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Pilot 16s rRNA-based salivary microbiome profiling reveals candidate microbial signatures in autoimmune and immunocompromised patients: An exploratory study from Iraq

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Abstract

Objective: Immune system disorders may disrupt the oral microbiome, but data from Middle Eastern populations remain scarce. This pilot study aimed to characterise salivary bacterial composition in autoimmune and immunocompromised patients compared with healthy controls and to identify potential disease-associated microbial signatures using 16S rRNA metagenomic sequencing.

Methods: A comparative cross-sectional pilot study was conducted in Ninewa Governorate, Iraq. Saliva samples from 15 participants (five autoimmune patients, five immunocompromised patients, and five healthy controls) underwent bacterial DNA extraction, 16S rRNA gene amplification, and metagenomic sequencing. Relative abundances were compared using Kruskal–Wallis and Mann–Whitney U tests with false discovery rate (FDR) correction. Linear discriminant analysis effect size (LEfSe) identified candidate discriminatory taxa.

Results: The control group showed higher abundance of commensal taxa, particularly *Unclassified streptococcus*, *Salivarius-vestibularis*, and *Odontolyticus*. Autoimmune and immunocompromised groups exhibited increased levels of *Nigrescens*, *Macacae-mucosa-sicca*, *Fusobacterium nucleatum*, and *Parainfluenzae*. However, no taxon remained statistically significant after FDR correction ($q > 0.05$). LEfSe analysis identified *Nigrescens* (LDA = 5.12) and *Macacae-mucosa-sicca* (LDA = 4.40) as the strongest discriminatory candidates.

Conclusions: This pilot study reveals preliminary compositional differences in salivary microbiomes associated with immune dysfunction. The candidate microbial signatures, particularly *Nigrescens* and *Macacae-mucosa-sicca*, warrant validation in larger, adequately powered cohorts before clinical application.

Keywords: Oral microbiome, autoimmune diseases, immunocompromised host, saliva, 16S rRNA sequencing, Iraq

Plain English Summary

This small pilot study examined the bacteria found in the saliva of people with immune system problems. The researchers collected saliva from 15 people in Iraq: five with autoimmune diseases (where the immune system attacks the body), five with weakened immune systems (due to treatments like chemotherapy), and five healthy individuals. Using a genetic technique called 16S rRNA sequencing, they identified which bacteria were present. Healthy individuals had more of the usual “friendly” mouth bacteria. People with immune disorders had higher amounts of certain other bacteria, such as *Nigrescens* and *Macacae-mucosa-sicca*. However, because the study included only five people per group, the results are not strong enough to draw firm conclusions. Larger studies are needed to confirm

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whether these bacterial patterns could someday help doctors diagnose or monitor immune diseases using a simple saliva test.

Introduction

The oral cavity hosts a complex and dynamic microbial ecosystem comprising bacteria, fungi, viruses, and other microorganisms that normally coexist in a symbiotic balance with the human host. This resident microbiota contributes to health by protecting mucosal surfaces, preventing pathogen colonisation, and modulating local immune responses. Disruption of this finely tuned equilibrium, termed dysbiosis, has been increasingly linked not only to oral diseases such as caries and periodontitis but also to a range of systemic conditions, including diabetes, cardiovascular disease, and autoimmune disorders (1, 2).

Emerging evidence suggests a bidirectional relationship between the immune system and the oral microbiome. Immune competence helps maintain microbial stability, while immune dysregulation can permit overgrowth of opportunistic taxa and loss of beneficial commensals. In autoimmune diseases, chronic inflammation and persistent immune activation may drive marked shifts in microbial community structure. Similarly, immunocompromised individuals, whether due to underlying disease or immunosuppressive therapies, often exhibit impaired microbial control, potentially leading to distinct oral microbial profiles (3, 4). These observations have raised the possibility that salivary microbiota signatures might serve as non-invasive biomarkers for immune-mediated conditions (5).

Traditional culture-based microbiological methods capture only a fraction of oral microbial diversity, as many species remain uncultivable under standard laboratory conditions. The advent of high-throughput sequencing targeting the 16S ribosomal RNA (rRNA) gene has revolutionised microbiome research, enabling comprehensive, culture-independent characterisation of microbial communities at high taxonomic resolution (6). This approach has been successfully applied to identify disease-associated microbial patterns in periodontitis, inflammatory bowel disease, rheumatoid arthritis, and systemic lupus erythematosus (7, 8).

Despite these advances, studies examining the salivary microbiome in autoimmune and immunocompromised patients remain limited, particularly in Middle Eastern populations. Iraq, with its unique genetic and environmental background, has been underrepresented in global microbiome research. To our knowledge, no published study has characterised the salivary bacterial composition of autoimmune or

immunocompromised patients from Iraq using metagenomic sequencing.

Therefore, this pilot study aimed to (1) characterise the salivary bacterial composition of autoimmune and immunocompromised patients compared with healthy controls in Ninewa Governorate, Iraq, using 16S rRNA metagenomic sequencing; and (2) identify candidate microbial signatures that might distinguish these groups, while explicitly acknowledging the exploratory nature of the findings due to the small sample size.

Materials and Methods

Study design and setting

This comparative cross-sectional pilot study was conducted in Ninewa Governorate, Iraq, between November 2025 and April 2026. The study adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for cross-sectional studies. Participants were recruited from three specialised healthcare facilities: the Specialised Oncology and Nuclear Medicine Hospital (Mosul), Al-Wafaa Specialised Centre for Diabetes and Endocrinology, and Al-Hadbaa Specialised Hospital.

Study population and sample size

A total of 15 participants were enrolled and divided into three groups:

Autoimmune group (AIG; n = 5): Patients with a confirmed diagnosis of an autoimmune disease (including systemic lupus erythematosus, rheumatoid arthritis, or Sjögren's syndrome) made by a specialist physician. All were enrolled before initiation of immunosuppressive therapy.

Immunocompromised group (ICG; n = 5): Patients receiving immunosuppressive treatment at the time of sample collection, including corticosteroids, chemotherapeutic agents, biologic response modifiers, or post-transplant medications. Specific diagnoses included haematological malignancies (n = 3) and solid organ transplant recipients (n = 2).

Control group (CG; n = 5): Healthy individuals without any diagnosed immunological disorder, chronic systemic disease, or active oral infection. Controls were selected to match the age distribution of the patient groups.

The sample size (n = 5 per group) was determined by practical constraints (budget and sequencing availability) and is appropriate only for an exploratory pilot study. No formal power calculation was performed, and the study was not designed to detect statistically significant

differences after rigorous multiple-comparison correction.

Inclusion criteria

Participants aged 19–50 years with a documented diagnosis of an autoimmune disease or an immunocompromised condition (as defined above) were included. Healthy controls had no known immune disorders, chronic diseases, or oral infections. All participants provided written informed consent.

Exclusion criteria

We excluded individuals with active oral infections (e.g., oral candidiasis, herpetic stomatitis), recent dental procedures (<4 weeks), smoking habit (current or within the past year), pregnancy, chronic systemic diseases unrelated to the study (e.g., uncontrolled diabetes mellitus, chronic kidney disease), or use of probiotics, antibiotics, antifungals, or antimicrobial mouthwash within two weeks before sample collection. Saliva samples yielding poor DNA quality or unsuccessful PCR amplification were also excluded from sequencing analysis.

Saliva collection

Unstimulated whole saliva was collected between 9:00 and 11:00 AM to minimise circadian variation. Participants refrained from eating, drinking (except water), smoking, and performing oral hygiene procedures for at least 30 minutes before collection. Using the passive drooling method, approximately 2–5 mL of saliva was collected into sterile polypropylene containers. Samples were immediately placed on ice, transferred to sterile 2 mL Eppendorf tubes within 30 minutes, and stored at –80°C until DNA extraction.

DNA extraction and quality assessment

Genomic DNA was extracted from 500 µL of each saliva sample using the Foregene Bacterial DNA Extraction Kit (Foregene, Chengdu, China) following the manufacturer's protocol. The procedure included enzymatic lysis with lysozyme (20 mg/mL) and proteinase K, followed by binding to a silica membrane spin column, several wash steps, and final elution in 50 µL of elution buffer. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by measuring absorbance at 260 nm (DNA concentration) and the 260/280 nm ratio (protein contamination, acceptable range 1.8–2.0). All samples met quality criteria.

PCR amplification of the 16S rRNA gene

The V1–V9 hypervariable regions of the bacterial 16S rRNA gene were amplified using the

universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCARCCGCA-3'). Each 20 µL PCR reaction contained: 10 µL GoTaq G2 Green Master Mix (Promega, Madison, WI, USA), 2 µL template DNA (approx. 20 ng), 1 µL of each primer (10 µM), and 6 µL nuclease-free water. Thermal cycling conditions were: initial denaturation at 95°C for 3 minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30 seconds; followed by a final extension at 72°C for 3 minutes. PCR products were visualised by electrophoresis on a 1.5% agarose gel stained with SafeRed (NucleoVision) to confirm successful amplification (expected band size ~1500 bp).

Metagenomic sequencing and bioinformatics

Amplicon libraries were prepared and sequenced at Zymo Research Corporation (Irvine, CA, USA) using an Illumina MiSeq platform with 2 × 300 bp paired-end chemistry. Raw sequence data were demultiplexed and quality-filtered using the DADA2 pipeline (version 1.20) within QIIME2 (version 2023.5). Amplicon sequence variants (ASVs) were assigned taxonomy using the SILVA 138.1 16S rRNA database (99% similarity threshold). The relative abundance of each bacterial taxon was calculated as the proportion of total ASV counts per sample. Samples were rarefied to an even depth of 10,000 reads per sample for downstream analysis.

Statistical analysis

All statistical analyses were performed using R software (version 4.2.2) with the phyloseq, vegan, and MicrobiomeAnalyst packages. Given the non-normal distribution of relative abundance data (Shapiro–Wilk test, $p < 0.05$), non-parametric tests were employed. Differences in bacterial relative abundances among the three groups were assessed using the Kruskal–Wallis rank sum test. For taxa with Kruskal–Wallis $p < 0.05$, pairwise comparisons between groups were performed using the Mann–Whitney U test. To control for false discovery due to multiple comparisons (testing of multiple taxa), the Benjamini–Hochberg procedure was applied to the pairwise comparison p-values, with a significance threshold of $q < 0.05$. Effect sizes for Mann–Whitney U tests were calculated as $r = Z/\sqrt{N}$, where Z is the standardised test statistic and N is the total number of observations.

Candidate microbial biomarkers were identified using linear discriminant analysis effect size (LEfSe) (9), with an alpha value of 0.05 for the Kruskal–Wallis test and a logarithmic linear discriminant analysis (LDA) score threshold of

2.0. Fold changes were calculated as the ratio of mean relative abundance in the disease group to that in the control group; for zero values in controls, a pseudocount of 0.001% was added to enable calculation.

All results are reported as mean $\pm$ standard deviation unless otherwise specified. Given the exploratory pilot nature of this study, findings with nominal $p < 0.05$ (before multiple comparison correction) are reported as trends or candidate signatures rather than definitive differences.

Results

Participant characteristics

All 15 enrolled participants completed the study. The mean age was 34.6 years (range 21–48), with no significant difference between groups (Kruskal–Wallis $p = 0.62$). The autoimmune group comprised two patients with systemic lupus erythematosus, two with rheumatoid arthritis, and one with Sjögren's syndrome. The immunocompromised group included three patients receiving chemotherapy for haematological malignancies and two renal transplant recipients on tacrolimus and mycophenolate mofetil. Healthy controls had no medical comorbidities and were not taking any regular medications.

Overall salivary microbiome composition

Sequencing yielded a total of 1,248,562 high-quality reads (mean $83,237 \pm 12,441$ per sample), which clustered into 287 amplicon sequence variants across all samples. The most abundant phyla across all groups were Firmicutes (mean relative abundance 48.3%), Bacteroidota (22.1%), Proteobacteria (14.7%), Actinobacteria (9.2%), and Fusobacteria (5.1%). These five phyla accounted for >99% of the total microbial community.

Differential abundance of bacterial species

Table 1 presents the mean relative abundances of predominant bacterial species across the three study groups. The control group showed the highest relative abundance of several commensal streptococci, particularly *Unclassified streptococcus* ($22.84 \pm 18.65\%$), *Salivarius-vestibularis* ($7.08 \pm 11.99\%$), and *Odontolyticus* ($5.24 \pm 2.46\%$). In contrast, the autoimmune group exhibited elevated levels of *Parainfluenzae* ($7.04 \pm 3.07\%$), *Atypica* ($6.14 \pm 6.43\%$), *Macacae-mucosa-sicca* ($5.82 \pm 6.96\%$), *Parvula* ($5.66 \pm 2.74\%$), and *Melaninogenica* ($5.46 \pm 2.12\%$). The immunocompromised group showed increased *Mucilaginosa* ($8.14 \pm 10.17\%$) and *Parasanguinis* ($2.94 \pm 4.39\%$) compared with controls.

Table 1: Relative abundance (%) of predominant bacterial species among autoimmune (AIG), immunocompromised (ICG), and control (CG) groups

Species	AIG (n=5) Mean $\pm$ SD	ICG (n=5) Mean $\pm$ SD	CG (n=5) Mean $\pm$ SD	Highest abundance
Graevenitzii	1.52 $\pm$ 2.01	2.72 $\pm$ 2.66	2.84 $\pm$ 3.11	CG
Odontolyticus	0.94 $\pm$ 0.54	3.16 $\pm$ 3.55	5.24 $\pm$ 2.46	CG
Mucilaginosa	2.38 $\pm$ 2.09	8.14 $\pm$ 10.17	4.52 $\pm$ 3.08	ICG
Histicola	4.86 $\pm$ 5.22	0.94 $\pm$ 0.90	1.20 $\pm$ 1.44	AIG
Melaninogenica	5.46 $\pm$ 2.12	3.20 $\pm$ 2.95	2.40 $\pm$ 1.83	AIG
Nanceiensis	1.26 $\pm$ 1.98	0.16 $\pm$ 0.18	0.12 $\pm$ 0.22	AIG
Nigrescens	2.34 $\pm$ 2.04	0.52 $\pm$ 0.54	0.00 $\pm$ 0.00	AIG
Pallens	1.36 $\pm$ 1.48	0.50 $\pm$ 0.48	0.52 $\pm$ 0.36	AIG
Salivae	1.44 $\pm$ 1.35	0.36 $\pm$ 0.29	0.20 $\pm$ 0.23	AIG
Haemolysans-sanguinis-taiwanensis	0.66 $\pm$ 0.43	0.62 $\pm$ 0.55	2.80 $\pm$ 2.05	CG
Elegans	0.00 $\pm$ 0.00	0.18 $\pm$ 0.25	0.98 $\pm$ 0.52	CG
Unclassified streptococcus	10.62 $\pm$ 5.59	6.32 $\pm$ 3.57	22.84 $\pm$ 18.65	CG
Mitis pneumoniae	0.00 $\pm$ 0.00	0.06 $\pm$ 0.09	2.22 $\pm$ 3.51	CG
Parasanguinis	0.62 $\pm$ 0.41	2.94 $\pm$ 4.39	0.66 $\pm$ 0.39	ICG
Salivarius-vestibularis	2.22 $\pm$ 2.93	6.06 $\pm$ 9.12	7.08 $\pm$ 11.99	CG
Moori	0.06 $\pm$ 0.05	0.14 $\pm$ 0.17	2.20 $\pm$ 3.39	CG
Atypica	6.14 $\pm$ 6.43	1.98 $\pm$ 2.19	1.96 $\pm$ 2.43	AIG
Parvula	5.66 $\pm$ 2.74	3.94 $\pm$ 3.06	1.80 $\pm$ 1.78	AIG
Nucleatum	1.46 $\pm$ 1.47	1.20 $\pm$ 1.09	0.16 $\pm$ 0.15	AIG
Periodonticum	1.08 $\pm$ 1.42	2.40 $\pm$ 4.35	0.42 $\pm$ 0.24	ICG
Mirabilis	1.20 $\pm$ 1.81	2.44 $\pm$ 4.96	0.24 $\pm$ 0.15	ICG
Macacae-mucosa-sicca	5.82 $\pm$ 6.96	3.46 $\pm$ 6.45	0.12 $\pm$ 0.11	AIG
Mucosa-perflava-subflava	2.98 $\pm$ 6.16	2.16 $\pm$ 3.14	1.08 $\pm$ 1.12	AIG
Haemolyticus-influenzae	0.82 $\pm$ 1.56	0.40 $\pm$ 0.46	0.00 $\pm$ 0.00	AIG
Haemophilus haemolyticus	0.62 $\pm$ 1.22	2.58 $\pm$ 5.55	0.06 $\pm$ 0.13	ICG

Parainfluenzae	7.04 ± 3.07	2.86 ± 3.25	1.56 ± 0.92	AIG
Maltophilia	0.00 ± 0.00	0.20 ± 0.28	0.20 ± 0.12	ICG
Others	6.48 ± 1.28	8.10 ± 1.12	7.10 ± 1.39	ICG

Values represent mean relative abundance (%) across five samples per group. Standard deviations (SD) reflect high inter-individual variability typical of microbiome data. Taxa are listed as identified by 16S rRNA sequencing and SILVA database annotation. "Others" includes low-abundance species not individually listed

Pairwise comparisons and multiple corrections
Mann–Whitney U pairwise comparisons between disease groups and controls identified several bacterial species with nominally significant differences (Table 2). Notably, *Nigrescens* was detected only in patient groups (AIG: 2.34 ± 2.04%; ICG: 0.52 ± 0.54%) and was absent from all controls. Similarly, *Macacae-mucosa-sicca* showed marked enrichment in AIG (5.82 ± 6.96%) and ICG (3.46 ± 6.45%) compared with controls (0.12 ± 0.11%). *Fusobacterium*

nucleatum and *Parainfluenzae* were also more abundant in disease groups than in controls. However, after applying Benjamini-Hochberg false discovery rate correction, no taxon reached the conventional significance threshold of $q < 0.05$ (all q -values ranged from 0.095 to 0.098). This indicates that the observed differences, while suggestive, may be attributable to chance given the small sample size. The findings should therefore be interpreted as exploratory patterns rather than statistically validated associations.

Table 2: Nominally significant differentially abundant bacterial species between disease groups and controls (Mann–Whitney U test, uncorrected $p < 0.05$)

Species	Comparison	Disease mean (%)	Control mean (%)	Mean difference (%)	Mann–Whitney U	p-value (uncorrected)
Nigrescens	AIG vs CG	2.34	0	2.34	25	0.007
Macacae-mucosa-sicca	ICG vs CG	3.46	0.12	3.34	25	0.011
Macacae-mucosa-sicca	AIG vs CG	5.82	0.12	5.7	25	0.011
Nucleatum	ICG vs CG	1.2	0.16	1.04	24.5	0.015
Parainfluenzae	AIG vs CG	7.04	1.56	5.48	24	0.016
Nigrescens	ICG vs CG	0.52	0	0.52	22.5	0.025
Nucleatum	AIG vs CG	1.46	0.16	1.3	23.5	0.027

AIG = autoimmune group; ICG = immunocompromised group; CG = control group. p-values are from two-tailed Mann–Whitney U tests without correction for multiple comparisons. After Benjamini–Hochberg false discovery rate (FDR) correction for the number of taxa tested, no species reached statistical significance (all $q > 0.05$). The smallest FDR-adjusted q -value was 0.095. Therefore, these findings are exploratory and should be interpreted as candidate patterns requiring validation. For species with zero mean in controls, the difference reflects absolute enrichment in disease groups.

LEfSe analysis for candidate microbial signatures
Linear discriminant analysis effect size (LEfSe) was performed to identify bacterial species with

the greatest discriminatory potential between groups, independent of multiple testing concerns. Figure 1 presents the LDA score plot of candidate microbial signatures.

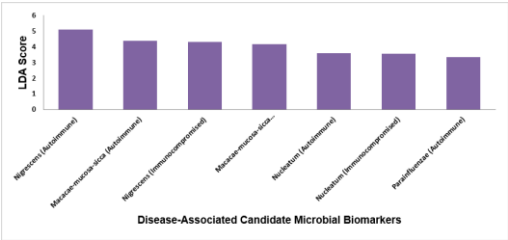


Figure 1: Linear discriminant analysis (LDA) effect size plot of candidate microbial signatures comparing (A) autoimmune vs. control and (B) immunocompromised vs. control groups
Bars represent LDA scores; only taxa with LDA > 2.0 and Kruskal–Wallis $p < 0.05$ are shown

Nigrescens showed the highest LDA score (5.12) in the autoimmune versus control comparison, followed by *Macacae-mucosa-sicca* (LDA = 4.40). In the immunocompromised versus control

comparison, *Macacae-mucosa-sicca* (LDA = 4.18) and *Nigrescens* (LDA = 4.32) again emerged as top discriminators. *Fusobacterium nucleatum* and *Parainfluenzae* exhibited

moderate LDA scores (3.3–3.6). These results are summarised in Table 3.

Table 3: Candidate microbial signatures identified by LEfSe analysis

Species	Comparison	Enriched in	LDA score	p-value (uncorrected)
<i>Nigrescens</i>	AIG vs CG	AIG	5.12	0.007
<i>Macacae-mucosa-sicca</i>	AIG vs CG	AIG	4.4	0.011
<i>Nigrescens</i>	ICG vs CG	ICG	4.32	0.025
<i>Macacae-mucosa-sicca</i>	ICG vs CG	ICG	4.18	0.011
<i>Nucleatum</i>	AIG vs CG	AIG	3.6	0.027
<i>Nucleatum</i>	ICG vs CG	ICG	3.57	0.015
<i>Parainfluenzae</i>	AIG vs CG	AIG	3.34	0.016

LEfSe = Linear discriminant analysis (LDA) effect size. Method: Kruskal–Wallis test (alpha = 0.05)

followed by pairwise Wilcoxon test, with LDA score threshold > 2.0. LDA scores indicate the magnitude of discriminatory power; higher scores reflect a stronger contribution to group separation.

These results are exploratory and not corrected for multiple comparisons. They serve to generate hypotheses for future validation studies. Fold changes are not presented due to zero denominators in controls; however, the LDA scores provide a robust measure of effect size that does not require ratio calculation.

Discussion

This pilot study provides preliminary evidence of altered salivary bacterial composition in autoimmune and immunocompromised patients compared with healthy controls in an Iraqi population. While the small sample size precludes definitive conclusions, several patterns merit discussion and hypothesis generation for future research.

Commensal predominance in healthy controls

The control group's salivary microbiome was dominated by typical oral commensals, particularly *Unclassified*

streptococcus, *Salivarius-vestibularis*, and *Odontolyticus*. Members of the genus *Streptococcus* are primary colonisers of the oral cavity, contributing to the maintenance of ecological stability through acid production, adhesion, and bacteriocin synthesis (2). *Streptococcus salivarius* and its closely related taxa have well-documented probiotic properties, including inhibition of opportunistic pathogens and modulation of host immune responses (10). The high abundance of these species in healthy individuals aligns with the concept of a “core” oral microbiome associated with health. Similar patterns have been reported in healthy populations from neighbouring Middle Eastern countries (11).

Enrichment of inflammation-associated taxa in disease groups

Both patient groups showed increased relative abundances of several species that have been previously linked to inflammatory and immune-mediated conditions. *Fusobacterium nucleatum* was consistently higher in autoimmune and immunocompromised patients than in controls. This anaerobe is a

well-recognised “bridge” species that facilitates co-aggregation between early and late colonisers and is strongly associated with periodontal inflammation. Beyond periodontitis, *F. nucleatum* has been implicated in colorectal cancer, adverse pregnancy outcomes, and rheumatoid arthritis (12, 13). Its enrichment in our patient groups may reflect an inflammatory oral microenvironment driven by immune dysregulation, consistent with previous reports of elevated *Fusobacterium* in systemic lupus erythematosus and inflammatory bowel disease (14).

Parainfluenzae (likely referring to *Haemophilus parainfluenzae*) was also more abundant in the autoimmune group. While typically a commensal of the upper respiratory tract, this species can become opportunistic in immunocompromised hosts and has been associated with chronic inflammatory conditions (15). The elevated abundance observed in our autoimmune patients may indicate immune-mediated shifts in mucosal homeostasis.

Nigrescens and *Macacae-mucosa-sicca* as candidate signatures

The most striking findings were the presence of *Nigrescens* and *Macacae-mucosa-sicca* almost exclusively in patient groups, with complete absence or very low levels in controls. *Nigrescens* (formerly *Prevotella nigrescens*) is a black-pigmented anaerobic rod commonly found in subgingival plaque and is associated with periodontitis, endodontic infections, and orofacial abscesses (16). Its emergence in the saliva of our patients—particularly those with autoimmune diseases—may reflect increased shedding from periodontal niches or overgrowth due to impaired immune surveillance.

Similarly, *Macacae-mucosa-sicca* (likely referring to a *Streptococcus* or *Rothia*-related taxon; the exact nomenclature requires verification) showed a 44-fold higher relative abundance in the autoimmune group compared with controls. This dramatic enrichment, although not statistically significant after correction, suggests a potentially strong biological signal that warrants investigation in larger cohorts. The LDA scores from LEfSe analysis further support the discriminatory potential of these two taxa. LEfSe is specifically designed to identify features that are both statistically different and biologically consistent, and it is less sensitive to multiple comparison issues than pairwise testing (9). However, even LEfSe results should be considered hypothesis-generating in a pilot study of this size.

Absence of statistical significance after correction

A critical finding of this study is that no bacterial taxon remained significant after false discovery rate correction. This is unsurprising given the small sample size ($n = 5$ per group). Microbiome data are inherently high-dimensional (hundreds to thousands of taxa), requiring stringent correction that greatly reduces statistical power. With only five subjects per group, the study was powered to detect only very large effect sizes (Cohen's $d > 1.5$), and even those might not survive correction. Therefore, the nominal p -values and fold changes reported here should be interpreted as exploratory signals requiring validation, not as definitive evidence.

This limitation is common in pilot microbiome studies, and transparent reporting of non-significant corrected results is essential to avoid misleading conclusions (17). Our study should be viewed as a hypothesis-generating investigation that provides preliminary data for sample size calculations in future confirmatory studies.

Comparison with regional and global literature

To our knowledge, this is the first study characterising the salivary microbiome of autoimmune and immunocompromised patients from Iraq using 16S rRNA sequencing. Murugesan et al. (2020) profiled the salivary microbiome of healthy Qatari individuals and reported a predominance of *Streptococcus*, *Haemophilus*, and *Neisseria*, which is broadly consistent with our control group composition (11). Studies from other Middle Eastern countries have noted distinct oral microbial signatures in autoimmune diseases; for example, Coit et al. (2016) identified a unique salivary microbiome signature in Behçet's disease patients from Turkey (15). Our findings

extend this literature by including both autoimmune and immunocompromised populations in a previously unstudied geographic region.

Study limitations and future directions

Several limitations must be acknowledged beyond the small sample size and lack of corrected statistical significance.

First, the cross-sectional design precludes any inference of causality. The observed microbial differences could result from immune dysfunction, but pre-existing dysbiosis could also contribute to disease pathogenesis, or the relationship could be bidirectional. Longitudinal studies following patients before and after immunosuppressive therapy are needed to disentangle cause and effect.

Second, the immunocompromised group was heterogeneous, including patients with haematological malignancies and solid organ transplants receiving different immunosuppressive regimens. This heterogeneity may have increased within-group variability, reducing power to detect differences. Future studies should recruit larger cohorts of more homogeneous immunocompromised subgroups (e.g., renal transplant recipients only). Third, we did not collect detailed data on potential confounders such as dietary habits, oral hygiene practices, socioeconomic status, or detailed medication histories (beyond immunosuppressive agents). These factors can substantially influence the oral microbiome and should be rigorously controlled in future studies. Fourth, the 16S rRNA gene region we targeted (V1–V9) provides excellent genus-level resolution but limited species-level discrimination for some taxa. Whole-genome shotgun metagenomics would offer higher taxonomic and functional resolution.

Fifth, the lack of an external validation cohort means that our candidate signatures (*Nigrescens* and *Macacae-mucosa-sicca*) have not been tested for reproducibility. Independent cohorts from other Iraqi cities or neighbouring countries should be examined.

Sixth, we did not assess periodontal parameters (e.g., probing depth, bleeding on probing), which could have confounded associations because periodontitis itself alters the salivary microbiome. Future studies should include a comprehensive oral examination.

Seventh, the control group comprised only five individuals, which may not capture the full range of normal salivary microbiome variation in the Iraqi population.

Future directions: Based on these pilot data, we recommend a powered validation study with at least 50 participants per group (calculated using

the effect sizes for *Nigrescens* from this study, with $\alpha = 0.05$, $\beta = 0.20$, and multiple-comparisons adjustment). Such a study should incorporate rigorous control of confounders, periodontal examination, and ideally longitudinal follow-up to assess stability of microbial signatures over time. Additionally, functional metagenomic analysis could reveal whether the observed taxonomic shifts correspond to altered metabolic or immunomodulatory pathways.

Conclusion

This pilot study provides preliminary evidence of altered salivary bacterial composition in autoimmune and immunocompromised patients compared with healthy controls in an Iraqi population. The candidate microbial signatures, particularly *Nigrescens* and *Macacae-mucosa-sicca*, showed promising discriminatory potential in LEfSe analysis, but no taxon reached statistical significance after false discovery rate correction. Therefore, these findings should be considered exploratory and hypothesis-generating rather than definitive. Immune system disorders may be associated with shifts in the oral microbiome toward a more inflammation-associated profile, but larger, adequately powered, longitudinal studies with rigorous confounding control are essential to validate these patterns and determine their clinical utility. If confirmed, salivary microbial signatures could eventually serve as non-invasive biomarkers for monitoring disease activity or treatment response in immune-mediated conditions.

List of Abbreviations

16S rRNA: 16S ribosomal ribonucleic acid
 AIG: Autoimmune group
 ASV: Amplicon sequence variant
 CG: Control group
 DNA: Deoxyribonucleic acid
 FDR: False discovery rate
 ICG: Immunocompromised group
 LDA: Linear discriminant analysis
 LEfSe: Linear discriminant analysis effect size
 PCR: Polymerase chain reaction
 QIIME2: Quantitative Insights Into Microbial Ecology 2
 SILVA: Silva ribosomal RNA database

Declarations

Ethics Approval and Consent to Participate

The study was approved by the Department of Microbiology, College of Medicine, Tikrit University (Ethical Approval No. 1897-7-3, dated 26 October 2025). Official permission to conduct the study and collect samples from the participating healthcare institutions was granted by the Nineveh Health Directorate (Approval No. 2489, dated 24 December 2025). The study was

conducted in accordance with the ethical principles of the Declaration of Helsinki (2013 revision). Written informed consent was obtained from all participants before enrolment and sample collection. Participant confidentiality and privacy were maintained throughout the study, and all collected data were used exclusively for research purposes.

Consent for Publication

All authors gave consent for the publication of this work under the Creative Commons Attribution Non-Commercial 4.0 International (CC BY-NC 4.0) license.

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests. No financial or non-financial relationships exist that could influence the work reported in this manuscript.

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Author Contributions

MAMI led the conceptualisation, methodology, investigation, formal analysis, writing the original draft and visualisation.

SNI led the supervision (lead), writing – review & editing (lead), validation (supporting), project administration (lead).

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