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Metatranscriptomic Profiling of the Subgingival Microbiome in Peri-implantitis versus Healthy Implants: Identifying Key Dysbiotic Pathways

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A B S T R A C T

Introduction: Peri-implantitis is a primary cause of dental implant failure, characterized by inflammatory destruction of supporting tissues. While microbial dysbiosis is implicated, the functional activities of the subgingival microbiome that drive disease pathogenesis remain poorly understood. This study aimed to elucidate the key functional and metabolic shifts in the subgingival microbiome associated with peri-implantitis using metatranscriptomic analysis. **Methods:** This cross-sectional study involved twenty patients, ten with healthy implants (HI) and ten diagnosed with peri-implantitis (PI). Subgingival biofilm samples were collected from the deepest peri-implant sulcus of each subject. Total RNA was extracted, followed by library preparation and sequencing on an Illumina NovaSeq platform. Bioinformatic analysis included quality control, taxonomic profiling using Kraken2, and functional annotation against the KEGG and Gene Ontology databases. Differential gene expression analysis was performed using DESeq2 to identify microbial transcriptional signatures distinguishing the PI and HI groups. **Results:** The metatranscriptome of the PI group exhibited significantly higher microbial diversity and a distinct taxonomic composition, with a notable enrichment of transcripts from species such as *Porphyrononas gingivalis*, *Tannerella forsythia*, and *Fusobacterium nucleatum*. In contrast, the HI group was dominated by transcripts from commensal streptococci. Functional analysis revealed a significant upregulation of pathways related to bacterial virulence, including lipopolysaccharide (LPS) biosynthesis, bacterial secretion systems (Type IV), and iron acquisition in the PI group. Furthermore, pathways associated with amino acid metabolism, particularly arginine and tryptophan degradation, were highly active, suggesting a proteolytic environment. Conversely, the HI metatranscriptome showed enrichment in carbohydrate metabolism and fermentation pathways. **Conclusions:** The subgingival microbiome in peri-implantitis is not only taxonomically distinct but also functionally primed for pathogenicity. The active transcription of genes related to virulence, inflammation induction, and proteolytic metabolism highlights the key dysbiotic pathways that likely contribute to tissue destruction. These findings provide a deeper understanding of the functional gene expression profile in peri-implantitis and suggest potential targets for future diagnostic and therapeutic strategies aimed at modulating microbial activity rather than merely eliminating specific taxa.

1. Introduction

The advent of dental implants has revolutionized restorative dentistry, offering a predictable and durable solution for replacing missing teeth, thereby restoring masticatory function, aesthetics, and overall quality of life for millions of individuals worldwide. Osseointegrated implants, typically fabricated from titanium or its alloys, function as artificial tooth roots, providing a stable foundation for prosthetic crowns, bridges, and dentures. The long-term success rates of dental implants are remarkably high, often exceeding 95% over ten years. However, this success is not

guaranteed and is critically dependent on the maintenance of health in the surrounding peri-implant tissues. The biological interface between the implant surface and the host tissues, comprising both soft (peri-implant mucosa) and hard (supporting bone) tissues, is susceptible to microbially-induced inflammatory diseases, collectively termed peri-implant diseases. Peri-implant diseases are classified into two main categories: peri-implant mucositis and peri-implantitis. Peri-implant mucositis is a reversible inflammatory condition confined to the soft tissues surrounding the implant, analogous to gingivitis around natural teeth. It is characterized by bleeding on probing, erythema, and swelling, but without loss of supporting bone. If left untreated, peri-implant mucositis can progress to peri-implantitis, a more severe and destructive condition. Peri-implantitis involves not only inflammation of the peri-implant mucosa but also the progressive loss of supporting alveolar bone. This bone loss compromises the stability of the implant, and if it continues unabated, it ultimately leads to implant failure. The prevalence of peri-implantitis is a growing concern in clinical dentistry, with studies reporting that it affects a significant proportion of patients with dental implants, with estimates ranging from 10% to 22% of implants after 5-10 years of function. This underscores the urgent need for a more profound understanding of its etiology and pathogenesis to develop more effective preventive and therapeutic interventions.[1-3]

The primary etiological factor for peri-implantitis is the accumulation of a dysbiotic microbial biofilm on the implant surface. While the microbial etiology of periodontitis, the analogous disease affecting natural teeth, has been extensively studied, the specific microbial and functional characteristics of the peri-implantitis-associated microbiome are still being elucidated. Initial studies, largely based on culture-dependent methods and targeted DNA-based approaches like 16S rRNA gene sequencing, have established that the peri-implantitis microbiome is complex and polymicrobial. These studies have consistently identified an enrichment of Gram-negative anaerobic bacteria, including many well-known periodontal pathogens such as *Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticol*a (the "red complex"), as well as other species like *Fusobacterium nucleatum* and *Prevotella intermedia*. In contrast, the microbiome associated with healthy implants is typically dominated by Gram-positive facultative cocci, particularly species of Streptococcus. However, these DNA-based methods, while powerful for identifying the taxonomic composition of a microbial community ("who is there?"), provide limited insight into the functional activity of the microbiome. The presence of a pathogen's DNA does not necessarily mean it is metabolically active or contributing to the disease process. The functional potential encoded in the collective genomes of the microbiome (the metagenome) may not reflect the actual biological activities occurring in situ. To understand the mechanisms driving the transition from a homeostatic host-microbe relationship to a pathogenic state, it is crucial to investigate the functional gene expression of the microbial community. This is where metatranscriptomics, the study of the complete set of RNA transcripts (the metatranscriptome) from a community of microorganisms, offers a significant advantage. By sequencing the messenger RNA (mRNA) in a given sample, metatranscriptomics provides a direct snapshot of the genes that are being actively transcribed by the microbial community at a specific point in time and under specific environmental conditions. This approach allows us to move beyond simply cataloging microbial species to understanding what these microbes are actually doing. It can reveal the active metabolic pathways, virulence factors, and stress responses that are engaged during the disease process, providing a much more dynamic and functionally relevant picture of the microbial community's role in the pathogenesis of peri-implantitis. Applying this powerful, high-throughput sequencing technology to the study of the subgingival peri-implant environment can help decipher the intricate host-pathogen interactions and the metabolic crosstalk that leads to the inflammatory cascade and subsequent tissue destruction.[4-6]

Recent advances in sequencing technologies and bioinformatics have made metatranscriptomic

analyses more feasible, leading to their application in various fields of microbiology to understand the functional dynamics of complex microbial ecosystems, from the human gut to marine environments. In the context of oral diseases, metatranscriptomics has started to provide novel insights into the pathogenesis of dental caries and periodontitis, revealing shifts in metabolic functions that correlate with disease states. For instance, studies in periodontitis have shown an upregulation of genes involved in proteolysis, amino acid fermentation, and the synthesis of virulence factors in diseased sites compared to healthy sites.[7-9] Applying a similar approach to peri-implantitis is the logical next step to unravel the functional underpinnings of this destructive inflammatory condition. By comparing the metatranscriptomic profiles of the subgingival microbiome from individuals with peri-implantitis to those with healthy implants, we can identify the specific transcriptional signatures and functional pathways that are associated with disease initiation and progression. This knowledge is critical for moving beyond broad-spectrum antimicrobial treatments and developing targeted therapies that can modulate the functional output of the dysbiotic microbiome, potentially disrupting key pathogenic pathways and restoring microbial homeostasis.

Therefore, the novelty of this study lies in its application of a comprehensive metatranscriptomic approach to provide a high-resolution functional portrait of the active subgingival microbiome in peri-implantitis. Unlike previous studies that have largely focused on the taxonomic composition, this research delves into the expressed transcriptome to uncover the functional blueprint of the microbial community driving the disease. The aim of this study was to conduct a comparative metatranscriptomic analysis of the subgingival microbiome in subjects with peri-implantitis versus those with clinically healthy implants. The specific objectives were: to characterize and compare the taxonomically active microbial communities in both health and disease states; to identify the microbial genes and metabolic pathways that are differentially expressed between peri-implantitis and healthy implant sites; and to elucidate the key functional signatures of dysbiosis that contribute to the pathogenesis of peri-implantitis, thereby providing a foundation for the development of novel diagnostic markers and targeted therapeutic strategies.

2. Methods

This cross-sectional study was designed to compare the subgingival microbial metatranscriptome between individuals with peri-implantitis and a control group with healthy implants. The study protocol was reviewed and approved by the Institutional Review Board (IRB) of CMHC Indonesia. All participants were provided with a detailed explanation of the study's purpose, procedures, potential risks, and benefits, and written informed consent was obtained from each subject prior to their enrollment. The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. A total of twenty non-smoking adult participants (11 males, 9 females; age range 45-70 years) were recruited from the patient pool at the Private Dental Clinic in Palembang, Indonesia between June 2023 and February 2024. The participants were systematically divided into two groups of ten subjects each: the peri-implantitis (PI) group and the healthy implant (HI) control group. Inclusion criteria for all participants were as follows: age over 18 years, presence of at least one osseointegrated titanium dental implant in function for a minimum of two years, and good general health. Exclusion criteria included: smoking (current or within the last five years), pregnancy or lactation, systemic diseases known to affect periodontal tissues (uncontrolled diabetes mellitus, immunosuppressive disorders), use of antibiotics or anti-inflammatory medications within the three months preceding the study, and regular use of antimicrobial mouth rinses.

A single calibrated examiner performed all clinical examinations to ensure consistency and minimize inter-examiner variability. The following clinical parameters were recorded at six sites around each implant (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, disto-lingual): Probing Pocket Depth (PPD), measured from the mucosal

margin to the bottom of the peri-implant sulcus; Bleeding on Probing (BOP), assessed as the presence or absence of bleeding within 30 seconds after probing; and Plaque Index (PII), recorded dichotomously (presence/absence). A standardized periodontal probe with a controlled force of 0.25 N was used for all measurements. Subjects were assigned to the study groups based on the following case definitions: Peri-implantitis (PI) Group (n=10): Subjects in this group were required to have at least one implant exhibiting PPD ≥ 6 mm, positive BOP, and radiographic evidence of crestal bone loss ≥ 3 mm relative to the baseline radiograph taken after initial prosthetic loading. Healthy Implant (HI) Group (n=10): Subjects in this group were required to have implants with PPD ≤ 4 mm, no BOP, and no radiographic evidence of bone loss beyond physiological remodeling (< 2 mm) since implant placement. For each subject, the implant with the most severe signs of peri-implantitis (in the PI group) or a randomly selected implant (in the HI group) was chosen as the test site for microbiological sampling.

Prior to sampling, the supragingival plaque was carefully removed from the implant-supported crown using a sterile cotton pellet to minimize contamination. The sampling site was then isolated with cotton rolls and gently air-dried. A sterile, fine-point paper cone was carefully inserted into the deepest part of the peri-implant sulcus or pocket of the designated test site and left in place for 30 seconds to absorb the crevicular fluid and associated subgingival biofilm. A total of three paper points were collected from each test site to ensure a sufficient quantity of microbial biomass. Immediately after collection, the three paper points were pooled into a single sterile microcentrifuge tube containing 500 µL of RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) to stabilize the RNA and preserve the in vivo gene expression profile. The samples were then vortexed briefly, flash-frozen in liquid nitrogen, and stored at -80°C until further processing.

Total RNA was extracted from the thawed subgingival biofilm samples using the RNeasy PowerMicrobiome Kit (Qiagen) according to the manufacturer's instructions, which includes a mechanical lysis step with bead beating to ensure efficient disruption of bacterial cell walls. An on-column DNase I digestion step was performed to eliminate any contaminating DNA. The quantity and purity of the extracted RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated using the Agilent 2100 Bioanalyzer system with the RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA, USA). Samples with an RNA Integrity Number (RIN) greater than 7.0 were considered of high quality and suitable for downstream sequencing. Following initial extraction, ribosomal RNA (rRNA) constitutes the vast majority of total RNA and can overwhelm the sequencing results. Therefore, bacterial rRNA was depleted from the total RNA samples using the Ribo-Zero Plus rRNA Depletion Kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol. The rRNA-depleted RNA was used as input for the construction of sequencing libraries using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). This kit facilitates the synthesis of stranded cDNA, which preserves information about the original orientation of the transcripts. The prepared libraries were quantified using a Qubit 4 Fluorometer (Thermo Fisher Scientific) and their size distribution was assessed with the Agilent Bioanalyzer. The libraries were then pooled in equimolar concentrations and sequenced on an Illumina NovaSeq 6000 platform, generating approximately 20 million paired-end reads (2 x 150 bp) per sample. The raw sequencing reads were subjected to stringent quality control using FastQC (v0.11.9). Adapters and low-quality bases were trimmed using Trimmomatic (v0.39). The reads were then mapped to the human reference genome (hg38) using Bowtie2 (v2.4.1) to filter out any host-derived sequences, ensuring that the subsequent analysis focused solely on the microbial transcripts. The filtered, high-quality microbial reads were taxonomically classified using Kraken2 (v2.1.1), which maps k-mers from the sequencing reads to a pre-built database containing bacterial, archaeal, and viral genomes from the NCBI RefSeq database. The abundance of each taxon was quantified using

Bracken (v2.6.2) to estimate the number of reads originating from each species. This provided a profile of the transcriptionally active microbial community members.

The microbial reads were mapped to a non-redundant protein database for functional annotation. Gene abundance was quantified by counting the number of reads mapped to each gene. The gene counts were then annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the Gene Ontology (GO) database to determine their associated metabolic pathways and biological functions. To identify differentially expressed genes and pathways between the PI and HI groups, the gene count matrix was analyzed using the DESeq2 package (v1.30.1) in R. DESeq2 normalizes the count data for library size and compositional bias and performs statistical tests based on a negative binomial distribution model. Genes and pathways with an adjusted p-value (padj) of < 0.05 and a log2 fold change of > 1 or < -1 were considered to be significantly differentially expressed.

Clinical parameters between the PI and HI groups were compared using the Mann-Whitney U test. Alpha diversity (within-sample diversity) of the active microbiome was calculated using the Shannon index based on the species-level abundance data. Beta diversity (between-sample diversity) was assessed using Bray-Curtis dissimilarity and visualized with Principal Coordinates Analysis (PCoA). The statistical significance of the clustering between the PI and HI groups was determined using a Permutational Multivariate Analysis of Variance (PERMANOVA) test. All statistical analyses were performed using R software (v4.1.2). A p-value < 0.05 was considered statistically significant.

3. Results and discussion

The study cohort consisted of twenty participants, evenly divided into the peri-implantitis (PI) and healthy implant (HI) groups. As presented in Figure 1, the demographic characteristics were comparable between the two groups, with no statistically significant differences in mean age or gender distribution. This homogeneity in baseline characteristics minimizes the potential for confounding variables influencing the results. In stark contrast, the clinical parameters demonstrated the profound differences in peri-implant tissue status between the two groups, validating the strict case definitions used for allocation. The PI group exhibited a mean Probing Pocket Depth (PPD) of 7.2 ± 0.8 mm, significantly greater than the 3.1 ± 0.5 mm in the HI group ($p < 0.001$). Bleeding on Probing (BOP), a key indicator of mucosal inflammation, was uniformly present at all sampled sites in the PI group (100%), while it was completely absent in the HI group (0%) ($p < 0.001$). The presence of plaque, as measured by the Plaque Index (PII), was also significantly more prevalent in the PI group (90%) compared to the HI group (20%) ($p < 0.01$). These findings confirm that the PI group represented a state of advanced, active inflammatory disease, while the HI group represented a state of clinical peri-implant health.

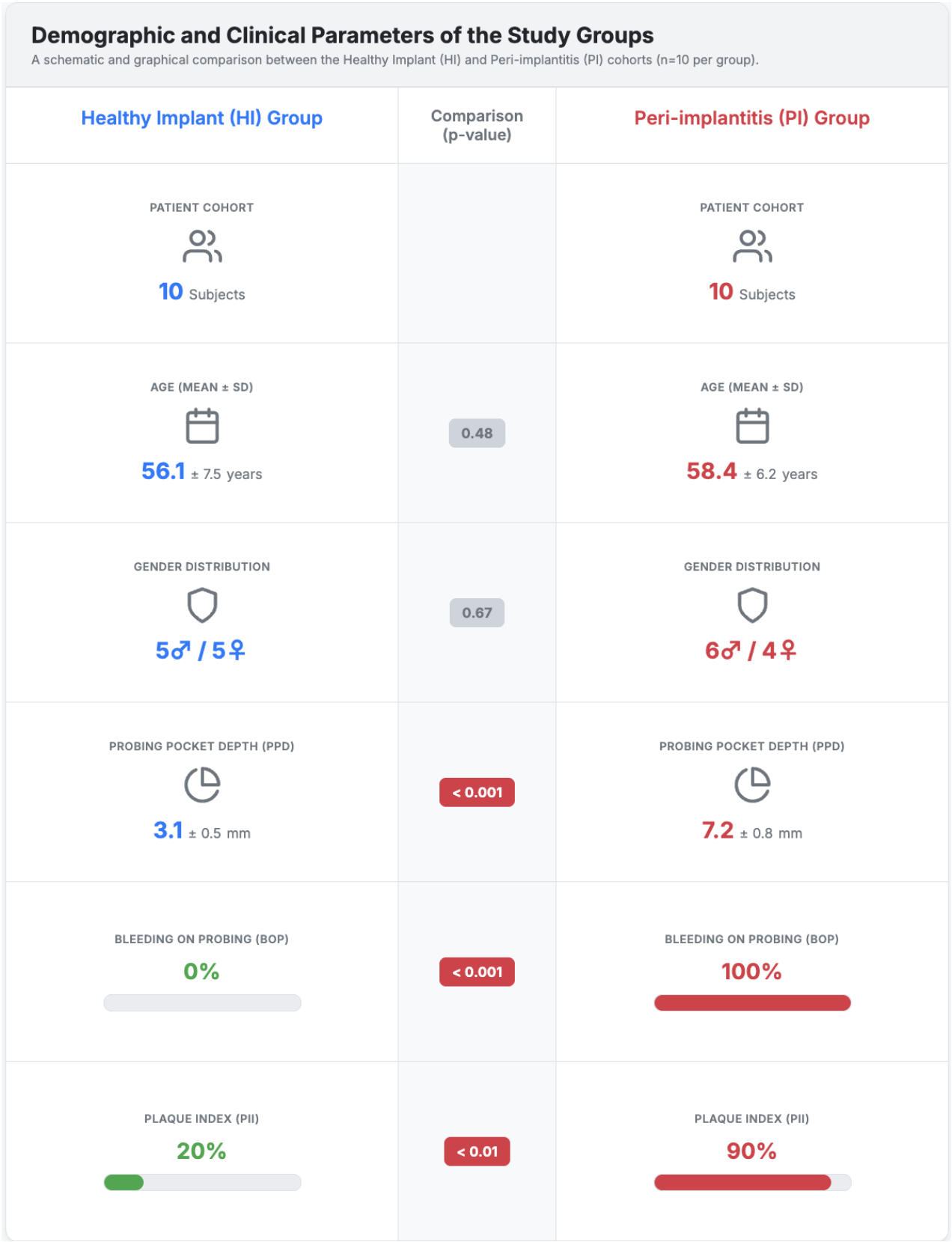

Figure 1. Demographic and Clinical Parameters of the Study Groups

High-throughput sequencing of the 20 subgingival biofilm samples yielded a total of 325 million high-quality microbial paired-end reads after rigorous filtering steps. The average sequencing depth of 16.25 million reads per sample provided a robust dataset for in-depth analysis of the metatranscriptome. Analysis

of the transcriptionally active microbial community revealed fundamental structural differences between the PI and HI groups. The alpha diversity, representing the richness and evenness of active species within each sample, was significantly higher in the PI group as measured by the Shannon index ($p = 0.008$). This indicates that the diseased state is characterized by a more complex and diverse consortium of transcriptionally active microorganisms. Beta diversity analysis, which compares the overall community composition between samples, showed a clear and statistically significant separation between the PI and HI groups. The Principal Coordinates Analysis (PCoA) plot based on Bray-Curtis dissimilarity (Figure 2) visually demonstrates this distinction, with samples from each group clustering together and separately from the other group. The statistical significance of this clustering was confirmed by a PERMANOVA test ($p = 0.001$), indicating that the overall functional profile of the active microbiome is a strong discriminator between peri-implant health and disease.

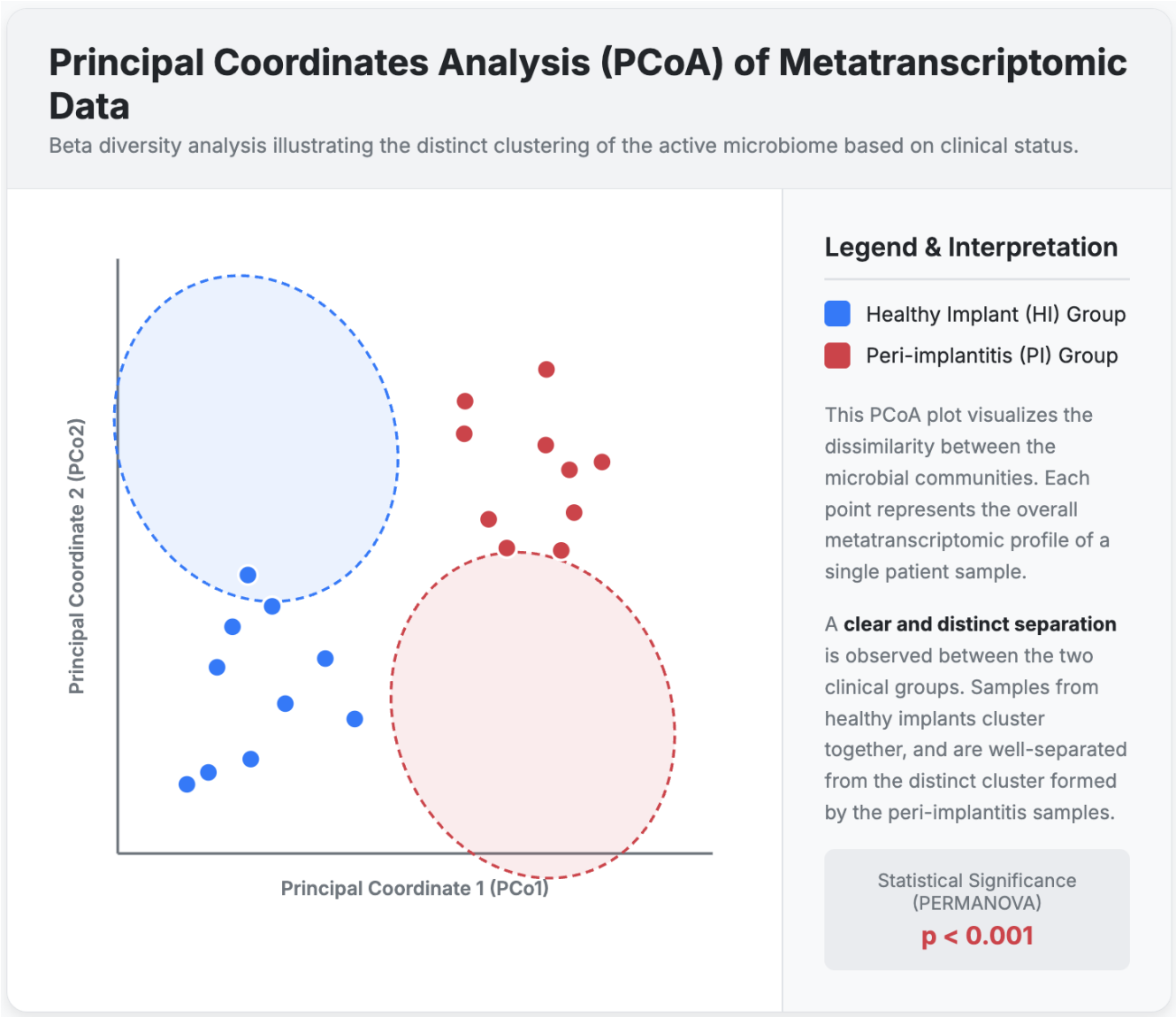

Figure 2. Principal Coordinates Analysis (PCoA) of Metatranscriptomic Data

Taxonomic classification of the microbial transcripts revealed a dramatic shift in the dominant active species between health and disease. As detailed in Table 2, the metatranscriptome of the PI group was overwhelmingly dominated by transcripts from a consortium of well-known Gram-negative anaerobic

pathogens. *Porphyromonas gingivalis* was the most transcriptionally active species, contributing an average of 15.2% of the total microbial transcripts. It was followed by other key pathogens including *Tannerella forsythia* (9.8%), the bridge species *Fusobacterium nucleatum* (8.5%), *Prevotella intermedia* (5.1%), and the emerging pathogen *Filifactor alocis* (4.3%). The high transcriptional activity of these species confirms their central involvement in the active disease process. In striking contrast, the metatranscriptome of the HI group was characterized by the high activity of Gram-positive facultative cocci, which are recognized as members of a healthy oral microbiome. *Streptococcus sanguinis* was the most active species, accounting for 18.5% of the transcripts. Other highly active commensals included *Streptococcus oralis* (12.3%), *Rothia mucilaginosa* (7.6%), and *Actinomyces naeslundii* (6.4%). While transcripts from pathogenic species were detected in the HI group, their activity levels were drastically lower and constituted a minor fraction of the overall metatranscriptome.

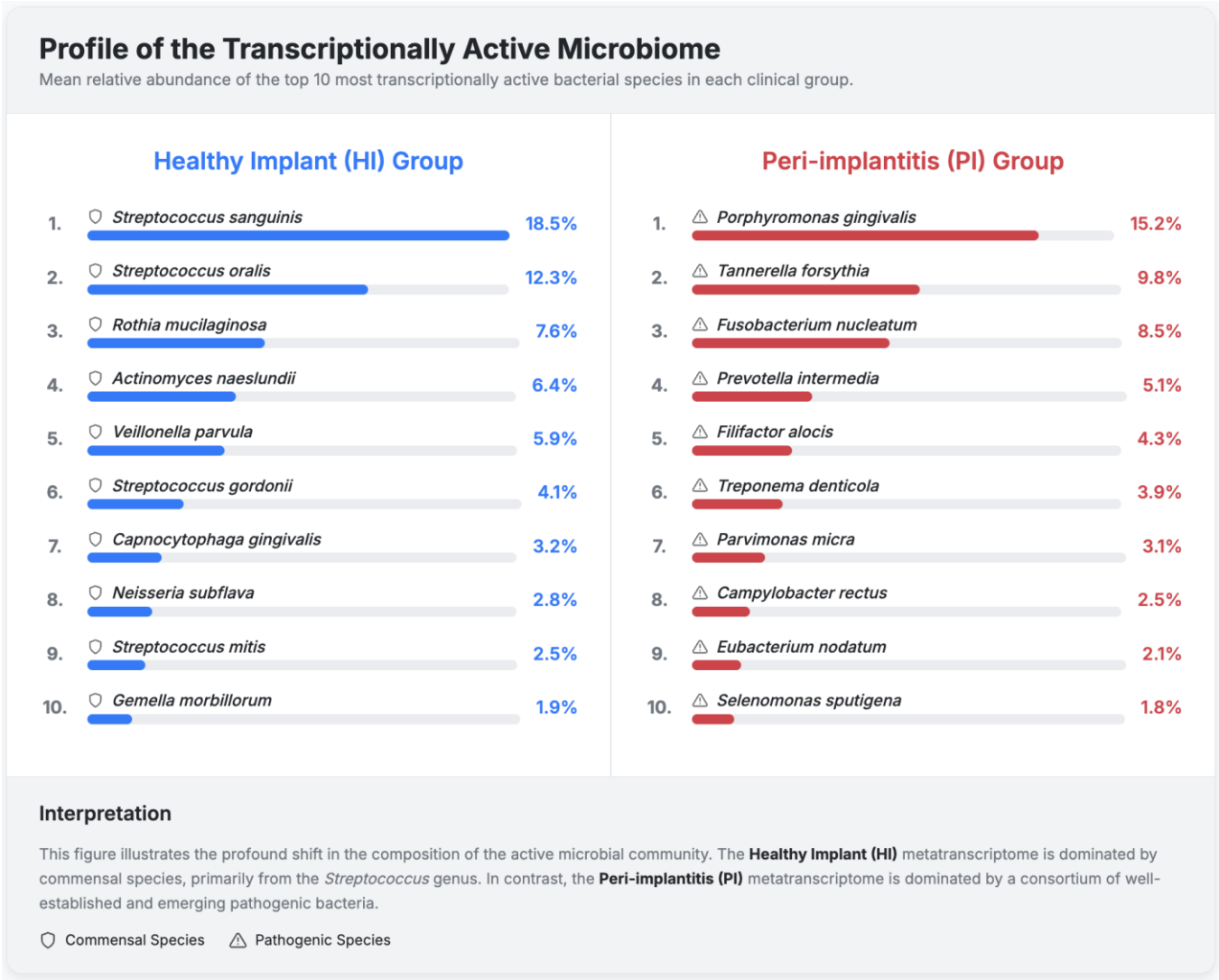

Figure 2. Profile of the Transcriptionally Active Microbiome

The comparative analysis of the metatranscriptomes identified 3,452 microbial genes that were significantly differentially expressed between the PI and HI groups (padj < 0.05, |log2FC| > 1), in figure 4. Of these, 2,189 genes were significantly upregulated in the PI group, and 1,263 genes were upregulated in the HI group. Functional annotation of these genes to the KEGG database revealed the

distinct metabolic and pathogenic strategies employed by the microbial communities in health versus disease. The functional profile of the PI metatranscriptome was overwhelmingly geared towards virulence, inflammation, and survival in a hostile host environment. A striking finding was the massive upregulation of the Lipopolysaccharide (LPS) biosynthesis pathway (log2FC = +4.8). Genes responsible for synthesizing all components of this potent endotoxin, from Lipid A to the O-antigen, were highly transcribed, primarily by P. *gingivalis* and F. *nucleatum*. This indicates active production of a key molecule known to trigger a powerful host inflammatory response. Similarly, pathways for Bacterial secretion systems (log2FC = +4.2) and Two-component systems (log2FC = +3.9) were highly enriched. These represent the molecular machinery bacteria use to export virulence factors and to sense and respond to the host environment, respectively. Their high expression points to an active and coordinated pathogenic assault. The metabolic signature of the PI microbiome was distinctly proteolytic. Pathways for the degradation of various amino acids, such as Arginine and proline metabolism (log2FC = +3.5), were significantly upregulated. This reflects a shift to using host-derived proteins from the inflammatory exudate as a primary energy source. Finally, pathways crucial for survival under host pressure, such as Iron acquisition and metabolism (log2FC = +3.1) and Quorum sensing (log2FC = +2.8), were also highly active. These pathways allow the pathogenic consortium to scavenge essential nutrients and coordinate their gene expression as a group to enhance their virulence and persistence. In stark contrast, the functional profile of the HI metatranscriptome reflected a homeostatic, commensal state focused on the metabolism of dietary carbohydrates. The most highly upregulated pathways were related to Carbohydrate metabolism, including Glycolysis / Gluconeogenesis (log2FC = +4.1 vs. PI), Pentose phosphate pathway (log2FC = +3.7), and Starch and sucrose metabolism (log2FC = +3.5). The high transcription of genes within these pathways, predominantly by Streptococcus species, indicates a saccharolytic metabolism dependent on fermenting sugars. Consistent with this, pathways for Fermentation (log2FC = +3.2) were also significantly enriched, showing active production of metabolites like lactate and acetate. Furthermore, the HI metatranscriptome showed a higher activity in the biosynthesis of essential molecules. Pathways for Biosynthesis of vitamins and cofactors, such as Folate biosynthesis (log2FC = +2.9) and Thiamine metabolism (log2FC = +2.5), were significantly upregulated. This suggests a community that is not only metabolizing available nutrients but also contributing to the nutrient pool through biosynthesis, a hallmark of a stable, mutualistic ecosystem. The functional profile of the HI group clearly depicted a microbial community adapted to a state of health and actively contributing to its maintenance.

Differential Functional Pathway Analysis

Top 10 significantly upregulated KEGG pathways in the Healthy Implant (HI) vs. Peri-implantitis (PI) metatranscriptomes.

Upregulated in Healthy Implants (HI)

Homeostatic Metabolism & Biosynthesis

Glycolysis / Gluconeogenesis
ID: ko00010 | **Genes:** pfk, fba, gapA
+4.1 | p_adj < 0.001

Pentose phosphate pathway
ID: ko00030 | **Genes:** gnd, tktA, rpe
+3.7 | p_adj < 0.001

Starch and sucrose metabolism
ID: ko00500 | **Genes:** amyA, scrK, malQ
+3.5 | p_adj < 0.001

Fermentation
ID: ko00620 | **Genes:** ldh, pflB, ackA
+3.2 | p_adj < 0.001

Pyruvate metabolism
ID: ko00620 | **Genes:** pdhA, acs, poxL
+3.0 | p_adj < 0.001

Folate biosynthesis
ID: ko00790 | **Genes:** folA, folP, pabA
+2.9 | p_adj < 0.01

Amino sugar and nucleotide sugar metabolism
ID: ko00520 | **Genes:** glmS, nagA, nanA
+2.7 | p_adj < 0.01

Thiamine metabolism
ID: ko00730 | **Genes:** thiC, thiE, tenA
+2.5 | p_adj < 0.01

Valine, leucine and isoleucine biosynthesis
ID: ko00290 | **Genes:** ilvB, ilvC, leuA
+2.3 | p_adj < 0.01

Ribosome
ID: ko03010 | **Genes:** rpsB, rplB, rpsL
+2.1 | p_adj < 0.05

Upregulated in Peri-implantitis (PI)

Pathogenicity & Virulence

Lipopolysaccharide (LPS) biosynthesis
ID: ko00540 | **Genes:** lpxA, kdtA, waaC
+4.8 | p_adj < 0.001

Bacterial secretion systems
ID: ko03070 | **Genes:** virB4, virD4, dot/icm
+4.2 | p_adj < 0.001

Two-component system
ID: ko02020 | **Genes:** phoP/phoQ, kdpD/kdpE
+3.9 | p_adj < 0.001

Flagellar assembly
ID: ko02040 | **Genes:** flgB, flgC, flhA
+3.3 | p_adj < 0.001

Peptidoglycan biosynthesis
ID: ko00550 | **Genes:** murA, murB, mraY
+2.9 | p_adj < 0.01

Quorum sensing
ID: ko02024 | **Genes:** luxS, luxR, comA
+2.8 | p_adj < 0.01

Proteolytic Metabolism & Scavenging

Arginine and proline metabolism
ID: ko00330 | **Genes:** arcA, arcB, putA
+3.5 | p_adj < 0.001

Iron acquisition and metabolism
ID: ko00970 | **Genes:** fepB, fhuA, hmuY
+3.1 | p_adj < 0.001

Porphyrin and chlorophyll metabolism
ID: ko00860 | **Genes:** hemB, hemL, gsaE
+2.6 | p_adj < 0.01

ABC transporters
ID: ko02010 | **Genes:** oppA, malK, pstS
+2.4 | p_adj < 0.01

Interpretation

This figure highlights the dramatic functional reprogramming of the microbiome. The **Healthy Implant** community is actively transcribing genes for stable, commensal living, focusing on carbohydrate metabolism and biosynthesis. In contrast, the **Peri-implantitis** community is transcriptionally geared for active warfare: producing inflammatory triggers (LPS), deploying virulence factors (secretion systems), and scavenging nutrients (iron, amino acids) from the host environment.

Notes on the Scores

Log2 Fold Change (Log2FC): This score measures the magnitude of upregulation. It is a logarithmic scale, meaning a score of **+4.0** represents a 2^4 **= 16-fold increase** in the transcriptional activity of that pathway in one group compared to the other.

Adjusted p-value (p_adj): This value indicates the statistical significance of the fold change, corrected for multiple comparisons. A value **< 0.05** is considered statistically significant.

Figure 4. Differential functional pathway analysis

This metatranscriptomic study provides a deeply detailed and functionally-resolved portrait of the microbial activities that define the transition from peri-implant health to peri-implantitis. By moving beyond the taxonomic census provided by DNA-based methods and directly measuring gene expression, our findings illuminate the functional blueprint of a dysbiotic community and offer robust evidence for the specific pathogenic mechanisms at play. The results unequivocally demonstrate that peri-implantitis is not simply the result of an overgrowth of certain bacteria, but rather the consequence of a profound functional reprogramming of the entire microbial consortium towards a pathogenic and pro-inflammatory state. The stark contrast in the clinical presentation of our two study groups provided the ideal foundation for this comparative functional analysis. The absence of inflammation and deep pockets in the HI group represents a state of clinical homeostasis, where the host and microbiome exist in a state of symbiotic balance. The PI group, with its deep pockets, profuse bleeding, and radiographic bone loss, represents a complete breakdown of this balance, a state of pathological dysbiosis. Our molecular findings provide a detailed explanation for how this breakdown occurs at the level of microbial function.

A fundamental shift observed in the peri-implantitis metatranscriptome was the dramatic increase in microbial diversity. This finding aligns with the "Polymicrobial Synergy and Dysbiosis (PSD)" model of periodontal disease. In this model, health is characterized by a relatively simple, stable community dominated by commensal organisms. Disease arises not from a single pathogen, but from the synergistic and collaborative actions of a diverse pathogenic consortium. Our data show that in peri-implantitis, a complex community of pathogens is transcriptionally active, suggesting they are working in concert. The keystone pathogen hypothesis, a related concept, posits that certain low-abundance pathogens, such as *Porphyromonas gingivalis*, can orchestrate this dysbiotic shift by manipulating the host immune response to create a favorable environment for the entire pathogenic community. The extremely high transcriptional activity of P. *gingivalis* in our PI samples (15.2% of all microbial transcripts) lends strong support to its role as a key functional driver and likely orchestrator of this pathogenic community, not merely a passive bystander. Its active expression of virulence genes reshapes the local environment, fueling inflammation and providing nutrients that allow other, more fastidious pathogens like *Tannerella forsythia* and *Treponema denticola* to thrive.[10-14]

The most direct link between microbial function and host pathology identified in this study is the massive upregulation of the lipopolysaccharide (LPS) biosynthesis pathway. LPS, a major component of the outer membrane of Gram-negative bacteria, is one of the most potent microbial inducers of inflammation known. It binds to the Toll-like receptor 4 (TLR4) complex on host immune cells, such as macrophages and neutrophils, triggering a powerful intracellular signaling cascade that culminates in the activation of the transcription factor NF-κB. Activated NF-κB drives the expression of a vast array of pro-inflammatory mediators, including cytokines like tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), as well as matrix metalloproteinases (MMPs). This cytokine storm is directly responsible for the clinical signs of peri-implantitis. TNF-α and IL-1β stimulate the recruitment of inflammatory cells, increase vascular permeability (leading to bleeding), and are potent inducers of osteoclastogenesis—the process of bone resorption. They achieve this by upregulating the expression of RANKL (Receptor Activator of Nuclear factor Kappa-B Ligand) on osteoblasts and other cells. RANKL then binds to its receptor, RANK, on osteoclast precursors, driving their differentiation and activation into mature, bone-resorbing osteoclasts. The extremely high level of transcription of LPS synthesis genes observed in our PI samples means that the dysbiotic microbiome is actively manufacturing and shedding massive quantities of this molecule, creating a sustained, high-level inflammatory stimulus that directly drives the progressive bone destruction that is the hallmark of peri-implantitis.[15,16]

Beyond simply triggering inflammation, the functional profile of the PI microbiome revealed a community perfectly adapted to thrive within that self-generated inflammatory environment. This is most evident in the profound metabolic shift from saccharolysis (carbohydrate breakdown), which dominated in the healthy group, to proteolysis (protein breakdown) in the diseased group. In health, the primary nutrient source for the subgingival microbiome is dietary carbohydrates. Commensal organisms like *Streptococcus sanguinis*, which were highly active in our HI group, ferment these sugars into weak acids like lactate. This process maintains a relatively neutral pH and creates an environment that is unfavorable for the growth of many pathogenic anaerobes. In contrast, the inflammation in peri-implantitis leads to an increased flow of gingival crevicular fluid (GCF), a serum exudate rich in host proteins like albumin and degraded collagen from the damaged connective tissues. Our data show that the PI microbiome has actively switched on the genetic machinery, such as the arginine deiminase system (highly active transcripts for *arcA* and *arcB*), to utilize these proteins as its primary source of energy and carbon. This proteolytic metabolism has two devastating consequences. First, it directly contributes to the breakdown of the host's tissue matrix. Second, the end products of amino acid fermentation are not weak acids, but toxic and inflammatory compounds such as ammonia, hydrogen sulfide (H_2S), and butyrate. Ammonia can raise the local pH, further favoring the growth of pathogenic bacteria, and is cytotoxic to host cells. H_2S is also highly cytotoxic and has been shown to induce apoptosis in gingival fibroblasts and osteoblasts. Butyrate can inhibit host immune responses and further contribute to tissue damage. Therefore, the active proteolytic metabolism of the PI microbiome creates a vicious cycle: inflammation provides protein nutrients, which the bacteria metabolize into products that cause more tissue damage and inflammation.[17,18]

The upregulation of bacterial secretion systems and two-component systems provides further insight into the sophisticated pathogenic strategies of the dysbiotic community. Secretion systems are essentially molecular syringes that pathogenic bacteria use to inject effector proteins and toxins directly into host cells or to release degradative enzymes into the extracellular space. The high expression of genes for Type IV secretion systems, for example, indicates active efforts to manipulate host cell functions to the bacteria's advantage. Two-component systems are the sensory apparatus of bacteria, allowing them to constantly monitor their environment for signals-such as changes in pH, nutrient availability, or the presence of host defense molecules-and rapidly alter their gene expression in response. The high transcriptional activity of these systems in the PI group signifies a highly adapted and intelligent community that is actively coordinating its pathogenic functions in real-time response to the host's attempts to control the infection. This includes activating genes for virulence, stress resistance, and nutrient acquisition, as seen with the significant upregulation of iron transport systems. Iron is a critical nutrient that the host deliberately sequesters (a process called nutritional immunity) to limit bacterial growth. The active transcription of high-affinity iron uptake systems (siderophores, heme transporters) by the PI microbiome is a classic virulence trait, demonstrating its ability to overcome this key host defense mechanism and successfully colonize the inflammatory site.[19,20]

In contrast, the functional profile of the healthy implant microbiome paints a picture of active maintenance of homeostasis. The high activity of carbohydrate metabolism and fermentation pathways by streptococci is not merely a passive state. As mentioned, the production of lactic acid helps maintain a pH that suppresses pathogens. Furthermore, many of these commensal streptococci, especially S. *sanguinis* and S. *gordonii*, are known to produce hydrogen peroxide (H_2O_2), which is directly bactericidal to many anaerobic pathogens. Therefore, the transcriptional signature of the HI group reflects a community that is actively defending its niche and contributing to host health by producing metabolites that antagonize the growth of potential pathogens. The enrichment of biosynthetic pathways in the HI group also suggests a more self-sufficient and stable

community, less reliant on scavenging resources from host tissue breakdown. This study provides a functional, mechanistic explanation for the clinical observations in peri-implantitis. The disease is characterized by an active microbial consortium that is transcriptionally primed for pathogenesis. This community actively produces massive amounts of inflammatory triggers like LPS, directly fueling the host's destructive immune response. Simultaneously, it shifts its entire metabolism to exploit the byproducts of this inflammation, consuming host proteins and producing toxic metabolites that perpetuate the cycle of tissue damage. It employs sophisticated sensory and secretion systems to interact with and manipulate the host environment, while actively overcoming host defenses like nutritional immunity. This detailed functional insight moves our understanding of peri-implantitis from a descriptive, taxonomic level to a mechanistic one, revealing the specific biological pathways that are the engines of disease progression. This knowledge is paramount for developing next-generation therapeutics that aim not just to kill bacteria, but to disarm their pathogenic functions and restore a healthy, symbiotic host-microbe relationship.[18,19]

4. Conclusion

The subgingival microbiome in peri-implantitis exhibits a distinct and significantly different transcriptional profile compared to that of healthy implants, characterized by increased microbial diversity and a predominance of transcripts from pathogenic species like *Porphyromonas gingivalis* and Tannerella forsythia. The metatranscriptome of the peri-implantitis-associated microbiome is functionally geared towards pathogenicity. There is a significant upregulation of genes and metabolic pathways involved in the biosynthesis of the inflammatory endotoxin LPS, bacterial secretion of virulence factors, proteolytic metabolism of host proteins, and acquisition of essential nutrients like iron. Conversely, the microbiome associated with healthy implants is transcriptionally dominated by commensal streptococci actively engaged in carbohydrate metabolism and fermentation, a functional state consistent with microbial homeostasis and active defense against pathogenic colonization.The identification of these key dysbiotic functional pathways provides a deeper understanding of the molecular mechanisms driving peri-implantitis pathogenesis and offers promising new targets for the development of advanced diagnostic tools and functionally-modulating therapeutic interventions.

5. References

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