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Functional profile of oral plaque microbiome: Further insight into the bidirectional relationship between type 2 diabetes and periodontitis

Nicoletta Favale[1] | Roberto Farina[2,3] | Alberto Carrieri[1] | Anna Simonelli[2,3] | Mattia Severi[2,3] | Silvia Sabbioni[4] | Leonardo Trombelli[2,3] | Chiara Scapoli[1]

[1]Department of Life Sciences and Biotechnology – Section of Biology and Evolution, University of Ferrara, Ferrara, Italy

[2]Research Centre for the Study of Periodontal and Peri-Implant Diseases, University of Ferrara, Ferrara, Italy

[3]Operative Unit of Dentistry, Azienda Unità Sanitaria Locale (A.U.S.L.), Ferrara, Italy

[4]Department of Life Sciences and Biotechnology – Section of Pathology and Applied Microbiology, University of Ferrara, Ferrara, Italy

Correspondence
Chiara Scapoli, Department of Life Sciences and Biotechnology – Section of Biology and Evolution, University of Ferrara, Ferrara, Italy.
Email: scc@unife.it

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Abstract

Increasing evidence support the association between the oral microbiome and human systemic diseases. This association may be attributed to the ability of many oral microbes to influence the inflammatory microenvironment. Herein, we focused our attention on the bidirectional relationship between periodontitis and type 2 diabetes using high-resolution whole metagenomic shotgun analysis to explore the composition and functional profile of the subgingival microbiome in diabetics and non-diabetics subjects with different periodontal conditions.

In the present study, the abundance of metabolic pathways encoded by oral microbes was reconstructed from the metagenome, and we identified a set of dysregulated metabolic pathways significantly enriched in the periodontitis and/or diabetic patients. These pathways were mainly involved in branched and aromatic amino acids metabolism, fatty acid biosynthesis and adipocytokine signaling pathways, ferroptosis and iron homeostasis, nucleotide metabolism, and finally in the peptidoglycan and lipopolysaccharides synthesis.

Overall, the results of the present study provide evidence in favor of the hypothesis that during the primary inflammatory challenge, regardless of whether it is induced by periodontitis or diabetes, endotoxemia and/or the release of inflammatory cytokines cause a change in precursor and/or in circulating innate immune cells. Dysbiosis and inflammation, also via oral–gut microbiome axis or adipose tissue, reduce the efficacy of the host immune response, while fueling inflammation and can induce that metabolic/epigenetic reprogramming of chromatin accessibility of genes related to the immune response.

Abbreviations: AA, amino acid; AMPK, AMP-activated protein kinase; APN, adiponectin; AT, adipose tissue; ATM, adipose tissue macrophages; CVD, cardiovascular diseases; FDR, false discovery rate; HFD, high-fat diet; IL-6, interleukin 6; IMP, inosine 5′-monophosphate; IR, insulin resistant; LDA, linear discriminant analysis; LPS, lipopolysaccharides; QC, quality control; ROS, reactive oxygen species; SFAs, saturated fatty acids; T2D, type 2 diabetes; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α; UMP, uridine 5′-monophosphate; WMS, whole metagenomic shotgun.

Nicoletta Favale and Roberto Farina equally contributed to the manuscript and share the first authorship.

Moreover, the presence of an enhanced ferroptosis and an imbalance in purine/pyrimidine metabolism provides new insights into the role of ferroptotic death in this comorbidity.

KEYWORDS
adipocytokine signaling pathways, BCAA, endotoxemia, ferroptosis, inflammation, metagenomics, oral microbiome, periodontitis, type 2 diabetes

1 | INTRODUCTION

Periodontitis is a common chronic inflammatory disease associated epidemiologically with several systemic diseases, including obesity, metabolic syndrome, cardiovascular diseases, and diabetes mellitus (Charupinijkul et al., 2021; Germen et al., 2021; Gobin et al., 2020; Jepsen et al., 2020; Sanz, Herrera, et al., 2020).

The bidirectional relationship between periodontitis and type 2 diabetes (T2D) is well documented (Barutta et al., 2022; Cardoso et al., 2018; Hajishengallis, 2022; Hajishengallis & Chavakis, 2021; Lamont et al., 2018; Sanz et al., 2018). A recent systematic review pooling data from 53 observational studies confirmed this bidirectional relationship by showing that T2D enhances the risk of developing periodontitis by 34%, while severe periodontitis increases T2D incidence by 53% (Wu et al., 2020). Similar results were obtained in another recent meta-analysis that only included prospective studies (Stöhr et al., 2021).

Periodontitis can have systemic effects favoring the development of insulin resistance (IR) and T2D predominantly by three mechanisms: (1) dissemination of periodontal bacteria and bacterial products from the periodontal tissues to the bloodstream, (2) induction/magnification of systemic inflammation via spillover of inflammatory cytokines and host response to the dissemination of bacteria/bacterial products, and (3) abnormalities in the gut microbiota and increased gut permeability induced by swallowed periodontal bacteria (Barutta et al., 2022; Schenkein et al., 2020).

One of the consequences of the spread of oral bacteria is the modification of the gut microbiota composition. The interaction between oral and gut microbiota is complicated, unstable, and interconnected (Acharya et al., 2017). The transmission of oral to gut and gut to oral microorganisms can shape and/or reshape the microbial ecosystem in both habitats and thus regulate the pathogenesis of different diseases (Park et al., 2021), especially in cases of oral–gut barrier damage (Khor et al., 2021).

Recent studies revealed that microbial/inflammatory factors can produce a form of memory in innate immune cells, enabling them to respond more effectively to a second challenge. This innate memory lasting several months has been named "trained immunity" (Netea et al., 2020; Penkov et al., 2019).

Mechanisms of trained immunity have been partially clarified. During the primary challenge, bacterial products and/or inflammatory cytokines trigger changes in cell metabolism of both mature myeloid cells and their progenitors, such as enhanced glycolysis, altered tricarboxylic acid cycle, and reduced mitochondrial oxidative phosphorylation (Netea et al., 2020). This leads to the accumulation of metabolites, such as fumarate, succinate, and acetyl-CoA, which can modulate the activity of chromatin-modifying enzymes, resulting in epigenetic changes that can increase accessibility to genes related to the innate immune response and allow the cell to respond more rapidly and robustly to a second, unrelated challenge (Netea et al., 2020; Saeed et al., 2014).

In 2019, Farina et al. (2019) used high-resolution whole metagenomic shotgun sequencing (WMS) to describe the taxonomical profile of the subgingival microbiome of patients, recruited in the metropolitan area of Ferrara (Italy), with a different health status regarding periodontitis and T2D. The work presented here, based on Farina's pilot study, aims to reconstruct the microbial signature of the different clinical subgroups by integrating taxonomic data with functional analyses in order to characterize not only the composition but also the functional activities of the oral microbiome in patients affected by both diseases, highlighting new features of the mechanisms underlying the bidirectional relationship between T2D and periodontitis.

2 | MATERIALS AND METHODS

2.1 | Sample collection, DNA isolation, and sequencing

The study design for this high-resolution WMS pilot study was approved by the Ethical Committee of Ferrara, protocol number: 150791. Each subject provided a written informed consent before participation. All clinical procedures were performed in full accordance with the Declaration of Helsinki and the Good Clinical Practice Guidelines.

Individuals recruitment, collection, and storage of subgingival plaque samples were performed at the Research Centre for the Study of Periodontal and Peri-Implant Diseases, University of Ferrara, Italy. Briefly, 12 adults (≥40 years) with north-Italian ancestry were recruited and assigned to one of the following groups (three individuals each): (i) t2d+p+ group: patients affected by moderate to severe periodontitis and T2D, (ii) t2d−p+ group: patients affected by moderate to severe periodontitis but no T2D, (iii) t2d+p− group: patients affected by T2D but no periodontitis, and (iv) t2d−p− group: healthy subjects.

For each eligible subject, four subgingival plaque samples were collected at four teeth. For the enrollment in the study, the following inclusion/exclusion criteria, related to periodontal/diabetic conditions, were evaluated, and subjects were recruited only if these criteria were strictly met.

The inclusion criteria were as follows:

- for t2d+ groups: diagnosis of T2D for at least 2 years according to the criteria of the American Diabetes Association (American Diabetes Association, 2014); insufficient metabolic control of diabetes (i.e., glycated hemoglobin serum level >7%); currently receiving stable doses of oral hypoglycemic agents and/or insulin under supervision of a diabetologist;
- for t2d− groups: no history of T2D diagnosis;
- for p+ groups: at least 20 teeth present; diagnosis of moderate to severe periodontitis, that is, at least 30% of sites with clinical attachment loss ≥3 mm (Armitage, 1999); at least four sites with probing depth ≥5 mm;
- for p− groups: no history of periodontitis, either treated or not (i.e., no interproximal clinical attachment loss >2 mm and no sites with probing depth >4 mm).

Exclusion criteria (valid for all groups) were as follows:

- current smoking or quit smoking less than 6 months prior to the screening visit;
- diseases or systemic conditions (in addition to T2D) with a documented influence on periodontal status;
- use of drugs with a documented influence on periodontal status (e.g., bisphosphonates, cyclosporine, phenytoin, nifedipine, calcium channel blockers, corticosteroids, and anti-inflammatory drugs);
- periodontal therapy within 12 months prior to the screening visit;
- local or systemic antibiotic therapy during the 3 months prior to the screening visit.

The general and clinical characteristics of the study population are presented in detail in Farina et al. (2019).

DNA was isolated and sequenced according to standard protocol for Illumina NextSeq 500 sequencer with 2 × 150-bp read layout, as extensively described previously (Farina et al., 2019).

2.2 | Bioinformatic analyses

2.2.1 | Pre-processing data

The quality control (QC) of the reads was done with FastQC (v. 0.10.1; Andrews, 2010). Trimmomatic (v. 0.36; Bolger et al., 2014) was used to trim from raw data bases with poor quality and read with length <100 bp. Parameters were defined as CROP:147, HEADCRO:3, SLIDINGWINDOW:5:20, MINLEN:100. Host DNA was aligned to the human reference genome (GRCh38) with the *mem* (Maximal Exact Matches) algorithm of BWA (v. 0.7.15, H. Li & Durbin, 2009).

2.2.2 | Taxonomical analyses

The taxonomic profile of the samples was built using two different approaches: the first involves aligning the non-human reads against the NCBI "nt" reference database using BLAST (Altschul et al., 1990), the parameters were set to include matches with an e-value $\leq 1 \times 10^{-6}$, a percentage identity of ≥95%, and a minimum length > 100 bp. A list of taxonomic identifiers was provided to the software to limit the search to taxa belonging to archaea, bacteria, fungi, and virus. Then, MEGAN6 (v. 6.21.5; Huson et al., 2007) with default parameters was used to perform the taxonomical analysis of the data, ranked for genus and species.

With the second method, after QC, the processed data were analyzed with MetaPhlAn 3.0 (Metagenomic Phylogenetic Analysis v. 3.0.11; Beghini et al., 2021). MetaPhlAn 3.0 allows to quickly profile the composition of metagenomes, aligning reads to a custom microbial database containing specific gene markers (mpa_v296_CHOCOPhlAn_201901), this type of database allows differentiation between similar species with high precision. The samples were analyzed with default parameters of MetaPhlAn 3.0 and merged in a single output table with the utility merge_metaphlan_tables.py.

To identify the major genera/species defining taxonomic profiles across clinical conditions, both MEGAN6 and MetaPhlAn 3.0 outputs were statistically analyzed through (1) White's non-parametric test performed by STAMP (v. 2.1.3; Parks et al., 2014); the non-parametric nature of this test allows for the handling of low sample size datasets and (2) the linear discriminant analysis EFfect Size (LEfSe) algorithm (https://huttenhower.sph.harvard.edu/galaxy/; Segata et al., 2011). LEfSe algorithm, combined a nonparametric Kruskal–Wallis test and the pairwise Wilcoxon rank-sum test with linear discriminant analysis (LDA), allowed the removal of the strong effect of false discovery rate (FDR) correction when several multiple comparisons are computed on a small sample size. To be more conservative, the threshold on the logarithmic LDA score was increased from 2 to 2.5 either for taxonomic and functional analysis.

2.2.3 | Functional analyses

As seen for the taxonomic characterization, two different methodologies were also used to create functional profiles.

A BLASTX analysis of QC processed data were performed through DIAMOND (v. 2.0.4; Buchfink et al., 2014). The KEGG classification contained in MEGAN6 Ultimate Edition (v. 6.21.11; Beier et al., 2017) was used to perform the functional analyses. Significant results were then explored through the KEGG PATHWAY online database (https://www.genome.jp/kegg/pathway.html).

With the second method, after QC, single functional profiles were computed starting from the processed data using HUMAnN 3.0 (HMP

Unified Metabolic Analysis Network, v. 3.0.0; Beghini et al., 2021). The samples were processed with default parameters and finally merged using the *human_join_tables.py script*.

Also for functional profile, both datasets were statistically analyzed using the LEfSe algorithm, with an LDA threshold of 2.5. All pathways found to be significantly enriched between clinical categories were investigated in depth with the MetaCyc website (https://metacyc.org/).

3 | RESULTS

3.1 | Taxonomical profiling of subgingival microbiome

Both methods used for taxonomical profile reconstruction, MEGAN6 and MetaPhlAn 3.0, proved to be quite consistent at genera level, with a concordance of 93.3% and 75% for p+ versus p− and t2d+ versus t2d−, respectively (Figure 1). At the species level, the degree of concordance decreases due to the different nomenclature system used by the two databases; therefore, only the top 15 species of each subgroup were considered to investigate the taxonomic profile of the subgroups with different clinical conditions. From the analysis (Figure 2), it was possible to describe a core microbiome, which is resilient to changes in the oral health status, consisting of *Actinomyces* sp. oral taxon 414, *Corynebacterium matruchotii*, *Selenomonas sputigena*, *Treponema denticola*, *Treponema socranskii*, and *Tannerella forsythia*.

While some of these strains, like *C. matruchotii*, were already described in the literature as a component of the oral plaque's core microbiome or associated with health status, such as *Actinomyces* sp. (Colombo & Tanner, 2019), others were historically associated with a different status of oral disease. For example, *T. denticola*, *T. forsythia*, and *S. sputigena* were related to orange and red periodontitis complex depending on their pathogenicity (Wirth et al., 2021), whereas *T. socranskii* was described in the literature as associated with gingivitis or mucositis (Colombo & Tanner, 2019).

All groups of affected subjects (t2d+p+, t2d+p−, and t2d−p+) were characterized by the presence of *Campylobacter rectus*, *Porphyromonas gingivalis* and *Porphyromonas endodontalis*. These three species have all been associated with periodontitis (Colombo & Tanner, 2019; Shi et al., 2020), with *P. gingivalis* and *C. rectus* historically part of the red and orange complexes, respectively (Socransky et al., 1998). In the present analysis, however, they appear to typify patients with an inflammatory condition, generically associated with p+ or t2d+ status, rather than specifically characterizing periodontitis.

The two groups of periodontitis subjects (t2d+p+ and t2d−p+) presented a prevalence of *Anaerolineaceae bacterium* oral taxon 439, *Prevotella intermedia*, and *Treponema maltophilum*. *Anaerolineaceae bacterium* oral taxon 439 and *P. intermedia* are part of the orange complex (Socransky et al., 1998; Wirth et al., 2021), whereas *T. maltophilum* was associated with only peri-implantitis (Colombo & Tanner, 2019).

T2d+p− and t2d−p+ groups of patients showed a high prevalence of *Actinomyces oris*, *Desulfomicrobium orale*, *Prevotella oris*, and *Treponema lecithinolyticum*. In the literature, *P. oris* has been reported as

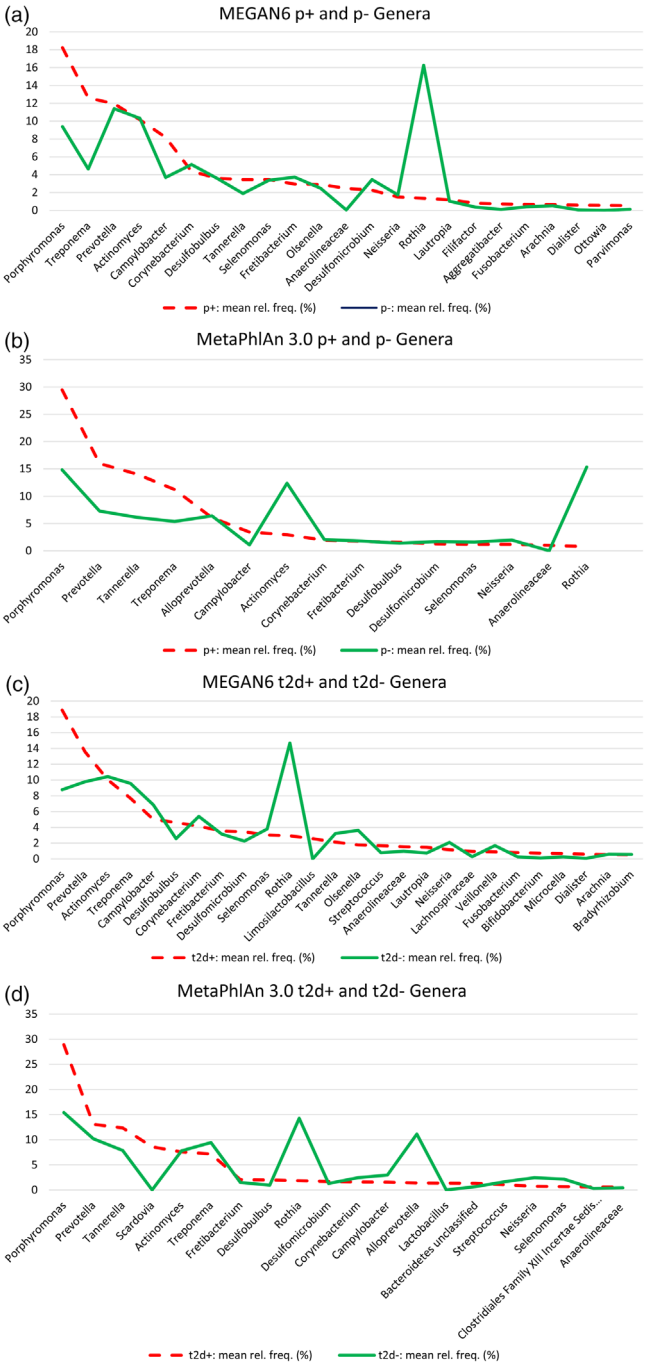

FIGURE 1 Distribution of bacterial genera with relative abundance higher than 0.5% in: subjects with periodontitis ($n = 6$) and without periodontitis ($n = 6$) in MEGAN6 (a) and MetaPhlAn 3.0 (b); type 2 diabetes patients ($n = 6$) and individuals without type 2 diabetes ($n = 6$) in MEGAN6 (c) and MetaPhlAn 3.0 (d) datasets.

associated with periodontal health status, while the other three strains were associated with mild or severe oral disease status: *A. oris* was associated with gingivitis, while *D. orale* and *T. lecithinolyticum* were related to periodontitis (Colombo & Tanner, 2019).

Healthy individuals (t2d-p-) showed the prevalence of *Veillonella parvula* and *Rothia dentocariosa*, which are stable components of the oral microbiome, associated with early colonizers complexes, purple

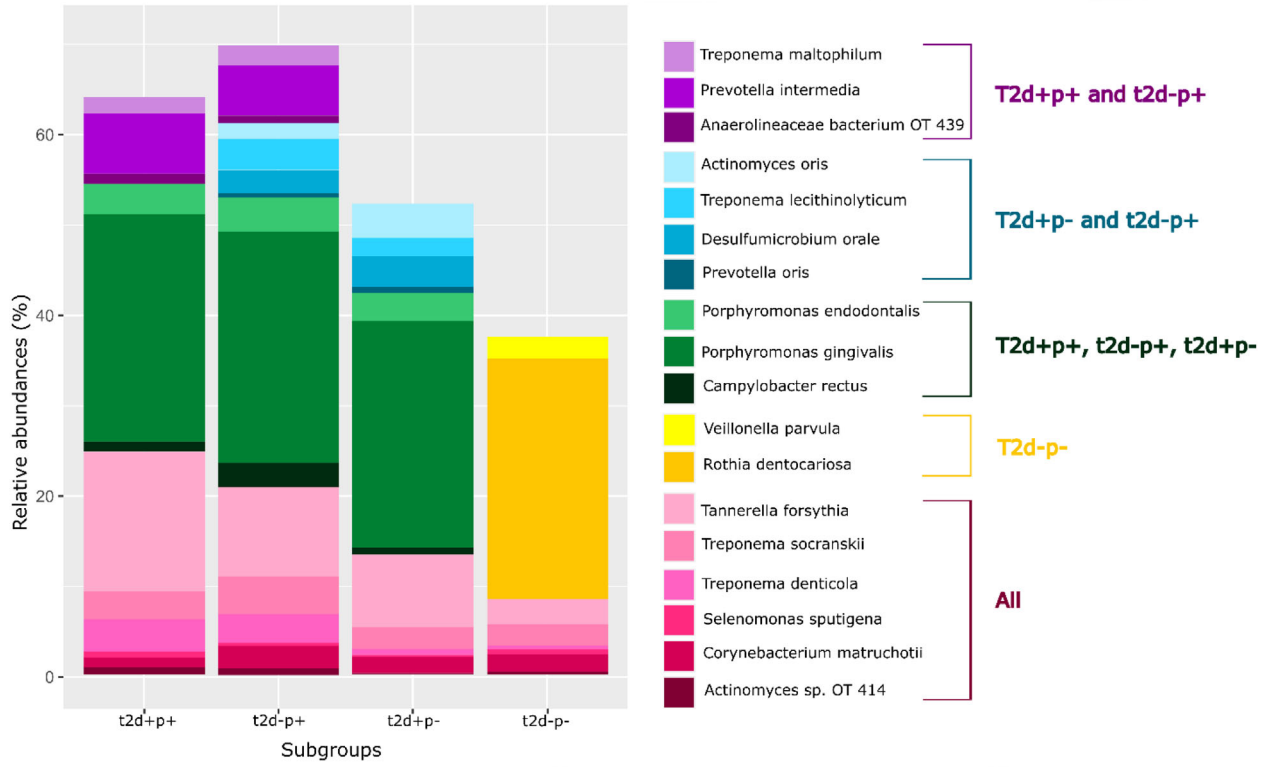

FIGURE 2 Species-level composition of the subgingival microbiome (top 15) among subgroups with different clinical conditions.

and yellow complexes, respectively (Knapp et al., 2017; Wirth et al., 2021).

3.2 | Differences in subgingival microbiome composition among different clinical conditions

To identify the most relevant genera/species defining taxonomic profiles across clinical conditions, both MEGAN6 and MetaPhlAn 3.0 outputs were analyzed with White's non-parametric test, performed by STAMP (Tables S1 and S2, respectively) and using the LEfSe algorithm (Figures 3 and 4, respectively).

From all statistical analyses, the results that overall consistently emerge concern (1) in subjects without periodontitis, the only consistent result involves the order of Micrococcales where the genus *Microcella*, represented by *Microcella alkaliphila*, and the genus *Rothia*, represented by *R. dentocariosa*, seemed to characterize a healthy periodontium (#3 in Figures 3 and 4; Table S1). In particular, *R. dentocariosa* is specifically enriched in p− subjects within t2d+ patients (Figure 5c); (2) the *A. bacterium* oral taxon 439 (#1 in Figures 3 and 4; Table S1) showed a significant higher relative abundance in periodontitis patients compared to individuals without periodontitis, both considering the overall population or the t2d+ subsamples, maintaining the significance also after FDR correction (p-value < 0.01). Precisely, t2d+p+ patients were characterized by the presence of *A. bacterium* oral taxon 439, absent both in t2d+p− and t2d−p− individuals (Figure 5c,e).

Several other species showed a relative abundancy significantly higher in periodontitis patients compared with p− subjects (Tables S1 and S2): *Campylobacter showae*, *Campylobacter rectus*, *Treponema maltophilum*, *Prevotella* spp. (*P. intermedia* and *P. melaninogenica*), *Neisseria* spp. (*N. elongata* and *N.* sp OT 104), *Filifactor alocis*, *Eubacterium* spp. (*E. minutum* and *E. nodatum*), *Parvimonas micra*, and *Dialister pneumosintes*.

Among these species: (1) a significant higher presence of *C. rectus* and *C. showae* (#2 in Figures 3 and 4) characterized p+ individuals against p− patients mainly in t2d− patients or as compared to t2d+p− individuals (Figure 5a,b,f); (2) *T. maltophilum* (#4a, Figure 4), together with *C. rectus* and *C. showae*, characterized p+ individuals and was mainly represented in t2d−p+ patients compared to t2d+p− individuals (Table S2); (3) *Prevoltella* spp. and the *Aggregatibacter* genus (#7 and #4, Figures 3 and 4) characterized p+ individuals, both t2d+ or t2d-, but are completely absent in t2d+p− (Table S1); (4) *F. alocis* and *Neisseria* spp. (#5 and #8, Figures 3 and 4) in p+ patients were observed primarily in non-diabetic subjects (Figure 5d); (5) *Eubacterium* spp. (#9 in Figures 3 and 4) is significantly highly abundant in p+ patients compared with p− subjects but does not show any specific distribution among the different clinical subgroups; (6) *P. micra* and *D. pneumosintes* characterized p+ individuals (#6 in Figure 4; Table S1), with *D. pneumosintes* significantly enriched in patients with comorbidity together with *A. bacterium* oral taxon 439 (Figure 5e).

With regard to two of the known strains belonging to the red and orange periodontitis complexes, *P. gingivalis* and *Fusobacterium nucleatum* respectively, White's non-parametric test and the LEfSe algorithm were not completely convergent. In particular, White's non-parametric

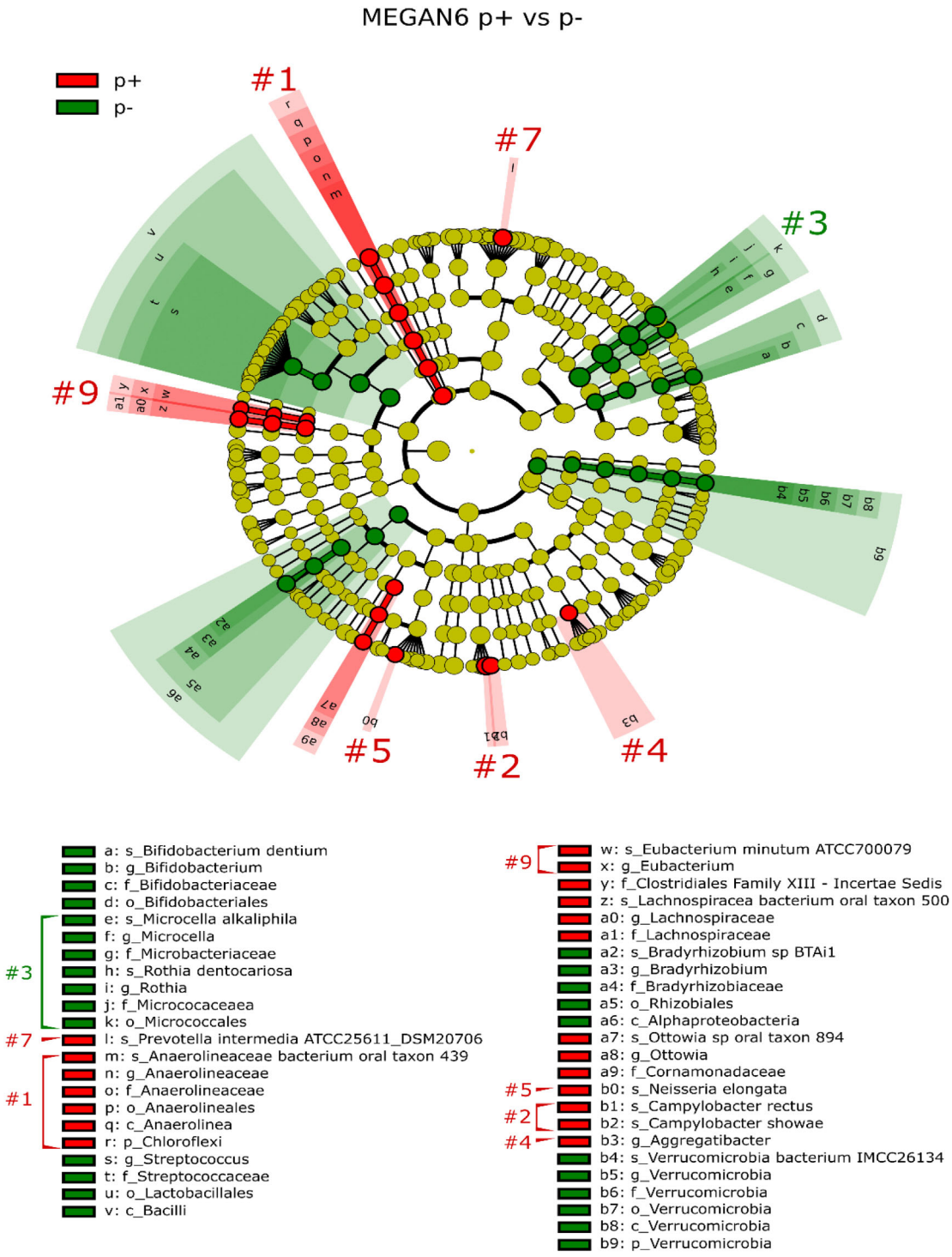

FIGURE 3 Cladogram plotted from the LEfSe analysis, based on MEGAN6 output, showing the taxonomic levels represented by rings with phyla in the outermost ring and species in the innermost ring. Each circle represents a member within that level. Taxa with enriched level in patients with periodontitis are colored in red, those enriched in subjects without periodontitis are colored in green, and not enriched taxa are ochre colored. Letters (a-b9) refer to the different taxa levels, while numbers (#1–#9) refer to taxonomical groups explained in the main text.

test identified a significant higher abundance of *P. gingivalis* in individuals p+ compared with p− subjects, both at the genus level and species level (Table S1), but this was limited to comparisons between t2d−p+ and t2d−p− subgroups (Figure 5b), and the significance was also confirmed in the MethaPhlAn 3.0 dataset (Table S2). These significances were cannot be detected in the analyses with LEfSe because in the latter, comparisons were limited to the largest cohorts (p+ vs. p−) and did not delve into subgroup details.

Regarding *F. nucleatum*, White's non-parametric test identified, within t2d- subjects, a significantly higher abundance of *F. nucleatum*

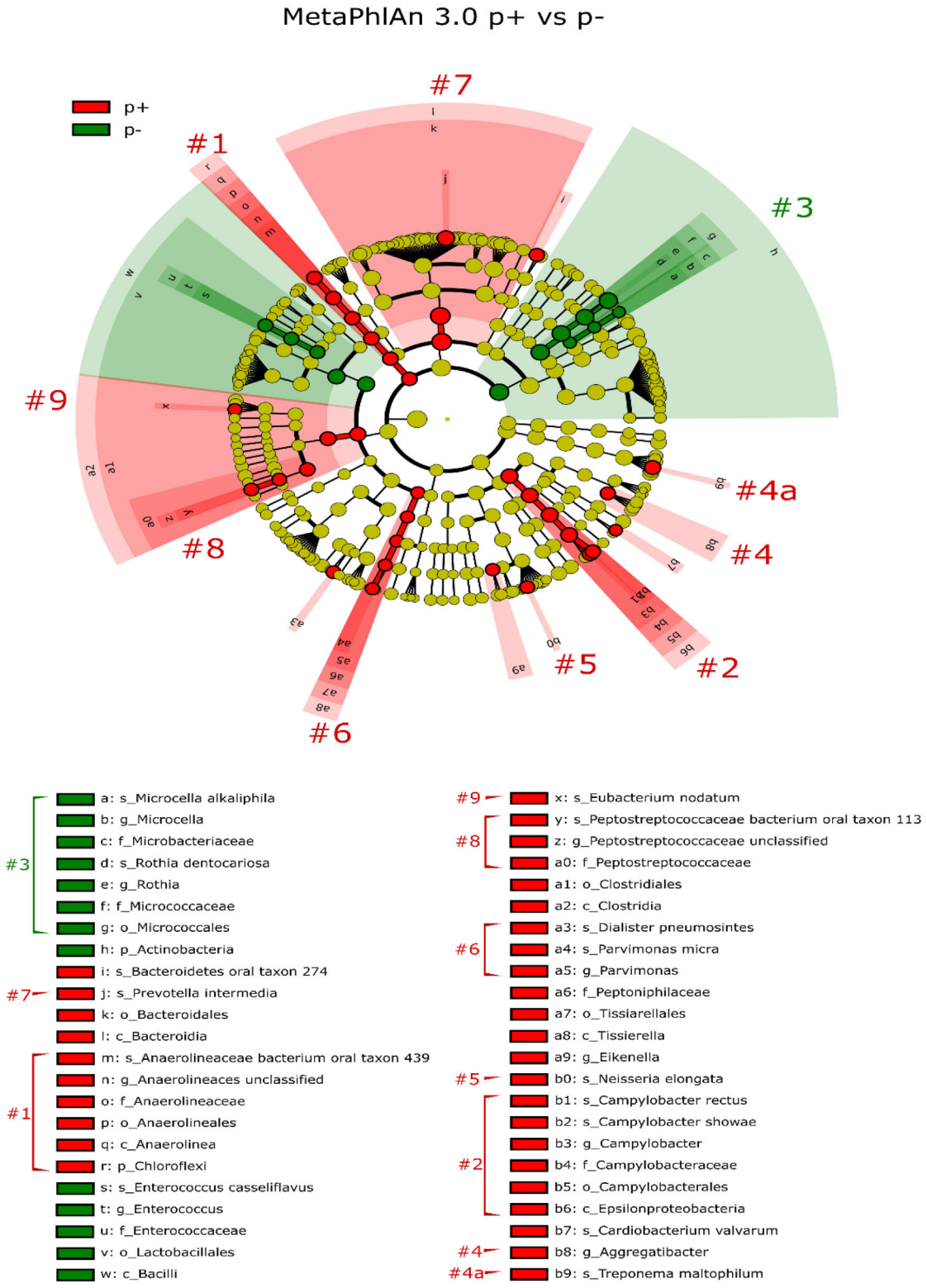

FIGURE 4 Cladogram plotted from the LEfSe analysis, based on MetaPhlAn 3.0 output, showing the taxonomic levels represented by rings with phyla in the outermost ring and species in the innermost ring. Each circle represents a member within that level. Taxa with enriched level in patients with periodontitis are colored in red, those enriched in subjects without periodontitis are colored in green, and not enriched taxa are ochre colored. Letters (a-b9) refer to the different taxa levels, while numbers (#1–#9) refer to taxonomical groups explained in the main text.

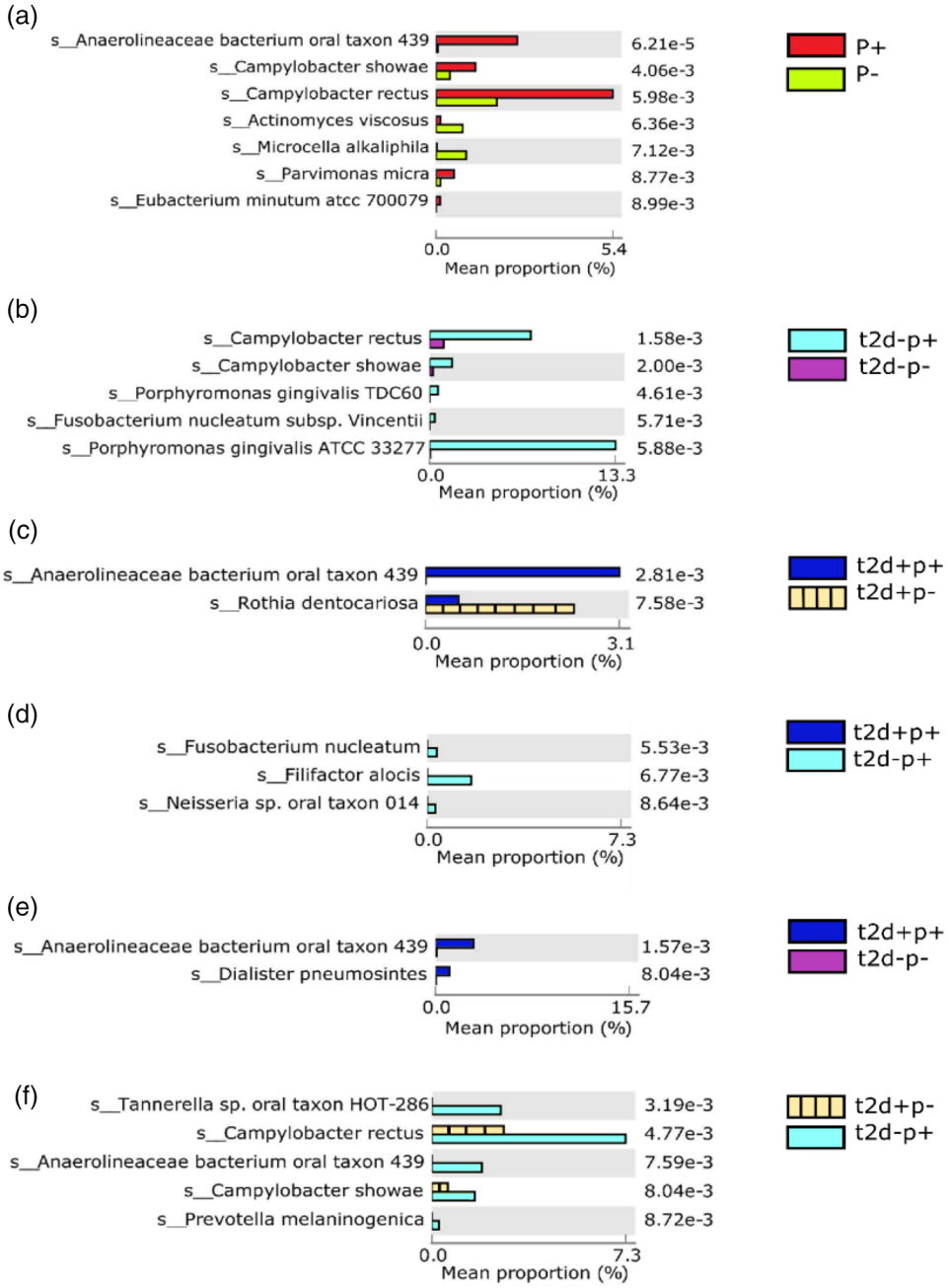

FIGURE 5 Comparison by White's nonparametric *t*-test of observed relative abundances (mean % proportions) between groups/subgroups for bacterial species observed in the MEGAN dataset. Only significant nominal differences ≤0.01 are reported on the right-hand scale.

subsp. vincentii in t2d−p+ patients than in t2d−p− subjects (Table S1; Figure 5b), whereas the LEfSe algorithm recognised *F. nucleatum* subsp. nucleatum as a significant discriminant between t2d+ and t2d− subjects regardless of periodontal disease (Figure 6).

Thus, as a general outline, we can conclude that statistical analyses indicated as significantly discriminant in p+ group strains attributed to orange and orange-associated complexes like *A. bacterium* oral taxon 439, whose results maintained significance even after FDR correction, *T. maltophilum*, *C. rectus* (and an emerging role of *C. showae*) and *P. intermedia* (Socransky et al., 1998; Wirth et al., 2021). Moreover, p− individuals were discriminated by the presence of *R. dentocariosa* and *M. alkaliphila*. As seen previously, *R. dentocariosa* is an early colonizer of plaque biofilm, while *M. alkaliphila* is a strain poorly characterized and never detected in oral plaque microbiome.

3.3 | Functional profiling for individuals among different clinical conditions

The results from the two different methods, MEGAN6-KEGG's analysis of DIAMOND data and HUMAnN 3.0 pipeline, used for the functional annotation of subgingival microbiomes were submitted to LEfSe

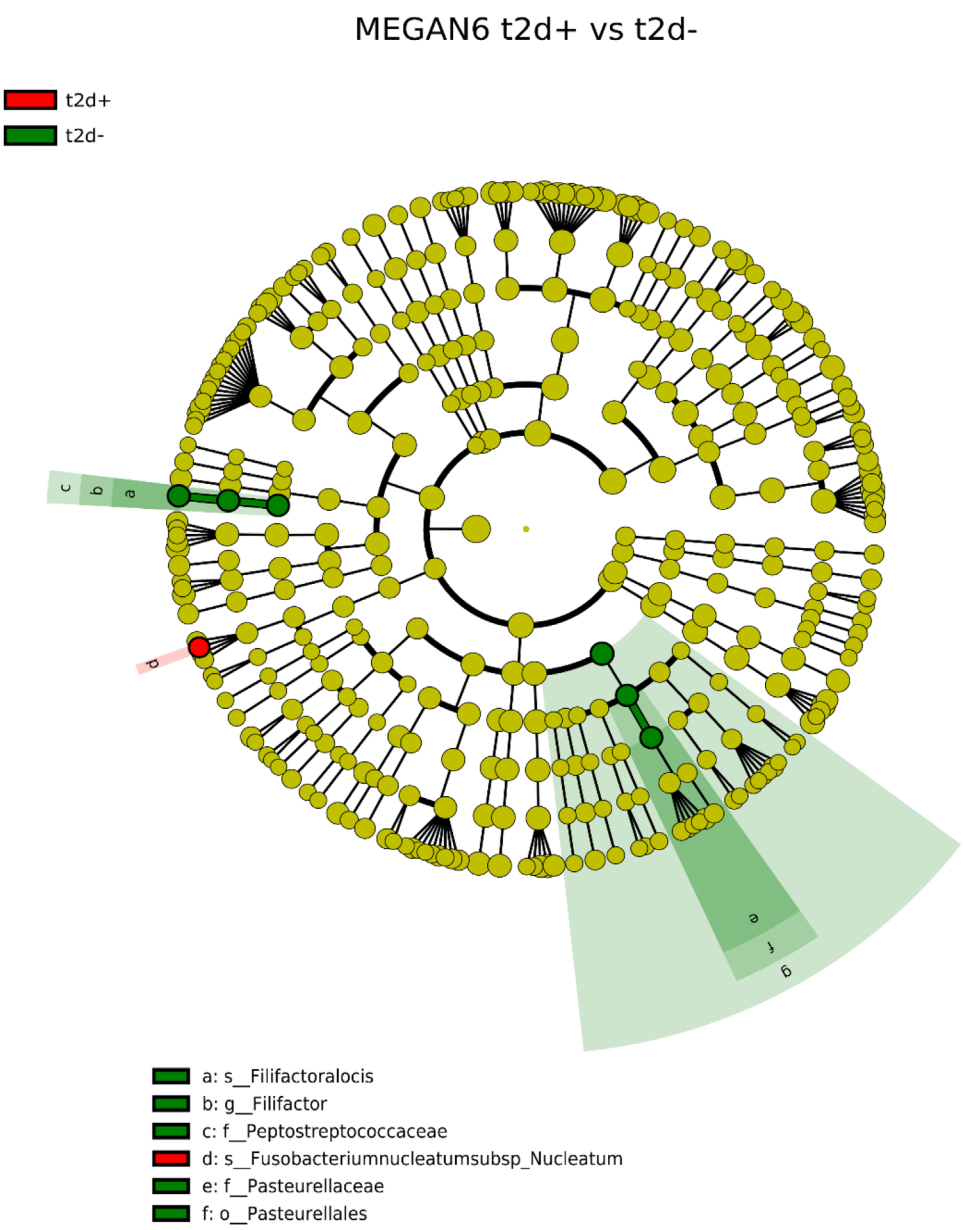

FIGURE 6 Cladogram plotted from the LEfSe analysis, based on MEGAN6 output, showing the taxonomic levels represented by rings with phyla in the outermost ring and species in the innermost ring. Each circle represents a member within that level. Taxa with enriched level in patients with type 2 diabetes are colored in red, those enriched in subjects without type 2 diabetes are colored in green, and not enriched taxa are ochre colored. Letters (a–g) refer to the different taxa levels.

to detect the pathways that can explain differences between subjects: (I) with and without periodontitis and (II) with and without T2D.

3.3.1 | Functional annotation of subgingival microbiome in individuals with and without periodontitis

MEGAN6-KEGG's analysis of DIAMOND data was performed both at the pathway level and protein level. Among the 459 pathways and 5442 proteins identified in the metagenome of the individuals analyzed, a total of 24 pathways and 11 proteins characteristic of p− individuals exceeded the 2.5 LDA threshold. In subjects affected by periodontitis, seven pathways and five proteins were found to be enriched. Results are detailed in Figure S1A,B for pathways and proteins, respectively. The HUMAnN 3.0's data annotation analysis led to the identification of a total of 5776 pathways among which LEfSe statistical analyses revealed nine pathways characteristic of p− individuals and 15 pathways enriched in p+ subjects (Figure S2).

By categorizing these pathways to higher classes (Table S3), p− cohort was predominantly enriched with path belonging to amino acid (AA) biosynthesis (13 pathways) and carbohydrate or energy metabolism (10 pathways), whereas the pathways for cell structure

biosynthesis (six pathways),and nucleoside and nucleotide metabolism (four pathways) were prevalent in the p+ cohort.

Overall, from the investigation of the two datasets, statistical analyses identified 33 and 22 inferred pathways significantly abundant in p− or p+, respectively, able to define distinct profiles between p+ and p− and to modulate differences between subgroups, especially between t2d+ and t2d− within p+ or p− subjects (Figure 7). Interestingly, among the biological/metabolic pathways significantly enriched in the p+ group, in addition to the most important groups mentioned above, there were also included pathways involved in: (1) ferroptotic death and/or iron homeostasis (three features: the whole path for ferroptosis [KEGG code ko04216] and two outer membrane receptor proteins TonB-dependent [KEGG code K02014 and K16089]); (2) the biosynthesis of preQ0 (the precursor for all 7-deazapurines, a class of purine-based secondary metabolites; MetaCyc code: PWY-6703) and chorismate metabolites (MetaCyc codes: ARO-PWY and PWY-6163); all these molecules are precursors to compounds often endowed with antibiotic or antiviral activity; (3) fatty acids biosynthesis and (4) adipocytokine signaling pathway (Figure 7).

3.3.2 | Functional annotation of subgingival microbiome in individuals with and without T2D

Comparing t2d+ and t2d− individuals, the LEfSe analyses were inconclusive at the protein level, but they were able to identify nine differently enriched attributes (seven in t2d− and two in t2d+) in the MEGAN6-KEGG's analysis of DIAMOND data (Figure 8a) and six significantly enriched pathways in HUMAnN 3.0's data annotation analysis, all characterizing the t2d+ group (Figure 8b).

Overall, from the comparison of subjects without and with diabetes, LEfSe reports for both dataset revealed very heterogeneous enriched pathways in the t2d− cohort, spanning along the KEGG's category, whereas significant features detected in t2d+ subjects were mainly related to synthesis of lipopolysaccharides (LPS), a major component of the outer membrane of gram-negative bacteria (three features), to some metabolic pathways (three features: glutathione, galactose, and pyrimidine metabolism) and to antibiotic synthesis (streptomycin biosynthesis).

4 | DISCUSSION

The oral microbiome is one of the most complex microbial communities in the human body, harboring more than 1000 species of microorganisms (Lamont et al., 2018). The association between periodontitis and T2D is already known, and the mechanisms through which periodontitis can be causally linked to diabetes and vice versa have been explored (Genco & Borgnakke, 2020; Kocher et al., 2018; Socransky et al., 1998; Wu et al., 2020).

In the present study, both taxonomical and functional aspects involved in the association between these two diseases and potentially underlying their bidirectional relationship have been investigated using high-resolution WMS analysis. The limited sample size, which represents an obvious limitation, partly also due to the cost containment considering the pilot study approach, is mainly attributable to the highly specific selection criteria used to enroll patients. In addition to the criteria detailed in Section 2, all subjects had to have established Northern Italian ancestry and clinical traits of periodontal or diabetic health or disease falling within extreme phenotypes, ideally characterized by extreme levels of biomarkers (both microbial and biochemical). To meet all these established selection criteria, the main difficulties were encountered in selecting subjects completely healthy and close in age to patients with T2D and periodontitis and, even more so, in recruiting patients with T2D with a completely intact and healthy periodontium.

In biomedical research, clinical trials often include experimental studies that present analytical problems related to small sample size; in metagenomics studies, one possible strategy to overcome this limitation is the pooling of microbiome samples before DNA amplification and metagenomic sequencing (Ray et al., 2019; Teufel & Sobetzko, 2022). Thus, our choice was to collect, for each participant, 16 samples (four subgingival plaque samples collected from four teeth and then pooled into a single specimen) by sampling subgingival plaque at sites all representative of each individual's periodontal condition, that is, for p− subjects, sampling was always performed at four sites randomly selected among those negative to bleeding on probing; in p+ patients, sampling was performed at four sites showing the deepest probing depth values among those positive to bleeding on probing.

This strategy, together with the WMS approach, allows the entire diversity of the microbiome to be analyzed at the community level and underrepresented species to be amplified and sequenced as well. Therefore, even with a small sample size, statistical errors could be corrected and statistically significant differences could still be found after the amplification of metagenomic data. It is important to emphasize here that the key statistical comparisons were made between the p+/− or t2d+/− groups (6 vs. 6 subjects), using two different methods and essentially highlighting the results that had concordance in the two methods. Subgroups comparisons were not made to obtain a statistical finding but to have a possible guidance of the interrelation between the two diseases.

4.1 | Main taxonomic outcomes

The analysis of the most abundant strains in the subgingival microbiome allowed the identification of a core microbiome composed, among the other, of *S. sputigena*, *T. denticola*, *T. socranskii*, and *T. forsythia* strains historically associated with a different status of oral disease (Figure 2). These strains were present crosswise in all the different clinical subgroups and appear to constitute a biofilm microbial population resilient to changes in the oral health status (Colombo & Tanner, 2019; Wirth et al., 2021; X. Li et al., 2022).

To identify the most relevant genera/species that define, regardless of abundance, taxonomic profiles among clinical conditions, two

FIGURE 7 Heat map of (a) MEGAN6-KEGG and (b) HUMAnN 3.0 datasets pathway analysis. The subgroups are indicated on the *x*-axis, and the differential pathways are represented on the *y*-axis. The gradation of the blue color reflects the low (light blue) or the high (dark blue) abundance of the biological/metabolic pathway.

different statistical tests were applied and indicated as significantly discriminant in p+ group strains belonging to orange and orange-associated complexes like *A. bacterium* oral Taxon 439, *T. maltophilum*, and *P. intermedia* (Socransky et al., 1998; Wirth et al., 2021). Interesting is the observation that *C. rectus*, *C. showae*, and *P. gingivalis* emerge with a significant higher abundance in p+ and specifically in t2d−p+ (Figure 5a,b).

Considering the diabetic status, *F. alocis* seems to characterize non-diabetic subjects both in the overall comparison between t2d+ and t2d− as well as within p+ patients (Figure 5d), whereas *F. nuclea-*

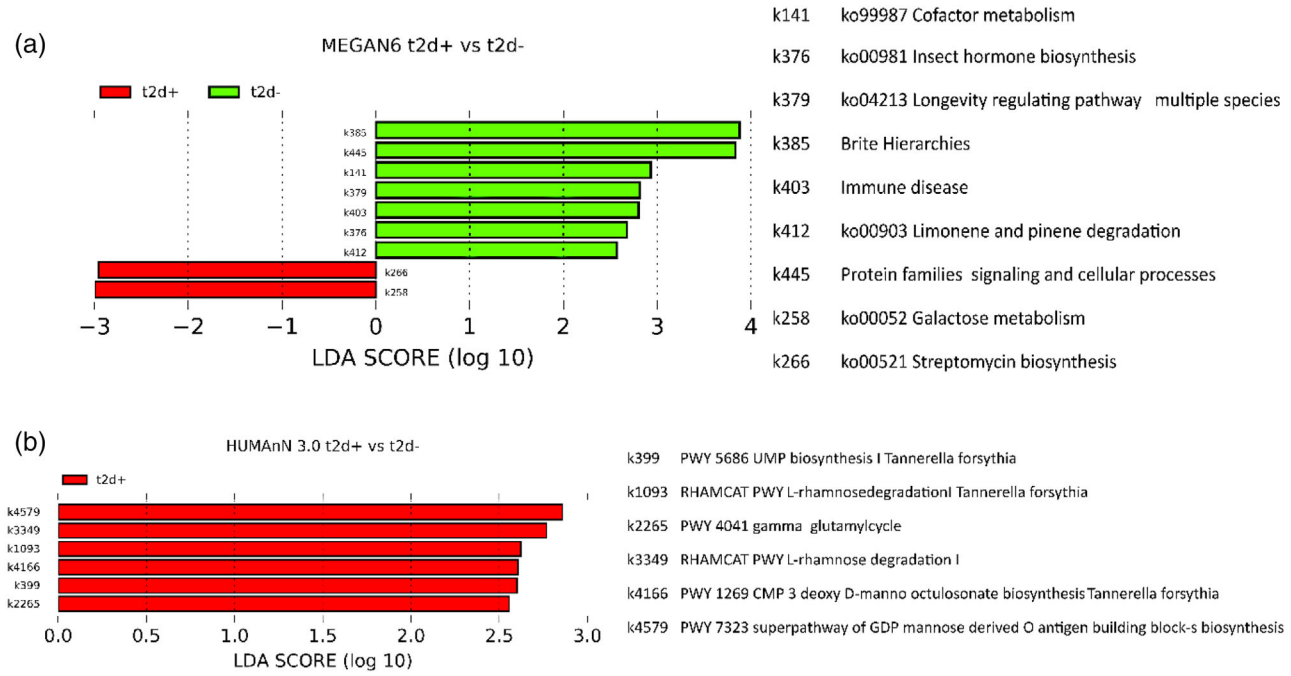

FIGURE 8 LEfSe results of functional pathway analysis between individuals with and without type 2 diabetis obtained from (a) MEGAN6 or (b) HUMAnN 3.0 datasets.

tum subsp. nucleatum show a significant higher abundance in t2d+ than t2d− subjects (Figure 6). Within t2d+ subjects, *R. dentocariosa* appears to play a protective role for the periodontium, being observed at significantly higher level in t2d+p− than in t2d+p+ subjects.

4.2 | Main functional outcomes

The first result to be highlighted concerns the several pathways involved in amino acid (AA) biosynthesis significantly enriched in p− subjects and, therefore, reduced in p+ patients. In detail, pathways of branched amino acids (BCAA [Ile, Leu, and Val]) biosynthesis/degradation and other essential/aromatic AA were particularly involved.

BCAAs represent not only important nutrients involved in proliferation during infection but also play a role both in the evasion of host defenses (Kaiser & Heinrichs, 2018) and some studies suggested a potentially causative role of the BCAAs, or their breakdown products, in metabolic disorders such as incident T2D and IR mice. In fact, in a model of *P. gingivalis*-induced periodontitis in high-fat diet (HFD)-fed mice, infection with the wild-type bacterium, but not with a BCAA aminotransferase-deficient mutant, resulted in increased serum levels of BCAAs (such as Leu, Ile and Val) and insulin resistance, as compared with uninfected HFD-fed mice (Tian et al., 2020). On the other hand, there are also evidences associating a reduction in BCAAs with metabolic disorders through the involvement of metabolic hormones, other than insulin and glucagon, in diabetic mice (Lian et al., 2015). Lian et al. (2015) demonstrated that adiponectin (APN) decreases circulating BCAA and branched chain keto acids (BCKA) by activating hepatic BCK dehydrogenase (BCKDH) through the downregulation of BCKD kinase and upregulation of protein phosphatase 2Cm. They concluded that impaired APN signaling is an important part of the underlying mechanism for disturbed BCAA catabolism in T2D.

In this regard, our data show that p+ patients, in addition to a dysregulation of metabolic pathways involved in BCAAs, aromatic AA, and glycine levels, also present a significant increase in fatty acid biosynthesis and adipocytokine signaling pathways (Figure 7), with the latter two closely interrelated metabolic pathways.

The adipocytokine signaling pathway refers to the sum of all proteins and factors responsible for the regulation of adipose tissue (AT) metabolism, which is the largest endocrine organ with the ability to produce and secrete a variety of hormones and factors including mainly APN, leptin, interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), and resistin. APN and leptin play an important regulatory role in the energy metabolism of glucose, sugars and fatty acids in cells through the AMP-activated protein kinase (AMPK) pathway. AMPK activation results in the inhibition of energy-consuming biosynthetic pathways (such as glycolysis/gluconeogenesis, cholesterol synthesis in the liver, and insulin secretion from β-cell) and activation of ATP-producing catabolic pathways (such as fatty acid uptake and oxidation in multiple tissues). AMPK activity is also expressed by modulating the transcription of specific genes involved in energy metabolism, thus exerting long-term metabolic control (Viollet et al., 2006).

From the present analysis, the enrichment in p+ and t2d+ subjects of adipocytokine signaling pathway and fatty acid biosynthesis together with the inhibition of cholesterol, sugar, and energy metabolic pathways (Figure 7) points to a dysregulation of AMPK activity in our patients, by confirming the role of AMPK as an important step to

counteract inflammation as already observed in various cells in animals with metabolic diseases such as diabetes and obesity (Viollet et al., 2010).

The importance of oral microbiota and its association with the intestinal microbiota recently received increasing attention (Chen et al., 2021; Kriebel et al., 2018). The oral cavity serves as an endogenous reservoir for gut microbial strains in both health and diseases such as rheumatoid arthritis, colorectal cancer, or type 1 diabetes (Schmidt et al., 2019). In periodontitis, swallowed periodontal bacteria can induce gut dysbiosis (Hajishengallis & Chavakis, 2021; Yamazaki et al., 2021) and recently metagenomic and proteomic studies showed that *P. gingivalis*-induced gut dysbiosis was paralleled by the downregulation of tight junction proteins (TJP-1, occluding) (Kashiwagi et al., 2021; Ohtsu et al., 2019), likely leading to enhanced gut permeability and favoring endotoxemia. The LPS-bacterial product has specifically been shown to translocate across the intestinal barrier and contribute to disease by inducing an increase in tight junction permeability also through toll-like receptor 4 (TLR4)-dependent mechanisms (Guo et al., 2013, 2015; Nighot et al., 2017), thus contributing to immune activation and inflammation that further disrupt the gut barrier (Page et al., 2022). Consistently, *P. gingivalis*- induced endotoxemia was associated with inflammation in important sites of insulin resistance, such as the liver and the adipose tissue (Sasaki et al., 2018). Mechanically, gut microbiota-derived LPS is taken up, then trafficked to AT, where it is internalized by adipocytes and adipose tissue macrophages (ATMs), resulting in AT expansion and the transition of macrophages from the anti-inflammatory M2 phenotype toward the proinflammatory M1 phenotype in a manner dependent on TLR4 signaling (Hersoug et al., 2016; Y.Y. Wang, Wang, et al., 2022). In AT also, a downregulation of genes that enhance insulin sensitivity has been observed (Arimatsu et al., 2014).

Again on the topic of LPS-produced damage, in the present study, the functional analyses highlighted, besides five metabolic pathways for the peptidoglycans synthesis enriched in p+ patients, also three pathways implicated in the biosynthesis of the O-antigen repeating units (OPS) in LPS. These three pathways, the GDP mannose-derived O-antigen building blocks biosynthesis, the CMP 3 deoxy-D-manno-Octulosonate biosynthesis, and the L-rhamnose degradation pathway, were all significantly enriched in t2d+ subjects pointing out a role for OPS as an additional virulence element in diabetic subjects.

The importance of the structure of the OPS for immunogenicity and virulence in bacteria has been recently demonstrated also in *Aggregatibacter actinomycetemcomitans*, where seven *A. actinomycetemcomitans* serotypes are recognized based on the antigenicity of the OPS (Monasterio et al., 2020). *Aggregatibacter actinomycetemcomitans* strains belonging to the serotype *b* are frequently isolated from subjects with severe periodontitis, while other serotypes are mostly isolated from milder periodontitis-affected patients or healthy individuals. The OPS of serotype *b* strains is structurally distinct from the OPS from the other serotypes, being composed of a disaccharide backbone of α-D-fucose and α-L-rhamnose. In this regard, the present results indicate that, in particular, the L-rhamnose degradation pathway was significantly enriched in t2d+ patients.

Another finding linking *A. actinomycetemcomitans* with the severity of periodontal disease comes from Ozuna et al. (2021), who presented for the first time that human neutrophils release epinephrine when challenged with *A. actinomycetemcomitans*, but not by *F. alocis*, a bacterium that occupies the same oral niche as *A. actinomycetemcomitans* and whose accumulation in the oral biofilm has been shown to be stimulated by the presence of specific strains of *A. actinomycetemcomitans* (Q. Wang et al., 2013). *Aggregatibacter actinomycetemcomitans* has also been found to be associated with *F. nucleatum*, whose growth is increased by hormones such as epinephrine and norepinephrine (Jentsch et al., 2013), with *Lactobacillus* spp., that expresses transporter systems for uptake of catecholamines (Lyte et al., 2018), and with other catecholamine-responsive species such as *Prevotella* spp. and *Leptotrichia* (Boyanova, 2017; Sandrini et al., 2015; Velusamy et al., 2019).

Interestingly, in the present taxonomic analyses, *Aggregatibacter* genus resulted significantly more abundant in p+ patients compared with healthy subjects (Figures 3 and 4), together with *F. nucleatum* and *F. alocis* (Figure 5). Therefore, it is likely that these bacteria work as a team to proliferate in the subgingival pocket, where the role of *A. actinomycetemcomitans* could be to induce the release of catecholamines by human neutrophils infiltrating the oral cavity, a release that could directly and differentially modulate the growth and composition of the subgingival microbiome.

An additional effect of the release of catecholamines or other leukocyte-produced molecules such as lactoferrin and transferrin is the sequestration of iron from the oral cavity making this essential nutrient for survival unavailable to bacteria (Freestone et al., 2000; Sandrini et al., 2015). However, some bacteria have evolved to subvert this mechanism by producing iron scavenging molecules, known as siderophores (Andrews et al., 2003; Chatterjee & O'Brian, 2018; Rhodes et al., 2007). Gram-negative bacteria have multiple siderophore-mediated iron acquisition pathways consisting of an outer membrane receptor, which is dependent upon the presence of a complex of three membrane proteins TonB, ExbB, and ExbD, and their ability to couple a proton gradient with siderophore transport (Bradbeer, 1993). Present analyses indicate that in p+ patients the enhanced pathway used to introduce iron into the cell used by bacteria present in the subgingival plaque seems to be the siderophores TonB dependent, given that among the proteins significantly enriched in p+ patients, there were two outer membrane receptors that were TonB dependent.

Furthermore, in the present analysis, among the pathways significantly enriched in the p+ group, were included three features involved in ferroptotic cell death and/or iron homeostasis. Ferroptosis, which is characterized by iron-dependent lethal lipid peroxidation, has been found to participate in the development of several inflammatory-based diseases, such as atherosclerosis, stroke, intracerebral hemorrhage, and ischemia/reperfusion injury (Abdalkader et al., 2018; Yang et al., 2014). This unique cell death is characterized by iron-dependent reactive oxygen species (ROS) and oxidized lipid contents in the cell membrane. A very recent study, using bioinformatics and a subsequent qRT-PCR validation analysis, indicated that ferroptosis served as a crucial target in the pathological mechanism and treatment of

periodontitis with T2D, and identified IL-1β, IL-6, NFE2L2, and ALOX5 as core ferroptosis-related genes (Pan et al., 2022).

In this regard, an additional noteworthy observation emerging from our results is that glutathione metabolism was enriched in p− subjects, thus reduced in p+ patients, and is known that the metabolism of glutatione is involved in the biosynthesis of a regulator of ferroptosis inhibition.

Finally, a further significant finding emerging from the functional analysis is related to nucleoside/nucleotide metabolism enriched in p+ and t2d+ subjects. In p+ subjects, the analysis showed an altered pyrimidine metabolism with a reduction in the degradation pathway accompanied by an enrichment of the salvage pathway. In addition, pyrimidine metabolism also resulted enriched for the biosynthesis of uridine 5′-monophosphate (UMP, the pyrimidine precursor) in t2d+ patients. UMP is the first product of de novo pyrimidine pathway and its pivotal role in the tissue and species specificity might highlight a possible pathogenic role of this metabolic pathway as already diagnosed in several other diseases (Smolenski et al., 1993; Webster, 2001).

Again in p+ patients, we observed that inosine 5′-monophosphate (IMP, the purine precursor) biosynthesis pathway was significantly enriched in p+. Recently, Lovászi et al. (2021) demonstrate that IMP is able to suppress the TNF-α production and augment the IL-10 production in endotoxemic mice.

The presence, shown here, of an imbalance in purine/pyrimidine metabolism in p+/t2d+ patients together with the other considerations proposed for the BCAAs, fatty acids, and adipocytokine metabolic pathways already discussed above, could point in the direction of a relevant role of a prolonged hyperactivation of the innate immune system induced by endotoxemia via oral–gut axis.

5 | CONCLUSIONS

To understand how bacteria cause disease, it is important to know how they colonize the host, how they evade the immune system or create an environment of immune tolerance, and also when, why, and how they transform into a pathogenic dysbiotic form.

From the taxonomic profile presented here, it was possible to outline a core microbiome, which is resilient to changes in the oral health status, composed not only of early colonizers but also showing the presence of species like *T. denticola*, *T. forsythia*, *S. sputigena*, or *T. socranskii* commonly considered periodontal pathogens. Instead, periodontitis patients were significantly characterized by the presence of *C. rectus* together with a significant contribution of low abundant bacteria such as *A. bacterium* oral taxon 439, *C. showae*, and *P. intermedia*. *Anaerolineaceae bacterium* oral taxon 439 also characterized, in p+ patients, t2d+ status, while *R. dentocariosa* appears to play a protective role in the periodontium of t2d+ subjects.

From a functional perspective, in the last decades, the pathogenetic interactions between periodontitis and T2D have been better understood but still much remain to be clarified (Barutta et al., 2022). The main functional findings presented in this study highlight a stimulating role of novel metabolic pathways involved in the complex inflammatory dialogue.

The first, intriguing, result is represented by the significantly different abundance of metabolic pathways related to BCAAs and other essential/aromatic amino acids and glycine levels, together with a significant increase in fatty acid biosynthesis and adipocytokine signaling pathways. All these features indicate an important role of the microbiome in stimulating systemic inflammation, promoting both hepatic and AT insulin resistance.

In this regard, some recently published molecular-based experimental work offers support for the functional analysis results reported here. Very recently, it has been proved that oral administration with heat-inactivated *Escherichia coli* during suckling improved intestinal resistance to *Salmonella typhimurium* infection in the weaned rat. The evidence was characterized by stronger inflammatory response due to enhanced inflammatory cytokine and higher antimicrobial capacity in the gut innate immune system, suggesting possible involvement of trained immunity (Cui et al., 2023). Another interesting finding stems from the demonstration, both in vitro and in vivo, that exposure to diets enriched in saturated fatty acids (SFAs) confers a hyper-inflammatory response to systemic LPS-induced endotoxemia by altering the composition of the hematopoietic stem cell compartment, with an increased response of bone marrow macrophages, monocytes, and splenocytes that leads to enhanced expression of inflammatory cytokines in the blood in a secondary LPS challenge (Seufert et al., 2022). Key insights from this study suggest the potential of SFAs to directly impact innate immune metabolism and epigenetics associated with inflammatory pathways.

A further interesting result is the increased abundance of features related to LPS biosynthesis, imbalance of nucleotide metabolism, significantly enriched in p+ and t2d+ patients, and ferroptosis enriched in p+ patients. These metabolic pathways might be involved in the two-way relationship between periodontitis and diabetes regarding the "T2D to periodontitis" direction. In fact, it has been demonstrated, for *P. gingivalis* (Nunes et al., 2020), that microbiota-derived circulating inflammagens, LPS and gingipain proteases, might be trafficked to AT and internalized by adipocytes and ATMs, thus affecting the phenotype of ATMs switch from the anti-inflammatory state to the pro-infammatory state, fueling inflammation and enhancing the host response to the bacterial challenge also within the oral tissues. Moreover, diabetes is able to increase periodontal destruction by both enhancing apoptosis of bone-forming cells and augmenting gingiva tissue degradation by increasing release of metalloproteinases and ROS by neutrophils and fibroblasts. The presence, shown here, of an enhanced ferroptosis, together with an imbalance in purine/pyrimidine metabolism observed in p+ and t2d+ subjects, might support the presence of a metabolic switch that eventually commits the cell to programmed cell death (Stasolla et al., 2004). Recent studies indicated that ferroptosis might represents a crucial target to identify new drugs with a potential clinical application value, opening interesting aspects also in the field of clinical treatment of periodontitis with T2D (Pan et al., 2022).

Also on this topic, some recent experimental data seem to corroborate the functional analyses reported here. The link between periodontitis and cardiovascular diseases (CVD) is well known, and although many studies have found associations between periodontitis and CVD, the relationship is still debated (Sanz, Marco del Castillo, et al., 2020). Recently, it has been shown that ferroptosis is involved in the new-onset atrial fibrillation with LPS-induced endotoxemia in a rat model (Fang et al., 2021) and that Resveratrol, through upregulation of miR-149 and of the high mobility group box 1 gene, can inhibit ferroptosis and improve myocardial damage in mice with LPS-induced endotoxemia (X. Wang, Simayi, et al., 2022). Thus, our results could indicate a possible role of ferroptotic death also for periodontitis and T2D comorbidity.

Further experimental evidence (Hersoug et al., 2018) shows that LPS is involved in the transition of macrophages from the M2 to the M1 phenotype in AT. LPS are taken up by adipocytes and may activate caspase-4/5/11. This may induce a highly inflammatory type of programmed cell death (i.e., pyroptosis), which also occurs after infection with intracellular pathogens. Large adipocytes are more metabolically active and potentially more exposed to LPS than small adipocytes. Thus, LPS might be involved in defining the adipocyte death size and the formation of crown-like structures. The adipocyte death size is reached when the intracellular concentration of LPS initiates pyroptosis. The mechanistic details remain to be elucidated, but the observations indicate that adipocytes are stimulated to cell death by processes that involve LPS from the gut microbiota.

All together, the results of the present study point to new features that may underlie the complex interplay between T2D and periodontitis, also suggesting the importance of these findings not only for potential dietary interventions but also for the treatment of inflammatory diseases exacerbated by metabolic dysfunction in humans.

AUTHOR CONTRIBUTIONS

Conceptualization: Chiara Scapoli and Leonardo Trombelli. *Methodology*: Nicoletta Favale, Roberto Farina, Alberto Carrieri, Silvia Sabbioni, Chiara Scapoli, and Leonardo Trombelli. *Formal analysis*: Nicoletta Favale, Alberto Carrieri and Chiara Scapoli. *Patients' recruitment and sampling*: Roberto Farina, Anna Simonelli. Mattia Severi and Leonardo Trombelli: *Writing—original draft preparation*: Nicoletta Favale and Chiara Scapoli. *Writing—review and editing*: Nicoletta Favale, Roberto Farina, Alberto Carrieri, Anna Simonelli, Mattia Severi, Silvia Sabbioni, Chiara Scapoli, and Leonardo Trombelli. *Supervision*: Chiara Scapoli and Leonardo Trombelli. *Funding acquisition*: Chiara Scapoli, Silvia Sabbioni, and Leonardo Trombelli. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequencing data are available under NCBI BioProject PRJNA976261.

ORCID

Chiara Scapoli https://orcid.org/0000-0003-4058-4787

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