain conditions (Persson et al. 1990, Mohanakrishnan et al. 2008, Schreiber et al. 2010). Various periodontopathogens generate significant amounts of hydrogen sulphide (Persson et al. 1990), and *P. gingivalis*, *Streptococcus anginosus* and *Fusobacterium nucleatum* produce the highest amounts of this substrate by degradation of cysteine compounds (Yoshida et al. 2009). Data on the microbiota of GAgP, described elsewhere (Faveri et al. 2008, 2009), have shown that the proportions of *F. nucleatum* ssp. *polymorphum* and *P. gingivalis*, both hydrogen sulphide producer bacteria, were increased in GAgP subgingival samples in comparison with chronic periodontitis samples, whereas the proportion of *Actinomyces naeslundii* 1, a fermenter organism, was increased in chronic periodontitis. Although no definitive conclusion can be drawn before functional studies are carried out, this study hypothesizes that the proportion of positive sites for *Archaea* may be negatively correlated with the levels of methanogen competitors or antagonists and positively to the levels of fermenters, which differ between chronic and aggressive periodontitis, and between diseased and healthy periodontal sites (Socransky & Haffajee 2005, Faveri et al. 2009). Therefore, the shifts in the subgingival microbiota observed from healthy to disease and variations from one to another type of periodontitis may also be influenced by *Archaea*.

As a conclusion, the present data suggest that *M. oralis* is the predominant archaeal phylotype in the subgingival biofilm of both healthy and aggressive periodontitis subjects and it might be considered a component of the normal oral microbiota. Despite the relatively low diversity and amount of *Archaea* in the subgingival sites, the higher proportions of methanogens in subjects with GAgP when compared with PH individuals might indicate that *Archaea* may play a role in the ecological shift of the microbiota of aggressive periodontitis subjects.

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Clinical Relevance

Scientific rationale for the study: Previous investigations suggested that *Archaea* were restricted to deep pockets in periodontitis subjects, but their diversity and amount in samples of subgingival biofilm of GAgP and PH subjects have not been determined.

Principal findings: Archaea, and particularly *M. oralis*, may be considered as part of the resident subgingival microbiota of humans. However, their levels and proportions, although low, are higher in disease than in healthy individuals.

Practical implications: The increased amount of *Archaea* in healthy and diseased sites within the GAgP group may indicate its use as a biomarker of altered microbiota.