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# Differences in the periodontal microbiome of successfully treated and persistent aggressive periodontitis

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## Abstract

**Aims:** The primary aim of this investigation was to analyse the periodontal microbiome in patients with aggressive periodontitis (AgP) following treatment.

**Methods:** Sixty-six AgP patients were recalled on average 7 years after completion of active periodontal treatment and had subgingival plaque samples collected and processed for 16S rRNA gene sequencing analyses.

**Results:** Of 66 participants, 52 showed persistent periodontal disease, while 13 participants were considered as "successfully treated AgP" (no probing pocket depths >4 mm) and 1 was fully edentulous. Genera associated with persistent generalized disease included *Actinomyces*, *Alloprevotella*, *Capnocytophaga*, *Filifactor*, *Fretibacterium*, *Fusobacterium*, *Leptotrichia*, *Mogibacterium*, *Saccharibacteria [G-1]*, *Selenomonas and Treponema*. "Successfully treated" patients harboured higher proportions of *Haemophilus*, *Rothia*, *and Lautropia* and of *Corynebacterium*, *Streptococcus* and *Peptidiphaga* genera. Overall, patients with persistent generalized AgP (GAgP) revealed higher alpha diversity compared to persistent localized AgP (LAgP) and stable patients ($p < .001$). Beta diversity analyses revealed significant differences only between stable and persistent GAgP groups ($p = .004$).

**Conclusion:** Patients with persistent AgP showed a more dysbiotic subgingival biofilm than those who have been successfully treated. It remains to be established whether such differences were predisposing to disease activity or were a result of a dysbiotic change associated with disease recurrence in the presence of sub-standard supportive periodontal therapy or other patient-related factors.

KEYWORDS

aggressive periodontitis, dysbiosis, microbiome, supportive periodontal therapy

## 1 | INTRODUCTION

Aggressive periodontitis (AgP) was introduced as a disease entity by the 1999 World Workshop classification (Lang et al., 1999) to define a specific condition characterized by rapid disease progression in otherwise systemically healthy patients with a positive family history of periodontal disease. Among secondary features, elevated proportions of bacteria such as *Aggregatibacter*

*actinomycetemcomitans* and *Porphyromonas gingivalis* were considered typical in some populations. In particular, an association between *A. actinomycetemcomitans* and AgP had been suggested in a large study in the United States using culture and immunofluorescence serotyping (Zambon, Christersson, & Slots, 1983) and confirmed by studies observing treatment response (Mandell, Ebersole, & Socransky, 1987) and antibody response (Albandar, DeNardin, Adesanya, Diehl, & Winn, 2001; Ebersole & Cappelli, 1994). The *JP2* clone of *A. actinomycetemcomitans* has been consistently associated with onset and progression of periodontal attachment loss in children and adolescents in North and West Africa (Haubek et al., 2008; Höglund et al., 2014) as well as in the United States (Fine et al., 2007). However, the great majority of comparative studies suggest that the microbial differences between AgP and Chronic Periodontitis are so limited (Lafaurie et al., 2007; Nibali et al., 2012; Riep et al., 2009) that they are no longer considered different disease entities (Tonetti, Greenwell, & Kornman, 2018); a conclusion supported also by evidence from other pathogenicity aspects (Tonetti et al., 2018).

The introduction of molecular techniques suggested a role for non-cultivable microbes as *Filifactor alocis*, *Centipeda* genus, *Mitsuokella* sp., *Selenomonas* genus, *Actinobacter baumannii* in the pathogenesis of periodontitis (Goncalves et al., 2012; Schlafer et al., 2010; Schulz et al., 2019) depicting a more variegated picture than previously thought, in line with the concept of polymicrobial synergy and dysbiosis (Hajishengallis & Lamont, 2014). However, the role of these putative periodontopathogenic bacteria has not been fully elucidated yet, and little is known about their potential to instigate periodontal disease recurrence during maintenance therapy.

The primary aim of this investigation was to analyse the periodontal microbiome in previously treated patients with AgP and to associate it with the presence of active periodontal disease or health. We also investigated the relationship between the AgP microbiome composition and association between regular supportive periodontal therapy (SPT), tooth loss and patient-related factors, such as oral hygiene, smoking and body mass index.

**Clinical Relevance**

*Scientific rationale for the study*: The level of dysbiosis associated with aggressive periodontitis disease recurrence is currently unknown.

*Principle findings*: Higher microbial diversity was detected in active versus stable aggressive periodontitis cases. Elevated differences at both genus and species levels were detected, mainly between persistent GAgP and successfully treated group. Overweight and irregular interdental brushing are associated with dysbiosis.

*Practical implications*: Increased dysbiosis, oral hygiene and obesity should be controlled to avoid dysbiosis-associated periodontal disease recurrence.

## 2 | MATERIALS AND METHODS

### 2.1 | Patient population

The study design and population have been previously described (Dopico, Nibali, & Donos, 2016). Briefly, patients seen in a dedicated AgP clinic were contacted and asked to participate in the study. Eighty-two patients were contactable of the original 330 with AgP, and 66 agreed to participate, gave informed consent and completed the study visit to the Eastman Dental Hospital (EDH), in London. The study was conducted in line with the principles outlined in the Declaration of Helsinki (2008) on experimentation involving human participants. Ethics approval for the conduct of the study was granted by the NRES Committee London—Queen Square (ref 13/LO/0874).

Inclusion criteria were (a) previous diagnosis of AgP—in the dedicated AgP clinic at the EDH between 1997 and 2011, and (b) age between 16 and 65 at recruitment. Exclusion criteria were (a) known infectious diseases such as Tuberculosis, HIV or hepatitis C, (b) comorbidities that would make it unsafe for the patient to travel or to undergo periodontal assessment and (c) pregnancy. Following initial diagnosis, patients had received active periodontal therapy (APT) consisting of oral hygiene instructions, non-surgical treatment, adjunctive antibiotics and/or surgical treatment according to clinical needs (Table 1). Following completion of APT, patients had a full periodontal assessment and were then discharged to their general dental practitioner for SPT.

At the study visit, following consent, patients were interviewed to obtain self-reported medical, dental and smoking histories. Participants' height and weight were measured, and body mass index (BMI) calculated. Patients were classified as "normal weight" if their BMI was <25 kg/m$^2$, "overweight" if their BMI was 25–29 kg/m$^2$ and "obese" if their BMI was ≥30 kg/m$^2$. Clinical data were collected at 6 sites per tooth by one calibrated examiner (JD) including full-mouth dichotomous plaque score (FMPS) (Guerrero et al., 2005), gingival index (Löe, 1967), tooth mobility (Laster, Laudenbach, & Stoller, 1975), tooth loss, furcation involvement (Hamp, Nyman, & Lindhe, 1975), probing pocket depth (PPD), gingival recession (distance from the cemento-enamel junction to the free gingival margin) and full-mouth dichotomous bleeding on probing score (FMBS, percentage of bleeding surfaces upon probing). Clinical attachment level (CAL) was measured as PPD plus recession.

### 2.2 | Clinical diagnosis

At the study visit, the following diagnoses could be assigned:

- *Persistent AgP*: in case of interproximal PPD and CAL ≥5 mm on at least two permanent teeth. Subdivided into either LAgP (at least one affected first molar or incisor, and no more than

**TABLE 1** Study population characteristics*

| | Frequency | Percentages |
|---|---|---|
| Gender | | |
| Male | 27 | 41 |
| Female | 39 | 59 |
| Systemic conditions | | |
| Diabetes mellitus | 2 | 3 |
| Cardiovascular disease | 5 | 8 |
| Overweight + Obese | 38 | 58 |
| Rheumatoid arthritis | 2 | 3 |
| Race/ethnicity | | |
| White | 39 | 59 |
| Asian | 7 | 11 |
| Black African/Caribbean | 17 | 26 |
| Other | 3 | 5 |
| Smoking status | | |
| Smoker | 8 | 12 |
| Non-smoker | 58 | 88 |
| Diagnosis | | |
| Successfully treated | 13 | 20 |
| LAgP | 8 | 12 |
| GAgP | 44 | 67 |
| Fully edentulous | 1 | 1 |
| Disease progression | | |
| Tooth loss (+) | 33 | 50 |
| Tooth loss (–) | 33 | 50 |
| | Mean ± SD (Range) | |
| Number of teeth | | |
| First visit | 26.3 ± 2.0 (21–28) | |
| Post-APT | 24.3 ± 4.6 (11–28) | |
| Last visit | 21.9 ± 6.5 (0–28) | |

Abbreviations: AB, antibiotics; APT, active periodontal therapy; IB, interdental brushes.

*Included participants for microbiome analyses.

three teeth other than first molars or incisors), or GAgP (affecting at least three permanent teeth other than first molars and incisors).

- *Successfully treated AgP*: no teeth with PPD ≥5 mm.

We defined "quality of SPT" during the follow-up period based on hospital records and patient interview as (a) no maintenance, (b) suboptimal or (c) optimal. Optimal maintenance was defined as those programs with at least three recall visits/year including oral hygiene advice and support, supra- and subgingival debridement and charting. Any SPT program, which did not fulfil the criteria for being considered optimal, was considered suboptimal. "No maintenance" was defined for patients who received no maintenance care at all after APT (Dopico et al., 2016).

## 2.3 | Subgingival plaque sampling

For every patient, samples from each quadrant were taken from the disto-buccal surfaces of all first molars with sterile curettes and pooled together. In case of absence of any of these teeth, the neighbouring teeth were chosen. The supragingival portion of the root surface of the site was carefully cleaned with a curette. With the area isolated from saliva (gentle air spray or cotton rolls), a sterile curette was inserted to the bottom of the pocket. After a single stroke, each microbiological sample was extracted from the pocket and then immediately placed into 1 ml of reduced transport fluid and stored at −80°C for future 16S rRNA gene sequencing analysis.

## 2.4 | Microbiological analysis

A site-based sub-analysis comparison of the periodontal and peri-implant microbiomes of 18 patients included in this study, reporting the microbiological techniques employed, has previously been published (Sousa et al., 2017). Briefly, plaque samples were processed for DNA extraction by bead and beating technique and then for sequencing analysis using the Illumina platform (MiSeq Desktop Sequencer; Reagent Kit v2; Illumina Inc., San Diego, CA, US). The PCR mixtures were prepared as described (Sousa et al., 2017). The products from the PCR were then quantified, purified and combined in equimolar ratios, to create a DNA library for 16S rRNA sequencing. The 16S rRNA gene fragments were sequenced by NGS (MiSeq Desktop Sequencer; Reagent Kit v2; Illumina Inc., San Diego, CA, US). DNA was extracted (Griffiths, Whiteley, O'Donnell, & Bailey, 2000) followed by individual PCR reactions for each sample in order to amplify the V5-V7 hyper-variable region with barcoded primers 785F (F 5′-GGATTAGATACCCBRGTAGTC-3′) and 1175R (R 5′-ACGTCRTCCCCDCCTTCCTC-3′).

## 2.5 | Statistical and bioinformatic analyses

A convenience sample was chosen for this study, based on the number of patients able to attend a re-evaluation appointment. Anonymized clinical data from all patients who took part in the study were entered into a database, proofread for entry errors and analysed by a statistical package (IBM SPSS [25.0]). Continuous variables are reported as means, percentages and standard deviations. The patient was the unit of analysis. Patients were grouped by disease activity (diagnosis of "persistent GAgP," "persistent LAgP" or "successfully treated AgP"). Further exploratory analyses were carried out by grouping patients by tooth loss during the follow-up period ("at least 1 tooth lost" vs. "none"), smoking status (current or not), BMI ("normal weight" versus. "overweight or obese") and interdental brushing ("daily" or "not").

Sequencing data were processed using QIIME 2 (Version 2018.11, https://qiime2.org/) (Bolyen et al., 2019). Raw sequences

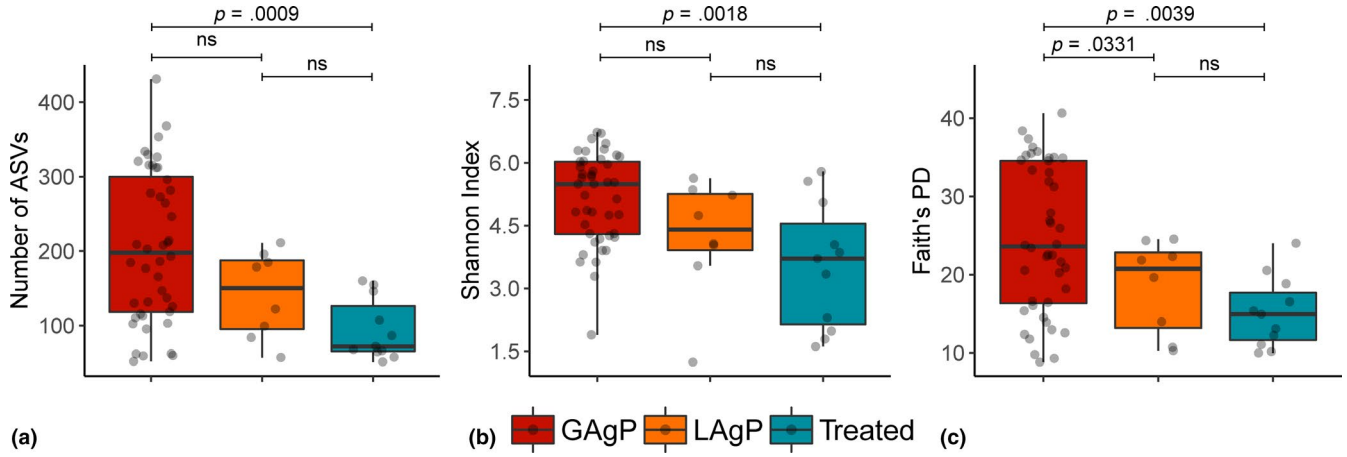


**FIGURE 1** Alpha diversity metrics boxplot diagrams of (a) number of ASVs of GAgP, LAgP and "successfully treated" groups, (b) Shannon index of GAgP, LAgP and "successfully treated" groups, (c) Faiths's PD of GAgP, LAgP and "successfully treated" groups. Alpha diversity is significantly ($p < .0018$) decreased in the "successfully treated" group

belonging to 65 samples were demultiplexed with q2-demux and then denoised using DADA2 plugin with default parameters to create Amplicon Sequence Variants (ASVs) (Callahan, McMurdie, & Holmes, 2017; Callahan et al., 2016). Two samples (TM1 and T63) were removed from the resulting feature table due to having extremely low number of sequences (<232 sequences). Alpha diversity metrics were calculated on the unrarefied feature table using native q2-diversity plugin and included Number of ASVs, Shannon index and Faith's phylogenetic diversity. For beta diversity, the feature table was rarefied at 2,477 sequences and used to calculate Aitchison (compositional) and weighted UniFrac (quantitative, accounts for phylogenetic information) distances (Aitchison, Barceló-Vidal, Martín-Fernández, & Pawlowsky-Glahn, 2000; Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011). These distance matrices were then fed to principal coordinate analysis to investigate clustering patterns based on the metadata groupings. A taxonomic classifier optimized for V5–V7 region of the 16S rRNA gene from the extended human oral microbiome database (HOMD extended, https://tinyurl.com/HOMD-Extended) was trained using q2-feature-classifier, and taxonomy was assigned using this classifier to ASVs at >85% confidence (Bokulich et al., 2018). With the unrarefied feature table, q2-gneiss plugin was used to determine taxonomic associations between healthy and disease samples at genus and species level (Morton et al., 2017). For this, a hierarchical tree was generated based on co-occurrence correlations among taxonomic units, followed by partitioning using simplicial linear regression accommodating available clinical data. From the top three balances created, y0 and y2 had the highest coverage of the taxonomic tree, and so were used to identify taxonomic associations. Furthermore, the unrarefied feature table was transferred to QIIME 1 platform to generate groupwise (health/disease, daily interdental brushing, body weight) taxonomic summaries using a *summarize taxa through plots.py* function (Caporaso et al., 2010). The data resulting from the analyses mentioned above were exported from QIIME 2 environment, and respective plots were generated in R v.3.5.0 using ggplot2 package v.3.1.0 (R Core Team, 2018; Wickham, 2016).

The statistical significance of the alpha diversity metrics, and the y0 and y2 balances among the persistent and stable periodontitis groups were tested using Mann–Whitney *U* test, and $p \leq .05$ was accepted as significant. For microbial profiles, statistical significance at phylum, genus and species level between GAgP, LAgP and successfully treated groups were tested using ALDEx2 package in R. Wilcoxon signed-rank test combined with Benjamini-Hochberg correction was reported, and $p < .05$ was accepted as significant (Fernandes, Macklaim, Linn, Reid, & Gloor, 2013; Fernandes et al., 2014). The clustering of these groups in beta diversity analyses was tested using groupwise PERMANOVA (999 permutations), and $p < .05$ was accepted as significant.

## 3 | RESULTS

Demographic characteristics of the 66 patients included in the study have been previously reported (Dopico et al., 2016). Relevant current study population characteristics are included in Table 1. Forty-nine patients had been diagnosed with GAgP and 17 as LAgP at first examination. Based on the current classification (Papapanou et al., 2018; Tonetti et al., 2018), 42 patients would have been diagnosed as generalized stage III grade C, 7 patients as generalized stage IV grade C and 17 as molar–incisor pattern stage III grade C at first examination. At the study visit, two patients reported diagnosis of diabetes mellitus, five reported cardiovascular disease and two reported rheumatoid arthritis—all of these were new diagnoses during the follow-up period. The majority of patients (58%) were Caucasians. The mean time in SPT was 6.97 years (95% CI 6.25–7.69, range 2–11 years). At the study visit, a total of 8 participants (12%) were current smokers. A large proportion of participants was overweight ($n = 20$, 31%) or obese ($n = 18$, 28%). Twenty-four percent of

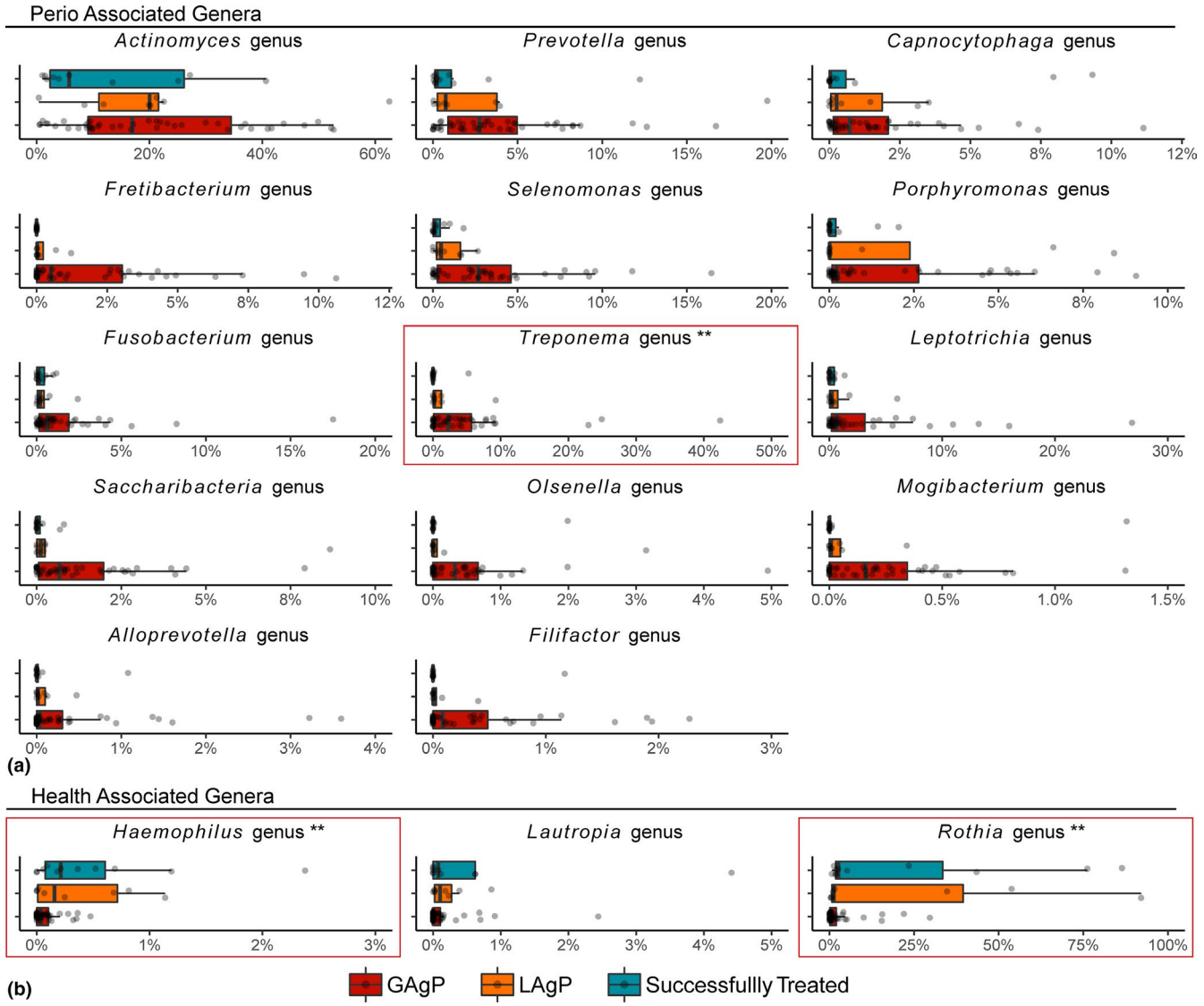


**FIGURE 2** Boxplot diagrams show the taxonomic analysis of periodontal disease (a) and health (b)-associated Genera by group: persistent GAgP (generalized aggressive periodontitis), persistent LAgP (localized aggressive periodontitis) and successfully treated (stable). Statistically signigificant genus was highlighted in red squares

patients underwent what we defined as "optimal" maintenance during the study follow-up. Half of the patients ($n$ = 33) lost at least one tooth during the follow-up period. Thirteen participants (20%) were diagnosed as "successfully treated," 52 (79%) were diagnosed as having persistent AgP (GAgP: $n$ = 44; LAgP $n$ = 8), while one was edentulous (Table 1). The mean PPD of all sampled sites was 4.06 mm (*SD* ± 0.86 mm), and the mean PPD in the deepest sampled pocket per patient was 5.26 mm (*SD* ± 1.75 mm).

## 3.1 | Community characterization

The alpha diversity measures of richness, evenness and phylogenetic divestity indicated clear differences appearing between the three groups. Overall, persistent GAgP samples revealed higher microbial richness and diversity compared to the other two groups (Figure 1). The mean number of ASVs in the GAgP group was 205 ± 101, in LAgP 141 ± 58, and in successfully treated group was 94 ± 41 (Figure 1a).

## 3.2 | Phylum level analysis

Samples taken from patients with "persistent AgP" (localized and generalized) had higher relative abundances of Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes, Fusobacteria, Synergistetes and Saccharibacteria phyla compared with patients with stable periodontitis (see Figure S1). Moreover, the Chloroflexi and Gracilibacteria phyla appeared in higher proportions in the GAgP group sample in comparison with the LAgP and "successfully treated" groups. The distribution of phyla appears to share similarities in the richness between samples across disease groups, with Actinobacteria and Firmicutes as dominant phyla.

However, a variety of Gram-negative phyla, such as Bacteroidetes and Fusobacteria, appears with higher proportions in the "persistent AgP" group. The obese group exhibited increases in Fusobacteria and Spirochaetes phyla when compared to the normal weight group.

### 3.3 | Genus and species level analysis

Two balances, y0 and y2, with the higest coverage of the taxonomic tree, showed significant differences between the GAgP and successfully treated groups. The underlying taxa from these balances at genus and species level were extracted (Figure S2). At genus level, GAgP patients had higher proportions of Gram-negative obligate anaerobes (putative periodontal pathogens) and of *Actinomyces*, *Alloprevotella*, *Capnocytophaga*, *Filifactor*, *Fretibacterium*, *Fusobacterium*, *Leptotrichia*, *Mogibacterium*, *Saccharibacteria [G-1]*, *Selenomonas*, *Porphyromonas* and *Treponema* genera (Figure 2), whereas LAgP patients presented higher proportions of *Rothia*, *Neisseria*, *Dialister* and *Saccharibacteria (TM7) [G-5]*. In the "successfully treated" group, there were higher proportions of *Haemophilus*, *Rothia* and *Lautropia* (Figure 3), and of *Corynebacterium*, *Streptococcus* and *Peptidiphaga* genera (see supplemental material 2 and 3).

At species level, persistent periodontitis was associated with periodontal bacteria including *Capnocytophaga granulosa* [HMT 325], *Actinomyces oris* [HMT 893], *Solobacterium moorei* [HMT 678], *Actinomyces naeslundii* [HMT 176], *Actinomyces odontolyticus* [HMT 701], *Fusobacterium nucleatum*, *F. alocis* [HMT 539], *Treponema socranskii* [HMT 769], *Treponema denticola* [HMT 584], *Leptotrichia wadei* [HMT 222], *Treponema* sp. [HMT 237] and *Selenomonas sputigena* [HMT 151] (Figure 3a). In comparison with sites from patients with

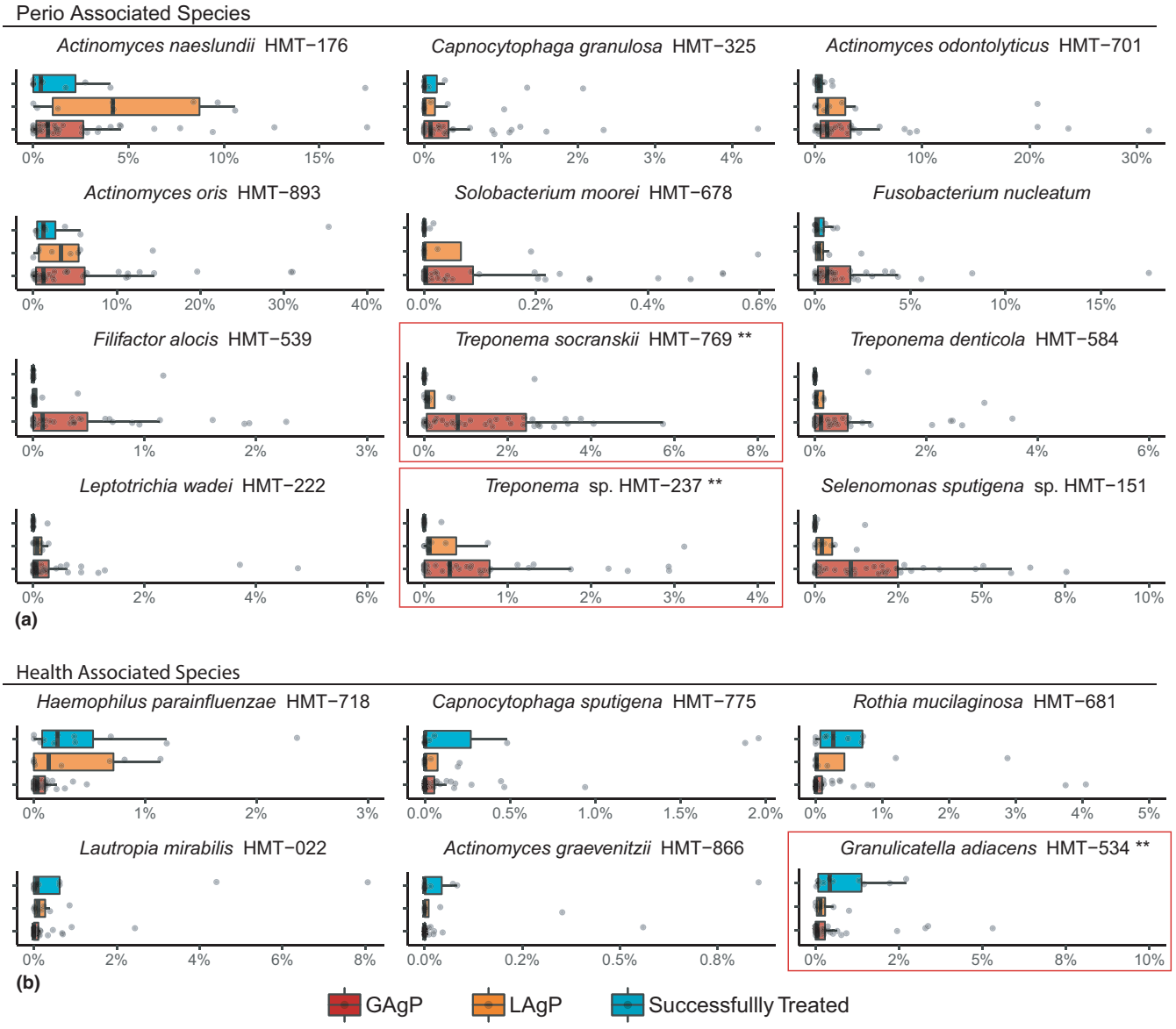


**FIGURE 3** Boxplot diagrams show the taxonomic analysis of periodontally periodontal disease (a) and health (b)-associated species by group: persistent GAgP (generalized aggressive periodontitis), persistent LAgP (localized aggressive periodontitis) and successfully treated (stable). Statistically signigificant species was highlighted in red squares

persistent GAgP, sites from successfully treated patients exhibited higher levels of *Actinomyces graevenitzii* [HMT 866], *Haemophilus parainfluenzae* [HMT 718], *Capnocytophaga sputigena* [HMT 775], *Rothia mucilaginosa* [HMT 681] and *Lautropia mirabilis* [HMT 022] (Figure 3b).

Notably, ALDEx2 testing did not reveal any statistically significant association between the LAgP and stable periodontitis at genus and species level analysis. However, *Treponema* genus ($p$ = .013) was found significantly higher abundance in the GAgP group, and *Rothia* ($p$ = .012) and Haemophillus ($p$ = .032) genera were in higher abundance in the successfully treated group. Similarly, at species level, *Treponema* sp. [HMT 237] ($p$ = .044) and *Treponema socranskii* [HMT 769] ($p$ = .038) were significantly higher in the GAgP group, while *Granulicatella adiacens* [HMT 534] ($p$ = .040) was significantly higher in the successfully treated group. In agreement with this, significant differences in beta diversity were detected between successfully treated and GAgP groups ($p$ = .004), but not between the LAgP and successfully treated groups ($p$ > .172) (Figure 4).

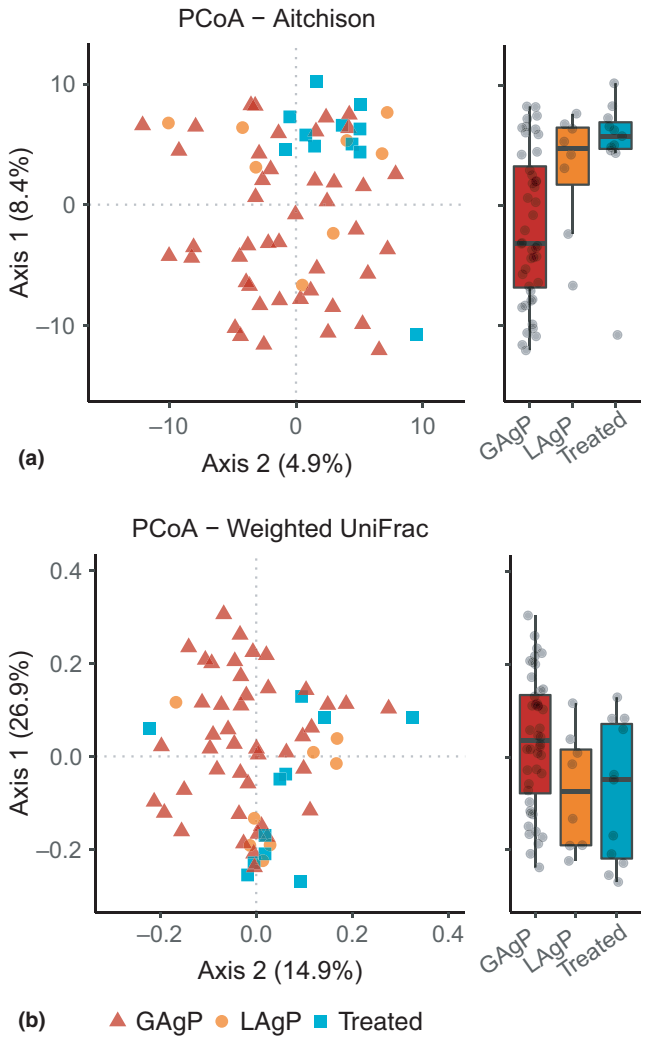


**FIGURE 4** Beta diversity: Principal coordinate analysis (PCoA) with distance matrices (a) PCoA Aitchison and (b) PCoA Weighted UniFrac, showing clustering of successfully treated versus persistent GAgP versus persistent LAgP. Only GAgP versus successfully treated resulted in significant differences. PERMANOVA of the Aitchison distances, $p$ = .004; of the Weighted UniFrac distances, $p$ = .013

## 3.4 | Analysis of patient factors associated with diversity

### 3.4.1 | Smoking

Higher proportions of *Corynebacterium*, *Gemella* and *Bacteroidales [G-2]* genera were observed in the non-smoker group, whereas the smoker group exhibited higher genera *Actinomyces*, *Prevotella*, *Porphyromonas*, *Neisseria*, *Clostridiales* and *Fretibacterium* (Figures S4 and S5). However, no statistically significant changes in alpha diversity were detected in smokers versus non-smokers ($p$ = .381).

### 3.4.2 | Tooth loss

No significant differences in alpha diversity were observed when comparing patients who had lost teeth during the study follow-up to patients who had not lost teeth ($p$ = .320).

### 3.4.3 | BMI

Statistically significant difference ($p$ = .042) in alpha diversity was observed comparing patients by BMI (overweight/obese vs. normal weight). Overweight/obese patients had higher proportions of *Actinomyces*, *Streptococcus*, *Selenomonas*, *Treponema*, *Leptotrichia*, *Veillonella*, *Capnocytophaga*, *Actinomycetaceae* and *Clostridiales* (Figures S4 and S5).

### 3.4.4 | Interdental cleaning

Statistically significant difference ($p$ < .001) in alpha diversity was observed between patients using daily interdental brushing in comparison with irregular interdental brushing. Patients using daily interdental brushing had higher proportions of *Actinomyces*, *Streptococcus*, *Corynebacterium*, *Rothia*, *Peptidiphaga* and *Veillonella* (Figures S4 and S5).

## 4 | DISCUSSION

This study explored the subgingival microbiome of patients affected by AgP, following an average of 7 years of SPT. It is important to emphasize that the majority of patients had not received what we defined as “satisfactory SPT” and 80% of them still had signs of ongoing or persistent disease, measured as multiple persisting sites with concomitant PPD and CAL ≥ 5 mm. Furthermore,

50% of patients lost at least one tooth during the SPT period, with an average tooth loss of 0.27 teeth/patient/year. We previously showed that having received surgical treatment, the performance of interproximal cleaning and shallower PPDs at start of SPT were found to be associated with lower annual tooth loss rates (Dopico et al., 2016). These results are in line with a recent study that reported an association between greater PPD reduction and frequent SPT recall visits in long-term maintenance periodontal patients (Müller Campanile et al., 2019). Diabetes mellitus, obesity and smoking have also been reported as risk factors influencing the recurrence of number of sites with deep PPDs, positive BOP and tooth loss (Kim, Choi, Kim, Jeong, & Lee, 2019; Müller Campanile et al., 2019).

This study shows, for the first time, clear differences in the subgingival microbiota between patients with persistent GAgP and patients considered "successfully treated." Patients with ongoing disease had a more diverse microbiota, compatible with increased dysbiosis, in agreement with previous studies which seem to suggest that increased microbial diversity at subgingival level is associated with disease (Cui, Liu, Xiao, Chu, & Ouyang, 2019; Dabdoub, Tsigarida, & Kumar, 2013; Sousa et al., 2017). More specifically, different bacterial species can be mapped according to the material and methods employed (Schulz et al., 2019) and these differences warrant further investigation.

Analysis by phyla revealed that *Chloroflexi* and *Gracilibacteria* were elevated in the persistent GAgP group when compared to the persistent LAgP and successfully treated groups, in agreement with the notion that Gram-negative bacteria are often elevated in periodontitis and particularly refractory periodontitis cases (Paster et al., 2001). The persistent GAgP-associated genera included *Actinomyces*, *Alloprevotella*, *Capnocytophaga*, *Filifactor*, *Fretibacterium*, *Fusobacterium*, *Leptotrichia*, *Mogibacterium*, *Saccharibacteria [G-1]*, *Selenomonas* and *Treponema*, while the "successfully treated" associated genera included *Haemophilus*, *Rothia* and *Lautropia*.

*Rothia*, a Gram-positive, aerobic, rod-shaped and non-motile bacterial genus from the phylum *Actinobacteria* was the dominant genus. Several other studies using next generation sequencing have previously shown a consistent association between increased *Rothia* levels and periodontal health compared with chronic periodontitis (Abusleme et al., 2013; Camelo-Castillo, Novoa, et al., 2015; Ikeda et al., 2019; Kirst et al., 2015). The latter study on 25 chronic periodontitis patients and 25 controls identified a disease-associated cluster driven by *Fusobacterium* and *Porphyromonas* and a health-associated cluster dominated by *Rothia* and *Streptococcus*, accompanied by alterations of the predicted functional capabilities of the periodontitis microbiome (Kirst et al., 2015). This finding is also in concordance with studies showing an increase in the relative abundance of *Rothia* following periodontal treatment and suggesting that *Rothia* is associated with therapeutic success in chronic and AgP (Belstrøm et al., 2018; Bizzarro et al., 2016; Colombo et al., 2012; Hagenfeld et al., 2018; Han, Wang, & Ge, 2017; Jünemann et al., 2012). The latter study in particular showed that successful periodontal treatment resulted in significantly higher relative abundance of *Streptococcus*, *Rothia* and *Actinomyces* in combination with a significant decrease in *Porphyromonas* and *Treponema* in subgingival plaque samples, demonstrating different shifts in relative abundance of health- and disease-associated bacteria following treatment (Belstrøm et al., 2018).

It was noteworthy that the *Saccharibacteria (TM7)* phylum was reduced in successfully treated participants compared with persistent AgP cases. This is in agreement with a microbiological analysis of chronic periodontitis patients, showing an increase in relative abundance of *Saccharibacteria* in periodontitis sites compared to healthy sites (Brinig, Lepp, Ouverney, Armitage, & Relman, 2003). Another microbiological study estimated the increase in the relative abundance of *Saccharibacteria* to be threefold higher in smokers compared to non-smokers (Camelo-Castillo, Mira, et al., 2015).

At the bacterial species level, persistent AgP was associated with some recognized periodontopathogenic bacteria such as *Fusobacterium nucleatum*, *F. alocis* and *Treponema denticola* as well as a series of potentially pathogenic bacteria (see Figure 3a), which is in agreement with a recent study on GAgP (Schulz et al., 2019). Another 16S study on 15 AgP and 15 healthy subjects identified species such as *F. alocis*, *Desulfobulbus* sp., *Fretibacterium* sp., *Porphyromonas gingivalis*, *Tannerella forsythia*, *Porphyromonas endodontalis*, *Peptostreptococcaceae* spp., *Parvimonas micra*, *Eubacterium nodatum* and *Eubacterium saphenum* as increased in AgP cases (Cui et al., 2019). In contrast, sites from successfully treated patients in the present study exhibited higher levels of *A. graevenitzii*, *C. sputigena*, *Rothia mucilaginosa*, *H. parainfluenzae and L. mirabilis* (Figure 3b). Interestingly, two of these bacteria (*H. parainfluenzae and L. mirabilis*) were also the most abundant in healthy children when compared with AgP (Shaddox et al., 2012), while *C. sputigena* has previously been associated with good response to periodontal treatment (Colombo et al., 2012). *Lautropia mirabilis* has also been found elevated in healthy subjects compared with chronic periodontitis patients in Japan (Ikeda et al., 2019). However, we should also point out that some bacteria normally associated with periodontal health, such as *A. oris*, *A. naeslundii and A. odontolyticus* (Abusleme et al., 2013), were also elevated in patients with persistent disease, which is difficult to explain, perhaps as a result of the study design and/or the analytic technique used.

Cigarette smoking has been associated with periodontal dysbiosis, regardless of periodontal phenotypes, possibly through alterations in the function of key periodontal pathogens (Hanioka et al., 2019). Although no statistically significant differences in alpha diversity were detected in the present study according to smoking status, non-smokers had an overall higher proportion of Gram-positive bacteria such as *Corynebacterium* and *Gemella* genera, while smokers had a tendency to a higher proportion of Gram-negative genera such as *Prevotella*, *Porphyromonas*, *Neisseria* and *Fretibacterium*. Another interesting finding in the present study is that the presence of the *Synergistetes* phylum (consisting of Gram-negative anaerobes) was increased in the smokers group compared to non-smokers. *Synergistetes* have

been implicated in necrotizing ulcerative gingivitis as well as in chronic periodontitis (Vartoukian, Palmer, & Wade, 2010), suggesting that smoking may predispose to a more pathogenic subgingival microbiota.

It is very important to note that daily interdental brushing was associated with a "healthier" subgingival microbiota, characterized by reduced diversity and comprising of higher proportions of *Actinomyces*, *Streptococcus*, *Corynebacterium*, *Rothia*, *Peptidiphaga* and *Veillonella*. This is not surprising, owing to the importance of oral hygiene in maintaining a healthy microbiota (Zhang et al., 2019). Interestingly, a large proportion of patients included in this study were overweight or obese. These conditions showed evidence of association with shifts in microbial communities subgingivally, measured as increased diversity. This is in agreement with a study showing an association between BMI and number of PPDs ≥4 mm and BOP in long-term maintenance patients (Müller Campanile et al., 2019). However, it is in contrast with an inverse correlation between BMI and diversity in subgingival plaque in diabetic patients (Tam et al. 2018). The association between obesity and subgingival microbiota mirrors the observation that obesity may predispose to gut dysbiosis, as observed in studies showing increased Firmicutes to Bacteroides ratio in obese individuals (Ley, 2010). Recently, the theory of an "obesogenic" microbiota was proposed, on the basis of experiments showing that bacteria from obese mice can be transferred to a germ-free recipient, resulting in increased adiposity as compared to transfer of a "lean" microbiota from lean mice (reviewed by Fak, 2016). However, we have to acknowledge a relatively small sample in the present study and possible residual confounding, as socio-economics factors were not accounted for.

A strength of this study is the comparison of the microbiota in a relatively large group of AgP patients with and without disease progression over a long follow-up period (7 years). Drawbacks are absence of microbial data at baseline and different degrees and intensity of SPT regimes and the relative abundance of unclassified data in the results. Harvesting a single pooled subgingival sample per patient from standardized sites (rather than necessarily the deepest pockets) also representes a limitation, as it may have missed dysbiotic plaque and pathogenic taxa present in deeper pockets. This can be avoided by the use of whole genome sequencing, which is relatively expensive compared to the technique used in the current investigation (16S rRNA gene sequencing).

Overall, this study suggests that subgingival dysbiosis is associated with progression of periodontitis and that factors such as poor oral hygiene and obesity may have a negative impact on the composition of the subgingival microbiota.

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## CONFLICTS OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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