The phylum *Synergistetes* in gingivitis and necrotizing ulcerative gingivitis

Angelica Baumgartner, Thomas Thurnheer, Helga Lüthi-Schaller, Rudolf Gmur and Georgios N. Belibasakis

**INTRODUCTION**

Gingivitis is a form of periodontal disease characterized by clinical inflammation and bleeding confined to the gingival tissues. Development of dental plaque on the tooth surface at the level of the gingival margin is commensurate with the occurrence of gingivitis, and its removal will result in reversal to clinically healthy status (Mariotti, 1999). Therefore, the level of oral hygiene control is a crucial factor for the development or prevention of the disease. It is estimated that ~90% of the global population is affected by gingivitis (Brown & Löe, 1993). In this paper, this disease form is therefore referred to as plaque-induced gingivitis. Necrotizing ulcerative gingivitis (NUG) is classified as a form of necrotizing periodontal diseases, which is distinct from plaque-induced gingivitis in terms of the course of clinical presentation and establishment (Armitage, 1999). In addition to the classical inflammatory symptoms of plaque-induced gingivitis, NUG is also characterized by tissue necrosis restricted to the gingival tissues (especially on the interdental papillae), rapid onset, pain and extensive bleeding. Onset of the disease is associated with certain immune-response-impairing risk factors, including psychological stress, smoking and human immunodeficiency virus infection (Rowland, 1999). It affects mainly young adults, and, depending on the population and socioeconomic status, the prevalence of the disease can be between 0.001 and 27% (Jenkins & Papapanou, 2001).

Microbiologically, plaque-induced gingivitis is characterized by the switch from Gram-positive aerobic to Gram-negative anaerobic bacteria. The proportion of Gram-positive rods, such as *Actinomyces oris/Actinomyces naeslundii* and *Actinomyces israelii*, is increased (Loesche & Syed, 1978), whereas there is a decrease in Gram-positive cocci, such as *Streptococcus oralis*, and an increase in Gram-negative species, such as *Tannerella forsythia* and *Prevotella intermedia* (Loesche & Syed, 1978; Tanner et al., 1998). Concerning the microbiology of NUG, earlier studies have shown that dental plaque from active sites exhibits high levels of spirochaetes and fusiforms (Plaut, 1894; Vincent, 1899; Listgarten, 1965; Heylings, 1967; Gmürt et al., 2004). Black-pigmented Gram-negative putative periodontal pathogens, such as *Porphyromonas gingivalis* and *Prevotella intermedia/Prevotella nigrescens*, were variably detected in dental plaque from patients with NUG, as shown by culture-based (Loesche et al., 1982; Falkler et al., 1987) or molecular (Paster et al., 2001; Gmürt et al., 2004) detection methods. This indicates the complexity, or lack of predictability, of the NUG-associated dental plaque microflora.

Abbreviations: 6-FAM, 6-Carboxyfluorescein; FISH, fluorescent in situ hybridization; NUG, necrotizing ulcerative gingivitis.
Synergistetes is a bacterial phylum widely distributed in the environment, consisting of Gram-negative anaerobes. Its members are postulated to be involved in a number of anaerobic digestion processes, and, so far, they have been identified in almost 100 different primarily anaerobic microenvironments, as diverse as the animal gastrointestinal track, the termite hindgut and petroleum reservoirs (Godon et al., 2005). They are also found as part of the human microbiota in health and disease (Horz et al., 2006; Vartoukian et al., 2007; Marchandin et al., 2010). In the case of the oral microflora, Synergistetes is recognized as one of 13 different phyla identified in the Human Oral Microbiome Database (Dewhirst et al., 2010). Synergistetes bacteria have been detected in high numbers in subgingival plaque from patients with chronic periodontitis and from the root canals of teeth with endodontic infections (de Oliveira et al., 2007; Siqueira & Rôças, 2007; Vianna et al., 2007; Vartoukian et al., 2009). In relation to periodontal status, Synergistetes bacteria are detected more frequently in periodontitis patients than in healthy subjects, and are more abundant in subgingival plaque obtained from diseased compared with from healthy sites (Vartoukian et al., 2009).

To date, human oral Synergistetes bacteria have been divided principally into one species (Jonquetella anthropi) and two clusters, the latter each consisting at present of one species and numerous non-cultivated phylotypes. These have been named clusters A and B (Vartoukian et al., 2009), and this classification is also used in the present work. In brief, the first cultivated oral Synergistetes cluster A species has been characterized recently, namely Fretibacterium fastidiosum. Its type strain, SGP1T, was found to be asaccharolytic, and its efficient growth was dependent on other oral bacteria, such as Fusobacterium nucleatum (Vartoukian et al., 2012). Cluster B contains at present the single species Pyramidobacter piscolens (Downes et al., 2009), whereas J. anthropi harbours the first characterized human clinical isolate of the phylum Synergistetes (Jumas-Bilak et al., 2007), but little is known about its association with oral diseases, except that one known clone was derived from an oral source (Vartoukian et al., 2009).

Although some information is now available on the presence of Synergistetes bacteria in periodontitis, the association of this phylum with NUG and any differences compared with plaque-induced gingivitis are not yet known. Therefore, the aim of this study was to evaluate the presence and levels of the known human oral Synergistetes bacterial clusters in dental plaque samples from patients with NUG and to identify any such differences compared with samples from patients with gingivitis.

METHODS

Subjects, clinical examination and sample collection. The dental plaque samples analysed in the present study comprised frozen aliquots of samples collected and used in three previous studies (Gmür et al., 2004, 2006; Züger et al., 2007) and had not been frozen/thawed more than twice before. Briefly, marginal plaque samples were collected from 21 subjects with gingivitis (36.9 ± 6.7 years) and 21 subjects with NUG (38.9 ± 6.3 years) who were otherwise systemically healthy. All subjects were of Chinese origin and were recruited from dental clinics in Beidaihe, Chengede, Shijiazhuang and Xi’an (People’s Republic of China) (Gmür et al., 2004). All subjects provided written and informed consent for sample collection for research purposes. They had at least 20 teeth, exhibited probing pocket depths ≤ 3 mm and had not received any antibiotics over the previous 3 months. During clinical examination, the plaque index, gingival index, bleeding on probing and probing pocket depth were noted. The diagnosis of NUG and differential diagnosis from gingivitis was based on the presence of gingival ulceration, pseudomembrane formation, loss of gingival papillae, pain and fetid odour (Rowland, 1999). The clinical periodontal parameters of the subjects have been provided elsewhere (Gmür et al., 2004). Samples collected from three to four affected sites were pooled together in 1 ml reduced transport medium containing 10 % glycerol and stored in liquid N2 until further use.

Analysis of dental plaque samples by fluorescent in situ hybridization (FISH). Aliquots of pooled supragingival plaque samples frozen in liquid N2, as described above, were used for this study. Once the samples had defrosted, they were vortexed for 1 min. The gingivitis samples were then diluted 1:5 and the NUG samples 1:10 in coating buffer (0.9% NaCl, 0.02% NaN3, 2.5 ± 10^- 8 % hexadecyltrimethylammonium bromide). Ten microlitres of these suspensions were then spotted on to individual 18- or 24-well epoxy-coated Adcell multi-well slides with a well diameter of 4 mm (Cel-Line; Erie Scientific Company). After air drying, the slides were fixed by 20 min incubation at 4 °C in 4% paraformaldehyde in PBS. The fixed slides were then processed for FISH.

To limit non-specific probe binding to the bacterial cell wall, each well was evenly covered with 9 µl Denhardt’s solution (diluted 1:50 in PBS; Fluka) in the presence of Protect RNA RNase inhibitor (diluted 1:500 in PBS; Sigma-Aldrich) and incubated for 1 h at 37 °C (Gmür & Lüthi-Schaller, 2007). Five specific oligonucleotide rRNA probes were used for members of Synergistetes cluster A, Synergistetes subcluster A1, Synergistetes subcluster A2 and Synergistetes cluster B and for J. anthropi, together with a universal probe that identifies most eubacteria. Two of these probes, EUB338 (Amann et al., 1995) and Syn-A1409 (Zijinge et al., 2010), have been validated and used previously. The remaining probes were designed and applied first by Lüdin (2011). The cluster classification of oral Synergistetes bacteria and the subclassification of cluster A members into A1 and A2 were based on the results of Vartoukian et al. (2009). The selected oligonucleotide probes were purchased from Microsynth and labelled with Cy3 or 6-carboxyfluorescein (6-FAM). The probes were designed according to Manz (1999) and were specificity tested by in silico hybridization using the ARB software environment and databases (http://www.arb-home.de) (Ludwig et al., 2004) and Ribosomal Database Project (RDP) II (http://rdp.cme.msu.edu) (Cole et al., 2007). The oligonucleotide sequences of the rRNA probes and targeted taxa are listed in Table 1. The in silico testing revealed no cross-reactivity other than with the targeted taxa. The SYN-A1409 probe detected almost 400 strains and clones in the RDP database (release 10, update 29 from 1 June 2012, sequences of ≥1200 bp), found either in the genus Thermovirga (36/123 entries positive) or in the cluster of unclassified Synergistetes bacteria (358/511 entries positive), including many non-oral non-human clones. In contrast, the probes designed for subclusters A1 and A2 exhibited a much narrower detection spectrum. SYN-A1-632 detected 2/123 Thermovirga bacteria and 25/511 unclassified Synergistetes bacteria, all from the human oral cavity except for a human skin clone and a few clones from the canine oral cavity, SYN-A2-207 detected only 13 clones, all among the 511 unclassified Synergistetes bacteria and all from the human oral cavity except for a human brain abscess-derived clone and a canine oral clone. Fretibacterium fastidiosum, was detected by
the SYN-A1409 and SYN-A2-207 probes (cluster A and subcluster A2, respectively) but not by the SYN-A1-632 probe (subcluster A1). Subcluster A2 was not detectable with the SYN-A1-632 probe, as there were at least two mismatches, making cross-reactivity between the two subclusters highly unlikely, even under non-stringent conditions. The SYN-B1149 probe detected 13 clones, again all part of the 511 unclassified Synergistetes bacteria, whereas the Jon219 probe detected all Jonquettella clones identified so far.

The 16S rRNA gene phylogenetic tree of the investigated Synergistetes bacterial clusters is provided in Fig. 1. The tree was reconstructed in the ‘tree builder’ function of the RDP (update 29), using the Neighbor weighted neighbour-joining tree building algorithm. All sequences used had a length of ≥1200 bp. The tree contained all species presently described for the various genera within the phylum Synergistetes, represented by their type strain, and the human oral clones described so far.

The final probe concentrations used for FISH were 5 ng µl−1 for Cy3 conjugates and 20 ng µl−1 for FAM conjugates, in the presence of 35% formamide. For hybridization, this solution was added to each well of the glass slide at a volume of 3–4 µl per well and incubated for 4 h at 46 °C and 100% atmospheric humidity. The slides were then transferred into washing buffer [70 mM NaCl, 20 mM Tris/HCl (pH 7.5), 5 mM EDTA, 0.01% SDS] for 30 min at 48 °C, air dried and covered with 50 µl mounting fluid [90% ultrapure glycerol, 10% 1,4-diazabicyclo(2.2.2)octane (25 mg ml−1), pre-dissolved in 10× PBS] and a coverslip. For the quantitative evaluation of these samples stained by FISH, an Olympus BX60 microscope (Olympus Optical) was used. To visualize the stained target cells, the microscope was equipped with phase-contrast optics, an HBO 103 W/2 mercury photo optic lamp (Osram), two filter sets (49004 Un-Mounted ET-Cy3 and 59004 Un-Mounted ET-FITC/TRITC; Chroma Technology) and an Olympus filter U-MNIBA for FAM/FITC fluorescence. Representative direct light and fluorescence images are provided in Fig. 2, where the morphology of the various Synergistetes bacterial clusters can be identified. For further validation of the Synergistetes bacteria-targeted probes, their cross-reactivity was evaluated by FISH against pure bacterial cultures of 18 well-characterized strains of 17 oral species. Strains had been grown beforehand in enriched fluid universal medium, as described previously (Gmüür & Guggenheim, 1983; Züger et al., 2007), harvested after growth to late-exponential phase, washed in 0.9% NaCl, pelleted and stored in a 50:50 mixture of ethanol and water at −20 °C until further use. FISH was carried out as described above. Scoring of the intensity of cell fluorescence was performed as described previously, with only grading of ≥2+ considered as positive (Gmüür et al., 2000). None of the Synergistetes bacteria-specific rRNA probes displayed measurable cross-reactivity with the tested strains (Table 2). Moreover, the morphology of weakly fluorescent cells (due to background fluorescence) did not match that of the Synergistetes bacteria identified in Fig. 2.

For the estimation of bacterial counts, a minimum of ten viewing fields per well were counted at 100× magnification and the total number of positive bacteria was calculated (ml suspension sample)−1, as described previously (Gmüür & Thurnheer, 2002). Microscopic pictures were taken with an Olympus E510 camera and stored electronically.

Statistical analysis. The differences in bacterial counts and prevalence between the two clinical groups were analysed by a Mann–Whitney test and unpaired t-test, respectively. The potential correlation between bacterial numbers and plaque index was investigated by Spearman’s correlation analysis. Differences were considered statistically significant at P<0.05.

RESULTS

Estimation of total eubacterial levels

The total bacterial numbers in the collected plaque samples were estimated using the EUB338 oligonucleotide probe, which identifies most eubacteria. Based on in silico hybridization, this probe was expected to recognize all human oral Synergistetes bacteria. Samples from NUG patients yielded a mean value of 2.3×10^9±1.4×10^8 bacteria, whereas this number was 4.6×10^7±2.0×10^5 in samples from plaque-induced gingivitis patients. This fivefold difference was statistically significant (P<0.0001). A Spearman correlation analysis showed that total eubacterial levels correlated positively with the plaque index (r=0.6872, P<0.0001).

Detection of Synergistetes cluster A bacteria

The presence of Synergistetes cluster A bacteria was further considered in the two groups. This cluster was detected in all samples from both patient groups (Table 3). However, it was found that dental plaque from NUG patients harbored 9.4-fold higher numbers of Synergistetes cluster A bacteria compared with the number found in gingivitis patients (Fig. 3), a difference that was statistically significant (P<0.0001). As this could be simply a result of the higher total bacterial numbers in NUG, the percentage of Synergistetes cluster A bacteria within the total bacterial population was calculated further. It was found that this cluster constituted 0.66±0.80% of the bacterial population in dental plaque from NUG patients and 0.26±0.21% in that from gingivitis patients (Table 3), accounting for a 2.5-fold difference between the two patient groups, which proved to be significant.

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Table 1. rRNA-targeted probe sequences and target taxa

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5’–3’)</th>
<th>Target taxa</th>
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<tbody>
<tr>
<td>EUB338–FAM</td>
<td>GCTGCCCTCCGTCCTAGGAGT</td>
<td>Most eubacteria</td>
</tr>
<tr>
<td>SYN-A1409–FAM</td>
<td>ACACCGGCCTGGGTTGTT</td>
<td>Synergistetes cluster A</td>
</tr>
<tr>
<td>SYN-B1149–Cy3</td>
<td>TCGATGGCAGTCGCGCGG</td>
<td>Synergistetes cluster B</td>
</tr>
<tr>
<td>SYN-A1–632–FAM</td>
<td>GCACCTCAGCTCAACTGCG</td>
<td>Synergistetes cluster A1</td>
</tr>
<tr>
<td>SYN-A2–207–Cy3</td>
<td>CCTTCCTCAGCGCTCCTC</td>
<td>Synergistetes cluster A2</td>
</tr>
<tr>
<td>Jon219–Cy3</td>
<td>CACAAGCTCCTCCATCAG</td>
<td>Jonquettella anthropi</td>
</tr>
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</table>
Quantitative differences within the Synergistetes cluster A bacteria were considered further by analysing the levels of bacteria belonging to subclusters A1 and A2 (Fig. 4). NUG patients exhibited higher numbers of both subclusters compared with gingivitis patients. The numbers of subcluster A1 and A2 bacteria were higher in NUG than in gingivitis by 12.8-fold and 12.9-fold, respectively. However, within each patient group, there were no significant differences between the numbers of bacteria in the two Synergistetes A subclusters.

When the percentages of Synergistetes subcluster A1 and A2 bacteria were considered, these were higher in NUG patients than in gingivitis patients, at levels comparable to the whole cluster A (Table 3). Within the same patient group, subcluster A2 was detected at higher percentages compared with subcluster A1, and accounted for 65 and 77% of the total cluster A population in gingivitis and NUG patients, respectively.

Detection of Synergistetes cluster B bacteria

The prevalence and levels of Synergistetes cluster B bacteria were also investigated. This cluster was detected in 100% of gingivitis and 81% of NUG samples (Table 3), and at 4.8-fold higher levels in NUG patients compared with gingivitis patients (Fig. 5), which did not prove to be
statistically significant ($P=0.285$). This difference appeared to reflect the difference in total bacterial numbers between the two disease groups. Indeed, when the percentage of this cluster B between gingivitis and NUG patients was compared, there was a 1.67-fold difference, which was not statistically significant ($P=0.358$) (Table 3).

Synergistetes cluster B bacteria represented $\leq 0.1\%$ of the total bacterial population and was present in lower numbers than cluster A members. When the numbers of the two clusters were compared, cluster A was 3.9-fold and 7.6-fold higher in gingivitis and NUG patients, respectively. Accordingly, the percentage of cluster A was 4.3-fold and 6.6-fold higher in gingivitis and NUG patients, respectively (Table 3).

**Detection of J. anthropi**

*J. anthropi* was detected at lower numbers (range $10^2$–$10^4$, close to detection limits) and less frequently than the other *Synergistetes* cluster members studied (Table 3). The mean numbers detected were $3.8 \pm 6.4 \times 10^3$ in NUG and $1.0 \pm 0.5 \times 10^3$ in gingivitis. In terms of percentages, *J. anthropi* represented $<0.01\%$ of the total bacterial population in either patient group (Table 3). However, due to the low frequency of detection and the low detection levels, no statistical analysis could be performed.

**DISCUSSION**

The present study investigated comparatively the presence and levels of bacteria belonging to the phylum *Synergistetes* in marginal dental plaque from patients with plaque-induced gingivitis and NUG. The well-defined clusters A and B of this phylum were considered specifically in this study. The data demonstrated that clusters A and B were both highly prevalent in gingivitis and NUG. However, members of *Synergistetes* cluster A were present at higher numbers and in greater proportions in NUG patients compared with gingivitis patients, with subclusters A1 and A2 being equally elevated. Hence, members of *Synergistetes*
Table 2. Absence of cross-reactivity in FISH experiments of the *Synergistetes* bacteria-targeted probes with various cultivable oral strains

Stringency conditions: experiments were performed at 46 °C in the presence of 35% formamide.

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<tbody>
<tr>
<td>Actinomyces oris (OMZ 745; own isolate)</td>
<td>4+</td>
<td>±</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Aggregatibacter actinomyctecomitans serotype b (OMZ 295; JP2)</td>
<td>4+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Aggregatibacter actinomyctecomitans serotype c (OMZ 247; ATCC 33384T)</td>
<td>4+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Aggregatibacter aphrophilus (OMZ 359; HK 322)</td>
<td>4+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Campylobacter rectus (OMZ 698, own isolate)</td>
<td>4+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fusobacterium nucleatum subsp. nucleatum (OMZ 598, F8)</td>
<td>3–4+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lactobacillus salivarius, subsp. salivarius (OMZ 525; ATCC 11741T)</td>
<td>4+</td>
<td>±</td>
<td>–</td>
<td>±</td>
<td>±</td>
<td>–</td>
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<tr>
<td>Lactococcus lactis subsp. cremoris (OMZ 1098; ATCC 19257)</td>
<td>4+</td>
<td>±</td>
<td>–</td>
<td>±</td>
<td>±</td>
<td>–</td>
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<tr>
<td>Leptotrichia buccalis (OMZ 531; ATCC 14201T)</td>
<td>4+</td>
<td>±</td>
<td>–</td>
<td>±</td>
<td>±</td>
<td>–</td>
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<tr>
<td>Porphyromonas gingivalis (OMZ 925; ATCC 33277T)</td>
<td>3–4+</td>
<td>±</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Prevotella intermedia (OMZ 248; own isolate)</td>
<td>3–4+</td>
<td>±</td>
<td>–</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Streptococcus mutans (OMZ 918; ATCC 700610)</td>
<td>4+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Streptococcus oralis (OMZ 607; SK 248)</td>
<td>4+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Streptococcus salivarius (OMZ 36; ATCC 13419)</td>
<td>4+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>Streptococcus sobrinus (OMZ 176; own isolate)</td>
<td>3–4+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>Tannerella forsythia (OMZ 1047; own isolate)</td>
<td>4+</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Treponema denticolta (OMZ 661; ATCC 35405T)</td>
<td>2–3+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Veillonella dispar (OMZ 493; ATCC 17748T)</td>
<td>4+</td>
<td>±</td>
<td>–</td>
<td>±</td>
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</table>

*Scoring of the intensity of cell fluorescence was performed as described previously (Gmürr *et al.*, 2000): –, no fluorescence; +/±, weak intensity; 1+, weak intensity but distinct; 2+, intermediate intensity; 3+, bright; 4+, very bright. In the enumeration of bacterial cells, only grading ≥2+ was counted as positive.
cluster A are more highly associated with NUG than with plaque-induced gingivitis, and subcluster A specificity is not decisive in this context. Interestingly, *Synergistetes* subcluster A2 bacteria were present in higher proportions than subcluster A1, but this applied to both disease groups. Indeed, this difference in relative proportions between the two subclusters was similar in the two disease groups, indicating that the observed abundance of subcluster A2 is not likely to be a crucial aetiological factor for NUG. Although *Synergistetes* cluster B bacteria were detected in higher absolute numbers in NUG patients compared with gingivitis patients, this numerical difference was not commensurate with a difference in their relative proportion within the total eubacterial population. Hence, the lack of quantitative differences between NUG and gingivitis denoted that the presence of *Synergistetes* cluster B bacteria does not constitute a differential aetiological factor between the two disease forms. In the case of the *J. anthropi* taxa, as its frequency and levels of detection were low in both NUG and gingivitis, no safe conclusions can be made about its association with these diseases. However, given the fact that it does not represent a major bacterial population in these samples, it is less likely to be a key aetiological factor for NUG.

To our knowledge, neither the presence of *Synergistetes* bacteria in NUG nor the relative comparison with plaque-induced gingivitis has been previously studied. However, it should be noted that, apart from the identification of *Synergistetes* in endodontic lesions (Machado de Oliveira et al., 2007; Siqueira & Rôças, 2007; Vianna et al., 2007; Vartoukian et al., 2009), there is also evidence on the association of members of this phylum with periodontitis (Vartoukian et al., 2009; Zijnge et al., 2010). *Synergistetes* bacteria are reported to constitute 0.1 % of the dental plaque flora of periodontally healthy sites or shallow periodontal pockets, but this percentage increases above 1 % as the pockets become deeper (Kumar et al., 2005; de Lillo et al., 2006). Sites with periodontal disease foster more diverse numbers of *Synergistetes* bacterial clones than healthy ones, whereas cluster A bacteria predominate over those of cluster B (Vartoukian et al., 2009). Collectively, these studies indicate that *Synergistetes* bacteria are more prevalent in patients with periodontal diseases than in healthy people. This trait is well in line with the low percentages of *Synergistetes* bacteria reported in this study, in which the samples were obtained from sites with a

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**Table 3. Prevalence of *Synergistetes* bacteria among subjects with gingivitis and NUG and percentage (mean ± st) of *Synergistetes* bacteria in the dental plaque of subjects with gingivitis and NUG**

The numerical values corresponding to 100 % (total eubacterial levels) were $2.3 \times 10^8$ and $4.6 \times 10^7$ organisms in NUG and gingivitis, respectively, against which the percentages of the different *Synergistetes* were calculated together with the fold differences between the two groups.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Prevalence of bacteria</th>
<th>Percentage of bacteria in dental plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gingivitis</td>
<td>NUG</td>
</tr>
<tr>
<td><em>Synergistetes</em> A</td>
<td>21/21 (100 %)</td>
<td>21/21 (100 %)</td>
</tr>
<tr>
<td><em>Synergistetes</em> A1</td>
<td>21/21 (100 %)</td>
<td>21/21 (100 %)</td>
</tr>
<tr>
<td><em>Synergistetes</em> A2</td>
<td>21/21 (100 %)</td>
<td>21/21 (100 %)</td>
</tr>
<tr>
<td><em>Synergistetes</em> B</td>
<td>21/21 (100 %)</td>
<td>17/21 (81.0 %)</td>
</tr>
<tr>
<td><em>J. anthropi</em></td>
<td>12/21 (57.1 %)</td>
<td>10/21 (47.6 %)</td>
</tr>
</tbody>
</table>

*The difference between the two patient groups was statistically significant ($P<0.05$).
shallow gingival sulcus (Gmür et al., 2004), as well as the increase of cluster A bacterial levels according to disease severity (i.e. NUG in the present case).

Little is known about the association of oral Synergistetes bacteria with other taxa implicated in periodontal diseases. To this extent, the present samples have been characterized previously for various other taxa. Higher proportions of fusiforms were identified in gingivitis, but higher proportions of spirochaetes (i.e. treponemes) were found in NUG (Gmür et al., 2004). *Fusobacterium nucleatum* subsp. *polymorphum* and *Fusobacterium periodonticum* were among the most prevalent fusobacteria (Gmür et al., 2006). A fraction of these samples was also analysed for the presence of the putative periodontal pathogen *T. forsythia* and associated *Tannerella* phyotypes. These were highly prevalent in both gingivitis and NUG patients but at rather low densities, potentially excluding any strong association with these forms of periodontal disease (Züger et al., 2007).

Taken together, although not studied concomitantly, there may be an association between the presence of spirochaetes and *Synergistetes* bacteria in NUG. Interestingly, the percentage of treponemes was reported to be twofold higher in NUG than in gingivitis (Gmür et al., 2004), in close agreement with the 2.5-fold difference in *Synergistetes* cluster A bacteria observed in the present study (Table 3). A comparable relationship with *Synergistetes* bacteria may also exist for *Porphyromonas gingivalis* and *Actinomyces gerencseriae*, which were detected in ≥95% of NUG samples but only one (5%) and three (14%) gingivitis samples, respectively (Gmür et al., 2004).

In conclusion, *Synergistetes* cluster A, but not cluster B, bacteria are represented in higher numbers and proportions in dental plaque from NUG patients than from plaque-induced gingivitis patients. These findings imply that cluster A bacteria of the phylum *Synergistetes* may be more strongly implicated as an aetiological factor for NUG than for plaque-induced gingivitis.

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**REFERENCES**


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