Quantitative analysis of classical and new putative periodontal pathogens in subgingival biofilm: a case–control study


Background and Objectives: A number of species/phylotypes have been newly implicated as putative periopathogens. The objective of this study was to explore associations among classical and new pathogens in subgingival biofilm and to assess their relative importance to chronic periodontitis.

Material and Methods: Pooled subgingival biofilm samples were obtained from 40 patients with chronic periodontitis and 40 healthy controls. Taqman q-PCR assays were used to determine the absolute and relative counts of Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Parvimonas micra, Filifactor alocis, oral Synergistetes and oral TM7s. Microbial associations were assessed using cluster analysis. Different statistical models were used to explore associations between microbial parameters and periodontitis.

Results: The median log and relative counts were lowest for TM7s (4.4 and 0.0016%, respectively) and highest for oral Synergistetes (7.2 and 1.4%, respectively). Oral Synergistetes clustered strongly with the red complex, particularly T. forsythia (100% rescaled similarity). All species/phylotypes except TM7s were significantly associated with periodontitis (Mann-Whitney test; \( p \leq 0.005 \)). However, P. gingivalis and F. alocis lost association after adjusting for confounders (ordinal regression). In receiving operator characteristic curve analysis, the log counts of oral Synergistetes were the best markers of periodontitis (82.5% sensitivity and specificity), followed by those of T. forsythia, P. micra and T. denticola. In prediction analysis, however, P. micra was the only microbial predictor of periodontal parameters.

Conclusions: Oral Synergistetes are presented here as new members of the red complex, with relative importance to periodontitis exceeding that of the classical members. P. micra is shown as an important periodontal pathogen warranting more attention.

Chronic periodontitis is characterized by the destruction of periodontal soft and hard tissues, resulting in progressive attachment loss, tooth mobility and, in severe cases, tooth loss. The tissue damage is mediated by destructive host immune responses believed to be orchestrated by a team of periodontal pathogens in subgingival
biofilm (1). What particular bacterial species constitute this team, however, has been subject to extensive research over the last five decades or so. Based on a modification of Koch’s postulates, findings from earlier studies employing cultivation-based and immunological techniques as well as experimental animals were used to identify a number of putative periodontal pathogens (2). Later application of the DNA-DNA checkerboard hybridization technique to screen tens of thousands of plaque samples did not only substantiate evidence for the etiological role of these putative pathogens, but also demonstrated that they coexist as complexes in subgingival biofilm (3).

By late 1990s, Porphyromonas gingivalis, Tannerella forsythia (previously Bacteroides forsythus) and Treponema denticola, the so-called red complex, became well established periodontal pathogens (3). Other recognized, but less important pathogens were members of the so-called orange complex, including Parvimonas micra (previously Peptostreptococcus micros), Fusobacterium nucleatum, Prevotella intermedia, Campylobacter rectus and Eubacterium nodatum. Since then, these species have been recognized as classical periodontal pathogens and their association with periodontitis has been documented in populations from different parts of the world using different techniques (4).

The last decade or so, however, has witnessed a paradigm shift towards a metagenomic approach to studying microbial composition of dental biofilm, employing open-ended molecular techniques such as clonal analysis (5–7) and, more recently, second-generation sequencing (or massive parallel sequencing) (8,9). This approach has uncovered the presence of a major uncultivable component in dental plaque constituting about 50% of its microbiota. This has not only doubled the richness of subgingival biofilm, which is currently estimated at 700 species (10), but also identified a number of new putative periodontal pathogens. These include uncultivable Treponemae (11) and oral phylotypes of the new bacterial phyla Synergistetes and TM7 (7,8,12). In addition, known species not recognized for long as putative periodontal pathogens, has been recently detected in association with chronic periodontitis with these molecular techniques; the main example being Filifactor alocis, which was first isolated in 1985 (6,7,13).

There is currently growing interest in exploring these new putative pathogens and their role in periodontitis, particularly oral Synergistetes (14,15), which now have cultivable members, including Fretilbacterium fastidiosum, Jonquetella anthropl and Pyramidobacter piscolens (16–18). However, there have been so far no attempts to assess their association with classical pathogens in subgingival biofilm and how this could relate to periodontal health status. The objective of this study was, therefore, to explore associations among classical and new putative pathogens in subgingival biofilm and to assess their relative importance to chronic periodontitis.

Material and methods

Study subjects, history and clinical examination

Forty patients with chronic periodontitis and 40 healthy controls were recruited at Al-thawra General Hospital and a private dental center in Sana’a City, Yemen. Subjects had to be 30–60 years old and have at least 20 remaining teeth. Exclusion criteria included previous periodontal treatment, history of antibiotic intake in the last 3 mo and any condition/disease known to modify subgingival microbial composition such as pregnancy, lactation, contraceptive pills intake and diabetes mellitus. History about oral habits, including cigarette smoking and qat chewing was also obtained.

Periodontal status of potential study subjects was assessed by a single examiner (A.A.) using the WHO’s community periodontal index (CPI) and clinical attachment loss (19). Plaque index (20) was recorded on index teeth. Chronic periodontitis was defined as having at least one index tooth per quadrant with pocket depth of ≥ 5 mm (CPI score 3; 3.5–5.5 band on the WHO probe invisible). Subjects having no CPI score > 2 were included as healthy controls. Written informed consent to participate in the study was obtained from each study subject.

Sample collection and DNA extraction

Subgingival plaque was sampled using sterile paper points (size 40; Megadenta, Radeberg, Germany), after removal of supragingival plaque with sterile cotton pellets. In each subject, the sample was pooled from four sites (the deepest pocket in each quadrant in the cases and one healthy site per quadrant in the controls) and stored in TE buffer at −20°C for later laboratory analysis (within 3 mo).

Before DNA extraction, the samples were thawed and centrifuged at 15,000 g for 1 min; the resultant pellet from each sample was then suspended in 180 μl lysozyme digestion buffer (25 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, 1% Triton X-100) containing 20 mg/mL lysozyme, and incubated at 37°C overnight. Finally, DNA was purified from the digest using the Purelink Genomic DNA extraction kit (Invitrogen, Carlsbad, CA, USA), eluted in 100 μl of the buffer supplied, and stored at −80°C.

Taqman q-PCR assays

Total bacteria, four classical pathogens (the three red complex species and one orange complex species), and three newly suspected pathogenic species/phylotypes (Table 1) were quantified in the samples using Taqman q-PCR assays (21). The primers and probes (sequences shown in Table 1) were designed in collaboration with Primerdesign, a UK-based company specialized in development and manufacturing of q-PCR kits. The primers/probe sets for oral Synergistetes and TM7 were designed to provide maximum coverage of reference 16S rRNA gene sequences of each phylum in the Human Oral Microbiome Database (HOMD; http://www.homd.org/index.php). Coverage of the primers/probe sets for
both taxa is described as a footnote to Table 1.

The specificity of primers/probe sets was assessed by blasting their sequences against reference oral bacterial 16S rRNA gene sequences in HOMD as well as the eubacterial sequence database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LOC=BlastHome). In the latter case, one phylum was taken at a time, limiting blasting to database sequences obtained from human oral samples while keeping the default settings of the primer stringency.

The q-PCR reaction setup and amplification were performed as previously described (22). Each reaction comprised 10 μL Precision Mastermix (Primerdesign, Southampton, UK), 1 μL primers/probe mix, 4 μL water, and 5 μL DNA sample. Runs were performed on an ABI 7000 real-time PCR platform (Applied Biosystem, Foster City, CA, USA) using the following amplification program: an initial enzyme activation step at 95°C for 10 min, and 40 cycles of a denaturation step at 95°C for 15 s, and an annealing/extension step at 60°C for 1 min. Each run included a negative template control. The plasmid-based positive controls provided with the kits were used for construction of standard curves for absolute quantification (Fig. S1). Relative counts were calculated by normalizing absolute counts of test species/phylotypes to total bacterial counts (22).

### Statistical analysis

Microbiological data included log-transformed absolute counts and relative counts (% total bacteria) of each of the tested species/phylotypes. The data were non-normally distributed (Kolmogorov–Smirnov statistic), so were summarized as medians and interquartile ranges. Detection rates were reported as percentages (prevalence). The Pearson correlation and squared Euclidean distance were calculated for each pair of the tested species/phylotypes based on relative counts. The resultant similarity coefficients, rescaled to 0–100%, were then used for clustering using average link- age. The significance of differences in absolute and relative counts between cases and controls was first sought using the Mann–Whitney test, applying Bonferroni’s correction for multiple comparisons (eight comparisons for absolute counts and seven for relative counts). It was then further challenged by ordinal logistic regression (OLR) to adjust for clinical confounders (age, sex, oral habits and mean plaque index), also correcting for multiple comparisons; the complementary log-log and negative-negative log functions were used for absolute and relative counts, respectively. Both the Pearson and deviance statistics were used to confirm goodness-of-fit of the OLR models. Receiving operator characteristic (ROC) curve analysis of microbial counts was performed to assess their usefulness as markers of periodontitis. Clinical and microbial predictors of chronic periodontitis

<table>
<thead>
<tr>
<th>Test species</th>
<th>Sequences 5’–3’</th>
<th>Target gene</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total bacteria</strong></td>
<td>F-primer: AAACCTAAAGGATGACGGGGG&lt;br&gt;R-primer: TTGGCTTGGTGCCGGAATC&lt;br&gt;Probe: FAM-CTGCGTCGTCGTCGTA-BHQ</td>
<td>16S rRNA</td>
<td>205</td>
<td>(22)</td>
</tr>
<tr>
<td><strong>P. gingivalis</strong></td>
<td>F-primer: ACAGATCAAGGTGCTAAGTT&lt;br&gt;R-primer: TTAGTCCTTTCATATTTAC&lt;br&gt;Probe: FAM-CCTGCTGTTCCAAATGTTGTTAATACG-BHQ</td>
<td>fimA</td>
<td>85</td>
<td>(22)</td>
</tr>
<tr>
<td><strong>T. forsythia</strong></td>
<td>F-primer: GATAGGCTTAACACATGCAAGTC&lt;br&gt;R-primer: GTTGCGGGCAGGTTACATAC&lt;br&gt;Probe: FAM-TTACTACCCCGGCTCGGCAGTGA-BHQ</td>
<td>16S rRNA</td>
<td>99</td>
<td>(22)</td>
</tr>
<tr>
<td><strong>T. denticola</strong></td>
<td>F-primer: GGCATCGGCTGAAATAATRATG&lt;br&gt;R-primer: CTCGGTGACAGGTTACATAC&lt;br&gt;Probe: FAM-CAGGCCTCTTGTAATATATATGACGAGAGTTG-BHQ</td>
<td>16S rRNA</td>
<td>98</td>
<td>This study</td>
</tr>
<tr>
<td><strong>P. micra</strong></td>
<td>F-primer: TGGAGCAACTTACCTACGACAG&lt;br&gt;R-primer: GCCCTTCTACCCGATAAAC&lt;br&gt;Probe: FAM-ACCGCCTCTAGACACGACGACA-BHQ</td>
<td>16S rRNA</td>
<td>112</td>
<td>(22)</td>
</tr>
<tr>
<td><strong>Oral Synergistetes</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F-primer: GGAAGTACGGGTCCCAAGATTG&lt;br&gt;R-primer: GTAAGGTCTCCGTGTTTGCATC&lt;br&gt;Probe: FAM-ACCGCATGAGACACGACGACGACT-BHQ</td>
<td>16S rRNA</td>
<td>98</td>
<td>This study</td>
</tr>
<tr>
<td><strong>F. alocis</strong></td>
<td>F-primer: ACCTCAAGTGGCAAACATTTAT&lt;br&gt;R-primer: TACTCCCTTCTCTCCTGTTAATAC&lt;br&gt;Probe: FAM-GAAATCTTACGTCTTCTCTACG-GTTTAATATATATGACGAGAGTTG-BHQ</td>
<td>16S rRNA</td>
<td>101</td>
<td>(22)</td>
</tr>
<tr>
<td><strong>Oral TM7s</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F-primer: GCCTGGTGCTGAGATGTATT&lt;br&gt;R-primer: ATGCCCTCCCTCTCCTGCGG&lt;br&gt;Probe: FAMTAAGTCCACATCAACGAGACGACGACGACGACT-BHQ</td>
<td>16S rRNA</td>
<td>107</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>The primers/probe set covers *Fretibacterium fastidiosum*, *Fretibacterium* sp. oral taxons 358, 359, 360, 361, 362, 452 and 453, *Jonquetella anthropi* and *Pyramidobacter piscolis*.

<sup>b</sup>The primers/probe set covers TM7 oral taxons 347, 348, 350, 351, 355, 356 (I025) and 437.
(case vs. control) and periodontal indices (CPI and clinical attachment loss) were identified using logistic regression and OLR analyses, respectively. All statistical analyses were performed using srs version 20 (IBM, New York, NY, USA).

Results

General findings

The clinical features of the included cases and controls are shown in Table 2. The cases were significantly older, included significantly more smokers and had a significantly higher mean plaque index (MPI).

All q-PCR assays achieved excellent linearity ($R^2 > 99.6\%$) and a detection limit of 5–10 copies per reaction (Fig. S1); i.e., 100–200 copies per sample, assuming a DNA extraction efficiency of 100%. Blasting of primer pairs for the five tested species against NCBI returned no hits other than the intended targets, meaning that at least one primer of each pair had at least two 3’ or six mismatches to unintended targets. For oral Synergistetes and TM7s, blasting returned unintended targets from some phyla; however, at least either primer had at least one middle and one 3’ mismatch or 3’ nt 1 mismatch to ensure no or, at least, much lower amplification efficiency (23,24). In addition, probes also had enough mismatches to compromise detection of non-specific amplification (Table S1).

The detection rate was 100% for all species/phylotypes except P. gingivalis, T. denticola and oral TM7s that were detected in 71.2, 91.2 and 95% of the samples, respectively. The overall log and relative counts are presented in Fig. 1. The median log total bacterial count was 9.17 copies per sample. For individual species/phylotypes, the median log and relative counts ranged 4.38–7.18 DNA copies/sample and 0.0016–1.44%, respectively, being lowest for oral TM7s and highest for oral Synergistetes. All species/phylotypes were detected at a relative count of $<1\%$ in 75% of the samples except oral Synergistetes and F. alocis. The former exceeded 1% in 52% of the samples and 5% in 25% of them, reaching as far as 11.5% in outliers, while the latter was detected at $>1\%$ in 30% of the samples and at $>2\%$ in 15% of them, approaching 10% in outliers.

Associations among classical and new putative pathogens

The output of cluster analysis based on rescaled Pearson correlation coefficients is shown in Fig. 2. As expected, a red complex, including the three classical members, P. gingivalis, T. forsythia and T. denticola was formed. However, oral Synergistetes linked with T. forsythia at a rescaled similarity of 100%, joining the complex at shorter distance than P. gingivalis and T. denticola. Oral TM7s joined the orange complex member P. micra at a rescaled similarity of ~60%. The same clusters formed using squared Euclidean distance, although the rescaled similarities were smaller.

Microbial parameters in cases vs. controls

The absolute counts of test species/phylotypes in the cases and controls are summarized in Table 3. Using Mann–Whitney test, all taxa were detected at significantly higher levels in the cases than in the controls with the exception of TM7 that did not withstand correction for multiple comparisons ($p \leq 0.0063$). However, P. gingivalis and F. alocis failed to maintain significant association with periodontitis after adjustment for confounders using ordinal regression. Age accounted for much of the variation between the cases and controls for these two species. Similar findings were seen for the relative counts (Table 4). Based on significance level and effect estimate, oral Synergistetes showed the strongest association with periodontitis.

Prediction analysis

The output of predictive logistic regression is shown in Table 5. Only age and MPI were identified as predictors of periodontitis (model 1); however, when MPI was excluded on the assumption of being an intermediate factor, gender and the relative counts of oral Synergistetes and P. micra showed as additional predictors.
The odds ratio for the latter was extremely large (975). In the predictive OLR model (Table 6), the relative counts of \textit{P. micra} along with age and MPI were identified as predictors of both mean CPI and clinical attachment loss. Qat chewing was an additional predictor of clinical attachment loss.

**Discussion**

With the increasing number of reports implicating new putative pathogens, such as oral Synergistetes, oral TM7s and \textit{F. alocis} in the etiology of chronic periodontitis (14,15,25,26), it is becoming evident that scientists have missed, for quite long time, important members of the pathogenic subgingival consortium that may be as, or even more, important than the classical members. The current work is probably the first attempt to explore associations among the classical and new putative pathogens in subgingival biofilm and to compare directly their relative importance to chronic periodontitis. The test panel included members of the red complex and \textit{P. micra} as classical pathogens and oral Synergistetes, oral TM7s and \textit{F. alocis} as new putative periodontal pathogens. \textit{P. micra} was particularly chosen as a representative of the orange complex because it has been recently shown to have strong association with chronic periodontitis in a sample from the same population (27).

The study subjects were recruited as part of a larger epidemiological study in which periodontal health status was assessed using CPI, which is the main limitation of the present study (28). While some of CPI’s original limitations have been overcome by including clinical attachment loss measurements, the index remains inferior to full mouth measurement in assessing prevalence and extent of periodontitis (29). In addition, bleeding on probing in the index is given a score of 2, so it does not show in subjects with scores \( \geq 3 \). Bleeding on probing was not recorded as a separate clinical parameter, which is another limitation of the study.

The 16S rRNA gene copy number per genome varies from one species to another and is not known for some. Subsequently, the counts reported for some taxa may be an overestimation.

![Fig. 1. Box plots showing the overall median and interquartile range of log absolute (left) and relative (right) counts of the tested taxa in subgingival biofilm samples. The error bars represent data within 1.5 interquartile range above Q3 and below Q1. Circles and stars are outliers.](image1)

![Fig. 2. A dendrogram showing microbial clustering in subgingival biofilm. Pearson correlation coefficient was calculated for every pair of the tested taxa; the resultant similarities were then rescaled to 0–100% and used for clustering using average linkage. The coloring refers to the red and orange complexes described by Socransky et al. (3).](image2)
of their actual counts in the samples. Therefore, the absolute counts were reported in DNA copies rather than bacterial cell numbers. This, anyway, does not influence the validity of comparisons between subjects. For *P. gingivalis*, the assay was based on the fimA gene, which is known to be species-specific and has in fact been

<table>
<thead>
<tr>
<th>Species</th>
<th>Controls (n = 40)</th>
<th>Cases (n = 40)</th>
<th>$p_1^a$</th>
<th>$p_2^b$</th>
<th>B $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>9.01 (8.70–9.30)</td>
<td>9.20 (9.04–9.24)</td>
<td>NS</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td><em>P. gingivalis</em></td>
<td>4.50 (3.23–6.37)</td>
<td>6.88 (3.07–7.48)</td>
<td>&lt; 0.001</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td><em>T. forsythia</em></td>
<td>5.33 (4.44–6.23)</td>
<td>6.98 (6.55–7.16)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>1.80</td>
</tr>
<tr>
<td><em>T. denticola</em></td>
<td>4.96 (2.74–6.01)</td>
<td>6.79 (6.19–7.19)</td>
<td>&lt; 0.001</td>
<td>0.002</td>
<td>1.60</td>
</tr>
<tr>
<td><em>P. micra</em></td>
<td>5.51 (5.02–6.09)</td>
<td>6.58 (6.20–6.77)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>1.64</td>
</tr>
<tr>
<td><em>F. alocis</em></td>
<td>5.17 (3.70–6.86)</td>
<td>7.15 (6.53–7.56)</td>
<td>&lt; 0.001</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>Oral Synergistetes</td>
<td>6.16 (5.45–6.99)</td>
<td>7.93 (7.54–8.07)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>1.97</td>
</tr>
<tr>
<td>Oral TM7</td>
<td>4.07 (3.52–4.56)</td>
<td>4.62 (4.07–5.09)</td>
<td>NS</td>
<td>0.003</td>
<td>0.72</td>
</tr>
</tbody>
</table>

NS, not significant.
a$^a$ Value by Mann–Whitney test; significance at 0.0063 adjusting for multiple comparisons.
b$^b$ Value by ordinal logistic regression adjusting for demographic variables, oral habits and mean plaque index; significance at 0.0063 adjusting for multiple comparisons. Pearson and deviance statistics not significant (model well-fitted). Ordinal logistic regression rather than linear regression was used because the dependent variable was non-normally distributed.
cEffect estimate based on regression analysis; reported for significant differences only.

<table>
<thead>
<tr>
<th>Species</th>
<th>Controls (n = 40)</th>
<th>Cases (n = 40)</th>
<th>$P_1^a$</th>
<th>$P_2^b$</th>
<th>B $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. gingivalis</em></td>
<td>6.7E-03 (1.1E-04–4.7E-01)</td>
<td>3.8E-01 (1.2E-04–1.3E+00)</td>
<td>0.001</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td><em>T. forsythia</em></td>
<td>1.4E-02 (3.1E-03–2.8E-01)</td>
<td>3.8E-01 (1.9E-01–7.6E-01)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>1.17</td>
</tr>
<tr>
<td><em>T. denticola</em></td>
<td>1.0E-02 (6.2E-05–2.6E-01)</td>
<td>2.4E-01 (8.7E-02–6.5E-01)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>1.28</td>
</tr>
<tr>
<td><em>P. micra</em></td>
<td>3.4E-02 (1.1E-02–1.1E-01)</td>
<td>1.3E-01 (1.0E-01–3.5E-01)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>1.22</td>
</tr>
<tr>
<td><em>F. alocis</em></td>
<td>1.9E-02 (3.1E-04–9.2E-01)</td>
<td>5.2E-01 (2.6E-01–1.5E+00)</td>
<td>0.005</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>Oral Synergistetes</td>
<td>1.9E-01 (2.8E-02–1.1E+00)</td>
<td>4.1E+00 (1.7E+00–6.2E+00)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>1.47</td>
</tr>
<tr>
<td>Oral TM7</td>
<td>9.9E-04 (2.4E-04–4.7E-03)</td>
<td>2.0E-03 (4.5E-04–5.8E-03)</td>
<td>NS</td>
<td>NS</td>
<td>–</td>
</tr>
</tbody>
</table>

NS, not significant.
a$^a$ Value by Mann–Whitney test; significance at 0.007 adjusting for multiple comparisons.
b$^b$ Value by ordinal logistic regression adjusting for demographic variables and oral habits and mean plaque index; significance at 0.007 adjusting for multiple comparisons. Pearson and deviance statistics not significant (model well-fitted). Ordinal logistic regression rather than linear regression was used because the dependent variable was non-normally distributed.
cEffect estimate based on regression analysis; reported for significant differences only.

Fig. 3. Receiving operator characteristic curve analysis of microbial log absolute counts (left) and relative counts (right) as markers of periodontal health status. The area under the curve is shown next to the name of each species/phylotypes in the legend (absolute and relative count respectively).
previously used for quantification of the bacterium (30). The q-PCR assays used allowed detection of as low as 100 DNA copies of each of the tested taxa per sample, which is largely consistent with the sensitivity reported for the assay in similar studies (31–34). Counts of each taxa were normalized to total bacterial counts (relative quantification) to adjust for variations in sampling and thus improve reliability of comparisons between samples (31,35). However, specificity of the primers/probe sets was confirmed by in silico analysis alone, which is one aspect of limitation of the current study. Frequently, specificity is also checked by direct testing of the primers/probe against a panel of clinical strains from different phyla, which was not feasible in the current study. Another limitation was that the primers/probe set for TM7s, due to designing difficulties, covered only seven of the 12 clones in the HOMD; either primer had a single 3’ mismatch with five of TM7 genus 1. However, care was taken to include clone I025, which has been partially linked to periodontitis.

Almost the same amount of total bacterial DNA was recovered from the cases and controls, which initially sounds counterintuitive because deep pockets are supposed to harbor more bacteria than healthy gingival sulci. However, it must be noted that there is a limit to the amount of DNA that silica mini-columns, such as the ones used in the study, can bind. According to the manufacturer’s manual, this is about $10^9$ bacterial DNA copies. Therefore, column saturation probably explains the lack of difference between total bacterial DNA recovered from the cases and controls. This can, however, be considered as an advantage as it represents a form of normalization, i.e., the same amount of total DNA is extracted from all samples. This probably explains why there were almost no differences in the results obtained from the analysis of absolute and relative counts.

The relative counts of the classical pathogens hardly exceeded 1%, substantiating evidence that they represent a minority of subgingival bacteria (6,36,37). Oral TM7s, however, were detected at the lowest abundance representing 0.0016% of total bacteria on average. A previous study using a similar approach detected subgingival TM7s at an average relative count of 0.3%, which while still demonstrates a low abundance, is about 180 times higher (25). One possible explanation for this difference is that the current assay did not cover all oral TM7s. In addition, there could be ethnic differences in carriage of oral TM7s as demonstrated for other periodontal bacteria (38). *F. alocis* tended to be present at higher proportions, exceeding 2% in 15% of the samples. This is somewhat consistent with findings from clonal analysis studies in which *F. alocis* was found to represent 1.5–3% of all clones (6,12). Notably, however, oral Synergistetes constituted considerably higher proportions of total bacteria, exceeding 5% in 25% of the samples and reaching 11% in outliers, which is in line with the findings by Vartoukian et al. (14) who found, using fluorescent in situ hybridization, that Synergistetes formed 3–11% of total bacteria in subgingival biofilm.

For the first time, oral Synergistetes are reported here to cluster strongly with the red complex. In fact, they were the first to join the cluster by linking to *T. forsythia* at 100 rescaled similarity, indicating that they may be more important members of the red complex than *P. gingivalis* and *T. denticola*; the latter two joined the complex at distances comparable to those originally reported by Socransky et al. (3). Oral Synergistetes are divided into clusters A and B (39); the former includes species of the new

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**Table 5. Independent predictors of periodontitis: multiple logistic regression model**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>$p$</th>
<th>Odds ratio</th>
<th>Pseudo $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.064</td>
<td>1.41</td>
<td>0.707</td>
</tr>
<tr>
<td>Mean plaque index</td>
<td>0.011</td>
<td>2.6E+4</td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>&lt; 0.001</td>
<td>1.92</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>0.022</td>
<td>17.9</td>
<td></td>
</tr>
<tr>
<td><em>P. micra</em> relative count</td>
<td>0.009</td>
<td>975</td>
<td></td>
</tr>
<tr>
<td>Synergistetes relative count</td>
<td>0.059</td>
<td>1.48</td>
<td></td>
</tr>
</tbody>
</table>

*Disease status (case/control) as dependent variable and age, gender, oral habits, mean plaque index and relative counts as independent variables.

According to Cox and Snell (46).

Mean plaque index was excluded with the assumption of being an intermediate factor.

Males compared to females.

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**Table 6. Independent predictors of mean CPI and CAL: multiple ordinal logistic regression model**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>$p$</th>
<th>Effect estimate</th>
<th>Pseudo $R^2$</th>
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</thead>
<tbody>
<tr>
<td>Mean CPI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>&lt; 0.001</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Mean plaque index</td>
<td>&lt; 0.001</td>
<td>2.23</td>
<td></td>
</tr>
<tr>
<td><em>P. micra</em> relative count</td>
<td>&lt; 0.001</td>
<td>2.32</td>
<td></td>
</tr>
<tr>
<td>Mean CAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>&lt; 0.001</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Mean plaque index</td>
<td>&lt; 0.001</td>
<td>2.35</td>
<td></td>
</tr>
<tr>
<td><em>P. micra</em> relative count</td>
<td>&lt; 0.001</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>Qat chewing status</td>
<td>&lt; 0.001</td>
<td>1.48</td>
<td></td>
</tr>
</tbody>
</table>

CPI, clinical periodontal index; CAL, community periodontal index.

*Mean CPI or CAL as dependent variable and age, gender, oral habits, mean plaque index and relative counts as independent variables. Pearson and deviance statistics not significant (model well-fitted). Ordinal logistic regression rather than linear regression was used because the dependent variable was non-normally distributed.

According to Cox and Snell (46).
genus *Fretibacterium*, including *F. fastidiosum* (18), while the latter include *J. anthropi* and *P. piscolens* (16–18). As cluster B has been previously shown to be much less frequently detected (14), it is likely that cluster A is involved in the interaction with the red complex described here. TM7s linked with *P. micra* at a rescaled similarity of 60% suggesting it may be a member of the orange complex, but this needs to be explored further including other complex members. *F. alocis* did not show association with any of the tested taxa. In fact, *F. alocis* has been recently shown to be inhibited by *P. gingivalis*, and this could explain its failure to cluster with the red complex (40).

Differences in microbial counts between periodontitis and healthy subjects is usually sought using the Mann–Whitney test (Kruskal–Wallis for more than two groups) with correction for multiple comparisons. Using this statistical model, the classical pathogens, including *P. micra* were found to be significantly associated with periodontitis, as expected. The differences for *F. alocis* and oral Synergistetes were also significant, which is consistent with recent findings in the literature (14,26). Indeed, *F. alocis* has been found recently to possess virulence factors making it capable of inducing periodontal destruction (41,42). As an exception, oral TM7s did not show significant association with the disease; however, this is still in line with a previous study in which oral TM7s were shown to be associated with mild periodontitis only (25).

What is frequently missed by investigators, however, is that differences in microbial parameters between cases and control may be explained by variables other than the disease status itself. Therefore, we also challenged the differences using ordinal regression, adjusting for potential confounders. With this model, *F. alocis* and *P. gingivalis* lost association with periodontitis, with age explaining much of the variation between the cases and controls. In a previous study on a sample from the same population, *P. gingivalis* also did not show association with periodontitis (27), suggesting that this species may be less important in populations from South Arabia. In fact, subgingival microbial profile associated with periodontitis seems to differ significantly by geographical location independent of other factors known to modify subgingival microbial composition. For example, the relative abundances of major periodontal pathogens in subgingival biofilm have been shown to differ significantly among chronic periodontitis populations from different countries (38). Furthermore, different strains of *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* with variable virulence have been identified in different parts of the world. These findings indicate that the relative importance of periodontal pathogens in periodontitis varies among ethnic groups, which is probably related to host tropism (adaptation of specific bacterial strains to certain host genetic lineage) rather than geographical differences per se (4).

On the other hand, oral Synergistetes *T. forsythia*, *P. micra* and *T. denticola*, maintained highly significant differences, withstanding both adjustment for confounders and correction for multiple comparisons. The log counts of the same four taxa were also identified as good markers of periodontitis using ROC curve analysis. However, oral Synergistetes showed the highest effect estimate in the OLR analysis and the best sensitivity and specificity in ROC curve analysis, suggesting they may be playing a more important role in the disease than the classical pathogens. These findings substantiate the rapidly growing evidence for their strong association with periodontitis. In fact, certain Synergistetes, such as *F. fastidiosum* (15) and OTU 4.2 (14), has already been implicated in the etiology of the disease, and may soon occupy the forefront as putative periodontal pathogens. Even with the statistical adjustment for confounders, there still remains another limitation: the microbial parameters in the analyses above are treated as dependent variables and the disease parameters as independent (explanatory), which is not correct from a strict statistical point of view. We, therefore, performed predictive modeling with disease status or periodontal indices (CPI and clinical attachment loss) as dependent variables and the demographic variables, MPI, oral habits and microbial relative counts as independent (predictive) variables. Age and MPI were found to be the only predictors of periodontitis; however, when MPI was removed, *P. micra* and oral Synergistetes popped up as predictors of the disease. In the ordinal regression model, the relative counts of *P. micra* were found to be predictors of CPI and clinical attachment loss independent of age and MPI. Recently, *P. micra* has been shown to have the strongest association with periodontitis in a sample from the same ethnicity (27). A number of other recent studies have also identified it as a major pathogen in periodontitis (43,44).

The current findings support the existing view that periodontitis is orchestrated by a bacterial consortium rather than by a single pathogen (1). Members of the subgingival pathogenic “team” probably vary in their importance and some of them may be substitutable. For example, *P. gingivalis* showed lower association with periodontitis in the current study, and one plausible explanation is that its role was taken over by other pathogens in a subset of the patients. The findings also demonstrate that all putative pathogens are present in low abundance at health and that an increase in their proportions to a certain “threshold” is required for triggering periodontitis, which is compatible with the ecological plaque hypothesis (45). What ecological factors drive the pathogenic microbial shifts in subgingival biofilm are not yet elucidated but an increased subgingival plaque mass (high plaque index) can be one such factor. In fact, MPI in the current study masked the effect of the tested putative pathogens in the logistic regression analysis (Table 5) suggesting it may well be an intermediate factor.

In conclusion, oral Synergistetes are presented here as new members of the red complex, with relative importance to periodontitis exceeding that of the
classical members. Further work to identify which specific Synergistetes are involved is warranted. On the other hand, only P. micra showed up as a predictor of periodontal health parameters, which, along with findings from other recent studies, suggests that the role of this bacterium in periodontitis should be revisited.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Standard curves of the quantitative PCR assays.
Table S1. In silico analysis of oral synergistetes and TM7 primers/probe sets specificity.

References

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