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Revealing the Occurrence of Antimicrobial Resistance in Microbiome and Metabolic Profile of Orthodontic Patients with White Spot Lesions (WSL)

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Abstract

This study investigates antimicrobial-resistant (AMR) strains in the salivary microbiome of patients with White Spot Lesions (WSL) using a metagenomic approach. The aim is to better understand microbial-host interactions in dental caries and bacterial diseases in patients with fixed orthodontic appliances. Saliva samples from three WSL patients were collected and analyzed for bacterial diversity, AMR, and metabolic profiling. Metagenomic sequencing identified Acetobacter and Lactobacillus species as predominant in the saliva of WSL patients, with variations in microbial diversity between samples. WSLMic3 had lower Acetobacter and higher Lactobacillus compared to WSLMic1 and WSLMic2. Additionally, ammonia-oxidizing (89.8%) and sulfate-reducing bacteria (85.4%) were the most prevalent. AMR was assessed using the Kirby-Bauer disc diffusion method, revealing the challenge of antibiotic resistance in managing oral conditions. DNA extraction was performed with the ZR Microbe DNA MiniPrep™ kit, followed by metagenomic analysis using the GAIA 2.0 workflow and GLE module for genus-level identification. Alpha and Beta diversity indices (Chao1, Shannon, Simpson) were calculated, and pathway-level metabolic profiling was predicted using Gene Ontology (GO) terms. This study highlights the importance of profiling pathogenic strains linked to WSL and the role of AMR in oral microbiota. Identifying these strains could aid in developing targeted therapies to manage WSL more effectively and address AMR challenges in orthodontic patients.

Keywords: *Genetics, Metabolic Profiling, Metagenomics, Microbiome, Phylogenetic Distribution, Saliva, White Spot Lesion.*

Introduction

White Spot Lesions (WSL) represent a significant challenge in dental health, often heralding the early stages of dental caries [1]. These lesions, characterized by demineralization of tooth enamel, signify an imbalance in the oral microbiome and metabolic processes within the oral cavity [2]. Traditional diagnostic methods have provided insights into the presence of WSL; however, unraveling the intricate interplay between microbial composition and metabolic activity demands cutting-edge techniques [3]. Metagenomic analysis coupled with metabolic profiling emerges as a promising avenue to dissect the complex ecosystem of the oral microbiota and its metabolic landscape [4]. Metagenomic analysis offers a holistic view of the microbial communities inhabiting the oral cavity, transcending the limitations of culture-based techniques. By sequencing the collective DNA within a sample, metagenomics enables

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the identification and characterization of diverse microbial taxa, including both abundant and rare species [5]. In the context of WSL, this approach provides crucial insights into the dysbiotic shifts occurring within the oral microbiome, shedding light on potential microbial biomarkers associated with lesion progression [6]. Complementing metagenomic analysis, metabolic profiling delves into the functional dynamics of the oral microbiota by examining the repertoire of metabolites present in saliva [7,8]. Metabolites serve as the molecular fingerprints of microbial activity, reflecting the intricate metabolic exchanges occurring within the oral ecosystem [3,9]. By employing advanced analytical techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy, researchers can map out the metabolic signatures associated with WSL, unveiling metabolic pathways implicated in lesion development and progression [10]. Integration of metagenomic and metabolic data holds immense promise in elucidating the etiology and pathogenesis of WSL, offering a comprehensive understanding of the microbial-host interactions driving dental caries [11]. Moreover, this integrative approach paves the way for the development of targeted diagnostic tools and personalized therapeutic interventions aimed at mitigating the risk of AMR strains in WSL development and preserving dental health. In this study, we delve into the intricacies of metagenomic analysis and metabolic profiling applied to salivary samples with WSL to identify the AMR strains, by identification of such AMRs further study can be constructed to tackle these strains and search for effective antimicrobial alternatives and exploring the potential implications for clinical practice and public health. By unraveling the mysteries shrouding WSL through the lens of modern molecular techniques, this study aims to pave the way for novel strategies in preventive dentistry and personalized oral healthcare.

Materials and Methods

Sample Collection

Salivary specimens from three patients diagnosed with White Spot Lesions (WSL) were meticulously collected in sterile containers and promptly chilled on ice. For the culture-independent analysis, 10 ml aliquots of each sample were subjected to centrifugation at 12,000 x g, with the resultant pellets undergoing three wash cycles in phosphate-buffered saline (PBS) at pH 6.4 before being preserved at -80°C.

In the subsequent bacterial isolation and biochemical characterization, the saliva samples were serially diluted to 10^6 and inoculated onto Nutrient Agar (Himedia), followed by incubation overnight at 37°C. Post-incubation, colony-forming units (CFU/ml) were quantified utilizing the plate count method [12], and colonies with distinct morphologies were sub-cultured and maintained on Luria-Bertani Agar (Himedia) for further analysis. To identify the species, isolates were streaked onto Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) [13]. Biochemical characterization was conducted according to the protocols outlined in Bergey's Manual of Systematic Bacteriology. This included Gram staining and a series of biochemical assays, such as the IMViC tests (Indole, Methyl Red, Voges-Proskauer, and Citrate utilization), Mannitol motility, Triple Sugar Iron, and catalase testing [14,15].

Antibiotic Sensitivity Assay

The antibiotic susceptibility profiles of the bacterial isolates were determined utilizing the Kirby-Bauer disc diffusion methodology. After an overnight incubation of the cultures in nutrient broth at 37°C, 100 µl of each bacterial suspension was evenly distributed onto Mueller-Hinton Agar (Sigma, Switzerland). Subsequently, antibiotic discs from Himedia, encompassing a range of agents such as Ampicillin (10 µg), Erythromycin (15 µg),

Tetracycline (30 µg), Ceftriaxone (30 µg), Penicillin G (10 µg), Chloramphenicol (30 µg), Vancomycin (30 µg), Gentamicin (10 µg), Cefpodoxime (10 µg), Norfloxacin (10 µg), Nalidixic Acid (30 µg), Polymyxin B (300 µg), and Ceftazidime (30 µg), were meticulously positioned on the agar surface. The plates were incubated at 37°C for 24 hours. Following incubation, the zones of inhibition surrounding each antibiotic disc were measured with precision, and the data were interpreted in alignment with CLSI standards [16].

Metagenomic DNA Isolation and 16S rRNA Gene Amplification

DNA extraction from the trio of samples was executed utilizing the ZR Microbe DNA MiniPrep™ kit (Zymo Research), adhering to the manufacturer's specifications. Initially, 200 µl of the pellet was re-suspended in phosphate-buffered saline (PBS) and combined with 650 µl of lysis solution and a 2% v/v proteinase K solution. This mixture was introduced to ZR Bashing Beads and subjected to a 30-minute incubation period. The sample was then placed in a bead beater equipped with a 2 ml tube holder and processed at maximal speed for 10 seconds, followed by centrifugation at 10,000 x g for 1 minute.

Following this, 400 µl of the supernatant was transferred into a new tube and centrifuged at 8,000 x g for 1 minute. To this supernatant, 1,200 µl of DNA binding buffer was added. From this solution, 800 µl was further transferred to a separate tube and subjected to another round of centrifugation at 10,000 x g for 1 minute. The resultant DNA was eluted and prepared for subsequent analysis. The quantity and purity of the isolated DNA were evaluated using a NanoDrop at A 260/280 (NanoDrop ND-1000, USA), and the DNA's integrity was confirmed via 1% agarose gel electrophoresis. The DNA was then stored at -20°C until further use.

For PCR amplification, 40 ng of the extracted DNA was utilized to amplify the V3-V4 regions of the 16S rRNA gene with primers F5'-AGAGTTTGATCMTGGCTCAG3' and R5'-TACGGYTACCTTGTTACGACTT3'. The PCR reaction mixture included 2 µL of genomic DNA, 5 µL of 10× PCR buffer, 3.2 mM MgCl2, 0.5 mM of each deoxynucleotide triphosphate, 1 U Taq DNA Polymerase, 10 pM of each primer, and 37.1 µL of autoclaved distilled water (all reagents from Invitrogen Corporation, Carlsbad, CA, USA). The amplification protocol comprised an initial denaturation at 95°C for 3 minutes, followed by 25 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 2 minutes, concluding with a final extension at 72°C for 10 minutes and holding at 4°C.

The PCR products were assessed for quantity and purity using a NanoDrop at A 260/280 (NanoDrop ND-1000, USA) and were subsequently separated by agarose gel electrophoresis. Finally, the prepared libraries were subjected to sequencing using the Illumina MiSeq platform.

Metagenomic Analysis

Metagenomic exploration was undertaken using the GAIA 2.0 platform (https://metagenomics.sequentiabiotech.com/), a resource from Sequentia Biotech SL. This analysis harnessed the EPI2ME16S workflow, which enables genus-level taxonomic identification through the GLE (Graphical Logarithmic Examination) module and provides a detailed database for species and subspecies-level scrutiny. The approach involved executing a sequence similarity search of the 16S rRNA gene against a vast bacterial RNA gene sequence repository, with the resulting best matches classified according to identity percentage and query coverage.

Employing the GAIA 2.0 toolkit, we analyzed sequence diversity, disparity indices, and the genus-phylum classification for the trio of saliva microbiome samples from WSL patients (WSLMic1, WSLMic2, and

WSLMic3). Alpha and Beta diversity indices were derived and validated using Chao1, Shannon, and Simpson estimators. The relative abundance of genera, represented as operational taxonomic units (OTUs), was computed and compared across the WSLMic samples. Furthermore, pathway-level metabolic profiles were predicted based on Gene Ontology terms and subjected to comparative analysis to gain insights into the microbial community structure among the WSLMic1, WSLMic2, and WSLMic3 samples.

Results

Isolation and Biochemical Characterization of AMR Strains from WSL Saliva

After performing serial dilutions and plating, 26 distinct colonies with varying morphologies emerged following incubation at 37°C from the WSL salivary samples. The majority of these colonies displayed circular forms with smooth, entire margins and exhibited diverse shades of white. Of these 26 isolates, 22 were classified as Gram-positive and 4 as Gram-negative and 8 strains are determined to be AMR strains by antibiotic susceptibility test, with the predominant morphology being rod-shaped. Biochemical analyses and subsequent assays identified a significant prevalence of Acetobacter sp. among the isolates. Additionally, the majority of isolates demonstrated susceptibility to antibiotic testing, indicating the absence of pathogenic strains and corroborating the effectiveness of the sterile collection method employed.

Metagenomic Analysis of Basic Parameters

The microbial composition of salivary samples from three distinct WSL patients was investigated through Sanger Illumina 1.9 sequencing, focusing on the V3-V4 region of the bacterial 16S rRNA gene and the fungal ITS region. This comprehensive sequencing produced untrimmed reads totalling 25,880, 21,704, and 29,704, with sequence lengths spanning from 8 to 21,304, 8 to 17,460, and 1 to 17,460 for WSLMic1, WSLMic2, and WSLMic3, respectively. After trimming, the sequences amounted to 23,060, 18,206, and 19,625, with lengths ranging from 501 to 19,672, 501 to 14,205, and 503 to 14,205 for the respective samples. The average GC content across the samples was determined to be 54%, as compared to the A+T disparity index (Table 1, Fig 1). This uniform 54% GC content suggests a notable convergence in the genomic sequences among the samples.

Table 1. Sample Details of 16s RNA Gene Amplicon Region among Selected Three Different Saliva Microbiome Samples WSLMic1, WSLMic2 and WSLMic3

Samples	SRA Accession No.	Untrimmed		Trimmed		Mean GC	Encoding
		Total # of residues	Sequence range	Total # of residues	Sequence range		
WSLMic 1	SAMN13899470	25880	8-21304	23060	501-19672	54	Sanger/Illumina 1.9
WSLMic 2	SAMN13931401	21704	8-17460	18206	501-14205		
WSLMic 3	SAMN13931402	29704	1-17460	19625	503-14205		

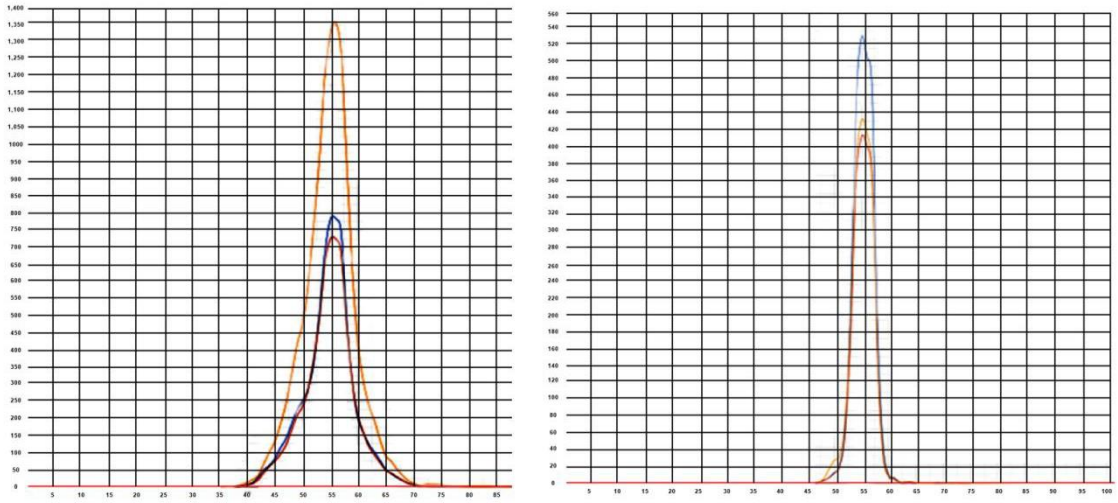

Figure 1. Appearance of untrimmed (left) and trimmed (right) reads of GC content found between WSLMic1, WSLMic2 and WSLMic3 samples. The uncovered region (sequence base pair) was removed in the trimmed reads.

Assessment of Diversity and Disparity Indices

The evaluation of microbial species richness (quantified by observed OTUs), dominance (measured by the Simpson index), evenness (reflected in the Shannon index), and singleton counts (represented by the Chao1 value)—all key indicators of alpha diversity—showed pronounced discrepancies among the three saliva samples from WSL patients (Table 2).

Table 2. Examination of Alpha Diversity in Three Varied Salivary Microbiome Samples: WSLMic1, WSLMic2, and WSLMic3

Samples	Observed	Chao1	Se. Chao1	Shannon	Simpson	Fisher
WSLMic1	102	102	0	0.81431	0.27908	13.73453
WSLMic2	110	110.2308	0.58765	0.89022	0.30487	15.56962
WSLMic3	110	110	0	1.10752	0.3945	15.37968

WSLMic2 and WSLMic3 exhibited analogous species richness, whereas WSLMic1 showed the lowest diversity, characterized by the minimal number of observed OTUs. Notably, WSLMic3 exhibited the highest degree of species evenness. Beta diversity metrics revealed substantial divergence between WSLMic1 and both WSLMic2 and WSLMic3, with a moderate level of diversity between WSLMic1 and WSLMic3. The smallest disparity was observed between WSLMic2 and WSLMic3. Genomic correlations of beta genetic diversity indicated that WSLMic1 was comparatively distinct from WSLMic2 and WSLMic3, with correlation values spanning from 0.061 to 0.078. In contrast, WSLMic2 and WSLMic3 displayed a close relationship, with their correlation value reaching 0.078 (Table 3).

Table 3. Evaluation of Beta Diversity in Three Distinct Salivary Microbiome Samples: WSLMic1, WSLMic2, and WSLMic3

Samples	WSLMic1	WSLMic2	WSLMic3
WSLMic1	0	0.078	0.061
WSLMic2	0.078	0	0.018
WSLMic3	0.061	0.018	0

Evaluation of Sequence Base-Pair Quality

The examination of sequence base-pair quality revealed minor variations in WSLMic1. The overall base-pair quality spanned lengths between 8 and 19. Additionally, the standard deviation bar graph for each sample suggested that base-pair quality might extend between 3 and 28 (Fig 2).

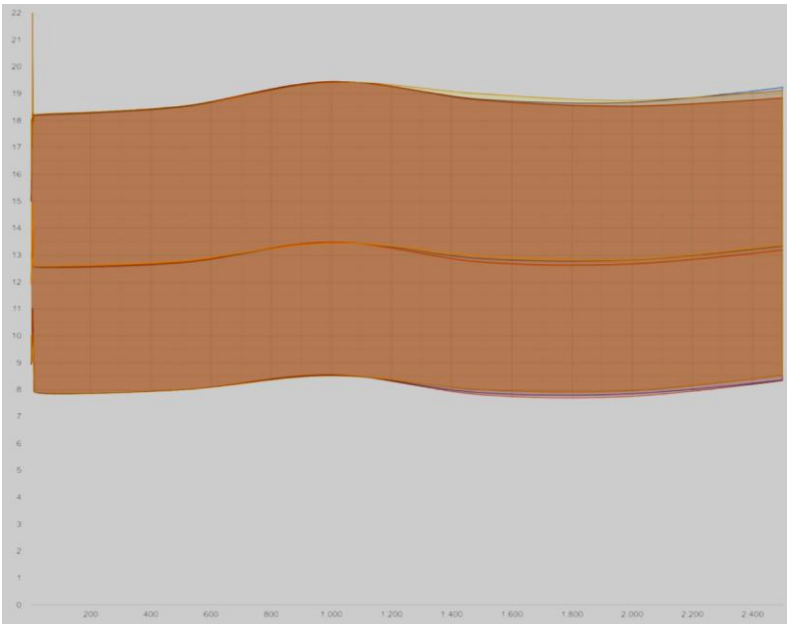

Figure 2. Prediction of sequence quality check/base upon WSLMic1, WSLMic2 and WSLMic3 samples.

Analysis of Sequence Base-Pair Length Distributions

Significant distributions of base-pair lengths—A, T, G, and C—were identified at the intervals of 100-550 bp, 1000-2000 bp, and 2500-3500 bp (Fig 3). This distribution pattern is attributed to the overlapping potential observed within the samples [17]. It is inferred that these sequence variations could occur within these specific ranges, possibly enabling genetic exchanges between species and impacting evolutionary transitions in varied ecological niches.

Figure 3. Prediction of sequence length distribution upon WSLMic1, WSLMic2 and WSLMic3 samples. The X-axis denotes the position of the base and the Y-axis denotes numbers of bacterial species found in the sample.

Sequencing Utilizing Small RNA Adapters

A compact small RNA adapter was utilized for sequencing fragmented DNA. This adapter, consisting of a short DNA fragment set, facilitates the identification of adapter sequences within genomes, streamlining the sequencing procedure through a method termed "tagmentation" [18]. Following sequencing, the adapter sequences were excised. The length distribution of adapter sequences across all three samples remained fairly consistent around 1000 base pairs. However, the concentration relative to sequence length varied within a range of 0.017 to 0.023 (Fig 4).

Figure 4. Prediction of sequence length distribution of adaptor content upon WSLMic1, WSLMic2 and WSLMic3 samples. The X-axis denotes the position of the base and the Y-axis denotes the percentage of adapter content.

Taxonomic Classification

The microbial profile differences among the three palm wine samples were evaluated by analyzing the relative abundances of dominant taxa at both the genus and phylum levels (Fig. 5). The bacterial phyla identified as most prevalent across the samples included Proteobacteria (91.6%), Firmicutes (5.6%), and Tenericutes (2.6%). Within the Proteobacteria phylum, the dominant genera were Acetobacter (89.6%), Gluconobacter (1%), Burkholderia (1%), and Pelobacter (0.3%). For the Firmicutes phylum, the prominent genera were Lactobacillus (4.6%), Candidatus Phytoplasma (1%), and Clostridium (1%). WSLMic3, in particular, displayed a diminished abundance of Acetobacter and a heightened abundance of Lactobacillus compared to WSLMic1 and WSLMic2 (Fig. 5).

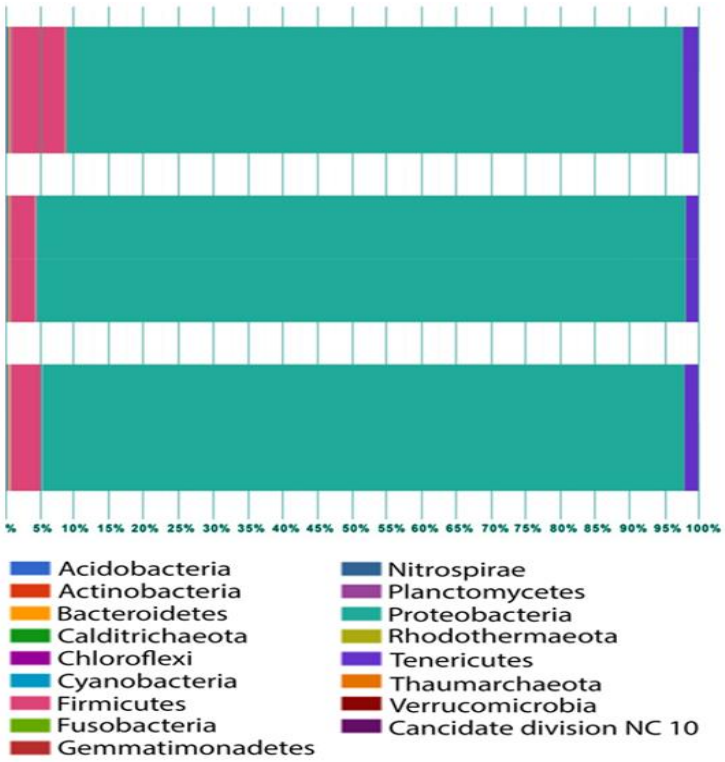

Figure 5. Construction of taxonomic plot analysis phylum (top) and genus level (bottom) upon WSLMic1, WSLMic2 and WSLMic3 samples

Assessment of OTU Relative Abundance

The distribution of OTU relative abundances across the three samples was analyzed and clustered using a heat map, revealing differences in the prevalence of dominant bacterial species (Fig. 6).

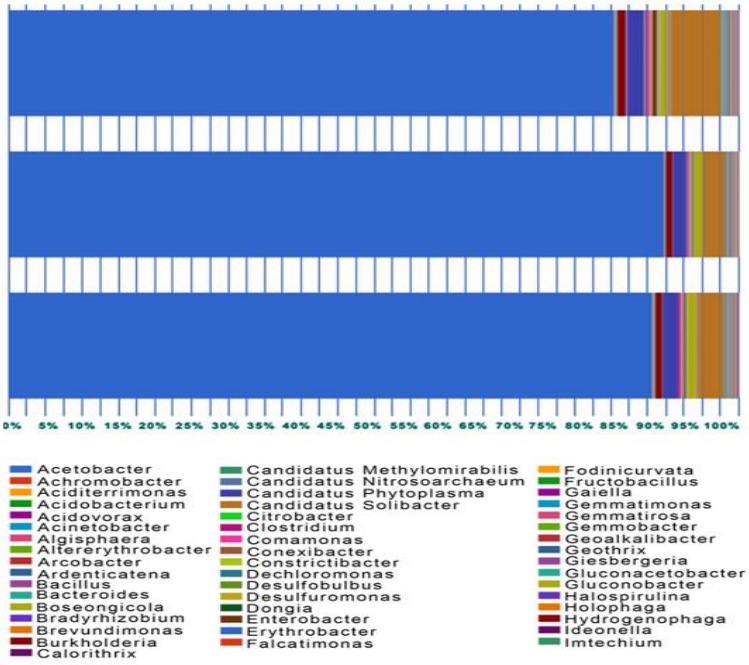

Figure 6. Heat map illustrating the comparative analysis of key bacterial species among the WSLMic1, WSLMic2, and WSLMic3 samples.

Cluster Profiling of OTU Relative Abundance

The WSLMic1 cluster exhibited a preponderance of genera such as Zymomonas, Sulfurospirillum, Erythrobacter, Bacillus, Aciditerrimonas, Arcobacter, and Oenococcus, indicating a rich microbial diversity. Conversely, the WSLMic2 cluster was dominated by Sulfurovum, Gluconacetobacter, Paeniciostridium, Acidobacterium, and Thiobacter. The WSLMic3 cluster, on the other hand, was predominantly represented by Leptotrichia, Acidovorax, Steroidobacter, Desulfuromonas, Enterobacter, Lactobacillus, Citrobacter, and Sulfitobacter. These observations point to the presence of several potentially probiotic strains within the WSL microbiota. Further, the top 10 operational taxonomic units with the highest enrichment across WSLMic1, WSLMic2, and WSLMic3 were analyzed based on their metabolic profiles (Fig. 7).

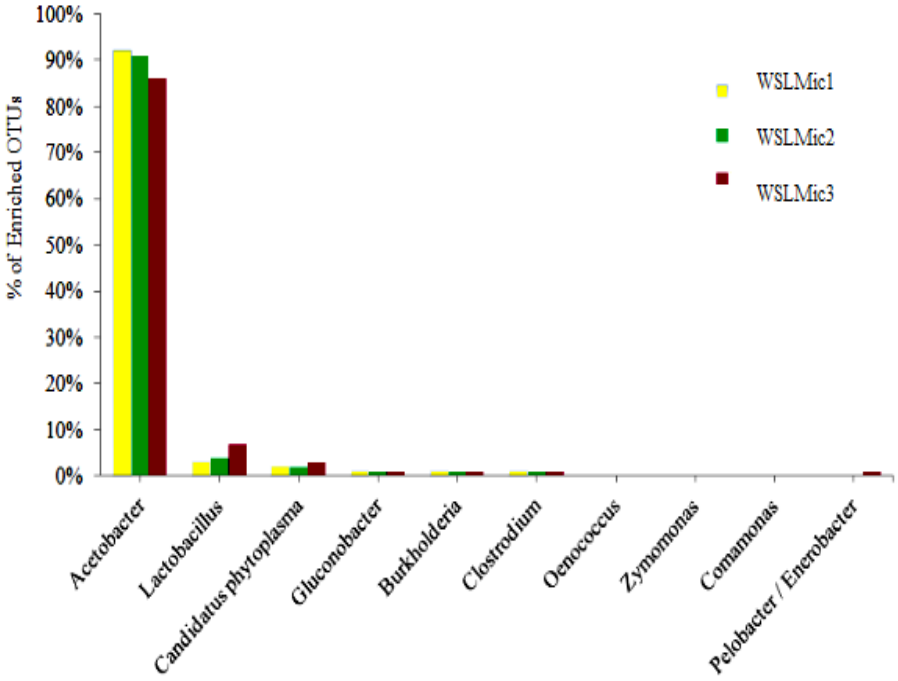

Figure 7. Top 10 enriched Operational taxonomic units found between WSLMic1, WSLMic2 and WSLMic3 samples based on its metabolic profiling.

Analysis revealed that *Acetobacter* sp. dominated the samples, representing 90% of the bacterial population. The remaining 10% included a diverse array of OTUs such as Lactobacillus, Candidatus, Gluconobacter, Burkholderia, Clostridium, Oenococcus, Zymomonas, and Enterobacter. These probiotic microorganisms could serve as advantageous agents within the human gut microbiota, potentially fostering improved health and bolstering immune responses.

Assessment of Functional Metabolic Profiling

Functional metabolic profiling of the WSL salivary microbial communities was carried out through gene ontology term analysis (Fig 8). The analysis revealed that ammonia oxidizers were the most prevalent, comprising 89.8% of the microbial community. This was followed by sulfate reducers at 85.4%, nitrite reducers at 84.2%, nitrogen fixers at 82.6%, atrazine-degrading organisms at 82%, and acid producers at 81.9%.

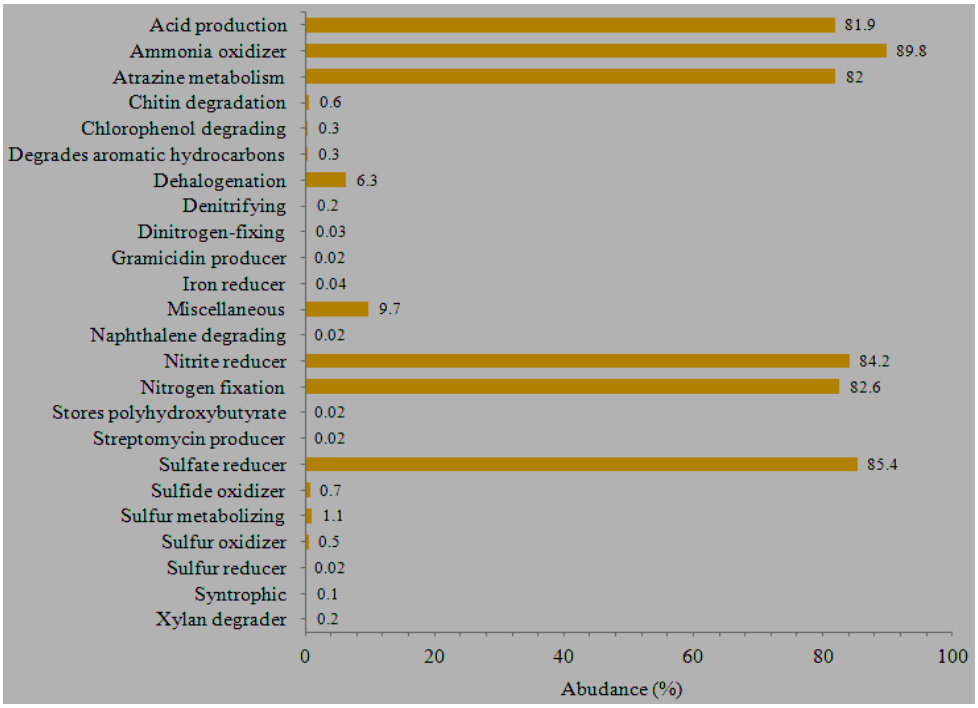

Figure 8. Visualization of metabolic profiling and the relative abundance of different metabolic pathways across the WSLMic1, WSLMic2, and WSLMic3 samples.

Discussion

The findings from the isolation and biochemical characterization of bacteria from WSL saliva provide valuable insights into the AMR microbial composition associated with white spot lesions. The predominance of Gram-positive isolates, particularly Acetobacter sp., suggests a potentially important role of these microorganisms in the etiology of WSL. Furthermore, the sensitivity of the isolates to antibiotics not only indicates the absence of pathogenic strains but also validates the efficacy of the sterile collection method employed, ensuring the reliability of subsequent analyses. Metagenomic analysis of the microbial communities within WSL salivary samples unveils the intricate diversity and composition of the oral microbiome [19]. The observed variations in species richness, dominance, and evenness among the samples highlight the dynamic nature of microbial ecosystems associated with WSL [20]. Particularly noteworthy is the dominance of Acetobacter within the microbial community, corroborating the findings from the isolation and biochemical characterization studies [20,21]. The presence of other genera such as Lactobacillus, Gluconobacter, and Burkholderia further underscores the complexity of microbial interactions within WSL [22-24].

Taxonomic classification reveals the predominance of Proteobacteria at the phylum level, with Acetobacter representing a substantial proportion of the bacterial community. Interestingly, differences in the relative abundance of Acetobacter and Lactobacillus among the samples suggest potential variations in microbial community structure and metabolic activity, which may influence WSL pathogenesis [25-28]. The relative abundance analysis of operational taxonomic units (OTUs) highlights the diversity of bacterial species within WSL salivary samples. Clustering based on relative abundance elucidates distinct microbial profiles associated with each sample, emphasizing the potential probiotic properties of certain bacterial strains. Enriching Acetobacter and other probiotic genera underscores their significance in modulating oral microbial ecology and potentially influencing WSL development. Functional metabolic profiling

provides further insights into the metabolic capabilities of the microbial community inhabiting WSL salivary samples [29]. The prevalence of ammonia oxidizers, sulfate reducers, and nitrogen fixers underscores the metabolic versatility of oral microorganisms. Moreover, the presence of acid producers suggests a potential link between microbial metabolic activity and enamel demineralization, warranting further investigation into the role of metabolic pathways in WSL pathogenesis.

Overall, the integration of isolation, biochemical characterization, metagenomic analysis, and functional metabolic profiling offers a comprehensive understanding of the microbial ecology and metabolic landscape associated with WSL. These findings pave the way for targeted interventions aimed at modulating oral microbial communities and preserving dental health, ultimately advancing strategies for the prevention and management of WSL and related dental conditions.

Conclusion

Metagenomic analysis significantly enhances our grasp of microbial diversity in environmental samples. This study aimed to characterize the microbial diversity and metabolic profile of saliva with white spot lesions (WSL). Biochemical assays revealed a high abundance of Acetobacter species. Using 16S amplicon sequencing, we assessed the alpha and beta diversity of the WSL microbiome, comparing it to culture-based results. WSLMic3 had lower Acetobacter levels and higher Lactobacillus levels compared to WSLMic1 and WSLMic2, with ammonia oxidizers (89.8%) and sulfate reducers (85.4%) being the most abundant microbes.

Many recent approaches have therefore been developed to combat white spot lesions, which involve a range of preventive pharmaceutical therapeutics to intercept minimally invasive procedures [29, 30]. The mineralization of artificial substitutes such as calcium carbonate, PRF, and nano-hydroxyapatite has demonstrated clinical benefits in various fields [31-33]. The most commonly encountered challenge includes oral microbial resistance to the currently available therapeutics. Identification and functional metabolic profiling of potential pathogenic WSL strains isolated from the saliva would enable the clinician to develop a database of pathogens responsible for WSL infection and create novel therapeutic agents against WSL management.

Conflict of Interest

Nil.

Acknowledgement

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