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Microbial whole genome sequencing analysis: from *de novo* single strains reconstruction to metagenomics to characterize bacterial communities

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Abstract

Microbial genomes analysis is the field that takes the greatest advantages from the breakthroughs in genomics and computational biology. Whole-genome sequencing strategies allowed the fulfilment of first complete genome sequence of a bacteria in 1995, since then technological improvements reduced the time required for sequencing and the cost per base pair while at the same time dramatically increased the quality of final products. These enhancement leads to the possibility to extend microbial genomes analysis ranging from the study of the single strain to whole microbial communities.

In this thesis two application of whole-genome sequencing are presented: I) the genome's reconstruction and characterization of *Acinetobacter johnsonii* ICE_NC and II) the analysis of the microbial community characterizing the oral plaque of human patients in different clinical conditions of periodontitis and type 2 diabetes.

Chapter 1. *Acinetobacter johnsonii* ICE_NC was isolated from industrial soil and showed a role in the biotransformation of bile acids (BAs). Here are described the use of hybrid *de novo* assemblies, that combine long-read Oxford Nanopore and short-read Illumina sequencing strategies, to reconstruct the entire genome of *A. johnsonii* ICE_NC strain and to identify the coding region for a 12α-hydroxysteroid dehydrogenase (12α-HSDH), involved in bile acids metabolism.

The hybrid *de novo* assembly of the *A. johnsonii* ICE_NC genome was generated using Canu and Unicycler, both strategies yielded a circular chromosome of about 3.6 Mb and one 117 kb long plasmid. Gene annotation was performed on the final assemblies and the gene for 12α-HSDH was detected on the plasmid.

Our findings illustrate the added value of long read sequencing in addressing the challenges of whole genome characterization and plasmid reconstruction in bacteria. These also allowed the identification of the *A. johnsonii* ICE_NC gene for the 12α-HSDH enzyme, whose activity was confirmed at the biochemical level.

Chapter 2. Periodontitis and type 2 diabetes have a long established bi-directional relationship. Twelve subjects, falling into one of the four study groups based on the presence/absence of poorly controlled type 2 diabetes and presence/absence of moderate-severe periodontitis, were selected. The high-resolution whole metagenomic shotgun sequencing was used to characterize the subgingival microbiome of patients with different health status regarding periodontitis and type 2

diabetes.

Taxonomic profile was analysed with a BLAST analysis combined with MEGAN6 or MetaPhlAn 3.0. Both outputs were statistically tested with STAMP and LEfSe.

The investigation of functional activities was performed by DIAMOND combined with MEGAN6 - KEGG and by HUMAnN 3.0; statistical analysis was carried out with LEfSe.

Taxonomic analysis results unlighted: I) the significant presence of *Anaerolineaceae bacterium* oral taxon 439 in periodontitis patients, II) that the presence of bacteria historically associated with onset and progression of periodontitis seems more linked to a general inflammatory status, rather than specifically with periodontal disease and III) the presence a core microbiome, that is resilient to changes in the oral health status.

Main results in functional investigation were that most of the significantly enriched pathways involved in amino acid biosynthesis and carbohydrate or energy metabolism belonged to patients without periodontitis. Pathways related to cell structure biosynthesis, fatty acid and lipid biosynthesis, nucleoside and nucleotide metabolism and ferroptotic death and/or iron homeostasis were significantly abundant in patients affected by periodontitis, diabetes or presenting comorbidity.

Abstract - Italiano

Il campo dell'analisi dei genomi microbici è quello che ha ottenuto i maggiori vantaggi dagli avanzamenti della genomica e della biologia computazionale. Le strategie di sequenziamento whole-genome hanno permesso il completamento della prima sequenza genomica completa di un batterio nel 1995, da quel momento gli avanzamenti tecnologici hanno portato alla riduzione dei tempi di sequenziamento, dei costi per base sequenziata e allo stesso tempo al drastico aumento della qualità dei prodotti finali. Questi miglioramenti hanno portato all'ampliamento dell'analisi dei genomi microbici, permettendo di spaziare da studi su singoli ceppi fino all'analisi delle comunità microbiche nella loro interezza.

In questa tesi sono presentate due applicazioni del sequenziamento whole-genome: I) la ricostruzione e caratterizzazione del genoma di *Acinetobacter johnsonii* ICE_NC e II) l'analisi delle comunità microbiche che caratterizzano la placca orale di pazienti in differenti condizioni per parodontite e diabete di tipo 2.

Capitolo 1. *Acinetobacter johnsonii* ICE_NC, isolato da suolo industriale, ha mostrato un ruolo nella biotrasformazione degli acidi biliari (BAs). Qui è descritto l'uso di strategie ibride per l'assemblaggio *de novo*, che combinano le strategie di sequenziamento long-read Oxford Nanopore e short-read Illumina, per ricostruire l'intero genoma di *A. johnsonii* ICE_NC per identificare la regione codificante per l'enzima 12α-idrossisteroide deidrogenasi (12α-HSDH), coinvolto nel metabolismo dei BAs.

L'assemblaggio ibrido *de novo* del genoma di *A.jonsonii* ICE_NC è stato generato usando Canu e Unicycler, entrambe le strategie hanno portato all'identificazione di un cromosoma circolare lungo circa 3.6 Mb e di un plasmide di circa 117 Kb. L'annotazione genica è stata eseguita sugli assemblaggi finali e il gene per 12α-HSDH è stato identificato nel plasmide.

I risultati ottenuti mostrano il valore aggiunto del sequenziamento long-read per superare le sfide nella caratterizzazione di genomi batterici completi e nella ricostruzione dei plasmidi. Inoltre è stato possibile identificare il gene codificante l'enzima 12α-HSDH in *A. johnsonii* ICE_NC, la cui attività era stata confermata a livello biochimico.

Capitolo 2. Parodontite e diabete di tipo 2 hanno una relazione bidirezionale nota da molto tempo. Sono stati selezionati 12 individui, ognuno appartenente a uno dei quattro gruppi di studio basati sulla presenza/assenza di diabete di tipo 2 scarsamente controllato e di parodontite da moderata a

severa. Per ogni soggetto eleggibile sono stati prelevati campioni di placca subgengivale in 4 siti, tutti rappresentativi delle condizioni parodontali dell'individuo. Per caratterizzare il microbioma subgengivale di questi pazienti è stato utilizzato il sequenziamento ad alta risoluzione dell'intero metagenoma.

Il profilo tassonomico è stato analizzato combinando BLAST con MEGAN6 e attraverso MetaPhlAn 3.0. Entrambi gli output sono stati sottoposti ad analisi statistica con STAMP e LEfSe. L'analisi delle attività funzionali è stata eseguita con DIAMOND combinato a MEGAN6 e attraverso HUMAnN 3.0; l'analisi statistica è stata eseguita con LEfSe.

I risultati dell'analisi tassonomica hanno evidenziato: I) la presenza significativa di *Anaerolineaceae bacterium* oral taxon 439 nei pazienti parodontitici, II) che la presenza di batteri storicamente associati all'insorgenza e al progredire della parodontite sembravano correlati maggiormente a un generale stato infiammatorio piuttosto che specificatamente alla parodontite e III) la presenza di un microbioma di base, resistente ai cambiamenti dello stato di salute orale.

I risultati principali dell'analisi funzionale hanno mostrato che i pathway legati alla biosintesi di amino acidi, al metabolismo energetico e dei carboidrati erano rappresentati significativamente nei pazienti non affetti da parodontite mentre i pathway relativi alla biosintesi di strutture cellulari, al metabolismo di acidi grassi, alla ferroptosi e al metabolismo di nucleosidi e nucleotidi erano abbondanti nei pazienti affetti da parodontite, diabete o entrambe le malattie.

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Microbial whole genome sequencing analysis

The first step in bacterial genome studies was made in 1995 with the sequencing of the complete genome of *Haemophilus influenza* (Fleischmann et al., 1995). From that moment, astonishing progresses were made in sequencing technologies that led to 34,401 genomic sequences in the NCBI genome database as of January 2022 (https://www.ncbi.nlm.nih.gov/genome/?term=prokaryotes).

Several improvements occurred to sequencing technologies throughout the years. The first revolution was the chemical chain termination method, followed by the Sanger sequencing process (Sanger et al., 1977). Over the years, the search for faster, more efficient and cheaper technologies led to another great advancement: the development of next generation sequencing technologies (NGS) that allowed sequencing output in the order of Gigabases (Gbp). Further, NGS avoids the bacterial cloning phase and is less expensive than Sanger's sequencing (van Dijk et al., 2018). Several NGS technologies were developed over the years (Mardis, 2017), but today's most used technology is the Illumina platform. Illumina allows sequencing short DNA fragments in paired-end reads (sequences) up to 300 bp. These short reads allow the identification of small variants or short indels (<50bp). However, larger structural variation cannot be detected nor repetitive genomic sequence within the genome can be disambiguated (Koren et al., 2013; Whiteford et al., 2005). Recently, the development of third generation sequencing (TGS) technologies allowed sequences of long fragments of DNA, over 200,000 bp, in real time and avoiding PCR amplification (Schadt et al., 2010).

Improvement of both NGS and TGS technologies lead to an increase in the resolution of bacterial genome studies, allowing to overcome the constraints posed when sequencing a single gene, like the widely used 16S rRNA. Sequencing specific DNA markers, such as the 16S, limits the reconstruction of the taxonomy of a bacterial strain or community (Hamady & Knight, 2009), as only a small portion of the variability will be studied. Conversely, whole genomic sequencing (WGS) enables a deeper analysis of bacterial genomes both in term of single strains and metagenomic samples. Single strain WGS permits the reconstruction of the genomic and the plasmid sequence, allowing the detection of variants between different strains of the same species and a better identification of the complete set of genes that are present. On the other hand, metagenomic WGS allows the study of the microbial community present in an environment, in a culture-independent way, while simultaneously studying prokaryotes, eukaryotes and viruses, in both a qualitative and quantitative taxonomic profiling of the sample. Furthermore, metagenomic WGS can also contribute to the detection of new genes in the community and the discovery of new lineages and species based on their genome sequence (Franzosa et al., 2015; Norman et al., 2015).

In this thesis, two different studies based on bacterial whole genomic sequencing (WGS) are presented in two chapters. The first chapter is a *de novo* reconstruction of a single strain genome from industrial soil. The second chapter is the analysis of the microbial community characterizing the oral plaque of human patients in different conditions of periodontitis and type 2 diabetes.

Chapter 1. Reconstruction of *Acinetobacter johnsonii* ICE_NC genome using hybrid *de novo* genome assemblies and identification of the 12α-hydroxysteroid dehydrogenase gene

1. Introduction

1.1 *Acinetobacter johnsonii*

Acinetobacter johnsonii is a Gram-negative coccobacillus belonging to the class of Gammaproteobacteria. It is a typical inhabitant of the aquatic environment, but can colonize a considerable amount of environments including soil, plant, animal and human (Guardabassi et al., 1999; Harald Seifert et al., 1997; Y. Shi et al., 2011; Wang et al., 2019). This species can cause human clinical infections, like bloodstream infections in immunocompetent patients and peritonitis (Rodríguez et al., 2014; H. Seifert et al., 1993).

Previous studies sequenced the genomes of strains isolated from various environments. Of the 45 genomes from *A. johnsonii* currently available on the National Center for Biotechnology Information (NCBI) database, 16 were assembled at complete level. The genome size varies between different strains, spanning from 2.67 to 4.14 Mbp, while GC content is usually about 41.5%. Recently, a comparative genomic analysis of isolates from clinical and environmental sources showed that *A. johnsonii* has great adaptability to different environments. Strains isolated from clinical and water environment showed a high heterogeneity in genome size (from 3.23 to 4.08 Mbp), while GC content was similar (around 41%), but these features were not significantly different between environmental and clinical isolates. Instead, strains from clinical sources revealed a higher number of core genes than strains from water samples. Furthermore, clinical-derived isolates accumulate more genes associated with translational modification whereas environmental-derived isolates possess more genes related to substances degradation (Jia et al., 2022). Therefore, this shows necessity of *A.johnsonii* to accumulate more genes linked to antimicrobial resistance and defence mechanism to adapt to human host.

1.2 *A. johnsonii* in industrial soil sample

In 2008, Giovannini et al. (Giovannini et al., 2008) reported the partial purification of 12α-hydroxysteroid dehydrogenase from *Acinetobacter calcoaceticus lwoffii* (now *A. johnsonii*). This strain was isolated from a soil sample of a chemical plant working with bile (Industria Chimica Emiliana, ICE). This enzyme belongs to the group of hydroxysteroid dehydrogenases (HSDHs), a

subgroup of NAD+- or NADP+-dependent oxidoreductases, and seemed very efficient in the quantitative oxidation of the C_7-OH function of chenodeoxycholic acid (Eggert *et al.*, 2014). Indeed, chenodeoxycholic acid is an intermediate in the ursodeoxycholic acid (UDCA) synthesis, that have important pharmaceutical applications related to its ability to solubilize cholesterol gallstones (Crosignani et al., 1996; Salen et al., 1980).

Despite several studies were made in the attempt to characterise 12α-HSDH from bacteria based on coding sequences (CDS) predicted by protein sequences, to date the 12α-HSDH coding gene have yet to be completely described and no information are available on 12α-HSDH gene in *Acinetobacter* (Aigner et al., 2011; Braun et al., 2011; Braun et al., 1991; Doden et al., 2018; Harris & Hylemon, 1978; Macdonald et al., 1979).

The *de novo* high resolution reconstruction of microbial genomes provides reference-free structural information and can identify loci not represent on available references or genes in non-chromosomic elements like plasmids. For example, the *de novo* genome assembly of an unauthorized genetically modified *Bacillus subtilis* strain allowed Berbers et al. (Berbers et al., 2020) to detect antimicrobial resistance genes localized on the plasmid.

1.3 Genome assembly

Starting in 1995 with the sequencing of *Haemophilus influenzae*'s genome (Fleischmann et al., 1995) genome sequencing of bacteria emerged as helpful approach to handle several microbiological issues. Nowadays, technological improvement allows to sequence microbial genomes quickly and in a cost-effective manner (Goldstein et al., 2019; Land et al., 2015). Illumina's sequencing platforms have long prevailed in the field of microbial genome sequencing due to their ability to generate extremely accurate reads (error rate <=1%) (https://emea.illumina.com/science/technology/next-generation-sequencing/plan-experiments/quality-scores.html) at reasonable cost.

Microbial genomes are usually characterized by repetitive genomic regions including ribosomal genes, transposons, toxins, CRISPR arrays, secondary metabolite biosynthetic gene clusters and others (Haubold & Wiehe, 2006; Kingsford et al., 2010). Illumina's short reads are not able to disambiguate repetitive regions longer than its read length (300 bp), therefore they often show weak performances during *de novo* assembly of microbial genomes, generating assembly graphs fragmented into contigs instead of single chromosomes (Koren et al., 2013; Whiteford et al., 2005).

Third-generation sequencing allow to obtain long fragment of DNA sequenced. The main companies for long read sequencing are Pacific Biosciences (PacBio) and Oxford Nanopore. The PacBio platform

can sequence DNA fragments of lengths averaging 10-25 Kb (Hon et al., 2020) with a high error rate, reduced over the years thanks to technological improvement. Because the errors are randomly distributed, the error rate can be reduced by increasing the sequencing coverage (Koren et al., 2013). Oxford Nanopore Technologies (ONT) offers various platforms for long read sequencing, characterized by high speed and low cost. ONT reads have a high error rate, dominated by false deletions and homopolymer errors (Sedlazeck et al., 2018). MinION is the most affordable sequencer offered by ONT (from 1000$). It is a small device, that can be connected directly to a personal computer through a USB port and can reach read length over 200,000 bp (Leggett & Clark, 2017, https://nanoporetech.com/how-it-works/advantages-nanopore-sequencing).

In bacteria, genome assemblies must take into consideration the potential presence of plasmids, these genetic elements that can span from roughly one thousand DNA base pairs to hundreds of thousands of base pairs (George et al., 2017). In contrast with Illumina, long reads allow to discriminate between plasmids and chromosomal sequences (Wick et al., 2017a).

Genome *de novo* assembly is a computational method that permits the reconstruction of genetic sequences from sequenced fragments. It is usually carried out using reads produced by a single sequencing technology. Several bioinformatic methods were developed to generate hybrid *de novo* genome assembly that allows to take advantage of both short and long read sequencing (Goldstein et al., 2019; Risse et al., 2015). Assemblies generated with hybrid *de novo* methodologies have a high accuracy and contiguity (Goldstein et al., 2019).

Two main strategies can be used to reconstruct a genome with hybrid *de novo* assembly. In the first, the assembly is generated with an assembler that can integrate both short and long reads. Illumina's data are initially used to generate a set of contigs and the long reads are then used to join contigs in larger elements called scaffolds. This strategy is often able to deconvolute regions of the assembly that short reads cannot resolve (repetitive genomic regions) (Wick et al., 2017b).

In the second approach, the assembly is generated directly from long (noisy) reads and the short reads are then used to remove errors (polishing process) from the assembly. The polishing phase does not increase the assembly contiguity but is able to enhance the base quality of the nucleotides composing the assembly (Koren et al., 2016; Walker et al., 2014).

2. Aim of the study

A. johnsonii is a gram-negative bacillus widely distributes in natural and clinical environments. With the rapid development of technology and the reduction of sequencing costs, more and more genomes are openly available. This made in-depth analysis of the genetic diversity, evolution and adaptability of specific bacteria at the genome level feasible.

Genome plasticity is a key determinant in *A. johnsonii* and its adaptability to different habitats is related to the presence of a large pan-genome that allows to the clinical-derived strains to accumulate more genes associated with translational modification, β-lactamase and to defence mechanisms, whereas to environmental-derived isolates to be more enriched of genes related to substances degradation.

At the Department of Life sciences and biotechnology of the University of Ferrara was available a strain of *A. johnsonii* isolated from a soil sample of a chemical plant. In 2008 Giovannini et al. described the role of this *A. johnsonii* ICE_NC strain in biotransformation of bile acids, but one of the key enzymes involved this activity of the bacteria, the 12α-hydroxysteroid dehydrogenase (12α-HSDHs), was only partially characterised and to date no information on 12α-HSDH gene in *Acinetobacter* are available.

In this chapter is described the reconstruction of *A. johnsonii* ICE_NC's genome through two different hybrid *de novo* assembly strategies with the aim, in addition to the characterization of the whole genome of the strain, to identify the 12α-HSDHs gene, making possible its cloning and the subsequent complete purification and characterization of the related protein, which has been shown to have pharmaceutical applications related to cholesterol gallstone solubilisation.

The whole-genome sequence was determined by combining Oxford Nanopore MinION and Illumina Nextesq 500 technologies. The dominant sequencing technology provided by Illumina, generates short-reads (75-300 kb) with low error rate (>1%) but presents the significant limitation that can't resolve genomic repetitive regions, that are common in bacterial genomes. The long read sequencers, like Oxford Nanopore MinION, allows the resolutions of repetitive regions, quickly and with low costs but still present difficulties in the basecalling process that result in a high error rate (about 10% for MinION platform).

Different hybrid *de novo* assembly strategies were applied in order to compare the efficiency of both MinION-only and Illumina-hybrid methods.

3.Materials & Methods

3.1 DNA isolation and preparation

The *A. johnsonii* strain (as described in Giovannini et al., 2008) in was grown at 28°C in LB medium for 18h. Whole genomic DNA was extracted from harvested cells using the GenElute™ Bacterial Genomic DNA Kit (Sigma), in accordance with the manufacturer's instructions.

Purified DNA was quantified by spectrophotometer (Shimadzu BioSpec-nano) and by Qubit (Life Technologies) fluorometric analyses.

3.2 Library preparation and sequencing

A) Nanopore MinION

Whole genome sequencing was performed on a MinION device loaded with a MinION Flow Cell (R9.4.1). A library was prepared using 1 µg of genomic DNA, first subjected to the NEBNext® Ultra™ II End Repair/dA-Tailing module (New England Biolabs) for end repair and dA-Tailing, in accordance with the manufacturer's instructions. Upon beads purification (AMPure XP beads, Beckman Coulter), DNA was ligated to adapters using Blunt/TA ligase (New England Biolabs), size selected using AMPure XP beads and finally loaded on the MinION cell.

B) Illumina NextSeq

The library was generated from 20 ng of genomic DNA, in accordance with the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina protocol (New England Biolabs). Prior to library preparation, DNA was fragmented using NEBNext® dsDNA Fragmentase for 30 min. NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers set 1) were used to produce the library and label it with specific molecular barcodes. Then, AMPure XP beads (Beckman Coulter) were used for library purification. Finally, the library was quantified using the High Sensitivity DNA Kit (Agilent Technologies) on the Bioanalyzer instrument (Agilent). Sequencing was performed with an Illumina NextSeq 500 sequencer with 2×150-bp read, using NextSeq® 500/550 Mid Output Kit v2.

3.3 Reads preparation

Nanopack's tools (De Coster et al., 2018) were used to manage the MinION raw reads: I) NanoPlot (v.1.21.0) to assess reads quality, II) NanoLyze (v.1.1.0) to remove reads mapping to the Lambda phage genome and III) NanoFilt (v.2.2.0) to filter fastq file based on a minimum quality cut-off (>10)

and minimum length (> 2,000 bp and > 20,000 bp). From the reads obtained with the MinION sequencer were generated two different datasets named subset_2k and subset_20k retaining respectively reads longer > 2,000 bp and > 20,000 bp. These two dataset were generated to avoid loss of plasmid material (subset_2k) and to evaluate the performance of very long reads in the whole chromosome assembly (subset_20k).

Estimation of the Nanopore MinION error rate was performed aligning the 2D reads of Lambda phage spike-in DNA (control DNA, CS, ONT) against the Lambda reference genome by using minimap2 (v.2.16-r922; Li, 2018), results were obtained by parsing BAM files with SAMtools (H. Li & Durbin, 2009) and using R scripts described in Fuselli et al. (Fuselli et al., 2018).

Quality control (QC) for Illumina reads was done with FastQC (v.0.10.1, Andrews, 2010). FastQC provide a QC report which spots potential issues within the raw reads, originating either in the sequencer or during the library preparation. To remove Illumina adapters,

AdapterRemoval (v.2.3.0, Schubert et al., 2016) was used.

3.4 Genome assembly

Two length filters were applied to MinION data in order to exclude reads shorter than 2 and 20 Kbp, respectively. The two resulting datasets were used to produce the *de novo* assemblies with two approaches that combine ONT data with Illumina data: Canu (v1.8, Koren et al., 2016) that generated *de novo* assemblies made up by long reads polished with short reads using Pilon (v. 1.23, Walker et al., 2014), followed by an Unicycler hybrid pipeline (v.0.4.8-beta, Wick et al., 2017b), that generates the assemblies from short reads. The long reads were then used to deconvolute the assembly graphs, decreasing the number of contigs. The assemblies quality assessment was performed using assembly-stats (https://github.com/sanger-pathogens/assembly-stats), that evaluate genome assemblies by computing various metrics, BUSCO (v. 5.0.0, Manni *et al.,* 2021) to estimate the completeness and Merqury (v. 1.3, Rhie *et al.*, 2020) to assess global completeness and assembly consensus quality value (QV).

3.5 Characterization of the complete genome and plasmid sequences

The final complete circular chromosome and the plasmid genome sequences of *A. johnsonii* ICE_NC obtained through Canu or Unicycler hybrid were analysed using Prokka (Seemann, 2014), to annotate protein coding genes. Additionally, a refined BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

analysis was performed to identify the different contigs and the presence of sequences ascribable to the 12 α-hydroxysteroid dehydrogenase.

Presence of prophages inserted in the chromosome was detected with PHASTER (https://phaster.ca/) (Arndt et al., 2016) while CRISPR arrays and CRISPR-associated genes (cas) where searched with CRISPRcasFinder (https://crisprcas.i2bc.paris-saclay.fr/) (Couvin et al., 2018).

VFanalyzer (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi) (Liu *et al.*, 2019), with the Virulence Factors Database, was used to find virulence factors across the chromosome and a BLASTP (version 2.10.0+, Altschul et al., 1997) analysis against the full Virulence Factors Database was performed to find putative virulence factors in the plasmid; eventually a ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/) (Bortolaia et al., 2020; Zankari et al., 2012) analysis was performed to search for potential antibiotic resistance genes.

4.Results

4.1 Sequencing

Results of whole-genome sequencing of *A. johnsonii* ICE_NC from Oxford Nanopore MinION and Illumina Nextesq 500 platforms are summarized in Table 4.1. The MinION device generated 2,737,448 reads totalling 12.5 Gbp, with a median read length of 2,191 bp (longest read was 198,250 bp long) and median read quality of 11.3. The Illumina sequencing resulted in 1.3 Gbp of throughput, consisting of 9,211,819 paired-end reads with median length of 141 bp and median read quality of 33.

Platform	Oxford Nanopore MinION	Illumina NextSeq 500
Total number of reads	2,737,448	18,423,620
Sequencing depth	1709	740
Median read length (bp)	2,191	141
Read length N50 (bp)	9,668	141
Longest read (bp)	198,250	141
Median Phred quality score	11.3	33

Table 4.1 Assemblies features of *A. johnsonii* ICE_NC.

The median error rate of the Nanopore sequencing (Figure 4.1) computed using Lambda DNA, was 2.3% for single nucleotide variations (SNV), 1.2% for insertion (INS) and 2.1% for deletions (DEL).

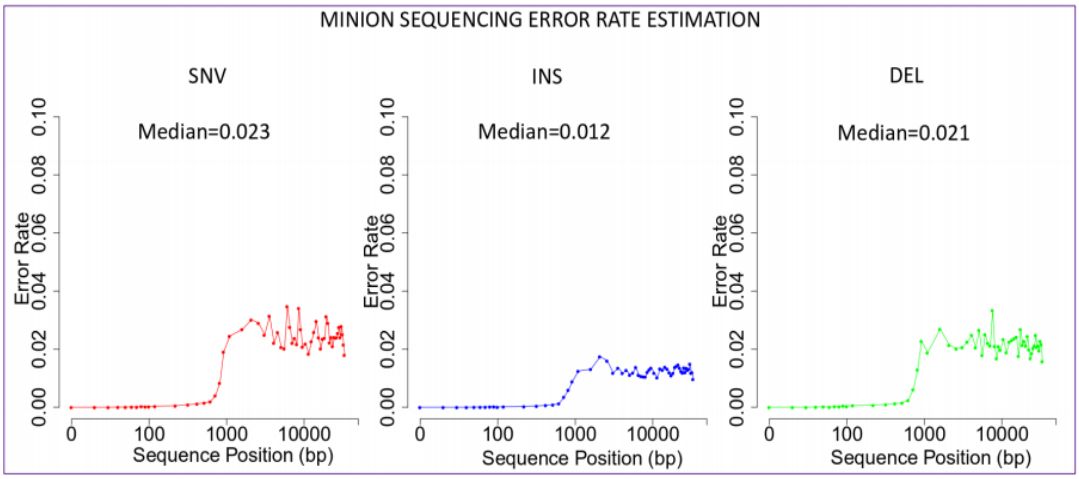


Figure 4.1 MinION sequencing error rate estimation. The three parameters were calculated as a function of sequence position.

4.2 Genome assembly

Considering the two filter cut-offs for long reads (reads shorter than 2000bp, subset 2K, or shorter than 20000bp, subset 20K), the assembly of the Oxford Nanopore's MinION reads with Canu 1.8 (Table 4.2) gave almost equal outputs for the two subsets, in terms of length of first and second longest contig. However, the total number of contigs varied among both subsets: the assembly of subset "2K" originated a total of 13 contigs, while the subset "20K" produced only 3 contigs (Table 4.2). For both assemblies, the longest contig corresponded to the of *A. johnsoni* ICE_NC, a sequence of about 3,674,300 bp (slightly different for the two subsets, Table 1).The second contig, of about 207,000 bp, represented a plasmid sequence. A BLAST analysis of the remaining contigs, for both assemblies, revealed that they were part of the main chromosomal contig.

The *de novo* Unicycler-hybrid assembly of the Illumina sequence reads plus the base-called Oxford Nanopore reads resulted in a complete circular chromosome of 3,583,655 bp plus a plasmid sequence of 117,458 bp identical for both subsets analysed (Table 4.2).

Analysis performed with QUAST confirmed that all assemblies did not contain any gaps (i.e., no N's, Table 4.2). Furthermore, Merqury estimates provided a value of gene completeness greater than 98% and a global completeness higher than 97%. Canu's assembly has a QV of 41.6, while the value for the Unicycler assembly was 68.

Sequencing platforms	**Oxford Nanopore-MinION**		**Illumina NextSeq 500 + Oxford Nanopore-MinION**	
Assembly method	Canu v. 1.8 + Pilon v.1.23		Unicycler v.0.4.8-beta	
Subset	2K	20 K	2K	20 K
Finishing quality	Scaffold	Scaffold	Closed complete genome + plasmid	Closed complete genome + plasmid
Number of contigs	13	3	2	2
Number of N's	0	0	0	0
Total length bp	4,121,247	3,974,922	3,701,113	3,701,113
GC%	41.17	41.21	41.24	41.24
N50	3,674,325	3,674,351	3,583,655	3,583,655
Complete BUSCOs	98.3%	98.3%	98.6%	98.6%

Table 4.2 Assemblies features of *A. johnsonii* ICE_NC.

4.3 Characterization of the complete genome and plasmid sequences

Both the final complete chromosome sequences of *A. johnsonii* ICE_NC obtained through Canu and Unicycler assemblies were annotated with Prokka (Table 4.3). For plasmid assemblies, a BLASTN alignment between the two 20K subsets obtained with Canu and Unicycler showed that the Canu contig of 207 Kb was a chimera produced by merging two sequences highly similar to the contig of 117 Kb obtained from Unicycler assembly. Thus, only the 117Kbp plasmid sequence obtained from the 20K Unicycler subset was annotated and characterized.

4.3.1 Complete genome characterization

The annotation of the chromosomal sequences obtained from the two 20k Canu or Unicycler assemblies revealed a comparable total number of genes, CDSs and RNA genes (Table 4.2) and consistent with literature (Jia *et al.,* 2021). Statistics about protein coding sequencing, putative genes and hypothetical protein showed sizeable differences: protein coding sequences were more abundant in the Canu assembly (2604 versus 1732 for Unicycler), while putative genes (18 versus 148) and hypothetical proteins (883 versus 1538) were more represented in the Unicycler assembly. These differences might be explained by the different size of the two assemblies: the greater size of the chromosome reconstructed with Canu, 100 Kb longer than the Unicycler assembly, is correlated with a greater completeness of gene elements, allowing the annotator a better recognition of known proteins, decreasing the number of predicted elements.

Assembly	**Canu20k chromosome**	**Unicycler20k chromosome**	**Unicycler20k plasmid**
Number of genes (total)	3,682	3,507	125
Total coding sequences (CDSs)	3,581	3,418	125
Protein coding sequences	2,604	1,732	61
Putative genes	18	148	3
Number of RNA genes	177	146	-
tRNA	100	88	-
tmRNA	1	1	-
Ribosomal RNA	76	57	-
Hypothetical proteins	883	1,538	61

Table 4.3. Annotation features of A. johnsonii ICE_NC genome using Prokka v.1.13.7.

Using the software PHASTER putative prophage regions were detected in both chromosome assemblies (Table 4.4). In the Canu assembly two regions were recognized as intact prophages (score >90), while the Unicycler assembly contained only one intact sequence, Entero Arya NC 031048, the same identified within the Canu's assembly.

The analysis of both chromosome assemblies with CRISPRFinder did not reveal any CRISPR array.

Resfinder's analysis uncovered one gene related to antibiotic resistance in both assembled sequences. The gene codes for a putative class D (blaOXA-309) β-lactamase protein.

The genomic sequences of *A. johnsonii* ICE_NC were analysed with VFfinder against the genus Acinetobacter. Several putative virulence factors were detected and the most represented categories were: iron uptake (28 items), biofilm formation (14 items) and immune evasion (8 items). These results were shared for both type of assemblies.

Assembly	**Canu20k intact region 1**	**Canu20k intact region 2**	**Unicycler20k intact region 1**
Region length (bp)	5,852	36,998	37,010
Score	110	120	110
Total proteins	11	58	57
Region position	993,621 – 999,473	3,558,700 – 3,595,698	877,031 – 914,041
Most common prophage	Stx2 c Stxa F451 NC 049924	Entero Arya NC 031048	Entero Arya NC 031048
GC (%)	46.27	40.30	40.29%

Table 4.4 Regions identified with PHASTER as intact prophage. The position in the genome, length, number of CDSs, most common tag and percentage of GC are indicated for each putative prophage.

4.3.2 Plasmid annotation

As the plasmid assembly by Canu turned out to be a chimera, only the plasmid sequence reconstructed by Unicycler was annotated and characterized. A genome atlas of this annotation is depicted in Figure 4.2.

The plasmid annotation with Prokka (Table 4.4) revealed a total of 125 genes, all classified as CDSs, 3 putative genes and 61 "hypothetical proteins". The characterization of the plasmid sequence revealed the presence of a putative prophagic region of 33,041 bp, with GC content of 37.89%

identified as Paenib Tripp NC 028930 and of 33 protein sequences corresponding to virulence factors. No CRISPR elements or antibiotic resistance genes were detected in the plasmid sequence.

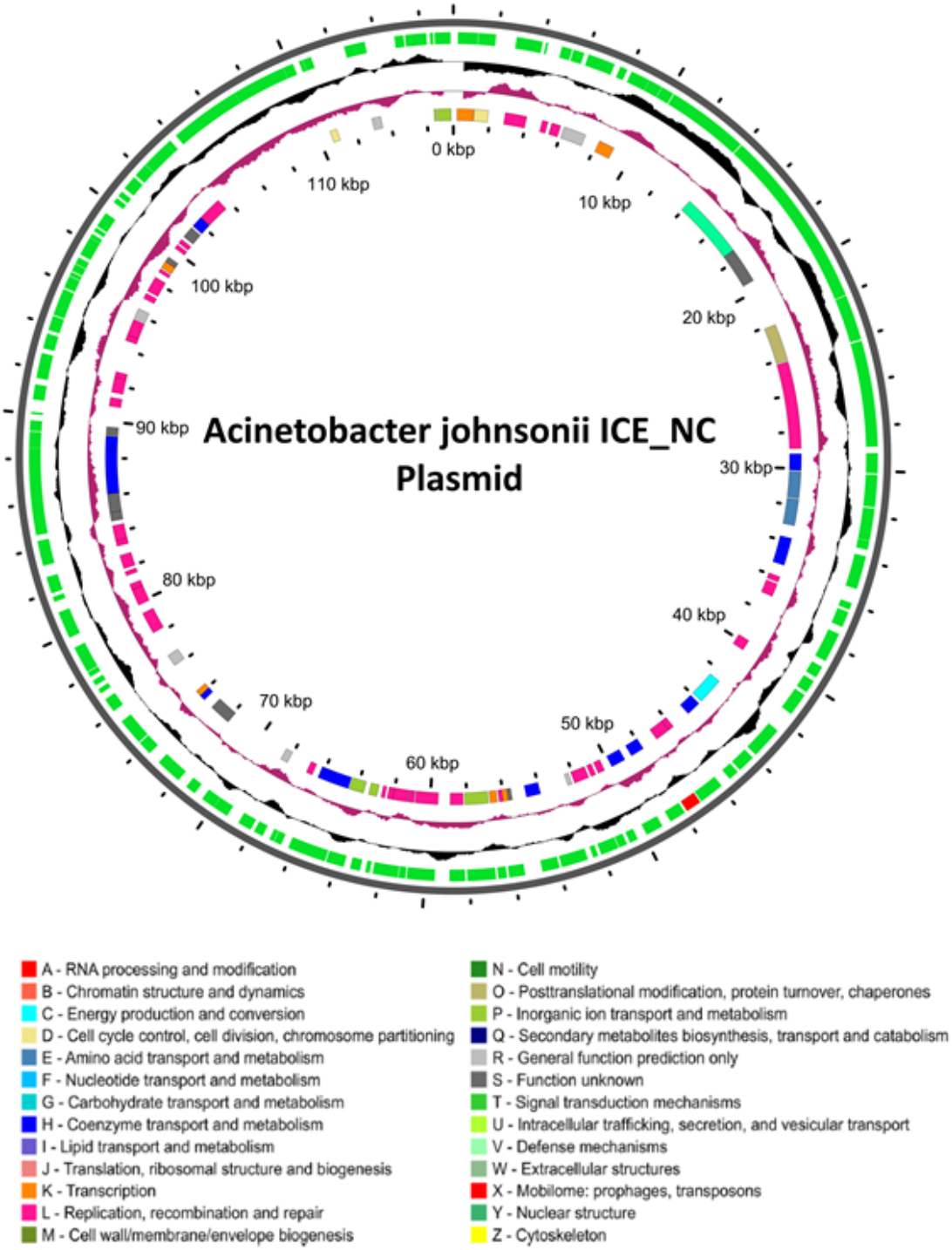


Figure 4.2 Features of the *A. johnsonii* ICE_NC plasmid built with the hybrid assembly annotated with Prokka, using the online server GView. Labelling from the outside to inside: backbone; CDSs (coloured in green with 12α-HSDH highlighted in red); GC content (GC-rich towards outside, GC-poor towards inside); GC skew (excess of guanine over cytosine towards outside, and vice versa) and CDSs coloured by COG categories (if assigned).

5. Discussion

Previous studies have isolated various strains of *A. johnsonii* from different environments, but only *A. johnsonii* ICE_NC showed a role in the biotransformation of bile acids, these are fundamental component of the cholesterol metabolism, particularly involved in elimination of cholesterol and regulation of its biosynthesis (Hofmann, 1999).

The work presented here aimed to characterize the genome of this strain in order to identify the 12α-HSDH gene. To achieve this, two different hybrid *de novo* assembly methods were employed. Both methodologies used here detected two genetic elements in *A.johnsonii* ICE_NC, one circular chromosome and one plasmid.

The plasmid's assembly of the Oxford Nanopore's MinION reads with Canu resulted in a chimeric sequence produced by the merging of two sequences highly similar to the contig of 117 Kb obtained from Unicycler assembly. Both Canu and Unicycler assembly strategies returned a chromosome of length coherent with the *A. johnsonii* genomes present in the NCBI database, respectively 3.6 and 3.5 Mb.

The annotation of both chromosomic assemblies allowed the identification of more protein coding sequencing in the Canu's chromosome, while in the Unicycler's chromosome there were more abundant putative genes and hypothetical proteins. This indicates that Canu allowed a better reconstruction of *A. johnsonii* ICE_NC chromosome. Furthermore, the number of genes identified was consistent with other *A.johnsonii*'s genomes available in literature (Jia et al., 2022). The annotation with Prokka of chromosomes and plasmid did not allow the detection of 12α-HSDH gene. This was expected because the sequence has yet to be completely described, but the gene was detected in the plasmid with a protein BLAST analysis using the predicted protein sequence of the Clostridium 12 α-HSDH gene as query.

Taken together, the results illustrate the added value of long read sequencing in addressing the whole genome characterization and the plasmid reconstruction challenge, and also demonstrate the ability to produce a highly accurate description of the 12α-HSDH gene in *A. johnsonii* ICE_NC.

6. Conclusions

Up to now, 45 genomes of *A. johnsonii* are available on the National Center for Biotechnology Information (NCBI) database, but only 16 of them, isolated from multiple sources (clinical or environmental isolates) were assembled to a complete level. A comparative genomic analysis performed on 16 isolates (Jia et al., 2022) demonstrated that to adapt to human host and to the high selective pressure of antibacterial agents, clinical-derived strains accumulate more functional genes associated with translational modification, β-lactamase and defence mechanisms. Members of the gastrointestinal microbiota have evolved 12α-HSDHs capable of oxidizing and epimerizing the 12α-hydroxyl group from host CA. However, the gene(s) encoding 12α-HSDH have not been identified in *A. johnsonii*.

The strain of *A. johnsonii* ICE_NC (previously named *A. calcoaceticus lwoffii*) was isolated from a soil sample collected at the ICE production plant (ICE, Industria Chimica Italiana) and it appeared to contain the enzymes able to perform the chemo-enzymatic synthesis of UDCA starting from CA (i.e. 7α- and 12α-HSDH) as judged by biochemical analyses with partially purified enzymes (Giovannini et al., 2008). Hybrid *de novo* genome assemblies methods allowed the reconstruct and annotation of one chromosome of about 3.6 Mb and one 117 kb long plasmid.

The utility of the approach used in this study was tested to identify and clone the gene for the *A. johnsonii* 12α-HSDH. The availability of the annotation for both the chromosome and the plasmid allowed the identification of the putative gene by sequence homology. These results would have been impossible to get by using next generation short reads sequencing alone, since short read WGS-based approaches do not allow a correct reconstruction of plasmids due to the many repeats and to the fact that plasmid sequences are sometimes even shared with the chromosomal DNA (Arredondo-Alonso et al., 2017; de Toro et al., 2014).

Chapter 2. Whole metagenome shotgun sequencing applied to investigate taxonomy and function of subgingival microbiome in absence/presence of periodontitis and type 2 diabetes.

1.Introduction

1.1 Periodontitis

Periodontitis is a chronic inflammatory disease, which leads to the progressive destruction of the dental support. A fundamental role is played by the interaction between pathogenic periodontal microbiota and the host immune response, modulated by environmental and genetic factors (Germen et al., 2021; Sanz, Herrera, et al., 2020).

Periodontitis is an important public health issue. In 2018 the World Health Organization estimated, in Europe, an incidence of periodontal disease in 5-20% of middle-aged adults (35-44 years) and up to 40% of people in the age group 65-74 years (https://www.euro.who.int/en/health-topics/disease-prevention/oral-health/data-and-statistics). The disease may lead to social inequalities and impair quality of life caused by tooth loss, dysfunctions in chewing and aesthetics (Eke et al., 2015; Papapanou et al., 2018).

Periodontitis through time

Periodontal disease afflict humanity since ancient times. Paleopathology studies identified this disorder in ancient samples dating back to different cultures such as ancient Greek, ancient Egyptians and early pre-Columbian America (Forshaw, 2009; Steckel, 2005). The firsts accurate medical treatises were written by Arabian scientists Albucasis and Avicenna during the Middle Age. Albucasis wrote a medical encyclopaedia, named *al-Tasrif*, used as medical text in European universities until the seventeenth century.

During Renaissance there was a development in anatomic and surgery studies, with progress in treatment for periodontitis ((Bailliére, 1840; Blum, 1887; Chernin et al., 1999), but it is only in the eighteenth century that modern dentistry was developed in Europe. The father of modern dentistry is considered to be Pierre Fauchard with the book The Surgeon Dentist published in 1728 (Tiwari et al., 2017), followed by the treaties of John Hunter and Thomas Berdmore in the middle of 1700 (Newman et al., 2018).

During the nineteenth century three breakthrough scientific findings have changed the studies of dentistry and periodontitis: the discovery of anaesthesia, the germ theory disease proved by Louis Pasteur, and the development of radiographs technologies promoted by the discoveries of Wilhelm Röntgen in 1895.

During nineteenth and twentieth centuries various dentists attempted to place several type of dental implants, made of porcelain, gold and other metals.

After the World War II the attention in the study and the treatment of periodontitis was focused more on the role of microorganisms and immunologic response (Newman et al., 2018).

Periodontitis genesis

The periodontium is a connective tissue that surrounds and supports the teeth, the term periodontium in fact means “around tooth” (Kumar et al., 2003). As described in Figure 1.1 it consists of the subsequent main tissues: gingiva, periodontal ligament, root cementum and alveolar bone proper which is in continuity with alveolar bone (Lang & Lindhe, 2015).

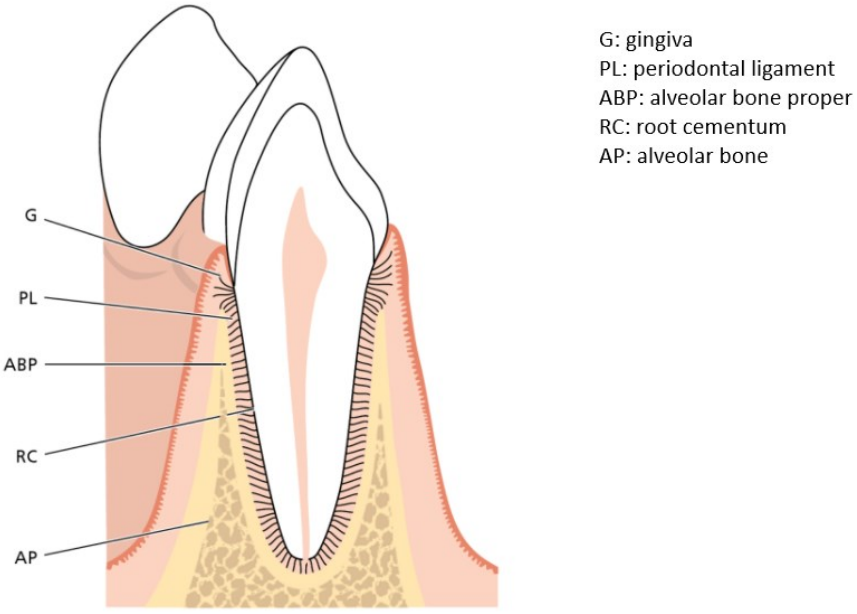


Figure 1.1 Periodontium structure from Lang & Lindhe (Lang & Lindhe, 2015).

Periodontitis is related to dental plaque formation, a six-stage process (Hope & Wilson, 2006):

1. Formation on tooth surface of pellicle, a layer composed of glycoproteins, phosphoproteins and lipids, but without bacteria.
2. Usual inhabitants of oral cavity, such as *Streptococcus sanguinis*, are the early colonizers of the pellicle. If not removed they can stable attach themselves with adhesion structure like pili.
3. Other microorganism, unable to bind directly the pellicle, can attach tooth surface coupling the first layer of colonizers.
4. Early colonizers become established, leading to an escalation in the dental plaque complexity, with formation of anaerobic zones.
5. Microbial diversity increase, leading to formation of microenvironments: spot with different rate of oxygen concentration, pH and secondary metabolite augmentation.
6. Some bacteria can detach from the biofilm, through enzyme that degrade the biofilm, in order to colonize other surfaces in mouth.

Dental plaque accumulations leads to the inflammations of the gingiva, a pathological status called gingivitis (Hassan et al., 2020; Reddy & Jeffcoat, 2000).

Gingivitis is characterized by redness, swelling, bleeding and formation of shallow pockets in the gums (https://www.ncbi.nlm.nih.gov/books/NBK279593/). Furthermore, there is an increase in gingival crevicular fluids that promote the colonization by pathogenic bacteria and untreated gingivitis often leads to the onset of periodontitis. Crevicular fluid is a physiological fluid as well as an inflammatory exudate which is located in the gum line. Irritation and inflammation of the gum tissues increase the flow and alter the constituents of the crevicular fluid (Barros et al., 2016).

Untreated gingivitis leads to development of periodontitis, where the persistence of the inflammatory state causes gingival bleeding and the gradual loss of periodontal tissue support, with the formation of periodontal pockets until the tooth comes out of its seat (Papapanou et al., 2018).

For the diagnosis of periodontitis several factors are taken into account: clinical attachment loss (CAL), proportion of sites that bleed on probing, the number and proportion of teeth with probing depth over certain thresholds, like buccal or oral CAL ≥3 mm with pocketing ≥3 mm is detectable at ≥2 teeth (Armitage, 1999; Holtfreter et al., 2015).

In addition to plaque there are other dental risk factors for development of periodontitis: ageing, smoking, systemic disease like deficiencies of neutrophil function, type 1 and 2 diabetes (Albandar et al., 2000; Deas et al., 2003; S.G. Grossi et al., 1995; Sara G. Grossi et al., 1994; Guzman et al., 2003; Van Dyke & Sheilesh, 2005).

Bacterial composition

Oral flora is colonized predominantly by Gram-positive, anaerobic bacteria, that help maintaining a stable oral condition. Healthy periodontium also presents gram-negative bacteria, anaerobic/facultative aerobic usually periodonto-pathogenic, but in a low abundance. These became problematic when their density increase.

The work of Socransky et al. (S. S. Socransky et al., 1998; Sigmund S. Socransky, 1970) allowed to identify specific groups of bacteria related to the pathogenesis of periodontitis, that are physiologically related and classified into complexes.

Complexes identified by Socransky et al. were codified by different colours corresponding to the different stages of the bacterial colonization. Many studies have been carried out to study the colonization dynamics of periodontitis bacteria through time, that led to the recognition of new strains associated with the periodontitis, but the colour classification remain valid. Figure 1.2 show an updated scheme of Socransky's microbial complexes (Wirth et al., 2021).

The base of the pyramids is constituted of early colonizers bacteria, able to adhere to the pellicle on tooth surface and that are not flushed out with crevicular fluid. Their presence allows the colonization by orange complex species, usually considered less pathogenic compared to the top species of red complex, usually ranked as the most pathogenic bacterial complex. Orange species are able to synthetise toxins and enzyme that contribute on progressive attachment loss and an increase in gingival pockets depth. These bacteria are "bridge-species" that contribute to the onset of anaerobic conditions and are crucial for the adhesion of highly pathogenic species to tooth surface.

Red complex bacteria are strictly anaerobic and they aggressively drive the destruction of the soft tissue and bone through the production of potent virulence factors. Their ability to penetrate the tissues makes their treatment extremely difficult (A. P. V. Colombo et al., 2009; S. S. Socransky et al., 1998; Wirth et al., 2021).

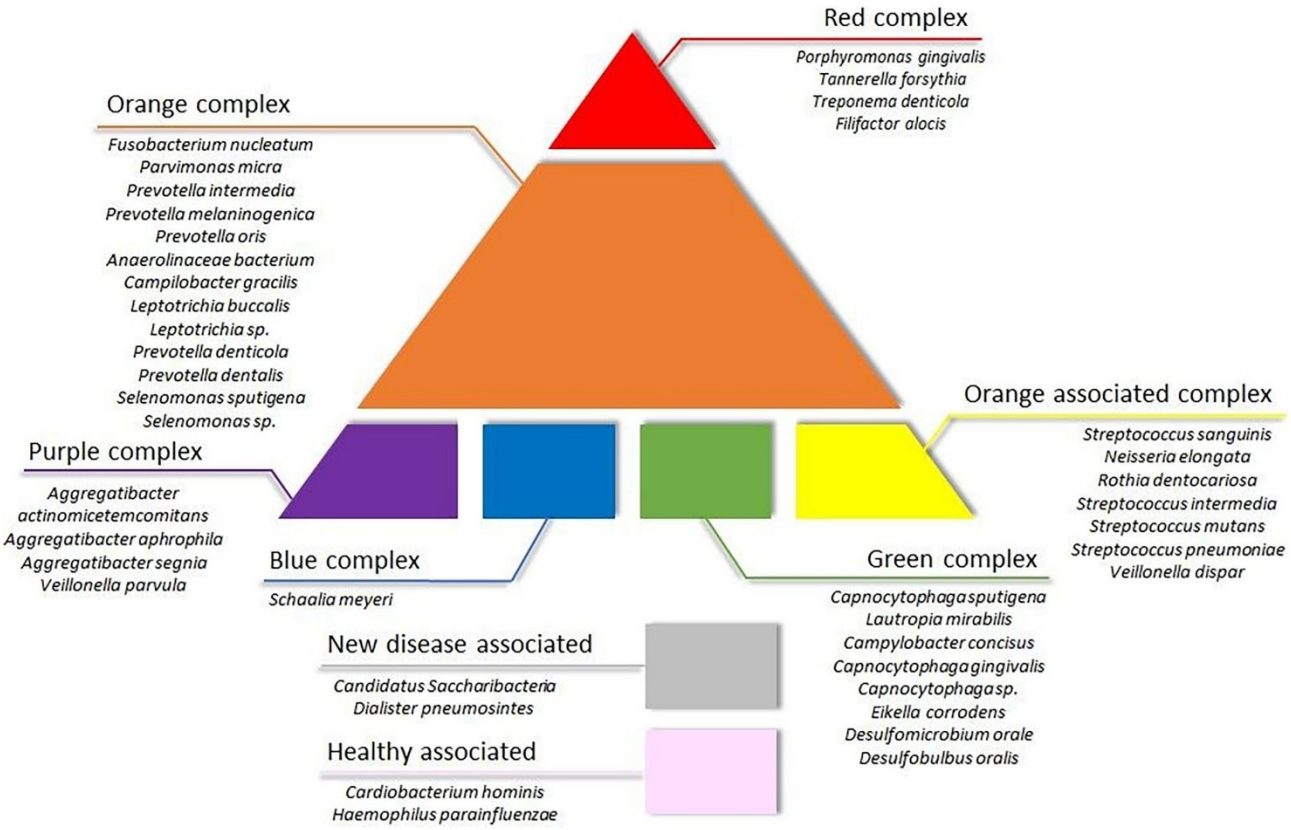


Figure 1.2 Classification of oral microbial taxa in microbial complexes. From Wirth et al. (2021).

Immune system

The bacterial component is indisputably the key factor in the onset of periodontitis, but the host's immune system also plays a fundamental role in the progression of the disease. In fact, it is the body immune system that define the extent of the inflammation (Darveau, 2010; Reddy & Jeffcoat, 2000).

There is a delicate balance between oral microbiota and local immune response at physiological conditions, reached up without a severe inflammatory response (D. T. Graves et al., 2019). After the colonization by periodonto-pathogenic bacteria the microbiota modifies its concentrations and metabolites, increasing pathogenicity of whole community. These altered conditions lead to the over-activation of host's immune response, with infiltration in the periodontium of a large amount of immune cells, activations of osteoclastic activity and destruction of hard and soft tissue.

The interaction between bacterial community and host's cells leads to the first large secretion of cytokines, that participate at the cytokine pro-inflammatory amplification, and differentiation and activation of different immune cells. The released cytokines can act as direct effector that leads to tissue disruption. The most represented cytokines in the inflammatory response against

periodontitis are interleukine-1 (IL-1) family, interleukine-6 (IL-6) family and tumor necrosis factor (TNF) (Pan et al., 2019).

Periodontitis and other disease

Oral health is a recognized key factor to improve quality of life, that allow to protect against noncommunicable disease (Tokyo declaration at who https://www.who.int/oral_health/tokyodeclaration_final.pdf?ua=1). Periodontal and systemic diseases share inflammatory features, and they seem to have a bidirectional relationship (Barutta et al., 2022).

Periodontitis can act as a risk factor for systemic disease through the entrance into the bloodstream of oral bacteria and inflammatory mediators (Hajishengallis, 2015).

Several chronic disease show associations with periodontitis including cardiovascular and respiratory disease, cancer, and diabetes.

Cardiovascular disease has a local and systemic inflammatory component (Tonetti & Van Dyke, 2013), some bacteria can infect vessel wall and can promote the development of a proinflammatory environment; they also can bring to autoimmunity against vessel cells and lead to atherosclerotic process (Leinonen & Saikku, 2002). In these circumstances, the inflammatory component of periodontitis can significantly increase the risk of cardiovascular disease (Spar et al 2006).

Periodontitis is also associated with development of pneumonia and respiratory disease (De Melo Neto et al., 2013; Terpenning et al., 2001). Oral cave contains various lung pathogenic bacteria, that can be aspirated and cause respiratory infection. Furthermore, inflammatory mediators that are characteristic of periodontitis, like cytokines, can be transferred to lungs and lead to local inflammatory processes (Paju & Scannapieco, 2007).

While associations between periodontitis and various neoplasm is still under investigation (Michaud et al., 2017; Tezal et al., 2007, 2009) there are some evidences that the inflammation can influence all cancer's stages (Inacio Pinto et al., 2015; Trinchieri, 2012) and that the cytokines involved in periodontitis response may be involved in the development of some type of cancer (E. Goldberg & L. Schwertfeger, 2010; Inacio Pinto et al., 2015).

The relationship between diabetes and periodontal disease is recognized and known as bidirectional (Taylor 2001, Cardoso et al 2018, Sanz 2018).

Various epidemiological studies supported an increased risk of periodontitis in patients with diabetes (both type 1 e 2), particularly when diabetes is poorly controlled (Genco & Borgnakke, 2020; Kocher et al., 2018; Romano et al., 2021; Zheng et al., 2021).

There are also evidence suggesting that individuals without diabetes, but with periodontitis, have higher blood glucose levels and a greater chance of developing pre-diabetes and diabetes (Chang et al., 2020; Graziani et al., 2018; Wu et al., 2020).

Diabetes can improve risk of periodontitis with a multifactorial mechanism that include: changes in oral microbiota, enhancing systemic inflammatory status and periodontal tissue destruction (Dana T. Graves et al., 2018; Ramamurthy & Golub, 1983; B. Shi et al., 2020; Taylor et al., 2013).

On the other hand periodontitis can lead to insulin resistance and progression of diabetes with propagation of oral bacteria and inflammatory mediators localized in the oral cave, and bringing to dysbiosis in gut microbiota (Abu-Ashour et al., 2018; Arimatsu et al., 2014; Hajishengallis & Chavakis, 2021; Konkel et al., 2019; Watanabe et al., 2008).

1.2 Type 2 diabetes

Type 2 diabetes is a chronical disease caused by the inability of cells to use insulin. Type 2, also called non-insulin dependent, is the predominant form of diabetes, in fact more than 95% of patients with diabetes suffer of type 2 (https://www.who.int/news-room/fact-sheets/detail/diabetes). About 32.3 million adults were diagnosed with diabetes in the European Union in 2019 and an additional 24.2 million were estimated to have undiagnosed diabetes (IDF, 2019).

Conditions attributable to diabetes have been described in ancient Egypt 3500 years ago (MacCracken & Hoel, 1997). Ancient Indian scientists, Charaka and Sushruta (400-500 A.D.), identified 2 different type of a disease previously defined *madhumeha* (“honey urine”) (FRANK, 1957; Tipton, 2008).

The first complete description, during the first century, was made by Aretaeus the Cappadocian, who neologize the word diabetes and declared *“… no essential part of the drink is absorbed by the body while great masses of the flesh are liquefied into urine”* (Sanders 2002). Diabetes was also described in “The Canon of Medicine” by Avicenna (980–1037 A.D) (Lakhtakia, 2013). Paul Langerhans identified the cells responsible of the synthesis of insulin in 1869, but the term insulin was coined in the early years of 1900 (Sakula, 1988).

Etiology of type 2 diabetes

Type 2 diabetes has a clear genetic component. Twin-based studies and family history analysis suggest that individuals with a first-degree relative with the disease has a likelihood of developing the disease increased up to 40% (Fonseca & John-Kalarickal, 2010). Furthermore, most supported inheritance model is polygenic mode, with important environmental component (Froguel & Velho, 2001). Among those, obesity, physical inactivity, dietary influence and aging are some well documented risk factors for type 2 diabetes (Fletcher et al., 2002; Ismail et al., 2021; Narayan et al., 2007).

Type 2 diabetes is characterized by the same symptoms of type I, related to chronic hyperglycaemia with disturbance of carbohydrate, fat, and protein metabolism resulting from an impaired insulin production and secretion by pancreatic beta-cells, as well as peripheral tissue insulin resistance (Leahy et al., 2015). This wide range of symptoms makes type 2 diabetes a very heterogenous disease that requires various type of treatments with a prognosis that is largely different between patients. While different treatments are available, it remains a strong cause of blindness, end-stage renal disease, lower limb amputation and cardiovascular disease (Brownlee, 2001).

1.3 Metagenomics

Microbial genomes study begun with the DNA sequencing of some phages in the late 1970s followed by the sequencing of the first bacterial genome*, Haemophilus influenza,* in the mid-1990s (Wooley et al., 2010). Nowadays, the NCBI microbial genome database contains the genomes of 376,572 Prokaryotes and 47,107 Viruses (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/).

Initially, microbial genomes studies were limited by the need of microorganisms cultivation as a source of the DNA to be sequenced, furthermore, the available sequencing technologies were extremely time-consuming and not cost-effective. The technological advancement of DNA extraction methods and the development of PCR allowed the analysis of microbial communities in their entirety, identifying the species that compose it through the amplification of specific genetic markers such as the 16S rRNA gene, which is found in all Bacteria and Archaea (Hamady & Knight, 2009). However, no more than the taxonomic profile can be inferred using these approaches.

In the second half of the 1990s, Stein et al (Stein et al., 1996) analysed several large heterogeneous fragments of DNA from planktonic marine Archaea. It was the first attempt to perform a metagenomic study.

In 1998, Handelsman et al. (Handelsman et al., 1998) had used for the first time the term metagenomics:

"The methodology [cloning of environmental DNA into E. coli *for phenotype screening] has been made possible by advances in molecular biology and Eukaryotic genomics, which have laid the groundwork for cloning and functional analysis of the collective genomes of soil microflora, which we term the metagenome of the soil."* (Handelsman et al., 1998).

According to the initial definition, the metagenomics is the study of the entire microbial genetic material present in an environment. This type of analyses is usually performed through the whole metagenomic shotgun sequencing.

Whole metagenomic shotgun sequency requires the fragmentation of the extracted DNA in small pieces (some hundreds of bp) that are then sequenced, and the reads obtained are stitched back together through bioinformatics tools in order to reconstruct the unknown community. Therefore, all genomic DNA present in a sample can be read and then used to build the taxonomic profile of the sample, to identify and to profile microbial genes, avoiding DNA amplifications and quantifying the abundances of different microorganism (Quince et al., 2017).

2. Aim of the study

Periodontitis and diabetes are two common chronic inflammatory diseases having an extensive worldwide epidemic impact and presenting a great increased in prevalence in the aging population. Several studies have demonstrated the relationship between periodontitis and the occurrence of several systemic diseases among which type 2 diabetes; this relationship may be mediated via inflammatory pathways and changes in oral flora.

The bidirectional relationship between periodontitis and type 2 diabetes is note and documented, and several mechanisms are proposed to explain the association between these two pathologies such as (1) altered host immune response, (2) subgingival floral changes, (3) genetic predisposition (4) microvascular changes, and (5) crevicular fluid changes, but few studies were made in order to characterise the composition and functional activities of the oral microbiome in patients affected by both diseases.

In 2019 Farina et al. (Farina et al., 2019) used high-resolution whole metagenomic shotgun sequencing to describe the taxonomical profile of the subgingival microbiome of patients, recruited in the metropolitan area of Ferrara (Italy), with different health status regarding periodontitis and type 2 diabetes.

The work presented in this thesis, starting from Farina's pilot study, aims to update the taxonomic analysis through the implementation of new databases and bioinformatics tools and then to build the microbial signature of different clinical subgroups analysed by integrating taxonomic data with functional analyses.

Two of the most widely used approaches in WMS studies, built on marker genes search, are: (I) MEGAN, based on BLAST (for taxonomy) or DIAMOND (for functional analysis) that search of shotgun reads against the NCBI nt or nr databases; (II) the bioBakery platform, that use MetaPhlAn3.0, for taxonomic profiling, and HUMAnN, for functional profiling, mapping reads on internal database

An obvious limitation of the study concerns the small sample size, mainly attributable to the difficulty of enrolling periodontally healthy diabetic subjects. In the attempt to partly compensate for the limitation due to the small sample size, specific selection criteria were used to select patients with extreme very different clinical traits of periodontal health or disease (thus resulting in two subgroups that were well representative of a fully intact and healthy periodontium or moderate to severe periodontitis). The results obtained from the analyses will need to be validated by future studies of larger cohorts.

3.Methods

3.1 Study population

The study was approved by the Ethical Committee of Ferrara, protocol number: 150791. 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 and already described in Farina et al. 2019 (Farina et al., 2019) . Briefly, a total of 12 subjects were recruited and assigned to one of the following groups (3 individuals each):

- t2d+p+ group: patients affected by moderate to severe periodontitis and type 2 diabetes (individuals 1-3-7)
- t2d-p+ group: patients affected by moderate to severe periodontitis but no type 2 diabetes (individuals 4-9-11)
- t2d+p- group: patients affected by type 2 diabetes but no periodontitis (individuals 2-6-12)
- t2d-p- group: healthy subjects (individuals 5-8-10)

Participants were all Caucasian of age ≥ 40 years, enrolled among temporary and permanent residents in the metropolitan area of Ferrara.

Inclusion criteria:

- **t2d+p+ group**
  - ✓ diagnosis of type 2 diabetes for at least two years according to the criteria of the American Diabetes Association ("Standards of Medical Care in Diabetes-2014," 2014);
  - ✓ insufficient metabolic control of diabetes (i.e., glycated haemoglobin serum level >7%);
  - ✓ currently receiving stable doses of oral hypoglycaemic agents and / or insulin under supervision of a diabetologist;
  - ✓ at least 20 teeth present;
  - ✓ diagnosis of moderate to severe periodontitis, i.e., at least 30% of tooth sampling sites with clinical attachment loss ≥3 mm (Armitage, 1999);
  - ✓ at least 4 sites with probing depth ≥5 mm.

- **t2d+p- group**
  - ✓ diagnosis of type 2 diabetes for at least two years according to the criteria of the American Diabetes Association ("Standards of Medical Care in Diabetes-2014," 2014);

- ✓ insufficient metabolic control of diabetes (i.e., glycated haemoglobin serum level > 7%);
- ✓ currently receiving stable doses of oral hypoglycaemic agents and / or insulin under supervision of a diabetologist;
- ✓ at least 20 teeth present;
- ✓ 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).

- **t2d-p+ group**
  - ✓ no history of type 2 diabetes diagnosis;
  - ✓ at least 20 teeth present;
  - ✓ diagnosis of moderate to severe periodontitis, i.e., at least 30% of sites with clinical attachment loss≥ 3 mm (Armitage, 1999);
  - ✓ at least 4 sites with probing depth≥ 5 mm.
- **t2d-p- group**
  - ✓ no history of type 2 diabetes diagnosis;
  - ✓ at least 20 teeth present;
  - ✓ 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:

- X Current smoking or quit smoking less than 6 months prior to the screening visit;
- X diseases or systemic conditions (in addition to type 2 diabetes) with a documented influence on periodontal status;
- X use of drugs with a documented influence on periodontal status (e.g., bisphosphonates, cyclosporine, phenytoin, nifedipine, calcium channel blockers, corticosteroids and anti-inflammatory drugs);
- X periodontal therapy within 12 months prior to the screening visit;
- X local or systemic antibiotic therapy during the 3 months prior to the screening visit.

The relative restricted study population is due to the difficulty to recruiting subject affected by type 2 diabetes and no periodontitis.

3.2 Collection and storage of subgingival plaque samples

For each eligible subject, 4 subgingival plaque samples were collected at 4 teeth. In t2d+ p− and t2d− p− subjects, sampling was always performed at 4 sites randomly selected among those negative to bleeding on probing. In t2d + p+ and t2d − p+ subjects, sampling was performed at the 4 sites showing the deepest probing depth values among those positive to bleeding on probing.

At each site selected for plaque sampling, saliva was blotted with a gauze or cotton roll, and supragingival plaque was removed with a curette or scaler. Subgingival plaque was collected with a curette and wiped onto a sterile, coarse endodontic paper point. A commercially available kit (OMR-110; DNA Genotek, Ottawa, ON, Canada) was used for the storage of plaque samples. For each subject, the four samples were stored together in a sterile microcentrifuge tube containing a fixative solution containing sodium dodecyl sulphate, glycine, N,N'-trans-1,2-cyclohexanediylbis[N-(carboxymethyl)-, hydrate, and lithium chloride, at a temperature comprised between 15 °C and 25 °C, until lab processing for extraction of the oral microbiome DNA.

3.3 Library preparation and sequencing Isolation of DNA – Library preparation and sequencing

DNA was isolated from frozen samples by using the Maxwell RSC DNA Blood Kit (Promega) according to the manufacturer's protocol. The concentration of DNA was determined with the Qubit 2.0 Fluorometer (Life Technologies) by using the Qubit dsDNA HS Assay Kit (Life Technologies). Prior to library preparation, DNA was fragmented using NEBNext® dsDNA Fragmentase for 30 min. Each library was generated from 20 ng of genomic DNA, as described in the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina protocol (New England Biolabs). NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers set 1) were used to produce the individual libraries and label them with specific molecular barcodes. Then, Agencourt AMPure XP beads (Beckman Coulter) were used for library purification. Finally, libraries were quantified by using the High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) on the Bioanalyzer instrument (Agilent), diluted and pooled together in equimolar amounts. Samples were sequenced with an Illumina NextSeq 500 sequencer with 2 × 150-bp read layout, using the NextSeq® 500/550 Mid Output Kit v2.

3.4 Data pre-processing and bioinformatics pipeline for metagenomics analysis

The bioinformatic pipeline developed for integrated metagenomic analysis is represented in Figure 3.1.

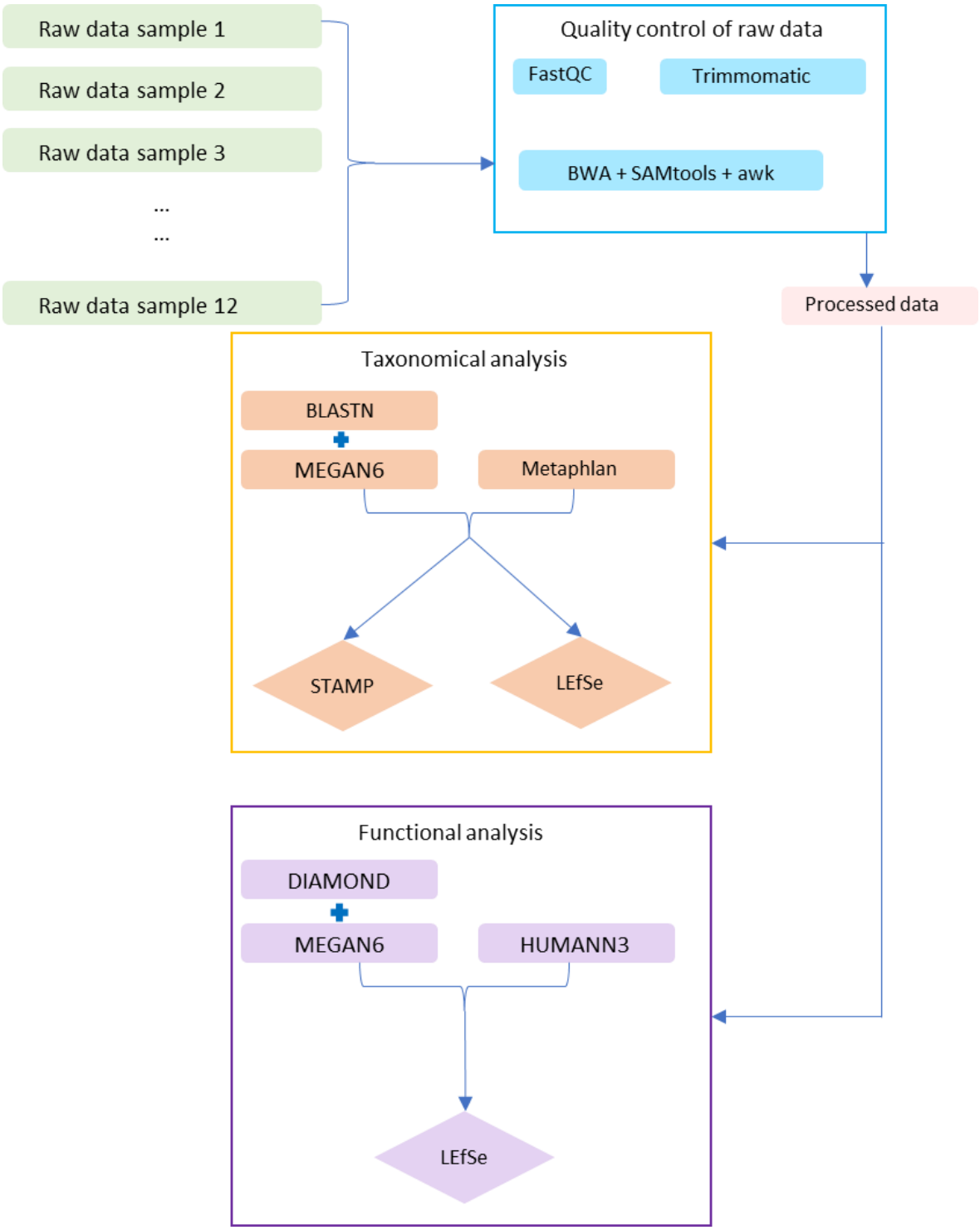


Fig. 3.1: Data pre-processing and bioinformatics pipeline for metagenomics analysis.

The Illumina BaseSpace cloud platform (https://basespace.illumina.com/) was used to convert the raw Illumina output to the Fastq format and to assign reads to each individual (demultiplexing) according to sequencing barcodes.

In the first step, the quality control (QC) of the reads was done with FastQC (S. Andrews, 2010). FastQC provide a QC report which spots potential issues within the raw reads, originating either in the sequencer or during the library preparation. The first check is based on the use of the Phred score (Patel & Jain, 2012), given for each base, denoting the probability of incorrectly calling the nucleotide. The Phred score is defined as:

$$Q\ Phred = -10\log10P(error)$$

A cut-off of 20, corresponding to a probability of the base being wrong equal to 1 in 100 (or an accuracy of 99%) is an empirical threshold that is commonly used to identify low confidence nucleotides from sequencing reads. According to this threshold, reads with an overall low quality (mean Phred Base quality score <20) are discarded and regions of the reads characterized by low quality nucleotides can be trimmed.

Besides the computation of basic statistics and the visualization of base quality score distributions, FastQC provide a full QC report which can point out issues originated during the sequencing process or during the library preparation process. A further important step in quality control supplied by the tool is the detection of contamination with sequencing adapters. Such adapters are artificial short DNA sequences that are added during the library preparation protocol and that are fundamental to trigger the sequencing process. These short exogenous sequences are automatically detected and removed from sequencing reads during the generation of fastq files but some adapter residuals (entire adapters or portions) may persist in the read pool. FastQC helps detecting the occurrence of adapters that then can be removed using other bioinformatic tools. Additionally, FastQC provides several information regarding read length distribution, GC-content, overrepresented sequences, k-mers abundance and other statistics that are useful to assess read quality.

Trimmomatic (v. 0.36, Bolger et al., 2014), was used to trim from raw data bases with poor quality and read with length < 100bp. Parameters were defined as CROP:147, HEADCRO:3, SLIDINGWINDOW:5:20, MINLEN:100. The CROP parameter remove bases from the end of the read until the specified value is obtained; HEADCRO cut a specified number of bases from the start of the read; SLIDINGWINDOWS performs a sliding window cut by cutting once the quality inside the window drops below a certain threshold; MINLEN discharge the read if it is below a specified length.

In this case number of bases to keep was 147, 3 bases were cut from the start of the reads, sliding window of 5 bases with quality score less than 20 was removed and reads with length less than 100 bases were discharged. Value of the parameters were chosen to retain the best quality core of reads.

Host DNA is the most abundant source of contamination in shotgun metagenome studies (McArdle & Kaforou, 2020), hence a fundamental step is the removal of such material. The exclusion of host DNA is fundamental because throughout the subsequent analysis steps the contaminant can generate false assignments to microbial taxa.

All the paired-end reads were aligned to a human reference genome (GRCh38) with the *mem* algorithm of BWA (v. 0.7.15, Li & Durbin, 2009). Reads displaying a concordant alignment against the human reference genome were discarded using SAMtools (v. 1.9, Li et al., 2009) and an in-house awk script.

The human reference genome GRCh38 contains, in addition to the reference, the "decoy" sequences: DNA viral sequences (e.g. Epstein-Barr Virus) and other human genomic material that is not part of the reference genome. In the alignment between metagenomes and the human genome it is important to also take into account these decoy sequences in order to be able to discard all the reads belonging to the human genome, which could then be erroneously assigned to microbial taxa.

3.5 Taxonomical analysis

The taxonomic profile of the 12 samples was build aligning the non-human reads against the NCBI "nt" reference database using BLAST (Altschul et al., 1990), a software based on a heuristic algorithm able to find regions of local similarity between sequences. The analysis was performed with the BLASTN utility that is designed to find pairwise similarity regions between DNA sequences in a nucleotide database (nt db) and a nucleotide query. The parameters were set to exclude matches with an e-value > $1x10^{-6}$, a percentage of identity ≥ 95% and a minimum length > 100 bp. A list of taxonomic IDs was provided to the software to limit the search to taxa belonging to Archaea, Bacteria, Fungi, Virus.

The blast2rma tool of the command line version of MEGAN6 Community Edition (v. 6.21.5, Huson et al., 2007) was used to convert the data from the BLASTN analysis to the rma6 format, and then imported in the graphical version of MEGAN6 which allows to merge the different individuals in a

single file (function “compare”). The *megan-nucl-Jan2021.db* nucleotide database was used in the conversion process to obtain a rma6 file.

MEGAN6 assigns reads to taxa with a naïve LCA-assignment algorithm: if the read specifically aligns only to the last taxonomy level, then it is assigned to that taxon. The less specifically a read hits taxa, the higher up in the taxonomy it is placed; reads that ubiquitously hit the taxonomy will be assigned to the root node of the NCBI taxonomy.

After the creation of the merged file containing all subjects, the taxonomic assignment was expressed as absolute abundance and then exported in tsv or txt format, in order to perform different statistical analyses. MEGAN6 automatically normalize data of each individual over the subject with the lowest number of assigned reads, in this case “ind5”.

Afterwards, processed data were analysed 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 allow distinguish between similar species with high precision. The software normalizes the total number of reads in each clade by the nucleotide length of its markers and provides the relative abundance of each taxonomic unit, taking into account any markers specific to subclades (Segata et al., 2012). The twelve samples were analysed with default parameters of MetaPhlAn 3.0 and merged in a single output table with the utility *merge_metaphlan_tables.py*.

3.6 Community complexity and diversity

Some widely used biodiversity indexes were calculated: the richness S index, the count of the number of different species in a sample (Magurran, 2021) and the Pielou evenness J index (Pielou, 1966) were used as more general community indexes (Chiarucci et al., 2011). Alpha- and beta-diversity were used to describe the microbiome diversity within and between groups (Lozupone & Knight, 2008). Alpha diversity deals with species within a sample and describe the relationships of their co-occurrence, e.g. a number of species and their abundance. Conversely, beta diversity tracks the differences in species composition among various samples, e.g. a number of shared species. Alpha-diversity was calculated with the Shannon H’ diversity index, and the within-sample Beta-diversity (intra-beta diversity) was evaluated with the Dβ index (as a component of total community diversity, Dγ) (Lozupone & Knight, 2008; Tuomisto, 2010).

The non-parametric Wilcoxon t-test and Kruskal-Wallis ANOVA test were applied to compare profiles organized into two or multiple groups, respectively.

White's non-parametric test was performed by STAMP (v. 2.1.3, Parks et al., 2014). It allows the analysis of differential abundance between samples organized in two treatment groups, ranked as genus and species. The non-parametric nature of this test allows for the handling of low sample size datasets.

Furthermore, taxonomical biomarkers were detected with LEfSe algorithm (https://huttenhower.sph.harvard.edu/galaxy/ Segata et al., 2011). LEfSe perform a linear discriminant analysis in 3 steps: first execute a Kluskal-Wallis sum-rank test to point out features with significant differential abundance in different classes, then perform a Wilcoxon rank-sum to test among subclasses, and eventually a linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant feature. To be more conservative, the threshold on the logarithmic LDA score was increased from 2 to 2.5 either for taxonomy and for functional analysis.

Both MEGAN6 and MetaPhlAn 3.0 output were statistically analysed through STAMP and LEfSe, in order to detected significantly differences between individuals with and without periodontitis (p+ vs p-) and affected or not by type 2 diabetes (t2d+ vs t2d-).

3.6 Functional analysis

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

A BLASTX analysis of quality control processed data were performed through DIAMOND (v. 2.0.4, Buchfink et al., 2014). This software allowed to align the processed reads against a protein database (NCBI nr reference). The *daa2rma* tool of the command line version of MEGAN6 Ultimate Edition (v. 6.21.11, Beier et al., 2017), was used to convert the output of the DIAMOND analysis to the *rma6* format, which allows a comparative analysis of all the sample with the graphical version of MEGAN6 Ultimate. The *megan-map-Jan2021-ue.db* database, containing KEGG, NCBI, GTDB, eggNOG, InterPro2GO and SEED, was used in the conversion process to obtain a rma6 file. The KEGG classification was used to perform a functional analyses. The KEGG output was exported in txt format in order to perform the statistical analysis with LEfSe, both at pathways and proteins level. LEfSe analysis was performed on groups: (I) p+ and p-; and (II) t2d+ an t2d-.

Significantly results were then explored through the KEGG PATHWAY online database (https://www.genome.jp/kegg/pathway.html). This database is a collection of pathway maps representing the current knowledge of molecular interactions, reaction and relation network for: metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases and drug development.

Starting from the processed data, single functional profiles were also computed with HUMAnN 3.0 (HMP Unified Metabolic Analysis Network, v. 3.0.0, Beghini et al., 2021). HUMAnN 3.0 allows to outline the abundance of metabolic pathways from metagenomics data. The twelve samples were processed with default parameters and finally merged using the *human_join_tables.py* script. HUMAnN 3.0 has 3 main output files: gene families, pathway abundance and pathway coverage, in which the different features (genes, pathways and coverage) are computed both at the community and species level.

In HUMAnN 3.0 there is a "compression constant" (k) that is equal to the total pathway abundance divided by the total abundance of genes that contributed to pathways; it is defined as UNINTEGRATED the abundance for the community, identified species, and the unclassified records equal to the total abundance of genes in that level that did not contribute to pathways (scaled by k).

Pathway abundance output were submitted to LEfSe to detect the pathways that can explain differences between groups: (I) p+ and p-; (II) t2d+ an t2d-.

All pathways found to be significantly enriched between clinical categories were investigated in deep with MetaCyc website (https://metacyc.org/). MetaCyc is a database of experimentally clarified metabolic pathway from various organisms. Both primary and secondary metabolism are included in this database, that also contain associated metabolites, reactions, enzymes and genes.

4. Results

4.1 Study population

Characteristics of the study population are reported in Table 4.1. Diabetic subjects had received the diagnosis of type 2 diabetes at least 3 years before participation to the study, and their glycated haemoglobin level was comprised between 7.1% and 8.0%. Subjects with periodontitis had a number of sites with probing depth ≥ 5 mm varying between 19 and 39 (t2d-p+ group) and between 18 and 29 (t2d+p+ group). The range of bleeding on probing was 8-25% in t2d-p- group, 17-20% in t2d+p- group, 28-49% in t2d-p+ group, and 30-44% in t2d+p+ group (Table 4.1).

Group	Patient	Gender	Age (years)	Years of type 2 diabetes diagnosis	Glycated haemoglobin (%)	Teeth present (n)	Sites with probing depth ≥ 5mm	Bleeding on probing score (%)
t2d+p+	#1	Male	67	1990	7.3	24	18	30
t2d+p+	#2	Female	60	2000	8.0	24	29	36
t2d+p+	#3	Male	70	2003	7.4	20	26	44
t2d+p-	#1	Male	47	2012	8.0	26	0	18
t2d+p-	#2	Male	54	1995	7.8	25	0	17
t2d+p-	#3	Male	56	2009	7.1	28	0	20
t2d-p+	#1	Female	62	-	-	24	19	49
t2d-p+	#2	Female	45	-	-	24	29	49
t2d-p+	#3	Female	51	-	-	22	23	28
t2d-p-	#1	Female	54	-	-	28	0	8
t2d-p-	#2	Male	47	-	-	26	0	25
t2d-p-	#3	Female	59	-	-	32	0	12

Table 4.1. Characteristic of the study population.

4.2 Quality control and pre-treatment of raw data

The whole metagenomic shotgun sequencing generated 947,943,558 reads (139.8 Gb sequenced). The quality control assessed using FastQC (S. Andrews, 2010) returned a very good level of sequencing quality for all 12 individuals. As an indication, in Figure 4.1 is shown the worst sequencing result. Nucleotides with a Phred score quality below the threshold of 20 were trimmed off, 4 and 3 bp were removed respectively at read's end and start to retain the best quality core of reads. Whole reads with an average Phred quality score lower than 20 were removed. Finally, reads shorter than 25bp were removed after trimming. No reads containing "N"'s were observed. This reduced the number of reads to 768,816,194.

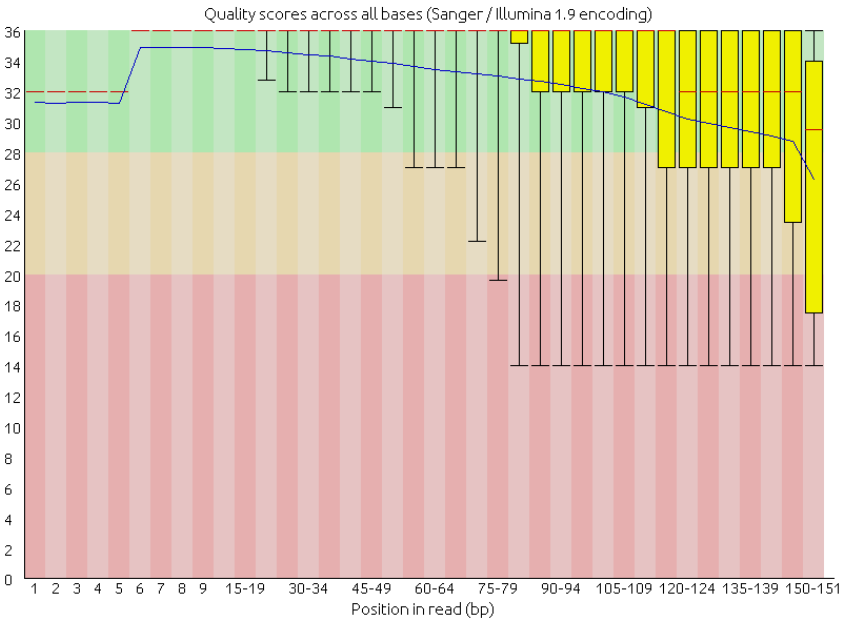


Figure 4.1 Per base quality score computed with FastQC.

Statistics of oral plaque samples after trimming and contaminant filtering are summarized in Table 4.2.

Host DNA span from 71.41% to 97.99 % of total reads. Average non-human reads content was 4,663,154 reads (7.55%). The sample with the highest number of sequences hit was ind8, with 16,416,896 reads (28.59%), while the lower non-human reads content were detected in ind12, with 1,054,246 reads (2.01%).

ID	Type	N° raw reads	N° tot reads after quality control	N° human reads	N° non-human reads	% human reads	% non-human reads
ind1	t2d+p+	78,015,504	65,284,068	60,973,858	4,310,210	93.40	6.60
ind3	t2d+p+	73,434,326	61,186,636	50,180,456	11,006,180	82.01	17.99
ind7	t2d+p+	73,339,766	58,469,398	53,146,190	5,323,208	90.90	9.10
ind2	t2d+p-	100,393,778	80,622,922	76,298,588	4,324,334	94.64	5.36
ind6	t2d+p-	81,205,598	67,873,856	66,192,640	1,681,216	97.52	2.48
ind12	t2d+p-	64,600,706	52,375,072	51,320,826	1,054,246	97.99	2.01
ind4	t2d-p+	85,131,290	66,338,258	64,940,522	1,397,736	97.89	2.11
ind9	t2d-p+	79,947,192	66,614,552	65,197,570	1,416,982	97.87	2.13
ind11	t2d-p+	79,463,624	66,644,814	62,981,872	3,662,942	94.50	5.50
ind5	t2d-p-	90,466,936	66,979,592	65,294,978	1,684,614	97.48	2.52
ind8	t2d-p-	68,614,856	57,425,098	41,008,202	16,416,896	71.41	28.59
ind10	t2d-p-	73,329,982	59,001,928	55,322,942	3,678,986	93.76	6.24

Table 4.2. Statistics of whole metagenome shotgun sequencing of oral plaque samples.

4.3 Community complexity and diversity

The consensus taxonomic composition of the reads identified using the naive LCA algorithm in MEGAN6, resulted in 70 genera and 156 species among all the 12 samples. Three genera were in common among all the samples: *Campylobacter, Actinomyces* and *Rothia*, while *Campylobacter gracilis* and *Actinomyces* sp. oral taxon 897 were shared at the species level. Table 4.3 shown the number of genera and species in the groups defined according to the different health status. T2d-p- showed the higher number of both genera and species, followed by t2d+p-, t2d-p+ with the lowest (and similar) number of genera and species. Minimum and maximum values of species detected in each group allow to recognize a wide variability among t2d-p- and t2d+p- groups, compared to t2d-p+ and t2d+p+ groups.

Group	Genera	Min	Max	Species	Min	Max
t2d-p-	53	25	28	109	41	66
t2d+p-	51	26	36	91	37	60
t2d-p+	33	28	28	69	49	50
t2+p+	34	22	29	71	41	50

Table 4.3. Genera and species detected in MEGAN6 dataset**.**

The taxonomic composition obtained by MetaPlhAn 3.0 detected a total of 69 genera and 182 species. Two genera were in common among all the samples: *Campylobacter* and *Rothia*, while the only species present in all samples was *Campylobacter gracilis*. Table 4.4 summarize number of genera and species classified by the different health status using MetaPlhAn 3.0. These data were noticeably heterogeneous: t2d-p- have the lowest number of genera but the highest of species; t2d+p+ showed the highest value in term of genera and a high number of species; lastly, the two intermediate disease conditions had a similar number of genera but a different number of species , with individuals t2d+p- presenting the smallest number of species detected. Minimum and maximum values of species detected in each group allow to observe a wide variability among the individuals of the t2d-p- group, much less marked in the subjects of the other groups. MetaPlhAn 3.0 allows to detect a higher number of genera and species in almost all groups (except t2d+p-) than MEGAN6.

Group	Genera	Min	Max	Species	Min	Max
t2d-p-	57	15	47	150	22	131
t2d+p-	39	19	27	74	36	43
t2d-p+	42	24	38	102	42	86
t2+p+	50	30	44	122	69	91

Table 4.4 Genera and species detected in MetaPhlAn 3.0 dataset**.**

Figure 4.2 schematizes the number, and the intersection, of genera and species detected within the 12 samples using the two different methods. MEGAN6 and MetaPhlAn 3.0 shared the 28.5% of genera and the 24.6% of species between those specifically detected. It is worth noticing that even if the number of genera detected through the two different pipelines were quite similar, MetaPhlAn 3.0 identified a higher number of species than MEGAN6.

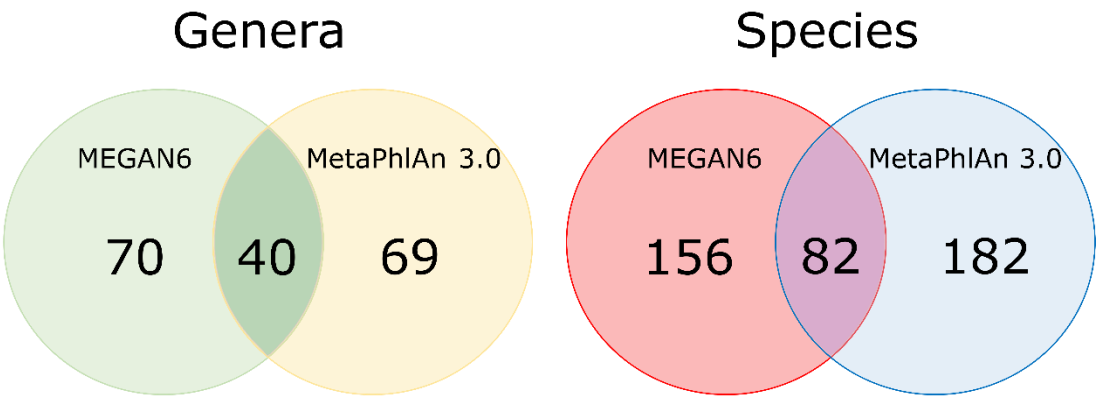


Figure 4.2 Venn's diagrams of genera and species detected with the two different pipelines.

For both taxonomic assignment methods used, all indices for diversity in microbial species did not differ significantly among groups (Krusla-Wallis p-value in Figure 4.3 and 4.4). The comparisons between periodontitic *versus* non periodontitic subjects, or diabetics *versus* non diabetic individuals highlighted how microbial richness was slightly higher in P- subjects.

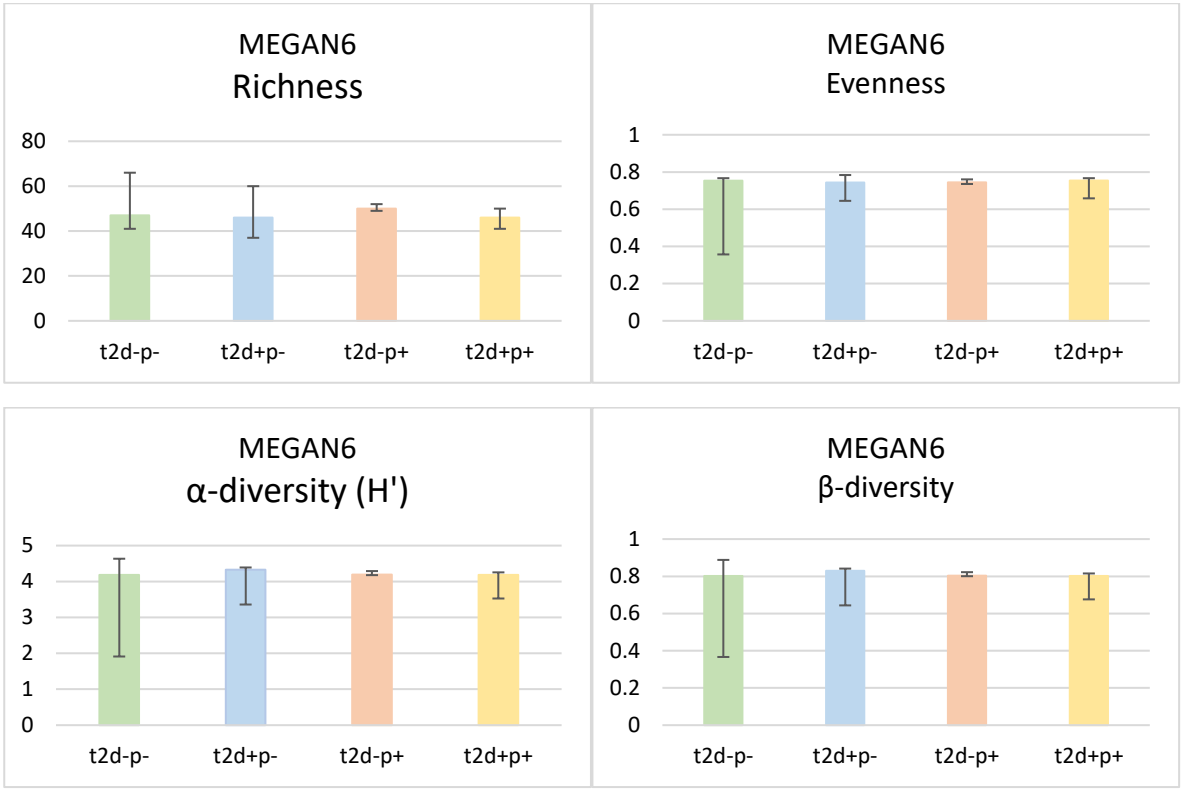


Figure 4.3 Intrapersonal diversity of the subgingival microbiome detected by MEGAN6. (A) Richness (p=0.443); (B) Evenness Index (J) (p=0.392); (C) alpha-diversity index (H') (p=0.270); (D) Beta-diversity Index (p=0.269). Data are represented as medians and minimum-maximum bars.

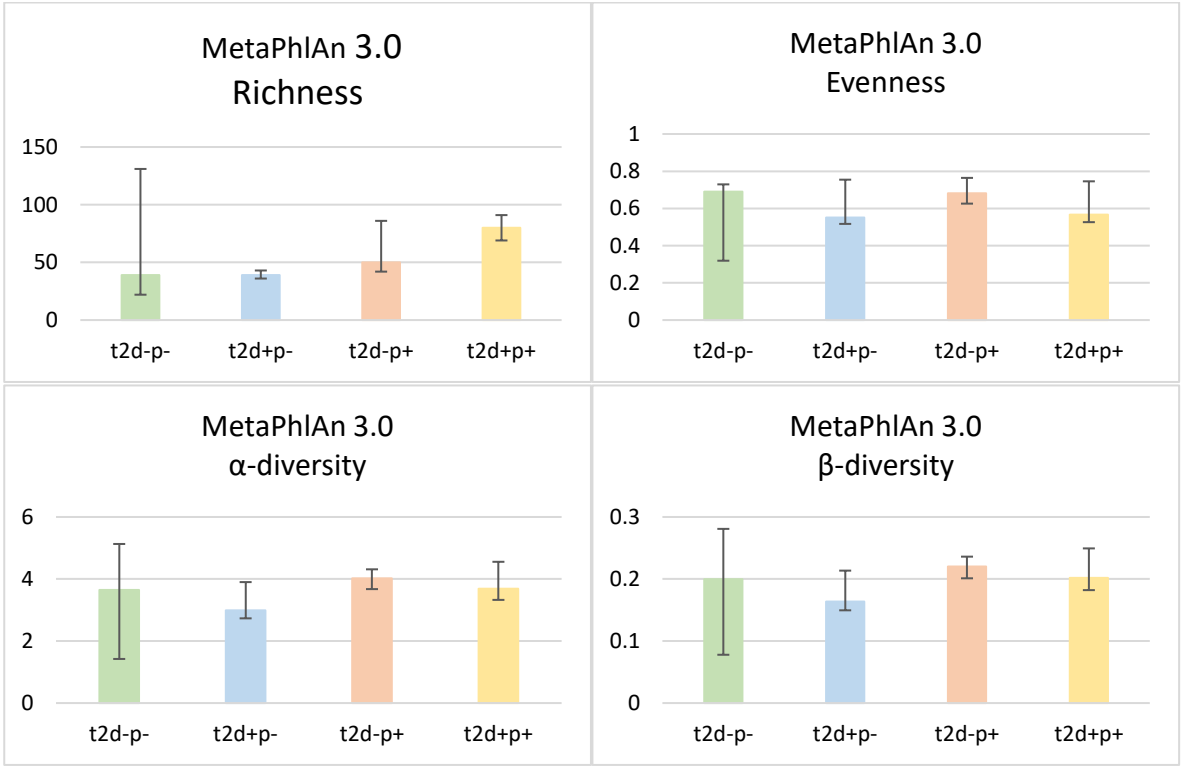


Figure 4.4 Intrapersonal diversity of the subgingival microbiome detected by MetaPhlAn 3.0. (A) Richness (p=0.289); (B) Evenness Index (J) (p=0.764); (C) alpha-diversity index (H') (p=0.557); (D) Beta-diversity Index (p=0.560). Data are represented as medians and minimum-maximum bars.

4.4 Taxonomical profiling of periodontal bacteria associated with periodontitis or type 2 diabetes

The distribution of differentially abundant genera and species detected in individuals with (n = 6) and without periodontitis (n = 6), or with (n=6) and without type 2 diabetes (n=6) were recorded for both methods.

4.4.1 MEGAN6

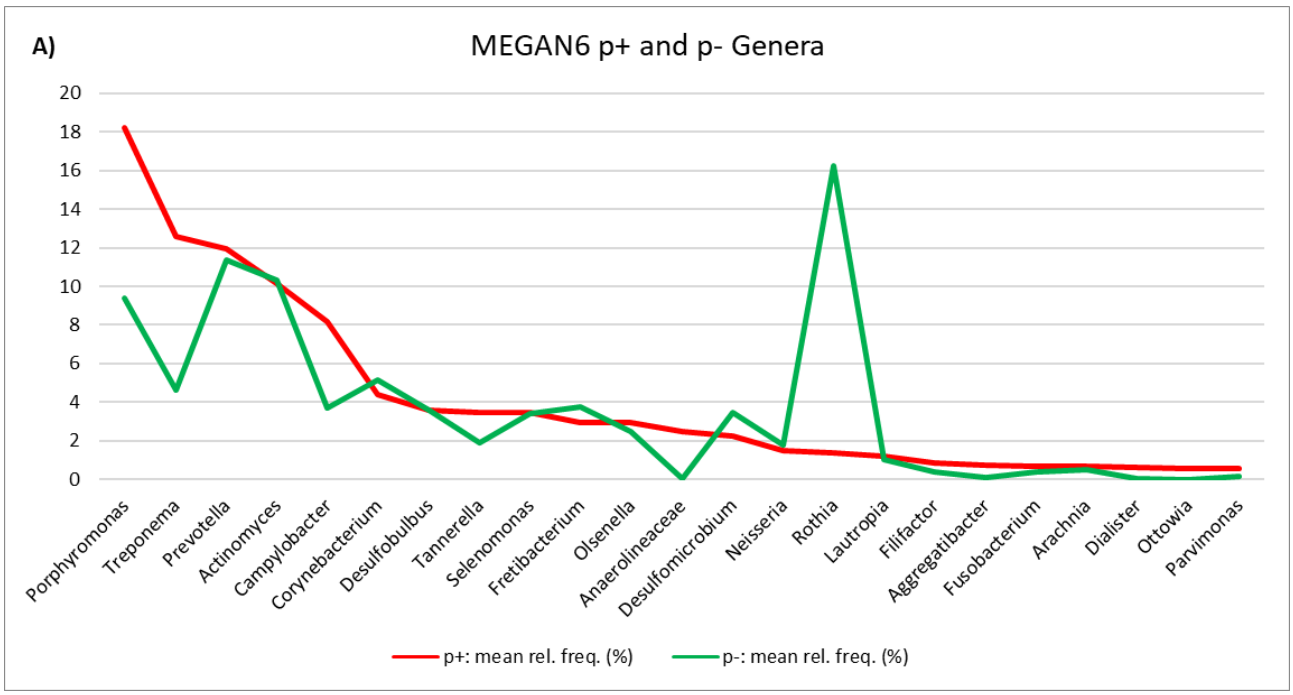
Figure 4.5 (a and b) shows the genera and species with abundance ≥ 0.5% in at least one groups, based on MEGAN6. The most abundant genera in subjects with periodontitis (p+) were *Porphyromonas*, *Treponema*, *Prevotella*, *Actinomyces* and *Campylobacter*. In individuals without periodontitis (p-) the most abundant were *Rothia*, *Prevotella*, *Actinomyces*, *Porphyromonas* and *Corynebacterium*.

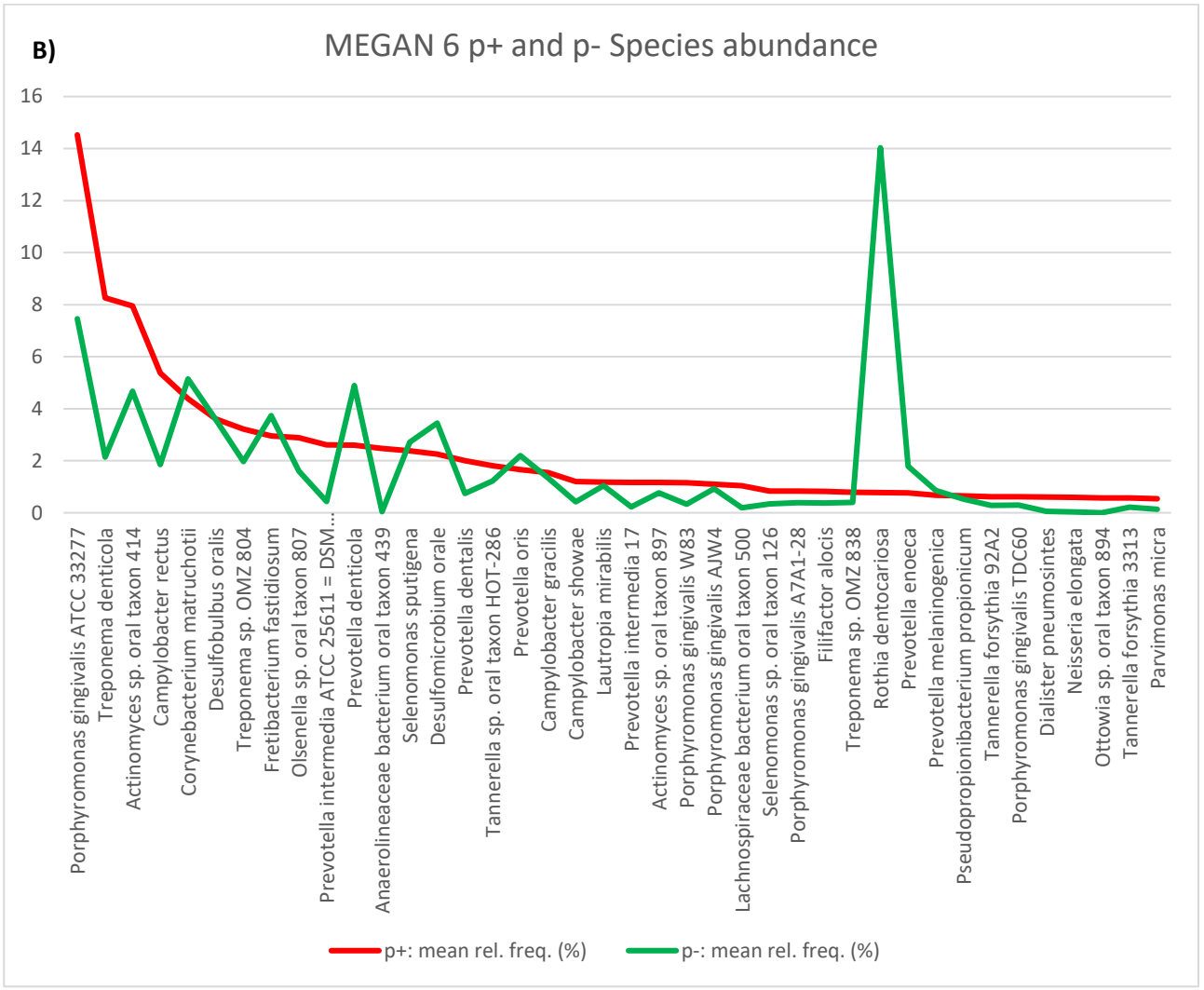
The most abundant species were *Porphyromonas gingivalis ATCC 33277, Treponema denticola, Actinomyces sp.* oral taxon 414 and *Campylobacter rectus* in p+ group, while in p- group were *Rothia dentocariosa, Porphyromonas gingivalis ATCC 33277* and *Corynebacterium matruchotii*.

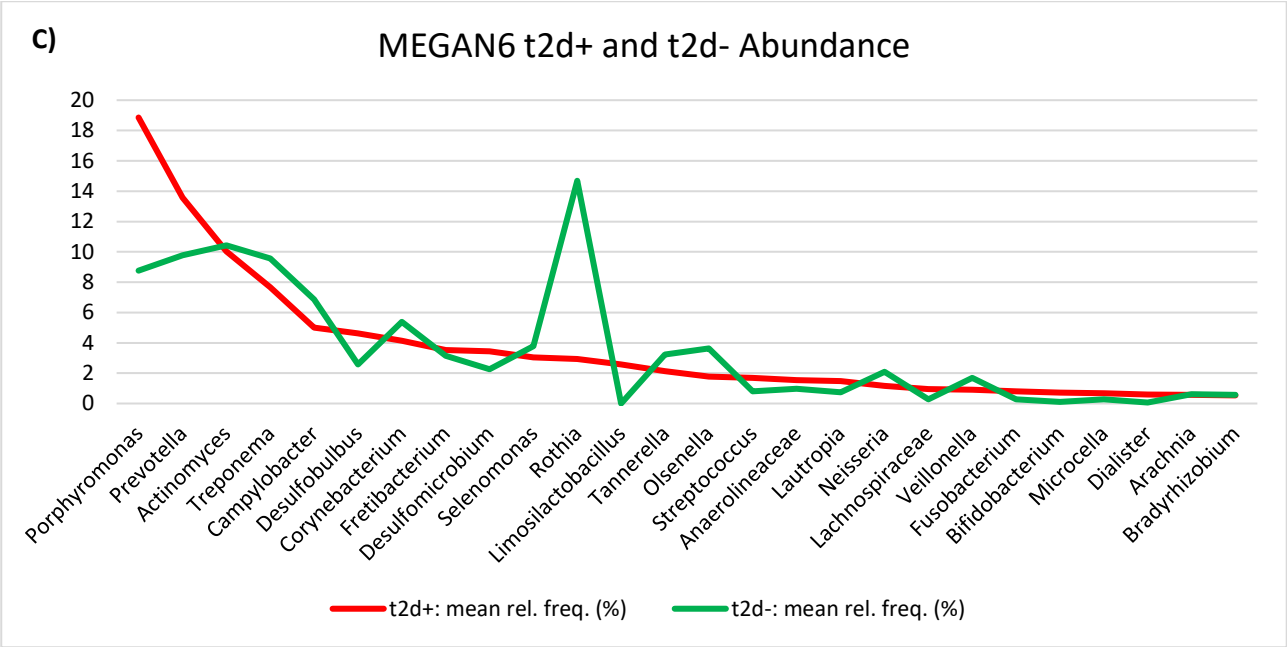
Figure 4.5 c and d shows the genera and species with abundance ≥ 0.5% in at least one groups, based on MEGAN6, respectively in individuals with (t2d+) and without type 2 diabetes (t2d-). Predominant genera were *Porphyromonas*, *Prevotella*, *Actinomyces* and *Treponema* in the t2d+ group and *Rothia*, *Actinomyces*, *Prevotella*, *Treponema*, *Porphyromononas*, *Campylobacter* and *Corynebacterium* in t2d- individuals.

The most represented species in t2d+ subjects were *P. gingivalis ATCC 33277, Actinomyces sp.* oral taxon 414*, P. denticola and T. denticola*, while t2d- individual has as frequent species *R. dentocariosa, P. gingivalis* ATCC 33277*, Actinomyces sp.* oral taxon 414 and *C. matruchotii*.

Genera *Porphyromonas*, with species *P. gingivalis ATCC 33277*, and *Prevotella* were abundant in all groups. Detailed tables showing the relative abundances are presented in supplementary table 1 and 2.


A)
MEGAN6 p+ and p- Genera
20
18
16
14
12
10
8
6
4
2
0
Porphyromonas
Treponema
Prevotella
Actinomyces
Campylobacter
Corynebacterium
Desulfobulbus
Tannerella
Selenomonas
Fretibacterium
Olsenella
Anaerolineaceae
Desulfomicrobium
Neisseria
Rothia
Lautropia
Filifactor
Aggregatibacter
Fusobacterium
Arachnia
Dialister
Ottowia
Parvimonas
p+: mean rel. freq. (%)
p-: mean rel. freq. (%)


B)
MEGAN 6 p+ and p- Species abundance
16
14
12
10
8
6
4
2
0
Porphyromonas gingivalis ATCC 33277
Treponema denticola
Actinomyces sp. oral taxon 414
Campylobacter rectus
Corynebacterium matruchotii
Desulfobulbus oralis
Treponema sp. OMZ 804
Fretibacterium fastidiosum
Olsenella sp. oral taxon 807
Prevotella intermedia ATCC 25611 = DSM...
Prevotella denticola
Anaerolineaceae bacterium oral taxon 439
Selenomonas sputigena
Desulfomicrobium orale
Prevotella dentalis
Tannerella sp. oral taxon HOT-286
Prevotella oris
Campylobacter gracilis
Campylobacter showae
Lautropia mirabilis
Prevotella intermedia 17
Actinomyces sp. oral taxon 897
Porphyromonas gingivalis W83
Porphyromonas gingivalis AJW4
Lachnospiraceae bacterium oral taxon 500
Selenomonas sp. oral taxon 126
Porphyromonas gingivalis A7A1-28
Filifactor alocis
Treponema sp. OMZ 838
Rothia dentocariosa
Prevotella enoeca
Prevotella melaninogenica
Pseudopropionibacterium propionicum
Tannerella forsythia 92A2
Porphyromonas gingivalis TDC60
Dialister pneumosintes
Neisseria elongata
Ottowia sp. oral taxon 894
Tannerella forsythia 3313
Parvimonas micra
p+: mean rel. freq. (%)
p-: mean rel. freq. (%)



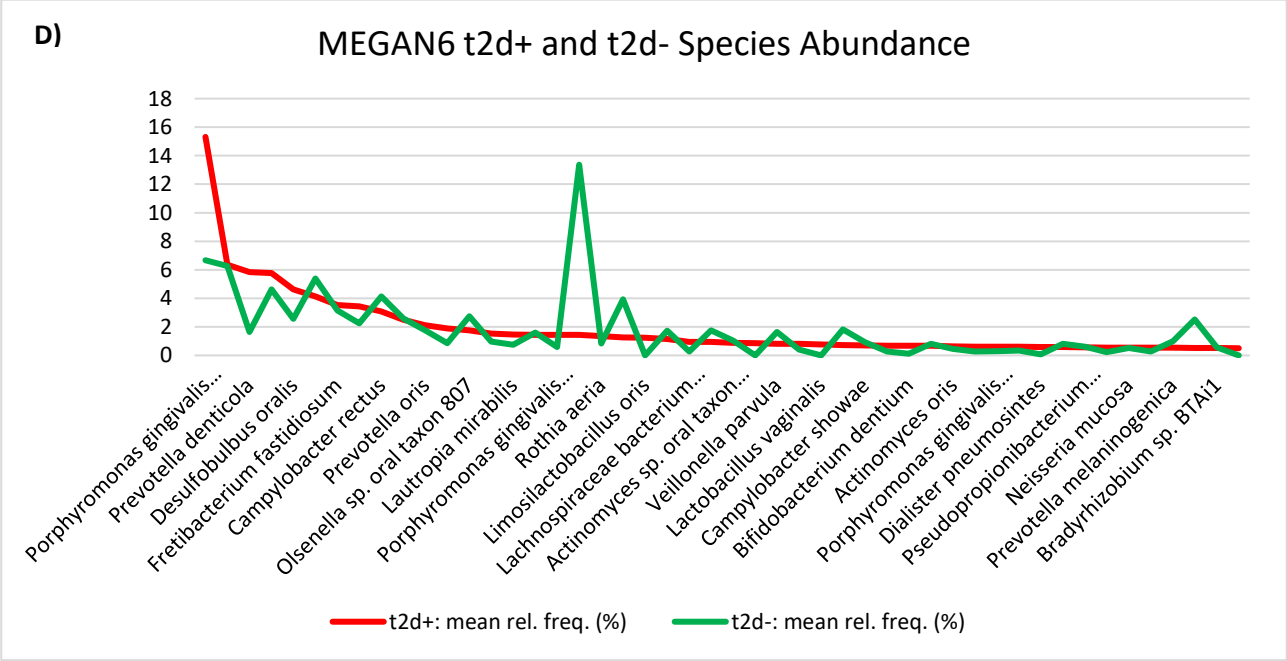


Figure 4.5 Bacterial genera and species, in MEGAN6 output, with relative abundance ≥ 0.5% in: **(A) GENERA and (B) SPECIES** in patients with periodontitis (n = 6) and individuals without periodontitis (n = 6); **(C) GENERA and (D) SPECIES** in patients with type 2 diabetes (n = 6) and individuals without type 2 diabetes (n = 6).

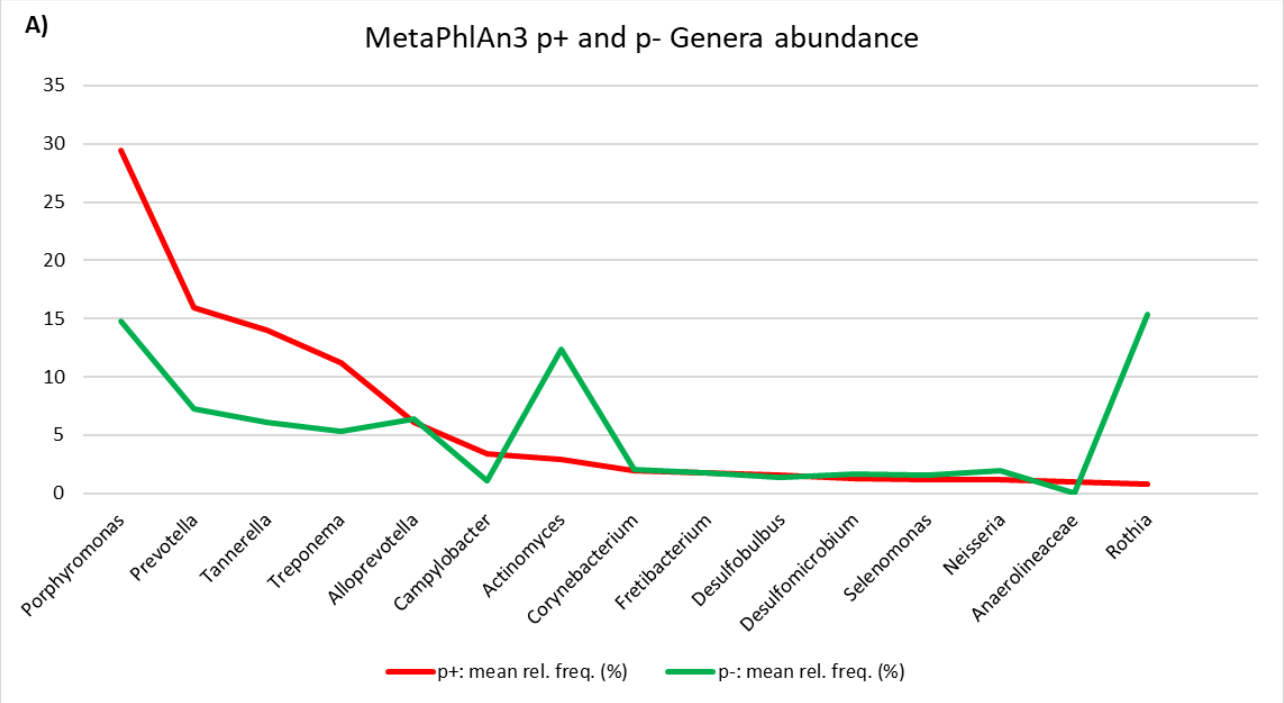
4.4.2 MetaPhlAn 3.0

Figure 4.6 shows the genera and species with abundance ≥ 0.5% in at least one groups, based on MetaPhlAn 3.0 analyses, respectively in individuals with (A) and without periodontitis (B) or in individuals with (C) and without type 2 diabetes (D).

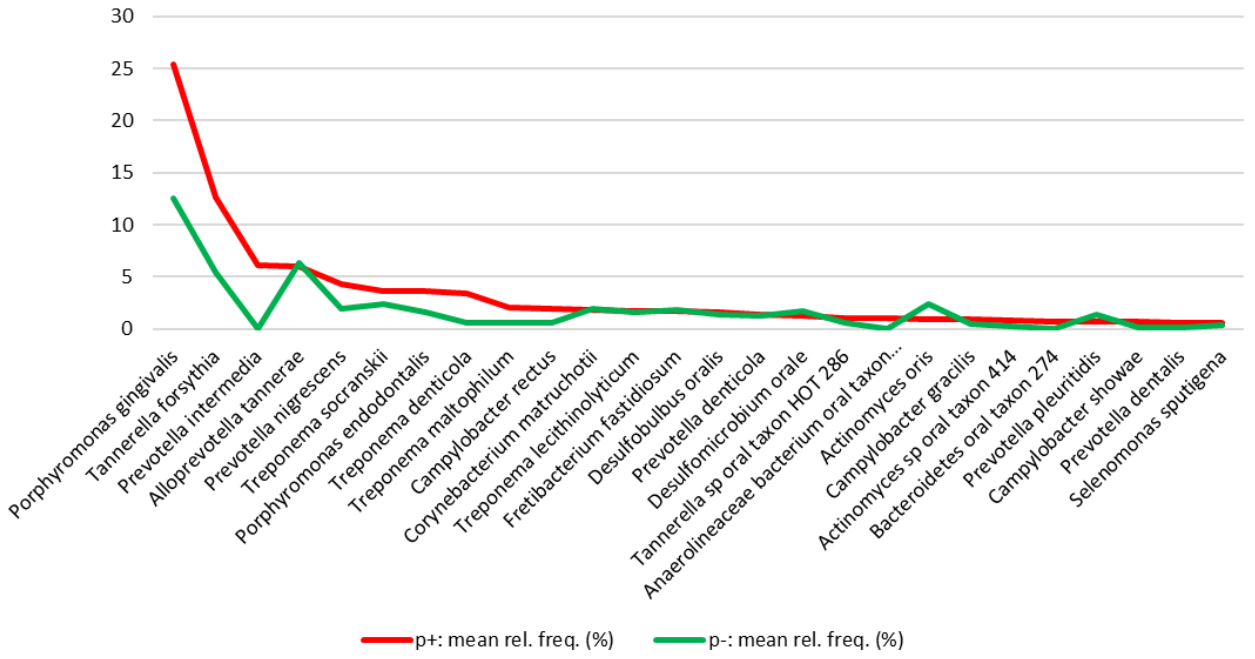
Most abundant genera in p+ subjects were *Porphyromonas*, *Prevotella*, *Tannerella, Treponema* and *Alloprevotella*. In individuals without periodontitis (p-) the most abundant were *Rothia*, *Porphyromonas, Actinomyces*, *Prevotella*, *Alloprevotella*, *Tannerella and Treponema*. The most abundant species were *P. gingivalis, Tannerella forsythia, Prevotella intermedia* and *Alloprevotella tannarae* in p+ group, while in p- group were *P. gingivalis, T. forsythia* and *A. tannarae.*

Concerning type 2 diabetes (Figure 4.6 c and d), the predominant genera were *Porphyromonas*, *Prevotella*, *Tannerella*, *Scardovia*, *Actinomyces* and *Treponema* in the t2d+ group and *Porphyromonas*, *Rothia*, *Alloprevotella*, *Treponema*, *Tannerella* and *Actinomyces* in t2d- individuals. The species most represented in t2d+ subjects were *P. gingivalis, T. forsythia* and *S. wiggsiae*, while t2d- individual has as frequent species *R. dentocariosa, P. gingivalis, Alloprevotella tannarae* and *T. forsythia*.

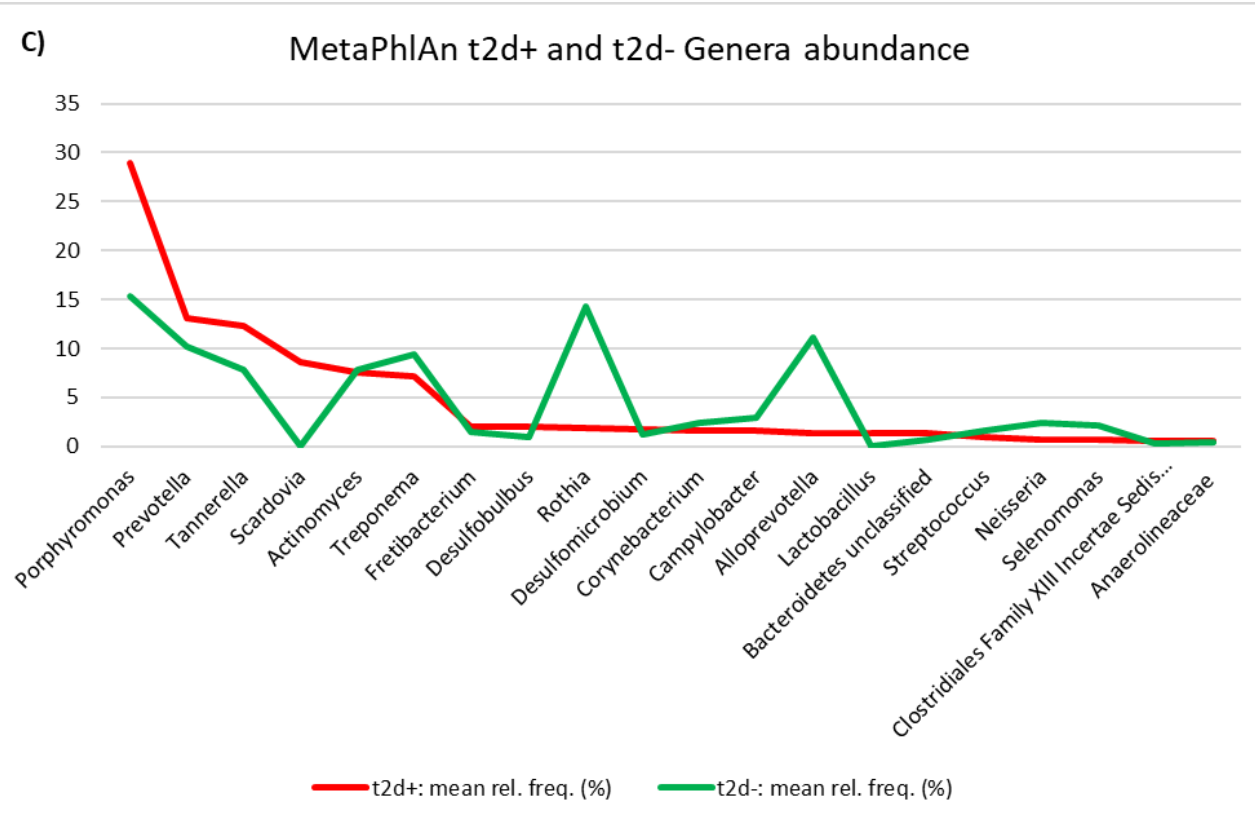
The genera *Porphyromonas*, *Prevotella*, *Tannerella* and *Treponema* were frequent in all groups, and the species commonly present in all class were *P. gingivalis* and *T. forsythia.* Detailed tables showing the relative abundances are presented in supplementary table 3 and 4.



B)


MetaPhlAn3 p+ and p- Species abundance
30
25
20
15
10
5
0
Porphyromonas gingivalis
Tannerella forsythia
Prevotella intermedia
Alloprevotella tannerae
Prevotella nigrescens
Treponema socranskii
Porphyromonas endodontalis
Treponema denticola
Treponema maltophilum
Campylobacter rectus
Corynebacterium matruchotii
Treponema lecithinolyticum
Fretibacterium fastidiosum
Desulfobulbus oralis
Prevotella denticola
Desulfomicrobium orale
Tannerella sp oral taxon HOT 286
Anaerolineaceae bacterium oral taxon...
Actinomyces oris
Campylobacter gracilis
Actinomyces sp oral taxon 414
Bacteroidetes oral taxon 274
Prevotella pleuritidis
Campylobacter showae
Prevotella dentalis
Selenomonas sputigena
p+: mean rel. freq. (%)
p-: mean rel. freq. (%)

C)


MetaPhlAn t2d+ and t2d- Genera abundance
35
30
25
20
15
10
5
0
Porphyromonas
Prevotella
Tannerella
Scardovia
Actinomyces
Treponema
Fretibacterium
Desulfobulbus
Rothia
Desulfomicrobium
Corynebacterium
Campylobacter
Alloprevotella
Lactobacillus
Bacteroidetes unclassified
Streptococcus
Neisseria
Selenomonas
Clostridiales Family XIII Incertae Sedis...
Anaerolineaceae
t2d+: mean rel. freq. (%)
t2d-: mean rel. freq. (%)

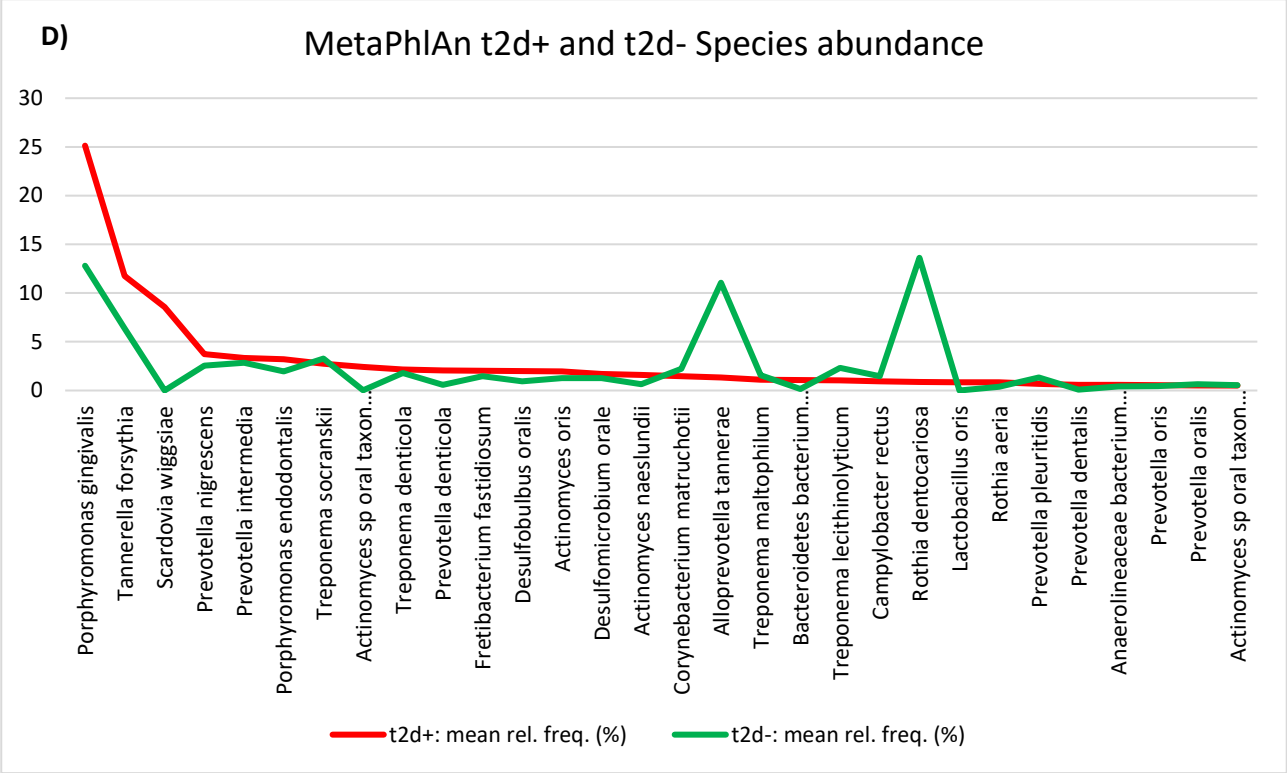


Figure 4.6 Bacterial genera and species, in MetaPhlAn 3.0 output, with relative abundance ≥ 0.5% in: **(A) GENERA and (B) SPECIES** in patients with periodontitis (n = 6) and individuals without periodontitis (n = 6); **(C) GENERA and (D) SPECIES** in patients with type 2 diabetes (n = 6) and individuals without type 2 diabetes (n = 6).

Taxonomical data categorised in term of p+ and p- groups did not take in account the contribution of type 2 diabetes and vice versa for t2d+ and t2d- groups. In tables 4.5 and 4.6 are briefly showed most abundant species detected, respectively with MEGAN6 an MetaPhlAn 3.0, in all clinical subcategories.

MEGAN6

t2d+p+	**t2d-p+**	**t2d+p-**	**t2d-p-**
Actinomyces sp. oral taxon 414	*Actinomyces sp.* oral taxon 414	*Actinomyces sp.* oral taxon 414	*Actinomyces sp.* oral taxon 414
Campylobacter rectus	*Campylobacter rectus*	*Campylobacter rectus*	
Porphyromonas gingivalis	*Porphyromonas gingivalis*	*Porphyromonas gingivalis*	
Desulfobulbus oralis		*Desulfobulbus oralis*	*Desulfobulbus oralis*
	Corynebacterium matruchotii	*Corynebacterium matruchotii*	*Corynebacterium matruchotii*
Treponema denticola	*Treponema denticola*		
Prevotella intermedia	*Prevotella intermedia*		
Prevotella denticola		*Prevotella denticola*	
Fretibacterium fastidiosum			*Fretibacterium fastidiosum*
	Treponema sp. OMZ 838	*Treponema sp.* OMZ 838	
	Desulfomicrobium orale	*Desulfomicrobium orale*	

	Olsenella sp. oral taxon 807		*Olsenella sp.* oral taxon 807
	Tannerella sp. oral taxon HOT-286		*Tannerella sp.* oral taxon HOT-286
Anaerolineaceae bacterium oral taxon 439			
Prevotella dentalis			
		Prevotella oris	
		Limosilactobacillus oris	
			Veillonella parvula
			Rothia dentocariosa
			Selenomonas sputigena
			Prevotella enoeca

Table 4.5 Most abundant species detected with MEGAN6 in all clinical subcategories.

MetaPhlAn 3.0

t2d+p+	t2d-p+	t2d+p-	t2d-p-
Tannerella forsythia	*Tannerella forsythia*	*Tannerella forsythia*	*Tannerella forsythia*
Porphyromonas gingivalis	*Porphyromonas gingivalis*	*Porphyromonas gingivalis*	
Porphyromonas endodontalis	*Porphyromonas endodontalis*	*Porphyromonas endodontalis*	
Treponema socranskii	*Treponema socranskii*	*Treponema socranskii*	
Treponema denticola	*Treponema denticola*		
Prevotella intermedia	*Prevotella intermedia*		
Fretibacterium fastidiosum			*Fretibacterium fastidiosum*
Prevotella nigrescens			*Prevotella nigrescens*
	Alloprevotella tannerae		*Alloprevotella tannerae*
Desulfobulbus oralis			
	Campylobacter rectus		
	Treponema lecithinolyticum		
		Desulfomicrobium orale	
		Actinomyces sp	
		Actinomyces oris	
		Scardovia wiggsiae	
		Actinomyces naeslundii	
			Veillonella parvula
			Rothia dentocariosa
			Actinomyces massiliensis
			Neisseria flavescens

Table 4.6 Most abundant species detected with MetaPhlAn 3.0 in all clinical subcategories.

4.5 Differences in subgingival microbiome composition for individuals with different clinical status

4.5.1 White's non-parametric t-test

The results of White's non-parametric t-test applied to compare relative abundance between groups are shown in Table 4.7 for MEGAN6 analyses and in Table 4.8 for the MetaPhlAn 3.0 output. In both tables, all genera and species with a nominal significance p-value equal or lower than 0.01 before FDR correction were included.

MEGAN6

Statistical analysis of patients with periodontitis (p+, n=6) compared to non-periodontitis subjects (p-, n=6) allowed to identify two significantly different genera: *Anaerolineaceae* (attributable to *A. bacterium* oral taxon 439) and *Microcella* (referable to *M. alkaliphila* species). These two genera/species specifically characterize periodontitis (*A. bacterium* oral taxon 439) and healthy subjects (*M. alkaliphila*).

Several other species showed a relative abundancy higher in periodontitis patients compared with p- subjects: *Campylobacter showae*, *Campylobacter rectus*, *Parvimonas micra* and *Eubacterium minutum.* In p- subjects *Actinomyces viscocus*, together with *M. alkaliphila,* were significantly most abundant.

When comparing the subgingival microbiome of individuals with and without type 2 diabetes, irrespective of periodontitis (n=6, for both groups), no significant inter-group differences in the relative abundance of genera or species were detected (table 4.7).

The investigation of subgingival microbiome in the different sub-categories of individuals (n=3, for each sub-subclass), showed that (table 4.7):

a) t2d-p+ vs t2d-p− were characterized by a significant higher abundance of *Porphyromonas* and *Campilobacter* genera, ascribable to the presence of *P. gingivalis* and *C. showae* or *C. rectus*. Among the species, also *Fusobacterium nucleatum* showed a higher abundance of in t2d−p+ compared to t2d−p− (p-value= 0.006);
b) t2d+p+ vs t2d+p− were characterized by a significant higher abundance of *Aggregatibacter* and *Anaerolineaceae,* at genus level, and by higher abundance of *A. bacterium* oral taxon 439 and lower abundance of *Rothia dentocariosa* at species level (table 2);

c) t2d+p+ vs t2d−p+, no significant inter-group differences were observed at genus level. Among species, *F. nucleatum*, *Filifactor alocis* and *Neisseria sp.* oral taxon 104 were observed only in non-diabetic subjects (p-values 0.006, 0.007 and 0.01 respectively).
d) t2d+p− vs t2d−p− no significant inter-group differences were observed both at genus or species level.

The subgingival microbiome of t2d−p− was then compared with the microbiome profile of t2d+p+ patients. At genus level, the main significant differences were observed for *Anaerolineaceae* (and, within this genus, *A. bacterium* oral taxon 439), *Fusobacterium, Dialister* (and, within this genus, *D. pneumosintes*) that were significantly more abundant in patients t2d+p+, whereas *Microcella* genus was significantly more abundant in t2d-p- subjects (table 4.7).

Thus, as a general outline (table 4.5), is possible to conclude that *A. bacterium* oral taxon 439 was significantly present in all groups of periodontitis subject (p+, t2d+p+ and t2d-p+) analysed against no periodontitis groups (p-, t2d+p-, t2d-p-). *Campylobacter rectus* and *C. showae* characterized t2d-p+ individuals compared to t2d+p-individuals and healthy subjects (t2d-p-).The *Microcella* genus (and, within this genus, *M. alkaliphila*) seems to characterize a healthy periodontium.

MetaPhlAn 3.0

In MetaPhlAn 3.0 results, few significant genera or species were highlighted in the different comparisons (table 4.8). At genus level, p+ group (n=6) showed significantly higher abundance of *Tannarella* compared with p- subjects (regardless of diabetic status, n=6). Among species, a higher presence of *Treponema maltophilum* characterized p+ individuals against p- patients. *T. maltophilum*, together with *C. rectus*, was also significantly represented in t2d-p+ patients as compared to t2d+p- individuals (table 4.8).

T2d+p+ patients were characterized by the presence of *A. bacterium* oral taxon 439, absent in t2d+p- and t2d-p- individuals.

Finally, *P. gingivalis* were detected with a significant higher relative abundance in t2d-p+ subjects compared with t2d-p- individuals.

To summarise the main results from both methods, the *A. bacterium* oral taxon 439 showed a higher relative abundance in periodontitis patients vs individuals without periodontitis, both considering the overall population or the type 2 diabetes subsample, maintaining the significance after FDR correction (Supplementary table 5 and 6). Both MEGAN6 and MethaPhlAn 3.0 identified a

significantly higher abundance of *P. gingivalis* in individuals with and without periodontitis but in absence of type 2 diabetes, and also for *P. gingivalis* the significance was confirmed, for MethaPhlAn 3.0 dataset, after FDR correction.

In addition to *P. gingivalis,* the output of MEGAN6 indicate that *C. rectus* and *C. showae* characterized t2d-p+ individuals. *Microcella* genus (and, within this genus, *M. alkaliphila*) seems to characterize a healthy periodontium.

Periodontitis patients vs individuals without periodontitis (genera)	Periodontitis mean ± s.d.	No periodontitis mean ± s.d.	Nominal p-val	Periodontitis patients vs individuals without periodontitis (species)	Periodontitis mean ± s.d.	No periodontitis mean ± s.d.	Nominal p-val
Anaerolineaceae unclass.	2.472 ± 1.018	0.045 ± 0.101	4.35E-06	Anaerolineaceae bacterium oral taxon 439	2.473 ± 1.019	0.045 ± 0.101	6.78E-05
Microcella	0.032 ± 0.070	0.921 ± 0.808	0.01	Microcella alkaliphila	0.032 ± 0.070	0.921 ± 0.808	0.008
				Campylobacter showae	1.202 ± 0.540	0.420 ± 0.328	0.004
				Campylobacter rectus	5.375 ± 2.554	1.853 ± 1.711	0.006
				Actinomyces viscosus	0.133 ± 0.163	0.805 ± 0.573	0.007
				Parvimonas micra	0.539 ± 0.364	0.129 ± 0.128	0.01
				Eubacterium minutum	0.124 ± 0.118	0 ± 0	0.01
t2d+p+ vs t2d-p+ (genera)	**t2d+p+ mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val**	**t2d+p+ vs t2d-p+ (species)**	**t2d+p+ mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val**
				Fusobacterium nucleatum	0 ± 0	0.368 ± 0.118	0.006
--	--	--	--	Filifactor alocis	0 ± 0	1.648 ± 0.561	0.007
				Neisseria sp. oral taxon 104	0 ± 0	0.297 ± 0.109	0.01
t2d+p- vs t2d-p- (genera)	**t2d+p- mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**	**t2d+p- vs t2d-p- (species)**	**t2d+p- mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**
--	--	--	--	--	--	--	--
Patients with type 2 diabetes vs individuals without type 2 diabetes (genera)	**type 2 diabetes mean ± s.d.**	**No type 2 diabetes mean ± s.d.**	**Nominal p-val**	**Patients with type 2 diabetes vs individuals without type 2 diabetes (species))**	**type 2 diabetes mean ± s.d.**	**No type 2 diabetes mean ± s.d.**	**Nominal p-val**
--	--	--	--	--	--	--	--
t2d+p+ vs t2d+p- (genera)	**t2d+p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val**	**t2d+p+ vs t2d+p- (species)**	**t2d+p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val**
Anaerolineaceae unclass.	3.078 ± 0.906	0 ± 0	0.003	Anaerolineaceae bacterium oral taxon 439	3.078 ± 0.906	0 ± 0	0.003
Aggregatibacter	0.171 ± 0.043	0 ± 0	0.001	Rothia dentocariosa	0.518 ± 0.520	2.356 ± 0.584	0.008
t2d-p+ vs t2d-p- (genera)	**t2d-p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val**	**t2d-p+ vs t2d-p- (species)**	**t2d-p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val**
Campylobacter	11.295 ± 2.695	2.427 ± 1.653	0.004	Campylobacter rectus	7.265 ± 0358	1.012 ± 1.333	0.002
Porphyromonas	17.468 ± 6.000	0.0721 ± 0.102	0.003	Campylobacter showae	1.608 ± 0.251	0.235 ± 0.189	0.002
				Porphyromonas gingivalis TDC60	0.597 ± 0.185	0 ± 0	0.005
				Porphyromonas gingivalis ATCC 33277	13.309 ± 4.469	0.039 ± 0.055	0.006
				Fusobacterium nucleatum subsp. Vincentii	0.368 ±0.118	0 ± 0	0.006
t2d+p+ vs t2d-p- (genera)	**t2d+p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**	**t2d+p+ vs t2d-p- (species)**	**t2d+p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**
Anerolineacea unclass.	3.074 ± 0.906	0.090 ± 0.128	0.003	Anaerolineaceae bacterium oral taxon 439	3.077 ± 0.906	0.090 ±0.128	0.001
Fusobacterium	0.835 ± 0.238	0.031 ± 0.044	0.002	Dialister pneumosintes	1.102 ± 0.487	0.033 ± 0.461	0.008
Dialister	1.1012± 0.487	0.033 ± 0.046	0.009				
Microcella	0.063 ± 0.089	0.531 ± 0.219	0.01				
t2d+p- vs t2d-p+ (genera)	**t2d+p- mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val**	**t2d+p- vs t2d-p+ (species)**	**t2d+p- mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val**
Tannerella	0.921 ± 0.662	3.576 ± 0.500	0.004	Tannerella sp. oral taxon HOT-286	0 ± 0	2.584 ± 0.730	0.003
Anaerolineaceae unclass.	0 ± 0	1.870 ± 0.726	0.007	Anaerolineaceae bacterium oral taxon 439	0 ± 0	1.87 ± 0.726	0.008
Aggregatibacter	0 ± 0	1.273 ± 0.385	0.003	Campylobacter rectus	2.695 ± 1.631	7.265 ± 0.358	0.005
				Campylobacter showae	0.604 ± 0.334	1.607 ± 0.251	0.008
				Prevotella melaninogenica	0 ± 0	0.270 ± 0.114	0.009

Table 4.7 Bacteria genera/species from MEGAN6 output having a nominal significantly difference ≤0.01 level at White's non-parametric t-test applied to compare relative abundance between groups.

Periodontitis patients vs individuals without periodontitis (genera)	**Periodontitis mean ± s.d.**	**No periodontitis mean ± s.d.**	**Nominal p-val**	**Periodontitis patients vs individuals without periodontitis (species)**	**Periodontitis mean ± s.d.**	**No periodontitis mean ± s.d.**	**Nominal p-val**
Tannerella	14.0169 ± 4.832	6.145 ± 4.722	0.009	Treponema maltophilum	2.066 ± 0.555	0.590 ± 0.947	0.005
t2d+p+ vs t2d-p+ (genera)	**t2d+p+ mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val**	**t2d+p+ vs t2d-p+ (species)**	**t2d+p+ mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val**
--	--	--	--	--	--	--	--
t2d+p- vs t2d-p- (genera)	**t2d+p- mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**	**t2d+p- vs t2d-p- (species)**	**t2d+p- mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**
--	--	--	--	--	--	--	--
Patients with type 2 diabetes vs individuals without type 2 diabetes (genera)	**type 2 diabetes mean ± s.d.**	**No type 2 diabetes mean ± s.d.**	**Nominal p-val**	**Patients with type 2 diabetes vs individuals without type 2 diabetes (species)**	**type 2 diabetes mean ± s.d.**	**No type 2 diabetes mean ± s.d.**	**Nominal p-val**
--	--	--	--	--	--	--	--
t2d+p+ vs t2d+p- (genera)	**t2d+p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val**	**t2d+p+ vs t2d+p- (species)**	**t2d+p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val**
Anaerolineaceae unclassified	1.147 ± 0.117	0 ± 0	0	Anaerolineaceae bacterium oral taxon 439	1.147 ± 0.117	0 ± 0	0
t2d-p+ vs t2d-p- (genera)	**t2d-p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**	**t2d-p+ vs t2d-p- (species)**	**t2d-p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**
Porphyromonas	29.823 ± 6.662	0.975 ± 1.378	0	Porphyromonas gingivalis	25.613 ± 4.984	0 ± 0	0
t2d+p+ vs t2d-p- (genera)	**t2d+p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**	**t2d+p+ vs t2d-p- (species)**	**t2d+p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val**
Anaerolineaceae unclassified	1.147 ± 0.117	0 ± 0	0	Anaerolineaceae bacterium oral taxon 439	1.147 ± 0.117	0 ± 0	0
t2d+p- vs t2d-p+ (genera)	**t2d+p- mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val**	**t2d+p- vs t2d-p+ (species)**	**t2d+p- mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val**
Prevotella	4.231 ± 3.532	10.037 ± 0.172	0.03	Treponema maltophilum	0.320 ± 0.408	2.255 ± 0.463	0.004
Campylobacter	1.326 ± 0.839	5.094 ± 2.306	0.04	Campylobacter rectus	0.790 ± 0570	2.716 ± 0.511	0.01

Table 4.8 Bacteria genera/species from MetaPhlAn 3.0 output having a nominal significantly difference ≤0.01 level at White's non-parametric t-test applied to compare relative abundance between groups.

4.5.2 LEfSe analyses

To identify the major genera/species defining taxonomic profiles across clinical conditions, both MEGAN6 and MetaPhlAn 3.0 outputs were also analyzed using the LDA Effect Size (LEfSe) algorithm. LEfSe emphasizes both the statistical significance and biological relevance of the detected metagenomic data, allowing the identification of differentially abundant features (genes or taxa) that characterize differences between two or more biological conditions.

In addition, the LEfSe algorithm, that combines a nonparametric Kruskal-Wallis test or pairwise Wilcoxon rank-sum test with LDA, allowed the removal of the strong effect of FDR correction when several multiple comparisons are computed on a small sample size.

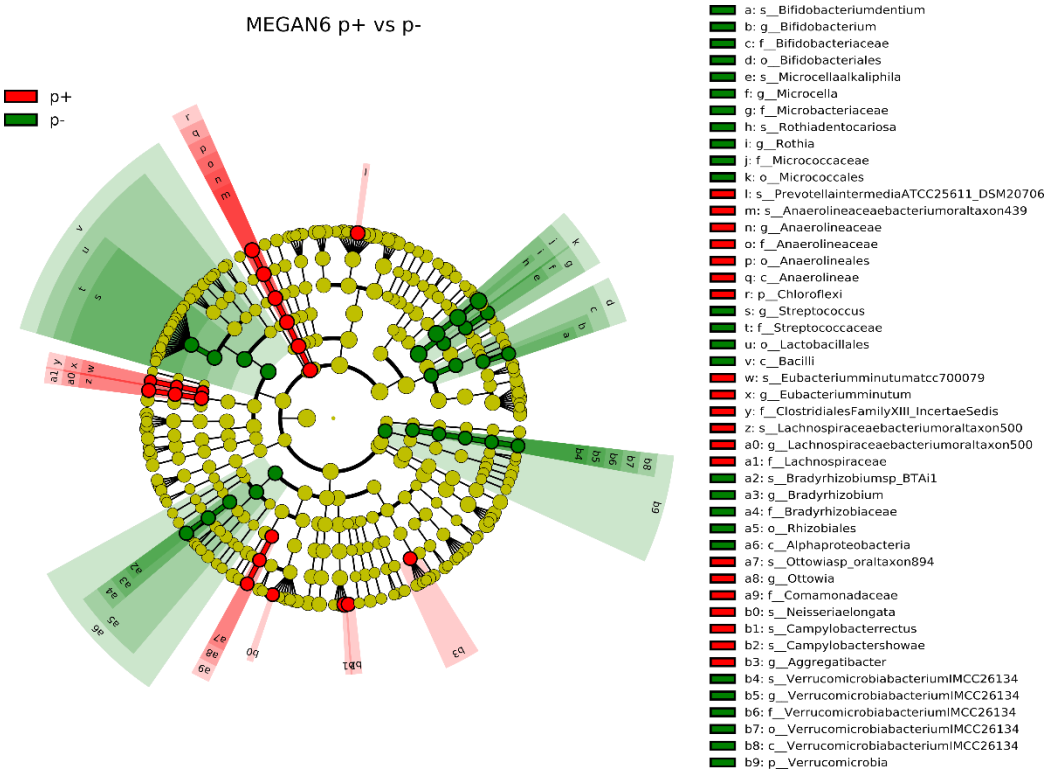
Results of LEfSe analysis on MEGAN6 and MetaPhlAn 3.0 output are represented in Figures 4.7, figure 4.8 and, for all comparison of clinical subcategories, in Supplementary figures 1 and 2. Here, only the main clinical classification (a) individuals with and without periodontitis; (b) subjects with and without type 2 diabetis, were presented.

(a) Subgingival microbiome in individuals with and without periodontitis (irrespective of type 2 diabetes): in both MEGAN6 and MetaPhlAn 3.0 comparison (figure 4.7A and 4.7B respecively), the LEfSe results showed that p+ patients, presented as characteristic features *A. bacterium* oral taxon 439, *P. intermedia*, *Neisseria elongata*, *C. rectus* and *C. showae*, whereas *R. dentocariosa* and *M. alkaliphila* were enriched in p- subjects (Figure 4.7A and 4.7B).

(b) Subgingival microbiome in individuals with and without type 2 diabetes (irrespective of periodontitis): LEfSe analyses, aimed to detect differentially abundant taxa across individuals with and without type 2 diabetes in MEGAN6 dataset, revealed that *F. nucleatum* was the only significative species enriched in t2d+ subjects, whereas no species were identified as characteristic in sample of individuals t2d- patients (figure 4.8).

Concerning MetaPhlAn 3.0 dataset, no significant results were detected by LEfSe analyses in the comparison between t2d+ and t2d- individuals.

A)



B)

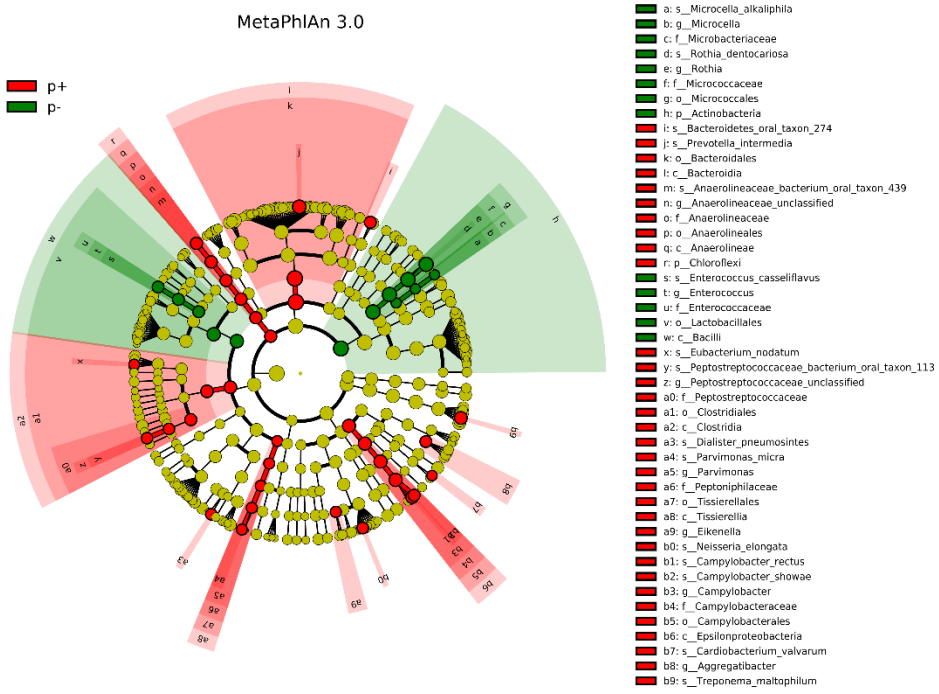


Figure 4.7 LEfSe results of comparison between individuals with and without periodontitis in taxonomic profiles made by: **a**. MEGAN6; **b**. MetaPhlAn 3.0.

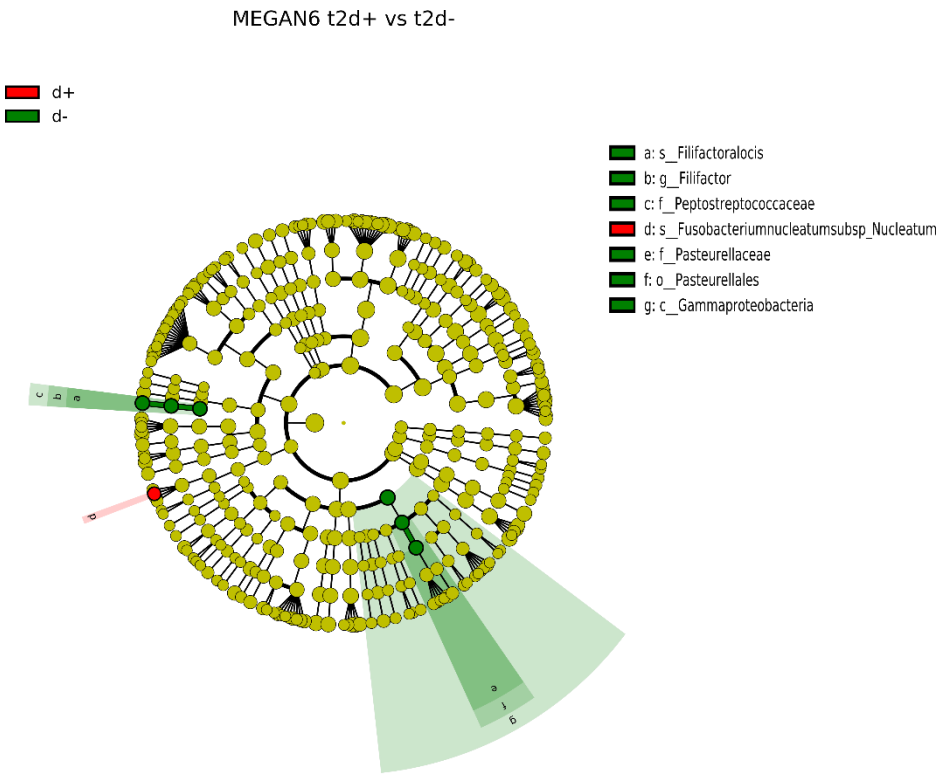


Figure 4.8 LEfSe results of comparison between individuals with and without type 2 diabetes in taxonomic profiles made by: **a**. MEGAN6; **b**. MetaPhlAn 3.0.

4.6 Functional analysis

Two different methodologies were used for the functional annotation: (a) the subgingival microbiomes were mapped on the NCBI nr reference database using DIAMOND and then classified using KEGG functional database of MEGAN6 Ultimate Edition; (b) the functional profiles of the twelve samples were also computed with HUMAnN 3.0 which annotates short reads using an accelerated version of the BLASTX algorithm on an internal databases of proteins.

Both annotations were then submitted to LEfSe in order to detect the pathway that can explain differences between subjects: I) with and without periodontitis, and II) with and without type 2 diabetes. Considering the high number of records in protein databases and the small sample sizes in the different clinical sub-classifications, functional analysis on these subgroups were not performed.

4.6.1 Functional annotation of subgingival microbiome in individuals with and without periodontitis

DIAMOND pipeline.

MEGAN6-KEGG's analysis of DIAMOND data were performed both at pathway and protein level, this allowed the identification of 459 pathways and 5442 proteins present in the metagenome of the twelve individuals.

Using LEfSe with a LDA score threshold at 2.5, a total of 24 pathways and 11 proteins characteristic of non-periodontitis individuals were detected. In subjects affected by periodontitis 7 pathways, 5 proteins were found to be enriched. Results are summarised in figure 4.9 (A) and (B), respectively for pathways and proteins.

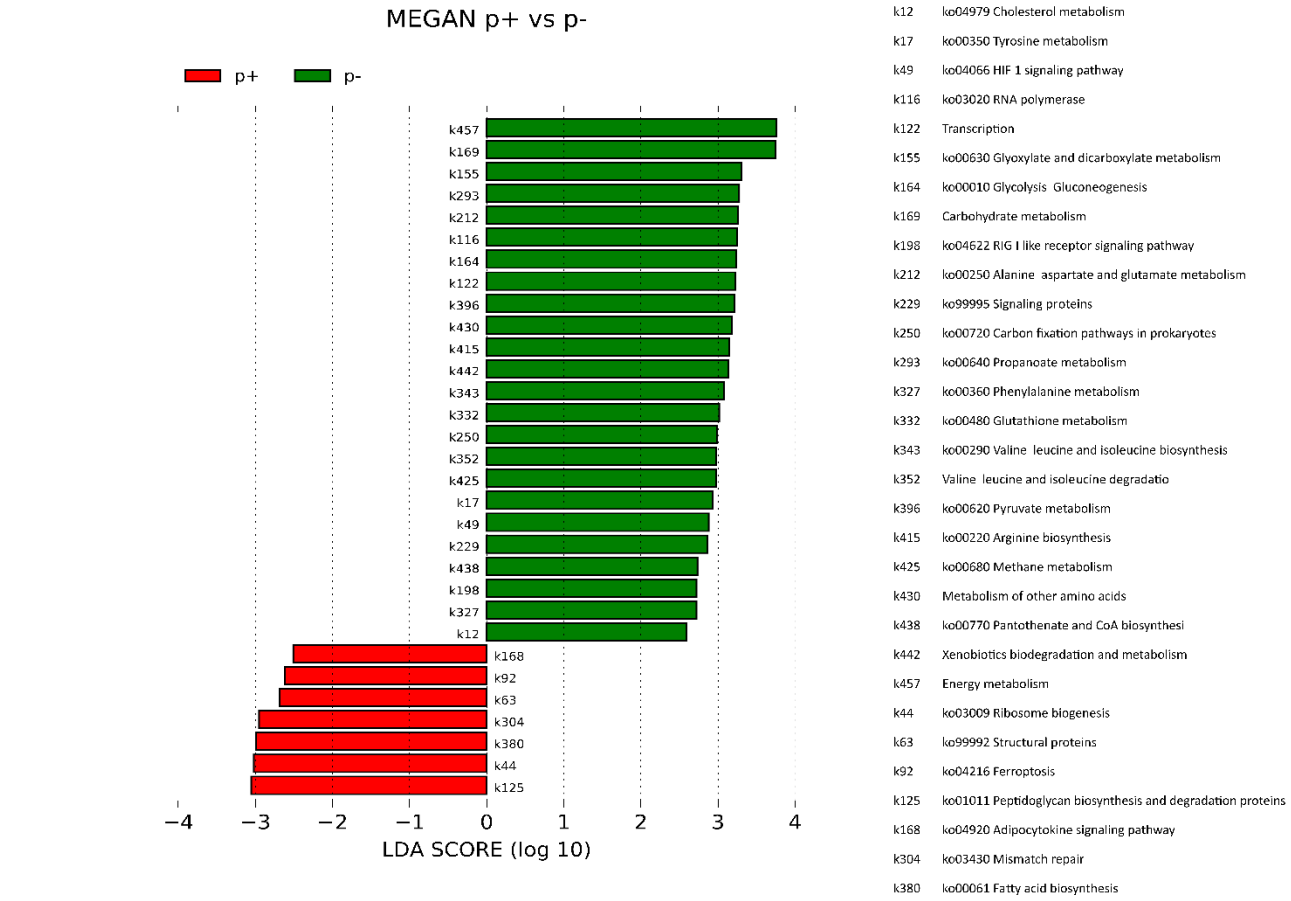
KEGG's annotation found many biological/metabolic pathways to be significantly enriched in the p- group:

- Carbohydrate metabolism group was significant as higher level of KEGG classification and was enriched for glycolysis / gluconeogenesis, propanoate metabolism, pyruvate metabolism, glyoxylate and dicarboxylate metabolism pathways; among proteins acetyl-CoA synthetase was significantly enriched.
- Energy metabolism was over-represented in healthy subjects and showed as characteristic pathways the carbon fixation metabolism in prokaryotes and methane metabolism, both has acetyl-CoA synthetase as relevant protein.
- Metabolism of other amino acids were significantly higher in p- subjects, as well as the pathways of glutathione metabolism and aminopeptidase N among proteins.
- Amino acid metabolism was not significant at higher level of KEGG classification, but some of its pathways were relevant: phenylalanine metabolism, both biosynthesis and degradation of valine, leucine and isoleucine, arginine biosynthesis, tyrosine metabolism and alanine, aspartate and glutamate metabolism; the latter was characterized by the proteins glutamate synthase (NADPH) large chain and glutamate dehydrogenase.
- Group not included in pathway or brite was characterized by signalling protein and specifically by GTP-binding protein.

The KEGG's annotation for features was found to be significantly enriched in the p+ group highlighted pathways of fatty acids biosynthesis, mismatch repair, peptidoglycan biosynthesis and

degradation and ferroptosis significantly present and characterised by long-chain acyl-CoA synthetase.

A)



B)

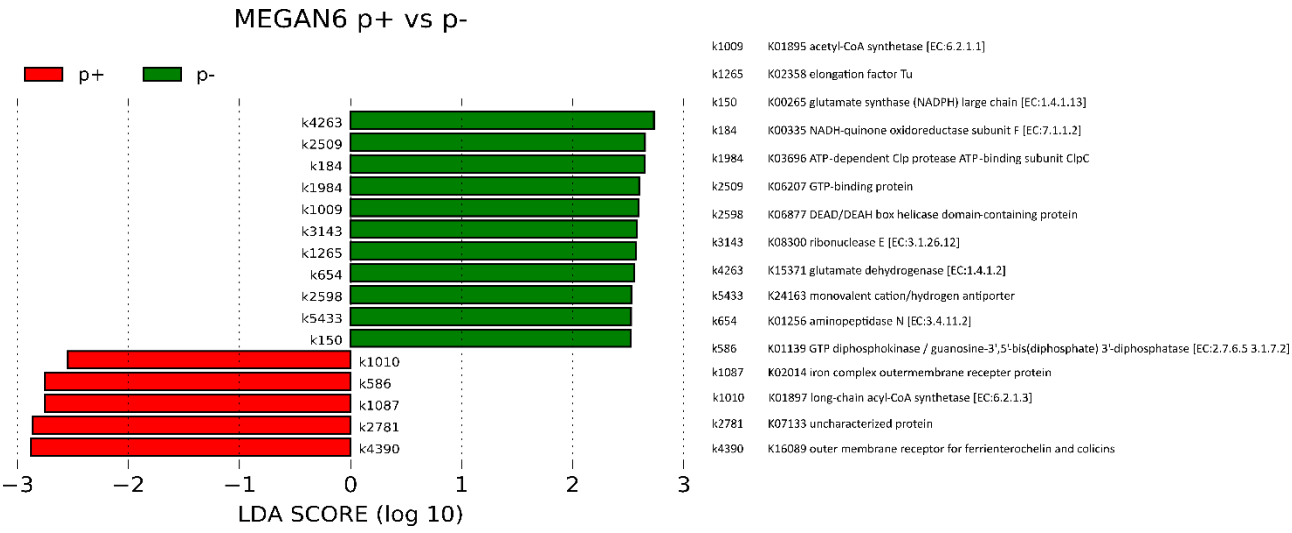


Figure 4.9 LEfSe results of MEGAN6's functional analysis between individuals with and without periodontitis for a) pathways and b) proteins.

HUMAnN 3.0's pipeline.

The HUMAnN 3.0's data annotation allowed to identify 5776 pathways present in the metagenome of the twelve individuals.

LEfSe analysis, with a LDA score threshold sets at 2.5, revealed 9 pathways characteristic of p- individuals and 15 pathways enriched in p+ subjects (Figure 4.10).

Individuals without periodontitis resulted characterized by:

- 5 pathways related to amino acids biosynthesis: superpathway of branched amino acids biosynthesis which leads to the production of L-leucine, L-Valine, and L-isoleucine. Single pathway of each branched amino acid are connected in this superpathway by a number of shared enzymes; superpathway of L-threonine biosynthesis that include all the processes of oxalacetate conversion in threonine, isoleucine, synthesized from threonine is an attenuator of this superpathway; superpathway of L-isoleucine biosynthesis I join the reactions that bring to formation of isoleucine starting from oxalacetate; superpathway of L-lysine, L-threonine and L-methionine biosynthesis II that leads to synthesis of lysine, threonine, methionine and pyruvate from aspartate; seleno-amino acid biosynthesis that bring to the reduction of selenate to selenometionine.
- 2 pathways associated with isoprenoids biosynthesis: isoprene biosynthesis I where isoprene is synthetised from pyruvate and D-glyceraldehyde 3-phosphate and methylerythritol phosphate pathway II very similar to previous pathway, but leads to the formation of different type of isoprenoids. Isoprenoids are involved in a wide variety of vital biological functions
- Superpathway of pyrimidine deoxyribonucleosides degradation where pyrimidine deoxyribonucleosides are used as resource of carbon and energy by production of Acetyl-coA.

Subjects with periodontitis were enriched for:

- 4 features related to peptidoglycan biosynthesis: peptidoglycan biosynthesis I (meso-diaminopimelate containing) is the way of synthesis of peptidoglycan most common in Gram-negative bacteria, as well as some Gram-positive bacteria; UDP-N-acetylmuramoyl-pentapeptide biosynthesis I (meso-diaminopimelate containing) that is a part of the previous superpathway; while peptidoglycan biosynthesis III (mycobacteria) and UDP-N-

acetylmuramoyl-pentapeptide biosynthesis II (lysine-containing) are variants of peptidoglycan biosynthesis I which differ in some intermediates. Peptidoglycan is the major structural polymer in most bacterial cell walls.

- 3 pathway connected to inosine-5'-phosphate (IMP): adenosine ribonucleotides *de novo* biosynthesis that leads to formation of ADP which can enter in the biosynthesis of ATP cycle from inosine-5'-phosphate and L-aspartate; inosine-5'-phosphate biosynthesis I IMP is a starting point for *de novo* synthesis of adenosine and guanosine.
- 2 pathway associated to chorismate biosynthesis: chorismate biosynthesis I and chorismate biosynthesis from 3-dehydroquinate differing for the starting point of synthesis, D-erythrose 4-phosphate and 3-dehydroquinate respectively. Chorismate is the precursor of many compounds like aromatic amino acids, tryptophan, alkaloids and folate.
- 2 pathway related to coenzyme A (CoA): superpathway of coenzyme A biosynthesis I (bacteria) and its subpathway phosphopantothenate biosynthesis I, also known as biosynthesis of vitamin B5, in which (R)-4'-phosphopantothenate is synthetized, it is a precursor for the synthesis of coenzyme A and acyl carrier protein. Coenzyme A (CoA) is a cofactor needed in a large number of enzymatic reactions central to intermediary metabolism, including the oxidation of fatty acids, carbohydrates, and amino acids.
- Super pathway of pyrimidine deoxyribonucleosides salvage an alternative pathway to synthetise pyrimidine deoxyribonucleosides, avoiding the energetic expensive *de novo* synthesis of ribonucleotides.
- Calvin-Benson-Bassham cycle is the major CO2 fixation pathway, founded in chloroplast and many autotrophic bacteria.
- NAD *de novo* synthesis I (from aspartate) also known as nicotinamide adenine dinucleotide biosynthesis, most prokaryotes use a *de novo* pathway to produce this coenzyme from aspartate. NAD is fundamental in redox reactions in catabolism.
- Pre Q0 biosynthesis which lead to the biosynthesis of an intermediate of 7- deazapurines, compounds that contain pyrrole-pyrimidine functional groups. These compounds often have antibiotic or antiviral activities.

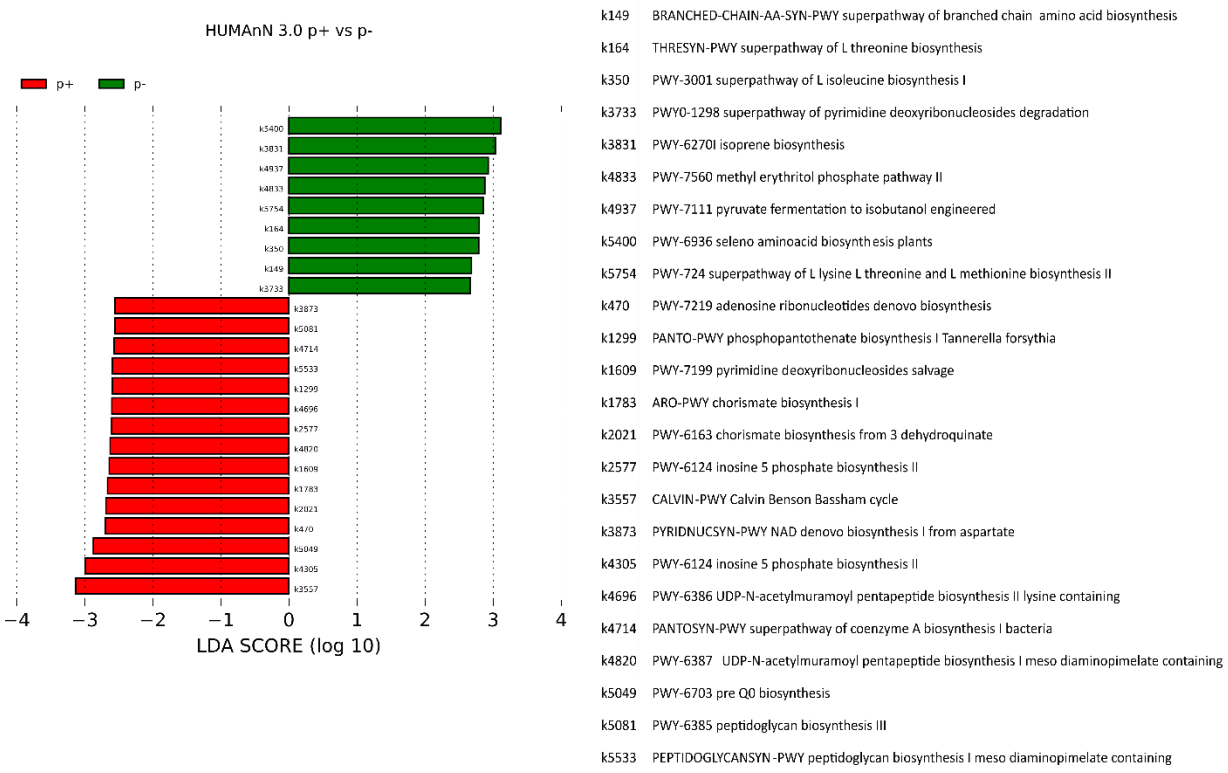


Figure 4.10 LEfSe results of HUMAnN 3.0's functional analysis between individuals with and without periodontitis.

4.6.2 Functional annotation of subgingival microbiome in individuals with and without type 2 diabetes

DIAMOND pipeline.

Nine attributes were identified in the LEfSe analysis (threshold 2.5) of pathways performed between individuals afflicted or not by type 2 diabetes (Figure 4.11). In this comparison, analysis at the protein level was inconclusive.

Significant features detected in t2d- individuals were very heterogeneous, spanning along the KEGG's category.

Only 2 pathways were discovered in t2d+ patients, related to antibiotic synthesis (streptomycin biosynthesis) and carbohydrate metabolism (galactose metabolism).

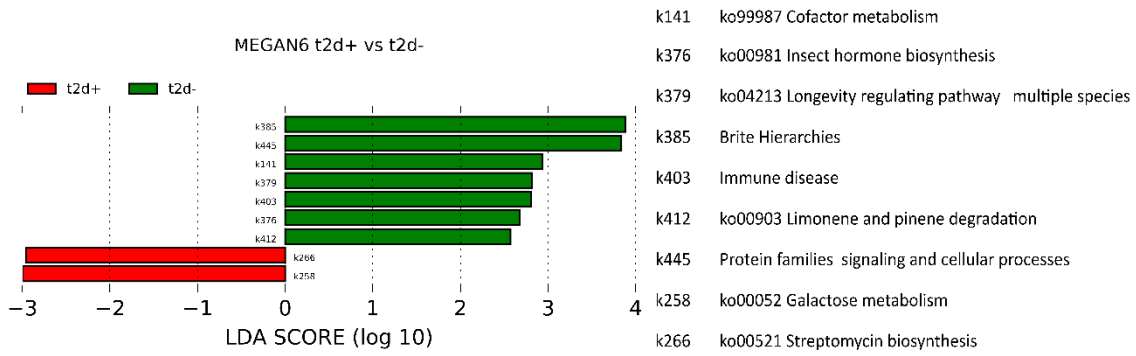


Figure 4.11 LEfSe results of MEGAN6's functional pathway analysis between individuals with and without type 2 diabetis.

HUMAnN 3.0's pipeline

When comparing t2d+ and t2d- individuals, LEfSe analyses (again with threshold 2.5) identified 6 significantly different pathways in the two groups, all characterizing t2d+ group (Figure 4.12).

Two features results related to synthesis of Lipopolysaccharides (LPS), a major component of the outer membrane of Gram-negative bacteria (superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis and CMP-3-deoxy-D-manno-octulosonate biosynthesis).

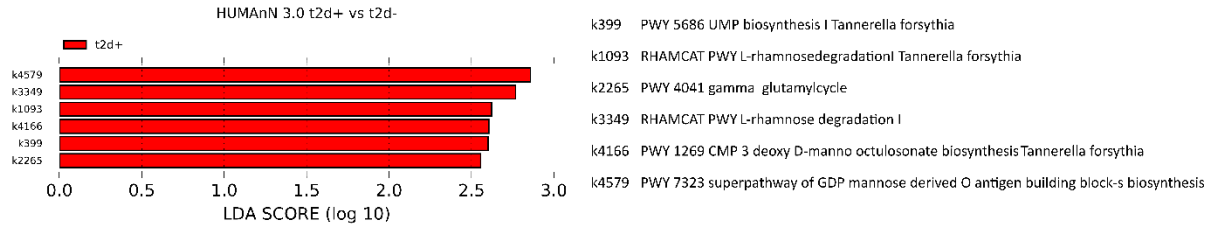


Figure 4.12 LEfSe results of HUMAnN 3.0's functional analysis between individuals with and without type 2 diabetes.

In order to give a brief overview results from both MEGAN6 and HUMAnN 3.0 analyses are summarised in table 4.9.

p+	p-	t2d+	t2d-
Amino acids metabolism			
	Superpathway of branched chain amino acid biosynthesis	Gamma glutamylcycle	
	Superpathway of L threonine biosynthesis		
	Superpathway of L isoleucine biosynthesis I		
	Superpathway of L lysine L threonine and L methionine biosynthesis II		

	Seleno aminoacid biosynthesis plants		
	Phenylalanine metabolism		
	Valine leucine and isoleucine biosynthesis		
	Valine leucine and isoleucine degradation		
	Arginine biosynthesis		
	Tyrosine metabolism		
	Alanine aspartate and glutamate metabolism		
	Metabolism of other amino acids		
	Glutathione metabolism		
Cell structures metabolism			
UDP N acetylmuramoyl pentapeptide biosynthesis I meso diaminopimelate containing		Superpathway of GDP mannose derived O-antigen building blocks biosynthesis	
UDP N acetylmuramoyl pentapeptide biosynthesis II lysine containing		CMP 3 deoxy-D -manno-Octulosonate biosynthesis	
Peptidoglycan biosynthesis III		L-rhamnose degradation I	
Peptidoglycan biosynthesis I meso diaminopimelate containing			
Peptidoglycan biosynthesis and degradation proteins			
Structural proteins			
Fatty acids metabolism			
Fatty acid biosynthesis			
Adipocytokine signaling pathway			
Carbohydrate metabolism			
	Pyruvate fermentation to isobutanol engineered	Galactose metabolism	
	Pyruvate metabolism		
	Glycolysis/Gluconeogenesis		
	Propanoate metabolism		
	Glyoxylate and dicarboxylate metabolism		
Energy metabolism			
Superpathway of coenzyme A biosynthesis I bacteria	Carbon fixation pathways in prokaryotes	UMP biosynthesis I Tannerella forsythia	
Phosphopantothenate biosynthesis I Tannerella forsythia	Methane metabolism		
	Pantothenate and CoA biosynthesis		
Nucleotide metabolism			
Adenosine ribonucleotides denovo biosynthesis	superpathway of pyrimidine deoxyribonucleosides degradation		
IMP inosine 5 phosphate biosynthesis II			
IMP inosine 5 phosphate biosynthesis II -			
pyrimidine deoxyribonucleosides salvage			
NAD *de novo* biosynthesis I from aspartate			
Fatty acid metabolism			
Fatty acid biosynthesis			
Adipocytokine signaling pathway			
Other			
Mismatch repair	Cholesterol metabolism	Streptomycin biosynthesis	Cofactor metabolism

Ribosome biogenesis	HIF 1 signaling pathway		Insect hormone biosymthesis
Chorismate biosynthesis I	RNA polymerase		Longevity regulating pathway
Chorismate biosynthesis from 3 dehydroquinate	Isoprene biosynthesis		Brite hierarchies
Calvin Benson Bassham cycle	Methyl erythritol phosphate pathway II		Immune disease
Pre Q0 biosynthesis	Processing Transcription		Limonene and pinene degradation
Ferroptosis	RIG I like receptor signaling pathway		Protein families signalling and cellular processes
	Signalling proteins		
	Xenobiotics biodegradation and metabolism		

Table 4.9 Summary of functional analysis results, with MEGAN6 an HUMAnN 3.0 in different health status regarding periodontitis and type 2 diabetes.

5. Discussion

The oral microbiome is one of the most complex microbial communities in the human body, harbouring more than 1,000 species of microorganisms (Lamont et al., 2018), and is closely related to oral and systemic health. Dental plaque biofilms are the primary etiologic factors of periodontitis, which is a common chronic oral infectious disease. Accumulating evidence has demonstrated that periodontitis is epidemiologically associated with several chronic diseases such as diabetes, cardiovascular diseases, metabolic syndrome and rheumatoid arthritis (Teles et al., 2021).

With the development of metagenomics and metatranscriptomics, the composition and structure of microbial communities as well as their overall functional characteristics can be fully profiled.

The association between periodontitis and type 2 diabetes 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; S. S. Socransky et al., 1998; Wu et al., 2020).

The aim of this chapter was to investigate, in addition to the microbial composition of the subgingival plaque biofilm, the functional aspects actually involved in the association between these two diseases and to discuss the potential mechanisms underlying their bidirectional relationship.

5.1 Main taxonomic outcomes

Both methods used for taxonomical profile reconstruction, MEGAN6 and MetaPhlAn 3.0, were quite concordant.

Analysing the abundance of species present in the different groups it was possible to describe a core microbiome, that is resilient to changes in the oral health status, composed of: *Desulfobulbus oralis*, *Corynebacterium matruchotii* and *Actinomyces* sp. In addition to these strain MetaPlhAn 3.0 detected as core bacteria also *Tannerella forsythia*, *Treponema socranskii* and *Alloprevotella tannerae*.

While some of these strains, like *C. matruchotii*, were described in literature as a component of the oral plaque's core microbiome (A. P. V. Colombo & Tanner, 2019), or associated with health status, such as *Actinomyces sp*. (A. P. V. Colombo & Tanner, 2019), others were historically associated with different status of oral disease. For example, *D. oralis and T. forsythia* were related to periodontitis complex depending on their pathogenicity, respectively green red complex (Wirth et al 2021),

T.socranskii is associated with mucositis an periodontitis (A. P. V. Colombo & Tanner, 2019; Na et al., 2020; Patini et al., 2018).

All groups, except healthy, were characterized by the presence of *Campylobacter rectus*, *Porphyromonas gingivalis* and *Porphyromonas endodontalis*. They were all associated with periodontitis or mucositis, specifically *P. gingivalis* is part of the red complex (Macuch & Tanner, 2000; S. S. Socransky et al., 1998; Wirth et al., 2021).

The group of periodontitis subjects (p+) presented a prevalence of *Treponema denticola*, *Prevotella intermedia*, *Prevotella dentalis* and *Anaerolineaceae bacterium* oral taxon 439. All these strains are components of the microbial complexes involved in advance stages of periodontal disease: *T. denticola* is part of the red complex, the other strains are all part of the orange complex (S. S. Socransky et al., 1998; Wirth et al., 2021).

The oral microbiome of patients affected by type 2 diabetes, was composed mainly of *Prevotella denticola* and *P. oris*. *Prevotella denticola* was historically associated with periodontal disease, it is part of the orange complex, while *P. oris* was associated with periodontal health status (A. P. V. Colombo & Tanner, 2019). Here *P. oris* was found predominantly in a group characterised by an inflammatory status, due to type 2 diabetes and comorbidity, confirming the association between this strain and an oral disease state already indicated by other studies (Lenartova et al., 2021; Riggio & Lennon, 2007).

Subjects not affected by type 2 diabetes showed a prevalence of *Alloprevotella tannerae*, *Tannerella sp.* oral taxon 286 and *Olsenella sp*. oral taxon 807. *Alloprevotella tannerae* is associated with periodontitis (A. P. V. Colombo & Tanner, 2019), *Tannerella sp.* oral taxon 286 is a strain phylogenetically close related to *T. forsythia*, but unlike this associated to health rather than disease (Vartoukian et al., 2016) while *Olsenella sp*. oral taxon 807 was described as component of dental calculus in ancient baboons (Ottoni et al., 2019).

The group of non-periodontitis individuals was characterized by the presence of strains associated with periodontal health status: *Actinomyces massiliensis, A. naeslundii* and *A. oris* (A. P. V. Colombo & Tanner, 2019).

Groups of patients in an intermediate inflammatory status, t2d+p- and t2d-p+, showed a prevalence of *Desulfomicrobium orale*, *Treponema sp.* OMZ 838 and *T. lecithinolyticum.* All these strains were described as associated with mild or severe oral disease status: *D. orale* is part of the green complex

(Wirth et al 2019), while treponeme bacteria were related to gingivitis and periodontitis (Chan et al., 2014, 2020; Wyss et al., 1999).

Healthy individuals showed the prevalence of *Veillonella parvula, Rothia dentocariosa, Prevotella enoeca and Selenomonas sputigena. Veillonella parvula* and *R. dentocariosa* are stable components of the oral microbiome, associated to early colonizers complexes, respectively purple and yellow complex (Knapp et al., 2017; Wirth et al., 2021), *P. enoeca* is a component of the human gingival crevice (Moore et al., 1994) while *Selenomonas sputigena* is related to periodontitis orange complex.

Two species were observed in comorbidity and healthy groups: *Fretibacterium fastidiosum* and *Prevotella nigrescens*, both described as related to peri-implant diseases (A. P. V. Colombo & Tanner, 2019).

Three strains poorly described as associated to periodontal disease were detected: *Limolisilactobacillus oris* in t2d+p-, *Scardovia wiggsiae* in t2d+p-, *Neisseria flavescens* in t2d-p-. Few studies described *L. oris*, while *S. wiggsiae* was related to caries pathogens (Kameda et al., 2020). Further *Neisseria flavescens* was never described in the oral microbiome, while it was detected in various other body parts (L. Huang et al., 2014; Shemesh et al., 2019; Solsi et al., 2020).

Statistical analyses indicated as significatively discriminant in p+ group strains associated with periodontitis, precisely attributed to orange and orange-associated complexes like *A. bacterium* oral taxon 439, *C. rectus*, *P. intermedia* and *Neisseria elongata* (S. S. Socransky et al., 1998; Wirth et al., 2021). Moreover, p- individuals were discriminated by the presence of *R. dentocariosa* and *Microcella 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.

5.2 Main functional outcomes

Overall, from the investigation of the two datasets (MEGAN6 and MetaPhlAn 3.0), LEfSe analysis identified 33 and 22 inferred pathways that were significantly abundant in subjects without periodontitis and periodontitis patients, respectively. From the comparison of subjects without and with diabetes, LEfSe report revealed fewer enriched pathways in the two cohorts, namely 7 and 8, respectively.

By categorizing these pathways to higher classes, most of them belong to amino acid biosynthesis (13 pathways), and carbohydrate or energy metabolism (10 pathways) for p- cohort. The pathways for cell structure biosynthesis (9 pathways; 5 in p+ and 4 in t2d+), fatty acid and lipid biosynthesis (3 pathways), nucleoside and nucleotide metabolism (5 for biosynthesis; 1 for degradation) and ferroptotic death and/or iron homeostasis (3 features) were abundant in the p+ in t2d+ affected cohorts.

Notably, most pathways identified both by MEGAN6 and MetaPhlAn 3.0 represented pathways involved in either synthesis of peptidoglycan, amino acid biosynthesis, or carbohydrate metabolism (glycolysis), which may be involved in supply of energy and molecules for biosynthetic processes and bacterial growth. It is important to outline that in addition to peptidoglycans, several other virulence factors were found in the present study.

In the next paragraphs, only the main metabolic factors affecting the specific clinical conditions will be discussed.

Pathways involved in cell structure biosynthesis

The first information that emerges from the functional analysis is how several features related to peptidoglycan (PGN) biosynthesis, in specific, the UDP-N-acetylmuramoyl-pentapeptide biosynthesis I and II pathways along with the peptidoglycan biosynthesis I and III sub-pathways were found to be significantly enriched in p+ patients by both MEGAN6 and MetaPhlAn 3.0. PGN is a vital cell wall component shared by all types of bacteria, while LPS is an essential structural element of the outer membrane present in Gram-negative bacterial cells. MetaPhlAn 3.0 also highlighted three pathways implicated in the biosynthesis of the O-antigen repeating units in lipopolysaccharides (LPS): the GDP mannose derived O-antigen building blocks biosynthesis, the CMP 3 deoxy-D-manno-Octulosonate biosynthesis, and the L-rhamnose degradation pathway, all significantly enriched in t2d+ subjects. It's been long known (Kotani et al., 1975) that these typical bacterial glycoconjugates, LPS representing the active entity of endotoxin, were shown to be major active cell components responsible for the immunestimulative function attributed to bacterial cells.

- *immunostimulative principle of bacterial peptidoglycan (enriched in p+ patients):*

Since the early observations by Freund on *Mycobacterium* cells in 1970s, it is known that the ability to induce the systemic activation of immunological responses in higher animals is common in bacteria (Hasegawa et al., 2006) and also PGN was proposed to be responsible for this immune-

adjuvant activity (Stewart-Tull, 1985). Chemically, PGN has a three-dimensional stable network structure whose composition and structure varies among different bacterial species, but in general consists of two segments of parallel, glycan threads alternating β-1,4-linked N-acetylglucosamine and N-acetyl muramate residues, held together by two small elastic peptide crosslinks. These crosslinks are usually composed of two pentapeptides that present considerable variation among species (Kusumoto et al., 2010).

In the present results two pathways related to the biosynthesis of the UDP-N-acetylmuramoyl-pentapeptide resulted significantly enriched in p+ patients: UDP-N-acetylmuramoyl-pentapeptide biosynthesis I, found in most of the Gram-negative organisms and some Gram-positive organisms, which contains meso-diaminopimelate is the third position; and the UDP-N-acetylmuramoyl-pentapeptide biosynthesis II, found in most of the Gram-positive organisms (phylum Firmicutes), which contains L-lysine in the third position.

Interestingly, in taxonomic analyses some firmicutes resulted significantly more abundant in p+ patients compared with healthy subjects: *Parvimonas micra*, *Eubacterium minutum*, *Dialister pneumosintes*, *Lachnospiraceae bacterium* oral taxon 500, *Peptostreptococcaceae bacterium*, *Eubacterium nodatum*.

- *immunostimulative principle of bacterial lipopolysaccharides (enriched in t2d+ patients):*

LPS consists of three main molecular components: lipid A, core oligosaccharide (core), and O-antigen or O-specific polysaccharide (OPS). While lipid A-core is universally present, not all bacteria produce O-antigen. LPS recognition stimulates strong innate immune responses, ranging from localized inflammation to disseminated sepsis (Opal, 2007). During infection, LPS, and in particular lipid A, is recognized by the Toll-like receptor 4 (TLR4) (G. Zhang et al., 2009)(Hsu et al., 2011) and, specifically in chronic periodontitis, Li et al. (C. Li et al., 2014) discovered that the ability of human periodontal ligament stem cells (hPDLSCs) to differentiate into osteoblasts was impaired by LPS through the nuclear factor kappa b (NF-κB) pathway activated via the TLR-4 signalling pathways (Song et al., 2017).

The biosynthesis of the lipid A molecule is intimately associated to that of the residues in the inner core (Stead et al., 2011) as lipid A is glycosylated with two residues of 3-deoxy-D-manno-oct-2-

ulopyranosonic acid (Kdo) and the inner Kdo serves as the point of attachment for the remaining core.

In the present analyses the superpathway CMP 3-deoxy-D -manno-Octulosonate biosynthesis, together with L-rhamnose metabolism, were significantly enriched in t2d+ patients.

It has been observed that several streptococcal species express a rhamnose cell wall polysaccharides, in which L-Rhamnose is the main building block for rhamnose polysaccharide (RhaPS) that are covalently anchored to peptidoglycan (Mistou et al., 2016; Zorzoli et al., 2019) and results critical or even essential for viability/virulence of a wide range of human pathogens (van der Beek et al., 2019). However, some Gram-negative bacteria when grown in a glucose-rich medium create a bacterial capsule composed of hetero-polymers of d-glucose, l-rhamnose, d-mannose, d-mannuronic acid and acetic acid (Martínez et al., 1995). This structure may contribute to protection from complement-mediated serum killing activity, preventing phagocytosis, facilitating interactions with other bacteria and host tissue (Norman et al., 2015).

A further element that emerges from the data presented in this thesis consists in the observation that in subjects t2d+ the metabolic pathway for GDP mannose derived O-antigen building blocks biosynthesis, resulted significantly enriched pointing out a role for OPS as an additional virulence element in diabetic subjects.

The OPS, which is the most surface-exposed LPS component, also contributes to pathogenicity by protecting invading bacteria from bactericidal host responses (Clay et al., 2008; Murray et al., 2006) as well as modulating macrophage recognition (Saldías et al., 2009), epithelial cell invasion (Duerr et al., 2009; West et al., 2005) and intracellular survival (Paixão et al., 2009). It has been observed in *P. gingivalis* that even very low circulating levels of LPS are sufficient to trigger an inflammatory response via the interaction with TLR2 and TLR4 signalling pathways, leading to low-grade systemic inflammation and resulting in insulin resistance (Blasco-Baque et al., 2016; Mei et al., 2020; Sasaki et al., 2018).

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), *A. actinomycetemcomitans* strains belonging to the serotype *b* are frequently isolated from subjects with severe periodontitis, while serotypes *a* and *c* are mostly isolated from milder periodontitis-affected patients and healthy individuals. The OPS from *A.*

actinomycetemcomitans serotype *b* is structurally distinct from the OPS from the other serotypes, being composed of a disaccharide backbone of α-D-fucose (D-Fuc) and α-L-rhamnose (L-Rha), linked by a non-reducing Kdo residue.

The serotype b in this bacteria, linked to severe periodontitis, affects both the maturation of APCs, the Th1, Th17, and Th22-pattern of the periodontal immune response, and the alveolar bone resorption during experimental periodontitis, playing a significant role in the virulence and immunogenicity of this highly virulent periodontal bacteria (Raja et al., 2014).

This metabolic aspect is one of the mechanisms linking diabetes to periodontitis. In fact, in diabetes the proinflammatory environment created by macrophages and inflammatory cytokines favours differentiation of naive CD4+ T cells in proinflammatory Th17 rather than in regulatory Treg (S. Zhang et al., 2021). The increases of the Th17/Treg lymphocyte ratio, raises the inflammatory cytokines levels (IL1, IL6,IL17) and fuelling inflammation as IL-17 plays a key role in periodontitis by both changing periodontal microbiota pathogenicity and driving periodontal bone loss (N. Huang et al., 2021).

Pathways involved in amino acid biosynthesis (enriched in p- subjects)

The second important finding emerging from the functional analysis is related to amino acid biosynthesis. Both MEGAN6 and MetaPhlAn 3.0 highlighted several pathways involved in amino acid biosynthesis significantly enriched in p- subjects: pathways of branched amino acids biosynthesis, which leads to the production of L-leucine, L-Valine, and L-isoleucine; pathways associated with the metabolisms of other amino acids: phenylalanine, lysine, threonine, methionine and arginine all essential amino acids; tyrosine, alanine, aspartate and glutamate not essentials elements.

- *pathways of branched amino acids biosynthesis*

The branched-chain amino acids (BCAAs [Ile, Leu, and Val]) are synthesized through a conserved pathway in Gram-negative and Gram-positive bacteria, where the level of synthesis is dependent on the availability of metabolites linked to central metabolism, including pyruvate, acetyl coenzyme A (acetyl-CoA), and oxaloacetate (Kaiser & Heinrichs, 2018).

BCAAs represent important nutrients in bacterial physiology, with roles that range from supporting protein synthesis to signalling and fine-tuning the adaptation to amino acid starvation. In some pathogenic bacteria, the adaptation to amino acid starvation includes induction of virulence gene

expression: thus, BCAAs support not only proliferation during infection, but also the evasion of host defences (Kaiser & Heinrichs, 2018).

The BCAAs are effectors of the global transcriptional regulators leucine-responsive regulatory protein (Lrp) in Gram-negative bacteria and CodY in Gram-positive bacteria (Sonenshein, 2005; Tani et al., 2002).

These transcriptional regulators are positioned at the intersection of metabolism and pathogenesis, and thus are able to coordinate not only the response to nutrient availability but also to regulate important virulence genes (Kaiser & Heinrichs, 2018).

A strong association of obesity and insulin resistance with increased circulating levels of branched-chain and aromatic amino acids and decreased glycine levels has been recognized in human subjects. Recently, essential branched-chain amino acids (BCAA) have been proposed as a link between *P. gingivalis*-induced periodontitis and insulin resistance. *P. gingivalis* can produce BCAA and a recent study has shown that worsening of HFD-induced insulin resistance by *P. gingivalis*-induced periodontitis was paralleled by an increase in BCAA plasma levels. Furthermore, a mutant form of *P. gingivalis*, unable to produce BCAA caused periodontitis, but did not induce insulin resistance (Tian et al., 2020).

Our data show that p+ patients present reduced enrichment of BCAAs metabolic pathways and this could be related to the fact that plasma concentrations of BCAAs are frequently elevated in obesity and type 2 diabetes (Kuzuya et al., 2008; She et al., 2007) and, since in our cohort of periodontitis subjects there are also subjects with t2d+ as comorbidity the presence of high level of circulating BCAA might acts with an inhibitory effect on the BCAAs metabolic pathways.

- *other amino acids related pathways*

Nine further pathways involved in amino acid metabolism (5 essential and 4 not essential amino acids) had significantly different signal intensities in the present cohorts. All these pathways showed lower relative abundance in periodontitis patients, indicating that subgingival community in p+ subjects might utilize some ammonia absorbed directly for physiological activities instead of biosynthesizing themselves (Y. Li et al., 2014). Periodontal microbes and host-derived proteases could degrade host periodontal proteins and proteins involved in the inflammatory and immune reactions into peptides and amino acids that the microorganisms can absorb and use as nutritional resources (Eley & Cox, 2003; Sandholm, 1986). Studies have shown elevated levels of peptides and

amino acids in periodontal population (Barnes et al., 2011; Rai et al., 2008). The increased peptides and amino acids would supply as a richer energy pool for the expansion of some specific organisms relying on oligopeptides as carbon and energy sources (Takahashi & Sato, 2002) that ultimately influences the microbial and functional structure.

- *Chorismate biosynthesis pathways (enriched in p+ subjects)*

The only pathways related with amino acid biosynthesis that resulted significantly enriched in p+ patients were two pathways associated to chorismate biosynthesis. Chorismate is an intermediate in the synthesis of the three aromatic amino acids: L-phenylalanine, L-tyrosine and L-tryptophan and its biosynthesis occurs via the shikimic acid pathway. Considered restricted to bacteria, fungi, yeasts, algae, plants and certain apicomplexan parasites, the lack of a shikimic acid pathway in metazoans, including humans, is evinced by their dietary requirements for aromatic compounds (Ratledge, 1982). Remarkable is the role that chorismate synthase plays in the biosynthesis of nucleotides, via the enzyme anthranilate phosphoribosyl synthase (APR synthase) that is a member of the phosphoribosyl transferase (PRT) involved in nucleotide biosynthesis and salvage apart from amino acid biosynthesis (Sinha & Smith, 2001). These amino acids serve as substrates in other pathways for secondary metabolites such as alkaloids, flavonoids, lignin, coumarin, indole derivatives and other phenolic compounds (Tzin et al., 2012).

Interestingly, in present data these chorismate pathways were the only features belonging to amino acid metabolism that resulted significantly enriched in periodontitis patients as well as were significantly enriched in p+ subjects all pathways related with nucleotide biosynthesis

Pathways involved in nucleoside/nucleotide metabolism (enriched in p+ subjects)

MetaPhlAn 3.0 analysis revealed a significant enrichment of the inosine monophosphate (IMP, a purine precursor) and adenosine-nucleotide biosynthesis pathways in p+ subjects. Again for periodontitis subjects, the analysis showed an altered pyrimidine metabolism with a reduction in the degradation pathway accompanied by an enrichment of the salvage pathway. As a last, pyrimidine metabolism also resulted enriched for the biosynthesis of Uridine 5'-monophosphate (UMP, the pyrimidine precursor) in t2d+ patients.

Cells produce ribonucleotides and deoxyribonucleotides via two pathways: the *de novo* synthesis, taking amino acids as substrates, and the "salvage" pathway by uptake of the circulating

purine/pyrimidines in the bloodstream. The relative contributions of the *de novo* and salvage pathways to nucleotide pool maintenance vary in different cells and tissues. Generally, the salvage pathway is the main deoxyribonucleotides sources for resting or fully differentiated cells, while the *de novo* pathway is necessary for high-proliferating cells to meet the improved requirement of purine/pyrimidines.

- *purines biosynthesis pathway*

IMP is the intracellular precursor for both adenosine and guanosine monophosphate but it can also serve as an extracellular signalling molecule, and can regulate diverse processes among which neutrophil function and inflammation. Recently, Lovászi and colleague (Lovászi et al., 2021) demonstrate that IMP suppressed tumor necrosis factor (TNF)-α production and augmented IL-10 production in endotoxemic mice. IMP exerted its effects through inosine metabolism, as IMP is degraded to inosine in the extracellular space by CD73 activity in lipopolysaccharide-activated macrophages.

Endotoxemia was associated with inflammation in important sites of insulin resistance, such as the liver and the adipose tissue (Sasaki et al., 2018). Repeated oral administration of *P. gingivalis* induced endotoxemia and both glucose intolerance and insulin resistance in high fat diet (HFD)-fed mice (Arimatsu et al., 2014; Blasco-Baque et al., 2016). In both liver and adipose tissues, there was overexpression of proinflammatory genes among which those encoding for TNF-alpha and IL-6, and a downregulation of genes that enhance insulin sensitivity.

An additional finding that might reinforce the hypothesis of a link between nucleotides metabolism and glucose uptake was presented by McCarthy and colleague (McCarthy et al., 2018). They showed that glucose metabolism leading to the generation of high-energy purine nucleotides, a process at the core of the Warburg effect, induces cell-surface expression of major histocompatibility class I chain-related protein A (MICA). However, raised glucose levels in diabetes have a limited effect on intracellular glucose concentration because of the lack of insulin, which is required to promote glucose uptake into cells. Thus, the extracellular glucose will only influence MICA levels in cells that can take up and metabolize the glucose through the purine synthesis pathway. Notably, CMV infection, which is associated with natural killer group 2D (NKG2D) ligand induction, is known to both up-regulate the glucose transporter (Yu et al., 2011) and induce Warburg metabolism (Landini, 1984).

In the present analysis, up-regulation of the purine biosynthesis pathway in p+ patients, together with the other considerations proposed for the metabolic pathways already discussed above, could point in the direction of a role of endotoxemia induced by the oral-gut axis. Recent metagenomic and proteomic studies showed that oral administration of *P. gingivalis* changed gut microbiota composition with an increase in Bacteroidales and a decrease in Firmicutes, thus suggesting that periodontitis-induced gut dysbiosis may cause endotoxemia, leading then to systemic inflammation and insulin resistance (Yamazaki et al., 2021). Prolonged hyperactivation of the innate immune system induced by trained immunity may provide explanation for the association between periodontitis and diabetes. Regardless of whether precursors and circulating innate immune cells are first affected by either periodontitis-induced or diabetes-induced inflammation, trained immunity can have a deleterious effect on both conditions and may provide a rationale for their bidirectional relationship (Hajishengallis & Chavakis, 2021).

- *pyrimidines biosynthesis pathway*

Pyrimidine is a basic and essential substrate not only for nucleic acids but also for a broad spectrum of key molecules such as phospholipid, glucose metabolism, and protein glycosylation. Furthermore, pyrimidine metabolism incorporates all enzymes involved in the synthesis, degradation, salvage, interconversion and transport of these related molecules (Lecca & Ceruti, 2008; Löffler et al., 2015).

The human intestinal mucosa contains all of the degradative enzymes necessary to convert dietary purines to uric acid before absorption. By contrast, dietary pyrimidines are readily absorbed and the liver contains all of the degradative enzymes for pyrimidine breakdown. Dietary purine and pyrimidine nucleotides are also partially degraded by gut bacteria to CO2.

To ensure their own survival, many pathogens redirect the metabolism of the cells they invade. Salvaging nucleotides requires substantially less energy than synthesizing them, so the balance between salvage and degradation is important both for optimizing the energy economy of bacteria and for maintaining levels of key elements. In the present analysis periodontitis patients showed a down regulation of the pyrimidine degradation pathway flanked by an enhancement of the salvage pathway.

The changes in the balance of salvage and degradation of pyrimidines were proposed to constitute a metabolic switch that eventually commits the cell to programmed cell death (Stasolla et al., 2004). The presence, shown here, of an imbalance in purine and pyrimidine metabolism in patients with periodontitis could be related to the enhanced ferroptosis pathway (discussed in the next

paragraphs) observed in the same subjects, and both mechanisms could be play a role in the inflammatory basis of the disease.

An additional upregulation in the pyrimidine metabolic pathway was observed in t2d+ patients and concern the UMP biosynthesis. Uridine 5'-monophosphate (UMP) is the first product of *de novo* pyrimidine pathway and its pivotal role in the tissue- and species specificity became evident when the disorder of pyrimidine metabolism was diagnosed in several diseases (Smolenski et al., 1993; Webster, 2001). Also in the present study, the level of UMP biosynthesis was significantly enriched in t2d+ patients highlighting once more a possible pathogenic role of this metabolic pathway.

Other functional pathways involved in periodontitis or type 2 diabetes (enriched in p+ subjects)

- *fatty acid biosynthesis pathways (enriched in p+ patients)*

MEGAN6 analysis shows a significant increase in fatty acid biosynthesis and adipocytokine signalling pathway in p+.

The adipocytokine signalling pathway refers to the sum of all proteins and factors responsible for the regulation of adipose tissue metabolism. Fat cells are an active endocrine and paracrine organ that secretes a variety of cytokines and biologically active substances that not only regulate energy balance in the body, but also participate in several processes such as inflammation, coagulation, fibrinolysis, insulin resistance, diabetes and atherosclerosis. Fat cells secrete dozens of cytokines, mainly include adiponectin APN, leptin, IL-6, TNF-α and resistin that regulate lipid metabolism.

In the previous section on the role of purine metabolism in determining endotoxemia it has been already discussed the link between periodontitis and diabetes via cytokine among which adipocytokines, molecules secreted by the adipose tissue that plays an important regulatory role in the energy metabolism of cell glucose, sugar and fatty acids, and participates in the regulation of cell proliferation and immune function.

- *Gamma-glutamyl cycle pathway (enriched in t2d+ patients)*

In the presents analyses emerged a significant enrichment of gamma glutamyl-cycle in t2d+ patients. Gamma-glutamyl transpeptidase (GGT) enzyme is ubiquitously present in all life forms and plays a variety of roles in diverse organisms. Higher eukaryotes mainly utilize GGT for glutathione degradation, and mammalian GGTs have implications in many physiological disorders also. GGTs

from unicellular prokaryotes serve different physiological functions in Gram-positive and Gram-negative bacteria (Saini et al., 2021).

In some Gram-negative pathogenic bacteria such as *Helicobacter pylori, Fransicella tularensis, Neisseria meningitidis,* and *Campylobacter jejuni*, the expression of GGT has been linked to their pathophysiology, and GGT has been ascribed as a formidable virulence factor (Ling et al., 2013).

Recently, the role of *Bacillus subtilis* GGT (BsGGT) as a novel virulence factor in the pathogenesis of bone resorption similar to mammalian GGT (Niida et al., 2004) has been demonstrated in a cell culture study (Kim et al., 2016). It was reported that the large subunit of BsGGT enhanced osteoclastogenesis activity via the upregulation of an osteoclast differentiation factor: the receptor activator of nuclear factor kappa-B ligand (RANKL), which interacted with surface receptors of precursor osteoblast cells and promoted the formation of osteoclast cells. Based on these findings, GGT has been hypothesized to act as a virulence factor in bone destruction, caused by periodontopathic bacteria (Kim et al., 2016).

Moreover, GGT has also been related to cardio-metabolic diseases such as obesity, hypertension and type 2 diabetes mellitus and elevated GGT levels predict the development of metabolic syndrome, cardio vascular disease events and mortality (Choi et al., 2018; Ruttmann et al., 2005).

In 2016, Jiang et al. (Jiang et al., 2016) using an immune-histochemical analysis revealed the presence of GGT-positive cells throughout the mandibular tissues, including the periodontal ligament (PDL) and pulp. The amount of GGT in PDL tissues was greater than that in the pulp, whereas no staining was observed in the alveolar bone. In addition, they identified a novel and selective inhibitor of GGT, named GGsTOP, that has a key role in glutathione homeostasis and in the maintenance of cellular reactive oxygen species (ROS). In addition, the inhibition of GGT activity by GGsTOP may affect intracellular ROS levels, and thereby influence the remodelling and renewal of human periodontal ligament cells to induce the fiber synthesis necessary to repair PDL tissues.

The enhancement of the GGT metabolism observed in the present data in t2d+ patients represents a further indication that diabetes might augments oral tissues degradation by increasing release of reactive oxygen species (ROS) by neutrophils and fibroblasts.

- *Ferropoptosis and outer membrane receptor for iron elements (enriched in p+ patients)*

In present analyses, some interesting pathways involved ferroptosis were seen enriched in the p+ subjects. Ferroptosis, which is characterized by iron-dependent lethal lipid peroxidation, has been

found to participate in the development of several diseases inflammatory based, such as atherosclerosis, stroke, intracerebral haemorrhage and ischemia/reperfusion injury (Abdalkader et al., 2018; Yang et al., 2014).

The term ferroptosis was coined in 2012 by Dixon et al. to describe the form of cell death induced by the small molecule erastin (Dixon et al., 2012) that inhibit cystine-glutamate antiporter system, blocking transportation of cystine into cytoplasm. This unique cell death is characterized by iron-dependent reactive oxygen species (ROS) and oxidized lipid contents in the cell membrane. This death pathway has been observed in *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* for which it was detected that ferroptosis may disrupt the integrity of tissue barrier and facilitate invasion of microbials (Shenid & Naqviid, 2021).

The metal iron is crucial for the development of ferroptosis, because iron may generate ROS by Fenton reaction in both physiological and pathological conditions (Mao et al., 2020). As part of the host immune response, iron is kept unavailable to bacteria by being sequestered by catecholamines, however some bacteria have evolved to subvert this mechanism by producing iron scavenging molecules, known as siderophores (S. C. 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, a periplasmic binding protein, and a complex of one or two cytoplasmic membrane proteins with an associated ATP-binding cassette, which together form an ABC transporter (Luscher et al., 2018). Siderophore uptake across the outer membrane is dependent upon the presence of a complex of three cytoplasmic 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 seems to be the siderophores TonB-dependent.

6. Conclusions

To understand how bacteria cause disease, it is important not only to know how they colonize the host, which partners they recruit to enable host-microbiota interactions, how they evade the immune system or create an environment of immune tolerance, but also when, why, and how they transform into a pathogenic dysbiotic form. Both human gut and oral cavity are dynamic and complex ecosystems harbouring an enormous number and variety of microorganisms including more than 1,000 species (Lamont et al., 2018; Neish, 2009) representing the largest microbiota reservoirs in the human body.

Periodontitis is associated epidemiologically with several non-communicable chronic diseases, including obesity, metabolic syndrome, diabetes and cardiovascular diseases (Charupinijkul et al., 2021; Jepsen et al., 2020; Saini et al., 2021; Sanz, Marco del Castillo, et al., 2020). A recent study has proven that transmission and subsequent colonization of the gut by oral bacteria is a common event (Schmidt et al., 2019) and the hypothesis of a potential causal link between the two diseases has been intensively investigated. Observational studies provided convincing evidence of a bidirectional relationship between periodontitis and diabetes data also reinforced by recent studies in experimental animals that identified mechanisms whereby periodontitis and diabetes can adversely affect each other (Gu et al., 2021; Sun et al., 2020).

Traditionally, studies on pathogenesis of periodontal microbiota involve a 'reductionist' approach to studying complex subgingival microbial communities by analysing individual bacterial species. As a result, a small group of predominantly Gram negative anaerobic bacteria, notably *P. gingivalis, T. forsythia and T. denticola*, have long been regarded as the main putative pathogens of periodontitis (Fischer et al., 2013; Seckin Ertugrul et al., 2013). However, more recent studies have shown that periodontitis may be a poly-microbial infectious disease where each individual within the community, even members in low abundance, might be involved in the disease occurrence and progression (Wirth et al., 2021).

A great advantage to define the complex regulatory networks that contribute to these diseases has emerged with the development of metagenomics and, more recently, metatranscriptomics through which the composition and structure of microbial communities as well as the overall functional characteristics of the flora can be fully profiled and revealed (N. Huang et al., 2021). Technological improvement of whole metagenome sequencing lead to a better understanding of the human

microbe, providing information applicable to public health. However, processing and analyse data from WGS is still challenging. There are two major approaches in pathogen detection with metagenomic analysis: the first approach, 16S rRNA metagenomic analysis, based on the analysis of specific gene regions “amplicons” has long been standard for microbial community sequencing, but it introduces bias and omits organisms and functional elements from analysis; the second approach relies on a whole metagenome shotgun (WMS) sequencing, which allows it to provide a full analysis of a metagenome, including genes, and pathways it contains. WMS increases significantly the specificity of pathogen detection, however the data processing is much more complicated.

Classification of WMS data relies on several strategies, including alignment (to all sequences or taxonomically unique markers), composition (k-mer analysis), phylogenetics (using models of sequence evolution), assembly, or a combination of these methods. Analysis tools focusing on estimation of abundance tend to use marker genes, which decreases the number of reads classified but increases speed (Lindgreen et al., 2016).

Two of the most widely used approaches in WMS studies, built on marker genes search, are: (I) MEGAN, based on BLAST (for taxonomy) or DIAMOND (for functional analysis) that search of shotgun reads against the NCBI nt or nr databases; (II) the bioBakery platform, that use MetaPhlAn 3.0, for taxonomic profiling, and HUMAnN 3.0, for functional profiling, mapping reads on internal database.

The work presented in this thesis showed the use of both approaches to describe the taxonomical composition and the functional activity of subgingival microbiome in patients with different health status regarding both periodontitis and type 2 diabetes.

A remarkably result was the concordance between the two methodologies, mainly in taxonomical analysis than functional investigation.

6.1 Main taxonomic outcomes

The taxonomical profiles reconstruction showed that: I) it was possible to describe a core microbiome, that is resilient to changes in the oral health status, composed mainly by early colonizers but also showing the presence of species like *T. forsythia* commonly considered periodontal pathogens; II) bacteria historically associated with onset and progression of periodontitis, *C. rectus, P. gingivalis* and *P. endodontalis* were always presents among the most

abundant species but seems more linked to a general inflammatory status, rather than specifically with periodontal disease; III) periodontitis patients were significantly characterised by the presence of bacteria associated to the red/orange complex: *T. denticola, P. intermedia, P. dentalis and A. bacterium* oral taxon 439, the latter being the unique bacterium that is always significant in statistical analysis; IV) the microbiome of subjects affected by type 2 diabetes, was mainly characterized by *P. denticola* and *P. oris*. *P. denticola* was historically associated with periodontal disease as part of the orange complex, while *P. oris* is controversially associated with periodontal health status (A. P. V. Colombo & Tanner, 2019), or to oral disease (Lenartova et al., 2021; Riggio & Lennon, 2007); V) *R. dentocariosa* and *M. alkaliphila* discriminated health oral microbiome.

Notably, three strain poorly described as associated to periodontal disease were detected: *Limolisilactobacillus oris* in t2d+p-, *Scardovia wiggsiae* in t2d+p-, *Neisseria flavescens* in t2d-p-. Few studies described *L.oris, S.wiggsiae* was related to caries pathogens (Kameda et al., 2020), while *N. flavescens* was never described in the oral microbiome, while it was detected in various body district (L. Huang et al., 2014; Shemesh et al., 2019; Solsi et al., 2020).

6.2 Main functional outcomes

Functional activity investigations lead to the detection of: I) an enrichment in amino acid biosynthesis (13 pathways), carbohydrate and energy metabolism (8 pathways) in non-periodontitis subjects, II) a significantly abundance in cell structure biosynthesis (9 pathways), fatty acid and lipid biosynthesis (2 pathways), nucleoside and nucleotide metabolism (8 pathways) and ferroptotic death and/or iron homeostasis (1 pathway) in the patients affected by periodontitis, diabetes or both diseases.

Overall, pathways associated with amino acid biosynthesis, carbohydrate and energy metabolism were found to be primarily enriched in healthy subjects. This may simply reflect a high level of metabolic activity of the bacterial biofilm, but with a substantial preservation of tissue homeostasis. Periodontal tissue homeostasis could be likened to an ‘armed peace’ between the host and the periodontal microbiota, with occasional microbial attacks that are readily subdued by immune defences.

The transition to periodontitis requires both a dysbiotic microbiota and a susceptible host which engage into a complex inflammatory dialog. The data presented here account for the association of specific pathological clinical conditions with metabolic activity of the subgingival microbiomes.

The first, expected, result is represented by the significantly different abundance of several features related to peptidoglycan and lipopolysaccharides biosynthesis. These products and cytokines present in the periodontium can enter into the bloodstream and trigger a systemic inflammatory response in the host. Moreover, periodontal cells damaged by the periodontal microbiota directly or by the host immune system indirectly, cause a nucleic acid releasing.

In the present analysis patients affected by periodontitis or diabetes had higher level of pyrimidine salvage pathway, showing that this could be a preferred route for the uptake of nucleosides/nucleobases, an important nutrient source for bacteria used not only for nucleic acid biosynthesis but also to be catabolized as carbon and energy sources.

It was also observed that balance of salvage and degradation of pyrimidines, as it was observed in the present study, was proposed to constitute a metabolic switch that eventually commits the cell to programmed cell death.

Notably, a further interesting observation emerging from the functional analysis was the increased abundance of features responsible for production of fatty acids and ferroptosis in periodontitis patients. Fatty acids can disrupt host defence systems by different mechanisms, such as induction of apoptosis in immune cells and also ferroptosis represents a cell death pathway characterized by iron-dependent reactive oxygen species.

Dysbiotic microbial communities exhibit synergistic interactions for enhanced colonization, nutrient procurement, and persistence in an inflammatory environment that promotes their adaptive fitness. From a microbial standpoint, the importance of inflammation lies in its providing a source of essential nutrients, although it can cause collateral damage to the periodontal tissues.

In the last decades the interactions between periodontitis and type 2 diabetes have been gradually better understood, with new scenarios being proposed on the mechanisms of the bidirectional relationship between these two diseases, but still much remains to clarify (Barutta et al., 2022). The study presented in this thesis represents a further investigation into the composition and activity of the subgingival microbiome, allowing a better exploration of features, both functional or taxonomical, enhanced or reduced in patients that might led to the identification of novel potential therapeutic targets.

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Supplementary materials

Supplementary information of Chapter 2

Supplementary table 1. Bacterial species, in MEGAN6 output, with relative abundance ≥ 0.5% in patients with periodontitis (n = 6) and individuals without periodontitis (n = 6).

Species	p+: mean rel. freq. (%)	p-: mean rel. freq. (%)
Porphyromonas gingivalis ATCC 33277	14.52589167	7.462613773
Treponema denticola	8.261467952	2.140459959
Actinomyces sp. oral taxon 414	7.949339527	4.671417814
Campylobacter rectus	5.374611044	1.853839594
Corynebacterium matruchotii	4.386908669	5.138342293
Desulfobulbus oralis	3.604432138	3.586735921
Treponema sp. OMZ 804	3.213520432	1.966184915
Fretibacterium fastidiosum	2.955027949	3.735419617
Olsenella sp. oral taxon 807	2.888385214	1.602704424
Prevotella intermedia ATCC 25611 = DSM 20706	2.607961713	0.437129711
Prevotella denticola	2.594708285	4.886518116
Anaerolineaceae bacterium oral taxon 439	2.473688593	0.045126622
Selenomonas sputigena	2.378476951	2.713577743
Desulfomicrobium orale	2.254855772	3.442352085
Prevotella dentalis	2.007438799	0.746409135
Tannerella sp. oral taxon HOT-286	1.81120404	1.221891583
Prevotella oris	1.660893064	2.198660049
Campylobacter gracilis	1.539712065	1.333018133
Campylobacter showae	1.202017038	0.419636218
Lautropia mirabilis	1.181288399	1.039817089
Prevotella intermedia 17	1.168483028	0.227844475
Actinomyces sp. oral taxon 897	1.166600112	0.759184057
Porphyromonas gingivalis W83	1.155841576	0.3311795
Porphyromonas gingivalis AJW4	1.101571406	0.925174755
Lachnospiraceae bacterium oral taxon 500	1.045467966	0.192453129
Selenomonas sp. oral taxon 126	0.837665691	0.341970268
Porphyromonas gingivalis A7A1-28	0.83498141	0.382517234
Filifactor alocis	0.823780364	0.376621574
Treponema sp. OMZ 838	0.79194738	0.399070166
Rothia dentocariosa	0.773297958	14.03106027
Prevotella enoeca	0.761200196	1.77997514
Prevotella melaninogenica	0.678166435	0.852284524
Pseudopropionibacterium propionicum	0.64649796	0.522557288
Tannerella forsythia 92A2	0.619523305	0.283305667
Porphyromonas gingivalis TDC60	0.616392304	0.292808572
Dialister pneumosintes	0.600242761	0.056242053
Neisseria elongata	0.597741235	0.032886525
Ottowia sp. oral taxon 894	0.575093941	0
Tannerella forsythia 3313	0.565520386	0.21594776

Parvimonas micra	0.539329936	0.129490492
s__Bacteroides heparinolyticus	0.493821097	0
s__Neisseria mucosa	0.478646818	0.590972437
s__Rothia aeria	0.462880332	1.712885448
s__Tannerella forsythia KS16	0.455901267	0.175945675
s__Aggregatibacter segnis	0.420763942	0.114974205
s__Veillonella parvula	0.366978656	2.092648196
s__Eikenella corrodens	0.347088028	0.16295236
s__Prevotella jejuni	0.340794674	0.124216078
s__Candidatus Saccharibacteria bacterium oral taxon 488	0.33873105	0.239377357
s__Treponema putidum	0.310193651	0.139347465
s__Fusobacterium nucleatum subsp. Nucleatum	0.304952575	0.037390086
s__Pauljensenia hongkongensis	0.295632932	0.564201943
s__Capnocytophaga gingivalis	0.281195061	0.192698509
s__Cardiobacterium hominis	0.279185212	0.190541005
s__Actinomyces sp. oral taxon 171	0.27221075	2.422057821
s__Neisseria subflava	0.228878799	0.450095515
s__Selenomonas sp. oral taxon 920	0.226966693	0.223656856
s__Actinomyces oris	0.201918768	0.893054201
s__Fusobacterium nucleatum subsp. Vincentii	0.183909097	0.203061978
s__Fusobacterium nucleatum subsp. Animalis	0.182094837	0.076879179
s__Actinomyces sp. oral taxon 848	0.180723836	0.032945606
s__Actinomyces naeslundii	0.179551833	0.670678685
s__Neisseria sp. oral taxon 014	0.148267529	0.472459086
s__uncultured bacterium	0.140491563	0.199934828
s__Actinomyces viscosus	0.132647646	0.80481272
s__Eubacterium minutum atcc 700079	0.124433649	0
s__Rothia mucilaginosa	0.116910624	0.518119763
s__Aggregatibacter actinomycetemcomitans D7S-1	0.116345508	0
s__Schaalia cardiffensis	0.116245741	0.044065471
s__Capnocytophaga sputigena	0.115458725	0.058305969
s__Candidatus Saccharibacteria bacterium oral taxon 957	0.104515888	0.022238094
s__Aggregatibacter actinomycetemcomitans D11S-1	0.090242768	0
s__Veillonella atypica	0.083078429	0
s__Prevotella scopos	0.082043738	0.058299695
s__Aggregatibacter aphrophilus ATCC 33389	0.076040035	0
s__Actinomyces slackii	0.068611695	0.04261059
s__Campylobacter curvus	0.062804973	0.059377693
s__Schaalia meyeri	0.062462502	0.023694453
s__Capnocytophaga endodontalis	0.057847195	0
s__Prevotella fusca	0.054219363	0.016565622
s__Neisseria sp. KEM232	0.052193835	0.038803381
s__Pseudomonas sp. S-6-2	0.048549735	0.341826865

s__Streptococcus anginosus	0.046445907	0.057305657
s__Schaalia odontolytica	0.045238551	0.491441878
s__Streptococcus cristatus	0.037195549	0.04973452
s__Streptococcus sanguinis	0.032836204	0.786203846
s__Olsenella uli	0.031936736	0.887291795
s__Microcella alkaliphila	0.031507574	0.92074869
s__Haemophilus parainfluenzae	0.02771996	0.177361013
s__Ketogulonicigenium vulgare	0.027108673	0
s__Streptococcus milleri	0.026773029	0
s__Pseudomonas aeruginosa	0.026679553	0.040993096
s__Phocaeicola dorei	0.026590104	0
s__Xanthomonas campestris pv campestris pv. str. CN03	0.024837697	0
s__Eikenella exigua	0.023785072	0
s__Leptotrichia buccalis	0.021965546	0
s__Capnocytophaga sp. FDAARGOS_737	0.020953518	0
s__Capnocytophaga leadbetteri	0.02082767	0
s__Aggregatibacter aphrophilus NJ8700	0.018479975	0
s__Treponema lecithinolyticum	0.018167765	0
s__Streptococcus gordonii	0.01755565	0.12161928
s__Xanthomonas campestris campestris pv. str. CN16	0.01578955	0
s__Acinetobacter baumannii	0	0.119217115
s__Acinetobacter johnsonii	0	0.019921438
s__Actinomyces radicidentis	0	0.017106533
s__Asticcacaulis excentricus	0	0.021123938
s__Bifidobacterium dentium	0	0.794727972
s__Bifidobacterium scardovii	0	0.033274293
s__Bradyrhizobium sp. BTAi1	0	1.08082387
s__Bradyrhizobium sp. ORS 285	0	0.022687018
s__Campylobacter concisus	0	0.02834353
s__Cryptobacterium curtum	0	0.048816206
s__Cutibacterium acnes	0	0.032752754
s__Enterococcus casseliflavus	0	0.043771046
s__Enterococcus gallinarum	0	0.024611192
s__Enterococcus sp. CR-Ec1	0	0.032347282
s__Ereboglobus luteus	0	0.02706796
s__Fusobacterium sp. oral taxon 203	0	0.09058278
s__Gemella haemolysans	0	0.102261349
s__Gemella morbillorum	0	0.053229614
s__Geosporobacter ferrireducens	0	0.016193683
s__Glycocaulis alkaliphilus	0	0.031731517
s__Halomonas hydrothermalis	0	0.024851691
s__Halomonas sp. BC-M4-5	0	0.063065815
s__Lactobacillus vaginalis	0	0.773131924
s__Lacunisphaera limnophila	0	0.057265123
s__Lancefieldella parvula	0	0.157922512

s__Leptotrichia sp. oral taxon 212	0	0.015963236
s__Ligilactobacillus salivarius	0	0.14629216
s__Limosilactobacillus fermentum	0	0.50182645
s__Limosilactobacillus oris	0	1.250237264
s__Limosilactobacillus reuteri	0	0.044070188
s__Meiothermus silvanus	0	0.038760624
s__Mesorhizobium terrae	0	0.296598842
s__Methylobacterium brachiatum	0	0.053425269
s__Methylobacterium radiotolerans	0	0.016233767
s__Methylorubrum populi	0	0.035915662
s__Neisseria cinerea	0	0.02586496
s__Neisseria flavescens	0	0.03480663
s__Neisseria gonorrhoeae	0	0.022570662
s__Neisseria meningitidis	0	0.083474426
s__Nibricoccus aquaticus	0	0.15869067
s__Nitrincola sp. KXZD1103	0	0.077222069
s__Opitutaceae bacterium TAV5	0	0.174828639
s__Opitutus sp. GAS368	0	0.035673382
s__Opitutus terrae	0	0.019766392
s__Pannonibacter phragmitetus	0	0.025242332
s__Parascardovia denticolens	0	0.083915894
s__Prevotella nigrescens	0	0.015776458
s__Prevotella sp. oral taxon 299	0	0.047956521
s__Proteiniphilum saccharofermentans	0	0.066526137
s__Pseudomonas litoralis	0	0.019922855
s__Pseudomonas pelagia	0	0.027850272
s__Pseudomonas putida group	0	0.022895637
s__Pseudomonas stutzeri	0	0.016950071
s__Ralstonia insidiosa	0	0.110012579
s__Rheinheimera sp. D18	0	0.041726795
s__Scardovia inopinata	0	0.241108244
s__Selenomonas sp. oral taxon 136	0	0.055872695
s__Selenomonas sp. oral taxon 478	0	0.041073003
s__Shewanella sp. W3-18-1	0	0.035353534
s__Streptococcus australis	0	0.023775433
s__Streptococcus intermedius	0	0.043414668
s__Streptococcus mitis	0	0.01908184
s__Streptococcus mutans	0	0.086554967
s__Streptococcus oralis ATCC 35037	0	0.017293505
s__Streptococcus oralis subsp. dentisani	0	0.041809371
s__Streptococcus oralis subsp. Tigurinus	0	0.050857717
s__Streptococcus parasanguinis	0	0.023929775
s__Streptococcus pneumoniae	0	0.051814993
s__Streptococcus salivarius	0	0.023609107
s__Streptococcus sobrinus	0	0.862836881
s__Streptococcus sp. LPB0220	0	0.033670034

s__Streptococcus sp. NPS 308	0	0.02937888
s__Veillonella dispar	0	0.047130449
s__Verrucomicrobia bacterium IMCC26134	0	0.703143115
unclassified Microbacterium	0	0.021643939

Supplementary table 2. Bacterial species, in MEGAN6 output, with relative abundance ≥ 0.5% in patients with type 2 diabetes (n = 6) and individuals without type 2 diabetes (n = 6).

Species	**t2d+: mean rel. freq. (%)**	**t2d-: mean rel. freq. (%)**
Porphyromonas gingivalis ATCC 33277	15.3143	6.674207
Actinomyces sp. oral taxon 414	6.346793	6.273964
Prevotella denticola	5.8453	1.635927
Treponema denticola	5.780629	4.621299
Desulfobulbus oralis	4.622504	2.568664
Corynebacterium matruchotii	4.140381	5.38487
Fretibacterium fastidiosum	3.536564	3.153884
Desulfomicrobium orale	3.442352	2.254856
Campylobacter rectus	3.089454	4.138996
Selenomonas sputigena	2.496573	2.595481
Prevotella oris	2.112641	1.746913
Prevotella dentalis	1.890675	0.863173
Olsenella sp. oral taxon 807	1.749226	2.741863
Anaerolineaceae bacterium oral taxon 439	1.538695	0.98012
Lautropia mirabilis	1.473031	0.748074
Prevotella intermedia ATCC 25611 = DSM 20706	1.443157	1.601934
Porphyromonas gingivalis AJW4	1.442872	0.583874
Rothia dentocariosa	1.436315	13.36804
Rothia aeria	1.347668	0.828098
Treponema sp. OMZ 804	1.257398	3.922307
Limosilactobacillus oris	1.250237	0
Campylobacter gracilis	1.147187	1.725543
Lachnospiraceae bacterium oral taxon 500	0.952669	0.285252
Actinomyces sp. oral taxon 171	0.942604	1.751664
Actinomyces sp. oral taxon 897	0.878978	1.046807
Streptococcus sobrinus	0.862837	0
Veillonella parvula	0.821117	1.63851
Porphyromonas gingivalis A7A1-28	0.816158	0.401341
Lactobacillus vaginalis	0.773132	0
Prevotella enoeca	0.719439	1.821736
Campylobacter showae	0.700368	0.921285
Microcella alkaliphila	0.68644	0.265816
Bifidobacterium dentium	0.683281	0.111447
Porphyromonas gingivalis W83	0.674768	0.812253

Actinomyces oris	0.641324	0.453649
Tannerella forsythia 92A2	0.619222	0.283607
Porphyromonas gingivalis TDC60	0.610933	0.298268
Actinomyces viscosus	0.60704	0.33042
Dialister pneumosintes	0.591125	0.06536
Prevotella intermedia 17	0.583433	0.812895
Pseudopropionibacterium propionicum	0.564918	0.604137
Tannerella forsythia 3313	0.554012	0.227456
Neisseria mucosa	0.548956	0.520663
Streptococcus sanguinis	0.545221	0.273819
Prevotella melaninogenica	0.543434	0.987017
Tannerella sp. oral taxon HOT-286	0.519352	2.513744
Bradyrhizobium sp. BTAi1	0.510675	0.570149
Limosilactobacillus fermentum	0.501826	0

Supplementary table 3. Bacterial species, in MetaPhlAn 3.0 output, with relative abundance ≥ 0.5% in patients with periodontitis (n = 6) and individuals without periodontitis (n = 6).

Species	**p+: mean rel. freq. (%)**	**p-: mean rel. freq. (%)**
Porphyromonas gingivalis	25.38214	12.55463
Tannerella forsythia	12.67147	5.415283
Prevotella intermedia	6.136502	0.042795
Alloprevotella tannerae	5.989305	6.38465
Prevotella nigrescens	4.351949	1.955035
Treponema socranskii	3.645572	2.384338
Porphyromonas endodontalis	3.585794	1.578625
Treponema denticola	3.367574	0.604093
Treponema maltophilum	2.065592	0.589883
Campylobacter rectus	1.915795	0.515948
Corynebacterium matruchotii	1.817797	1.883587
Treponema lecithinolyticum	1.738035	1.62942
Fretibacterium fastidiosum	1.72152	1.794363
Desulfobulbus oralis	1.55141	1.385848
Prevotella denticola	1.353194	1.293728
Desulfomicrobium orale	1.271758	1.68874
Tannerella sp oral taxon HOT 286	1.016075	0.615955
Anaerolineaceae bacterium oral taxon 439	0.99213	0
Actinomyces oris	0.896835	2.352172
Campylobacter gracilis	0.882635	0.467597
Actinomyces sp oral taxon 414	0.802305	0.260493
Bacteroidetes oral taxon 274	0.720797	0.050638
Prevotella pleuritidis	0.695603	1.352235
Campylobacter showae	0.634922	0.082055

Prevotella dentalis	0.599698	0.083987
Selenomonas sputigena	0.556843	0.37854

Supplementary table 4. Bacterial species, in MetaPhlAn 3.0 output, with relative abundance ≥ 0.5% in patients with type 2 diabetes (n = 6) and individuals without type 2 diabetes (n = 6).

Species	**t2d+: mean rel. freq. (%)**	**t2d-: mean rel. freq. (%)**
Porphyromonas gingivalis	25.13047	12.8063
Tannerella forsythia	11.73558	6.351172
Scardovia wiggsiae	8.573898	0
Prevotella nigrescens	3.743659	2.563326
Prevotella intermedia	3.338412	2.840885
Porphyromonas endodontalis	3.210014	1.954406
Treponema socranskii	2.741722	3.288188
Actinomyces sp oral taxon 448	2.426725	0.01576
Treponema denticola	2.170802	1.800865
Prevotella denticola	2.073284	0.573638
Fretibacterium fastidiosum	2.032674	1.48321
Desulfobulbus oralis	1.984787	0.952472
Actinomyces oris	1.971113	1.277894
Desulfomicrobium orale	1.68874	1.271758
Actinomyces naeslundii	1.593217	0.655902
Corynebacterium matruchotii	1.469962	2.231422
Alloprevotella tannerae	1.328274	11.04568
Treponema maltophilum	1.097837	1.557638
Bacteroidetes bacterium oral taxon 272	1.058635	0.16591
Treponema lecithinolyticum	1.038515	2.32894
Campylobacter rectus	0.95277	1.478973
Rothia dentocariosa	0.878897	13.61886
Lactobacillus oris	0.849103	0
Rothia aeria	0.838815	0.378575
Prevotella pleuritidis	0.695603	1.352235
Prevotella dentalis	0.581032	0.102653
Anaerolineaceae bacterium oral taxon 439	0.573282	0.418848
Prevotella oris	0.548765	0.460609
Prevotella oralis	0.525397	0.639838
Actinomyces sp oral taxon 414	0.50807	0.554728

Supplementary table 5. Bacteria genera/species from MEGAN6 output having a nominal significantly difference ≤0.01 level at White's non-parametric t-test applied to compare relative abundance between groups with FDR correction.

Periodontitis patients vs individuals without periodontitis (genera)	**Periodontitis mean ± s.d.**	**No periodontitis mean ± s.d.**	**Nominal p-val after correction**	**FDR**	**Periodontitis patients vs individuals without periodontitis (species)**	**Periodontitis mean ± s.d.**	**No periodontitis mean ± s.d.**	**Nominal p-val after correction**	**FDR**
Anaerolineaceae unclass.	2.472 ± 1.018	0.045 ± 0.101	0.01		Anaerolineaceae bacterium oral taxon 439	2.473 ± 1.019	0.045 ± 0.101	0.01	
t2d+p+ vs t2d-p+ (genera)	**t2d+p+ mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val after correction**	**FDR**	**t2d+p+ vs t2d-p+ (species)**	**t2d+p+ mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val after correction**	**FDR**
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t2d+p- vs t2d-p- (genera)	**t2d+p- mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction**	**FDR**	**t2d+p- vs t2d-p- (species)**	**t2d+p- mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction**	**FDR**
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Patients with type 2 diabetes vs individuals without type 2 diabetes (genera)	**type 2 diabetes mean ± s.d.**	**No type 2 diabetes mean ± s.d.**	**Nominal p-val after correction**	**FDR**	**Patients with type 2 diabetes vs individuals without type 2 diabetes (species))**	**type 2 diabetes mean ± s.d.**	**No type 2 diabetes mean ± s.d.**	**Nominal p-val after correction**	**FDR**
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t2d+p+ vs t2d+p- (genera)	**t2d+p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val after correction**	**FDR**	**t2d+p+ vs t2d+p- (species)**	**t2d+p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val after correction**	**FDR**
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t2d-p+ vs t2d-p- (genera)	**t2d-p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val after correction**	**FDR**	**t2d-p+ vs t2d-p- (species)**	**t2d-p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val after correction**	**FDR**
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t2d+p+ vs t2d-p- (genera)	**t2d+p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction**	**FDR**	**t2d+p+ vs t2d-p- (species)**	**t2d+p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction**	**FDR**
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t2d+p- vs t2d-p+ (genera)	**t2d+p- mean ± s.d.**	**No periodontitis mean ± s.d.**	**Nominal p-val after correction**	**FDR**	**t2d+p- vs t2d-p+ (species)**	**t2d+p- mean ± s.d.**	**No periodontitis mean ± s.d.**	**Nominal p-val after correction**	**FDR**
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Supplementary table 6. Bacteria genera/species from MetaPhlAn 3.0 output having a nominal significantly difference ≤0.01 level at White's non-parametric t-test applied to compare relative abundance between groups with FDR correction.

Periodontitis patients vs individuals without periodontitis (genera)	**Periodontitis mean ± s.d.**	**No periodontitis mean ± s.d.**	**Nominal p-val after correction FDR**	**Periodontitis patients vs individuals without periodontitis (species)**	**Periodontitis mean ± s.d.**	**No periodontitis mean ± s.d.**	**Nominal p-val after correction FDR**
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t2d+p+ vs t2d-p+ (genera)	**t2d+p+ mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val after correction FDR**	**t2d+p+ vs t2d-p+ (species)**	**t2d+p+ mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val after correction FDR**
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t2d+p- vs t2d-p- (genera)	**t2d+p- mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction FDR**	**t2d+p- vs t2d-p- (species)**	**t2d+p- mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction FDR**
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Patients with type 2 diabetes vs individuals without type 2 diabetes (genera)	**type 2 diabetes mean ± s.d.**	**No type 2 diabetes mean ± s.d.**	**Nominal p-val after correction FDR**	**Patients with type 2 diabetes vs individuals without type 2 diabetes (species)**	**type 2 diabetes mean ± s.d.**	**No type 2 diabetes mean ± s.d.**	**Nominal p-val after correction FDR**
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t2d+p+ vs t2d+p- (genera)	**t2d+p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val after correction FDR**	**t2d+p+ vs t2d+p- (species)**	**t2d+p+ mean ± s.d.**	**t2d+p- mean ± s.d.**	**Nominal p-val after correction FDR**
Anaerolineaceae unclassified	1.147 ± 0.117	0 ± 0	0	Anaerolineaceae bacterium oral taxon 439	1.147 ± 0.117	0 ± 0	0
t2d-p+ vs t2d-p- (genera)	**t2d-p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction FDR**	**t2d-p+ vs t2d-p- (species)**	**t2d-p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction FDR**
Porphyromonas	29.823 ± 6.662	0.975 ± 1.378	0	Porphyromonas gingivalis	25.613 ± 4.984	0 ± 0	0
t2d+p+ vs t2d-p- (genera)	**t2d+p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction FDR**	**t2d+p+ vs t2d-p- (species)**	**t2d+p+ mean ± s.d.**	**t2d-p- mean ± s.d.**	**Nominal p-val after correction FDR**
Anaerolineaceae unclassified	1.147 ± 0.117	0 ± 0	0	Anaerolineaceae bacterium oral taxon 439	1.147 ± 0.117	0 ± 0	0
t2d+p- vs t2d-p+ (genera)	**t2d+p- mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val after correction FDR**	**t2d+p- vs t2d-p+ (species)**	**t2d+p- mean ± s.d.**	**t2d-p+ mean ± s.d.**	**Nominal p-val**
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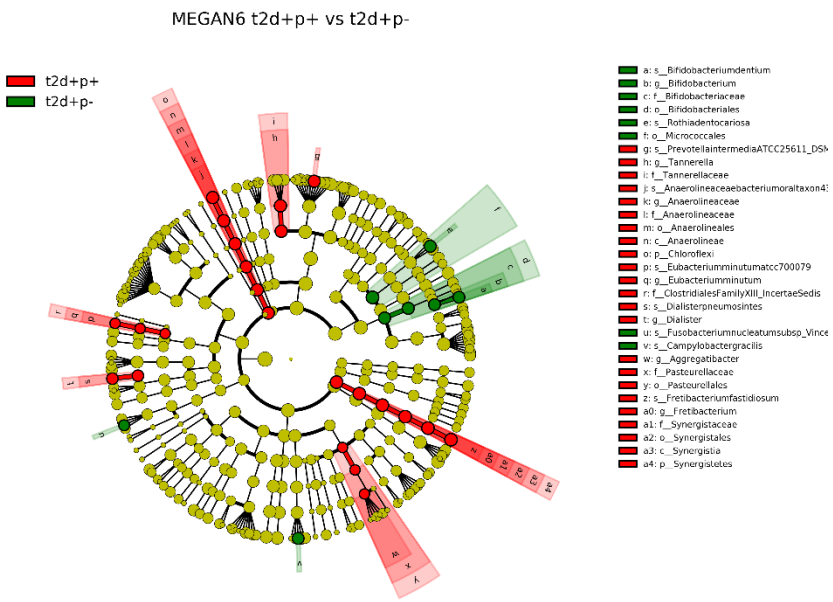
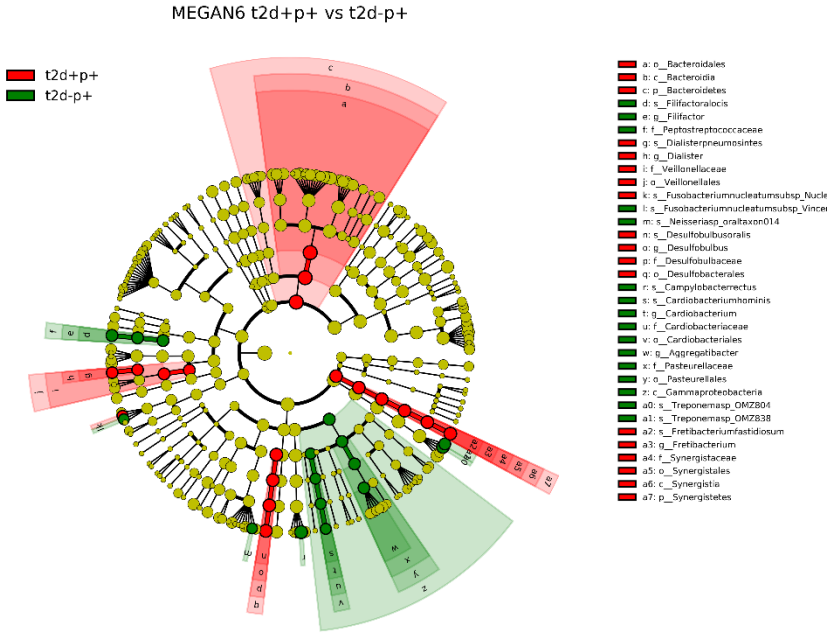
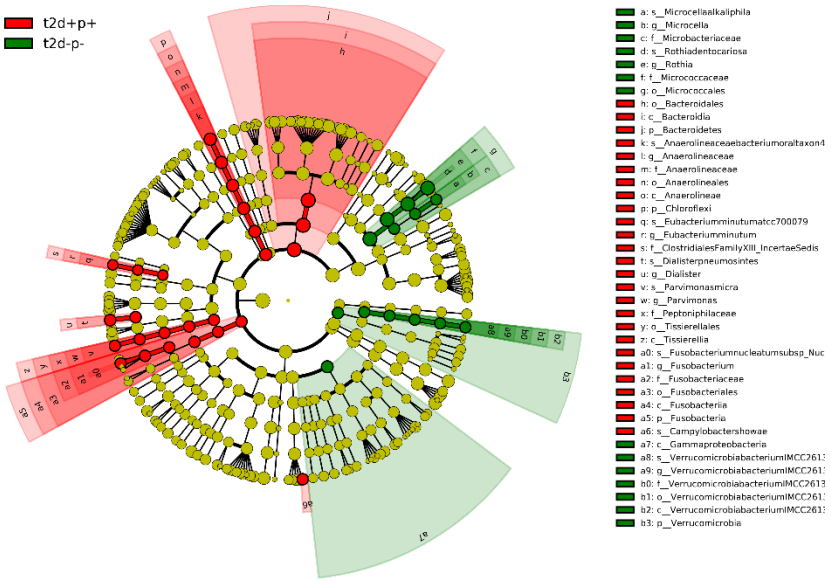
Figure 1. LEfSe comparison of clinical subcategories in MEGAN6's output

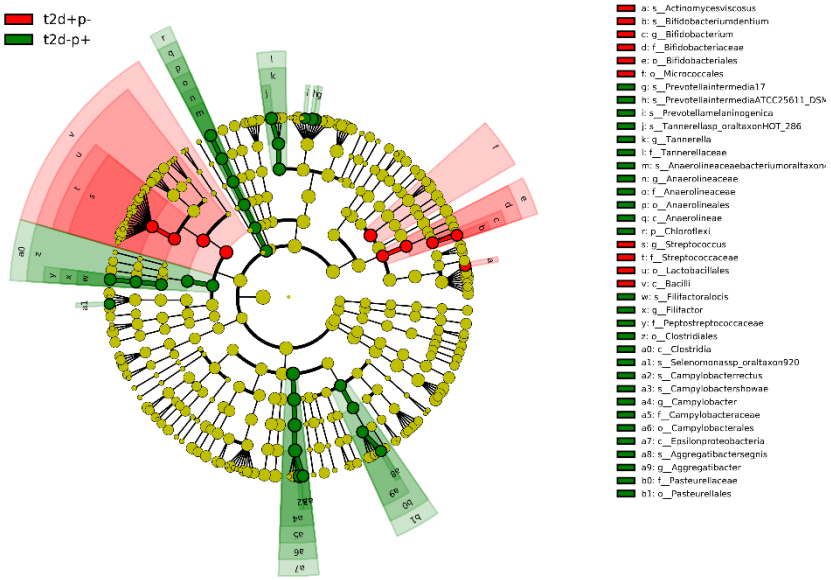




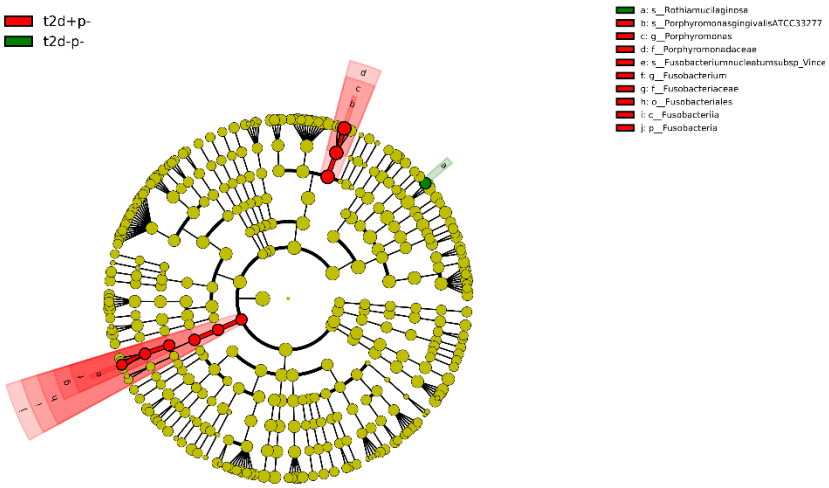
MEGAN6 t2d+p+ vs t2d-p-



MEGAN6 t2d+p- vs t2d+p+



MEGAN6 t2d+p- vs t2d-p-



MEGAN6 t2d-p+ vs t2d-p-

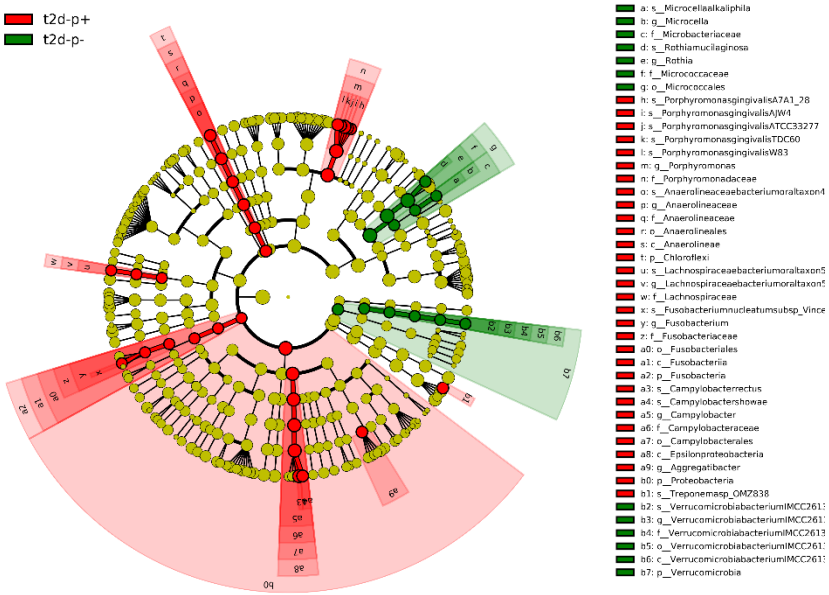
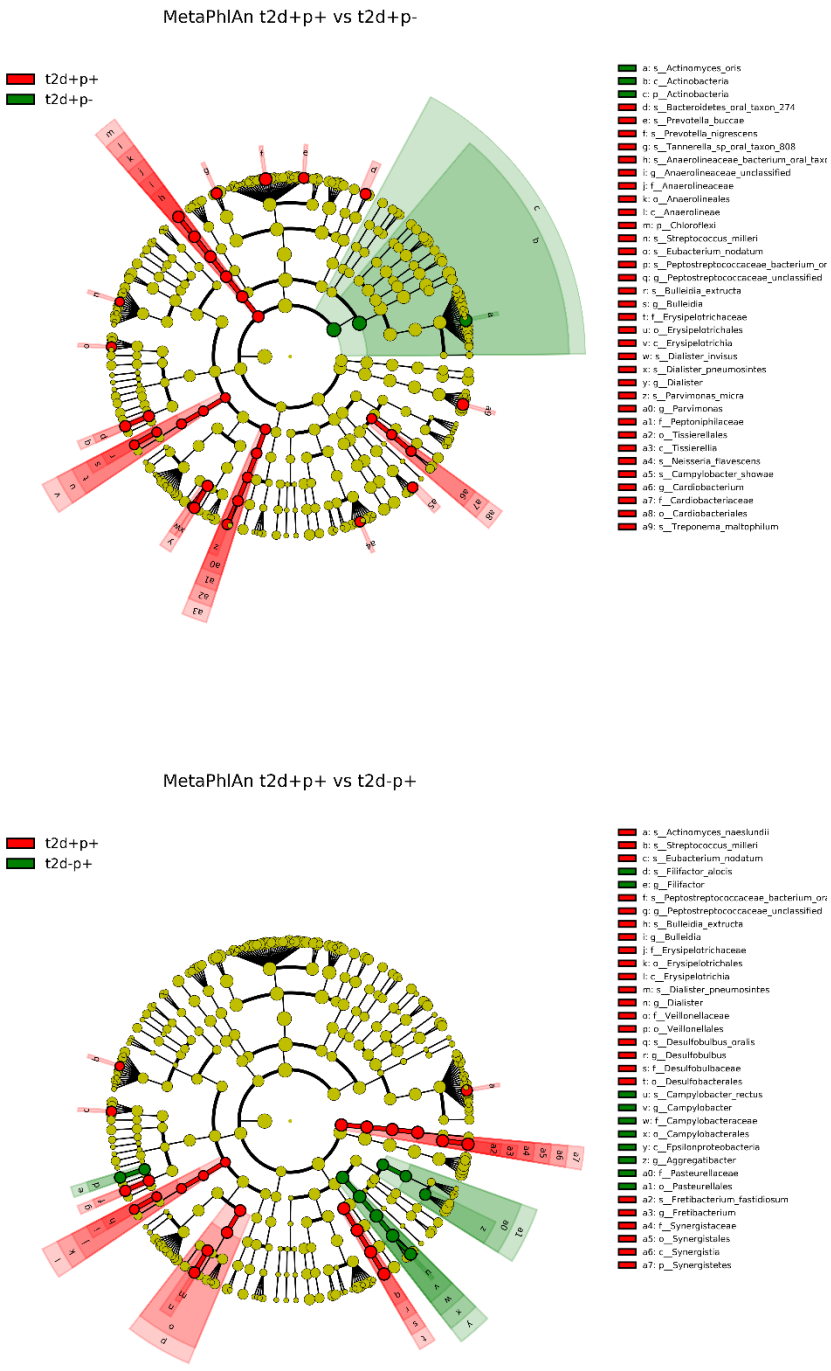
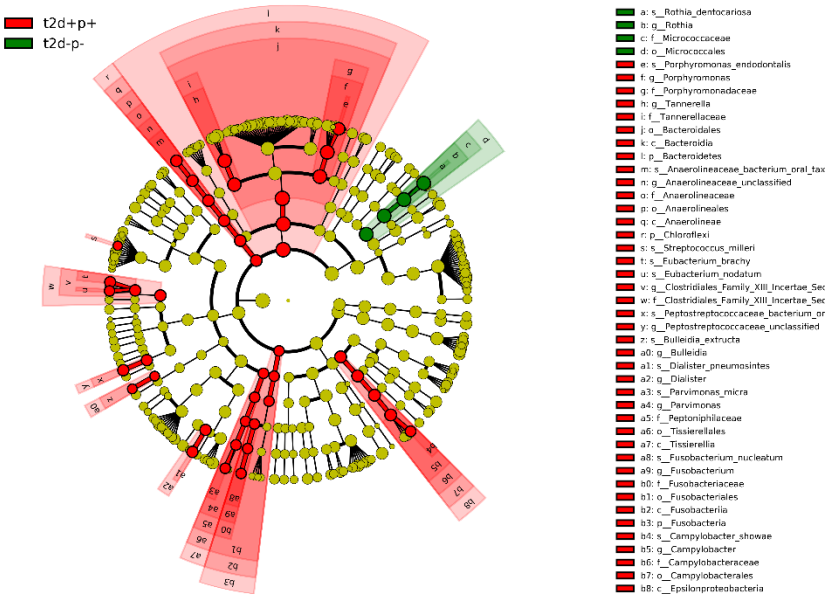


Figure 2. LEfSe comparison of clinical subcategories in MetaPhlAn 3.0's output.

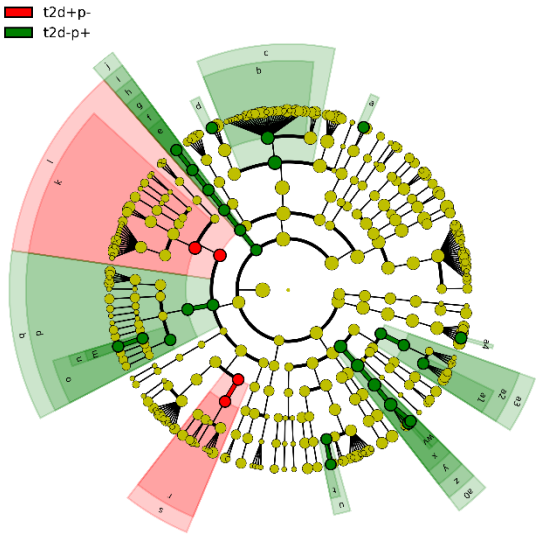


MetaPhlAn t2d+p+ vs t2d-p-



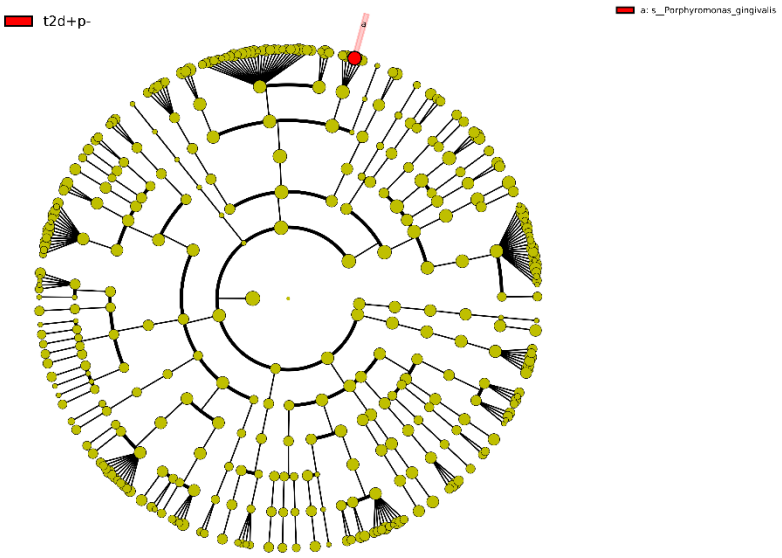
a: s__Rothia_dentocariosa
b: g__Rothia
c: f__Micrococcaceae
d: o__Micrococcales
e: s__Porphyromonas_endodontalis
f: g__Porphyromonas
g: f__Porphyromonadaceae
h: g__Tannerella
i: f__Tannerellaceae
j: o__Bacteroidales
k: c__Bacteroidia
l: p__Bacteroidetes
m: s__Anaerolineaceae_bacterium_oral_tax
n: g__Anaerolineaceae_unclassified
o: f__Anaerolineaceae
p: o__Anaerolineales
q: c__Anaerolineae
r: p__Chloroflexi
s: s__Streptococcus_milleri
t: s__Eubacterium_brachy
u: s__Eubacterium_nodatum
v: g__Clostridiales_Family_XIII_Incertae_Sec
w: f__Clostridiales_Family_XIII_Incertae_Sec
x: s__Peptostreptococcaceae_bacterium_or
y: g__Peptostreptococcaceae_unclassified
z: s__Bulleidia_extructa
a0: g__Bulleidia
a1: s__Dialister_pneumosintes
a2: g__Dialister
a3: s__Parvimonas_micra
a4: g__Parvimonas
a5: f__Peptoniphilaceae
a6: o__Tissierellales
a7: c__Tissierellia
a8: s__Fusobacterium_nucleatum
a9: g__Fusobacterium
b0: f__Fusobacteriaceae
b1: o__Fusobacteriales
b2: c__Fusobacteriia
b3: p__Fusobacteria
b4: s__Campylobacter_showae
b5: g__Campylobacter
b6: f__Campylobacteraceae
b7: o__Campylobacterales
b8: c__Epsilonproteobacteria

MetaPhlAn t2d+p- vs t2d-p+



a: s__Bacteroidetes_oral_taxon_274
b: g__Prevotella
c: f__Prevotellaceae
d: s__Tannerella_sp_oral_taxon_HOT_286
e: s__Anaerolineaceae_bacterium_oral_taxo
f: g__Anaerolineaceae_unclassified
g: f__Anaerolineaceae
h: o__Anaerolineales
i: c__Anaerolineae
j: p__Chloroflexi
k: o__Lactobacillales
l: c__Bacilli
m: s__Filifactor_alocis
n: g__Filifactor
o: f__Peptostreptococcaceae
p: o__Clostridiales
q: c__Clostridia
r: f__Veillonellaceae
s: o__Veillonellales
t: s__Eikenella_corrodens
u: g__Eikenella
v: s__Campylobacter_rectus
w: s__Campylobacter_showae
x: g__Campylobacter
y: f__Campylobacteraceae
z: o__Campylobacterales
a0: c__Epsilonproteobacteria
a1: g__Aggregatibacter
a2: f__Pasteurellaceae
a3: o__Pasteurellales
a4: s__Treponema_maltophilum

MetaPhlAn t2d+p- vs t2d-p-



MetaPhlAn t2d-p+ vs t2d-p-