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ORAL MICROBIAL SIGNATURES: FROM SEVERE INFECTIONS TO IMMUNE RESPONSES

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Cover illustration: The cover illustration represents the central themes of the thesis, focusing on the intricate interplay between the oral microbiome, infectious diseases, and host immunity. The interconnected nodes highlight key research areas, including bacterial diversity in oral infections, the influence of microbial secretions on MAIT cell activation and function, the role of the oral microbiome in shaping COVID-19 vaccination outcomes, and the characterization of oral fungal infections in head and neck cancer patients.

Oral Microbial Signatures: From Severe Infections to Immune Responses
Thesis for Doctoral Degree (Ph.D.)

By

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To my beloved Al-KHALIL family,

*My **father Khalil**, whose wisdom lights my way, and my **mother Nora**, whose love and prayers are my strength. To my **brother Ahmed**, my steadfast supporter, and my **sisters, Mona, Samira, Maha, Kholod, and Hanan**, my cherished companions. This work is a tribute to your boundless love and belief in me. You are my greatest inspiration.*

Popular Science Summary of the Thesis

The oral cavity is a dynamic ecosystem replete with diverse microbial communities, including bacteria and fungi crucial for oral and systemic health. While many microbes are beneficial, disruptions in their population balance (dysbiosis) can lead to severe infections and influence immune responses. Understanding the interrelations between oral microbes and the immune system is essential for advancing diagnostics, personalizing treatments, and improving health outcomes. This doctoral thesis examines oral microbial signatures—the unique microbial species composition of the microbiome— in different contexts, from severe infections to immune responses, to determine their clinical relevance and biological implications. By integrating advanced microbial profiling techniques with immune analysis, the thesis helps reveal the intricate connections between oral microbes, health, and disease.

The **first study** investigates bacterial diversity in severe oral infections, such as dentoalveolar abscesses and jaw osteomyelitis, using data collected over 11 years in Stockholm County. Advanced microbial identification with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry revealed notable shifts in pathogenic bacterial populations, including *Streptococcus anginosus* and *Granulicatella adiacens*, underscoring the need to monitor emerging pathogens in oral infections, and offering valuable insights for clinical management.

The **second study** examines oral fungal infections in head and neck cancer patients undergoing radiotherapy, a group particularly vulnerable to opportunistic pathogens. The study highlights the dominance of *Candida* species, including non-*albicans* strains such as *C. glabrata* and *C. tropicalis*. Fungi co-occurrence patterns and novel pathogen identification provide crucial information for managing oral fungal infections in immunocompromised individuals, and potential directions for improving therapeutic interventions.

The **third study** explores the relationship between the oral microbiome and immune responses to COVID-19 vaccination. The durability of salivary antibody responses in healthy individuals and those living with HIV were associated with distinct microbial signatures. Gram-positive bacteria, such as *Granulicatella* and *Rothia,* were abundant in high-responders to vaccination, while gram-negative anaerobes like *Prevotella* and *Leptotrichia* in low-responders, implicating the oral microbiome's significant influence on immune responses—a finding with potential implications for enhancing vaccine strategies.

The **fourth study** investigates the interaction between microbial secretions and mucosal-associated invariant T (MAIT) cells (key components of the immune system). The study reveals that the origin of microbial secretions—from the oral cavity, bloodstream, or pancreatic tumors—affects MAIT cell activation and cytokine production, highlighting the influence of microbial metabolites in modulating immune responses and their potential importance in oral infections and cancer-related immunity.

الملخص العلمي لأطروحة الدكتوراة

يعد تجويف الفم بيئة ديناميكية مليئة بمجتمعات ميكروبية متنوعة، تشمل البكتيريا والفطريات، والذي يلعب دورًا حيويًا في الحفاظ على صحة الفم والجسم. وعلى الرغم من أن العديد من هذه الميكروبات مفيدة، إلا أن الاختلال في توازنها قد يؤدي إلى التهابات خطيرة ويؤثر على استجابات الجهاز المناعي. إن فهم العلاقة التفاعلية بين الميكروبات الفموية والجهاز المناعي أمر بالغ الأهمية لتطوير تشخيص الحالات المرضية، وتخصيص العلاجات، وتحسين النتائج الصحية. تستكشف هذه الأطروحة البصمات الميكروبية الفموية في سياقات مختلفة، بدءًا من العدوى الحادة وصولًا إلى استجابات الجهاز المناعي، للكشف عن أهميتها السريرية وتأثيرها البيولوجي. ومن خلال الجمع بين تقنيات متقدمة لتحليل الميكروبات ودراسة الجهاز المناعي، تهدف هذه الأطروحة إلى تسليط الضوء على العلاقات المعقدة بين الميكروبيوم الفموي والصحة والمرض.

ركزت الدراسة الأولى على تحليل تنوع البكتيريا في التهابات الفم الحادة، مثل الخراجات السنية والتهاب العظم الفكي، باستخدام بيانات تم جمعها على مدى أحد عشر عامًا في مقاطعة ستوكهولم. وقد كشفت تقنية مطياف الكتلة MALDI-TOF عن وجود بكتيريا ممرضة بارزة، مثل *Streptococcus anginosus* و *Granulicatella adiacens*. تسلط هذه النتائج الضوء على التغيرات في أنماط الميكروبيوم الفموي، مما يعكس الحاجة إلى مراقبة مسببات الأمراض الناشئة في التهابات الفم، وتوفر رؤى قيّمة لتحسين العلاجات السريرية لهذه الحالات.

تناولت الدراسة الثانية الالتهابات الفطرية لدى مرضى سرطان الرأس والرقبة الذين يخضعون للعلاج الإشعاعي، وهم من الفئات الأكثر عرضة للممرضات الانتهازية. أظهرت النتائج هيمنة أنواع المبيضات (*Candida*)، بما في ذلك السلالات غير المبيضة مثل *C. glabrata* و *C. tropicalis* ومن خلال تحليل أنماط التعايش الفطري وتحديد مسببات الأمراض الجديدة، تقدم هذه الدراسة معلومات أساسية حول معالجة العدوى الفطرية الفموية لدى المرضى ذوي المناعة الضعيفة، مما يفتح آفاقًا جديدة لتحسين التدخلات العلاجية.

أما الدراسة الثالثة، فقد استكشفت العلاقة بين الميكروبيوم الفموي والاستجابة المناعية للقاح كوفيد-19، حيث كشفت عن بصمات ميكروبية مميزة تؤثر على استدامة الأجسام المضادة اللعابية لدى كل من الأشخاص الأصحاء والمصابين بفيروس نقص المناعة البشرية (HIV) ، والذين يعانون من استجابات مناعية ضعيفة. أظهرت النتائج أن الأفراد ذوي الاستجابة القوية للقاح لديهم غزارة في البكتيريا موجبة الجرام مثل *Granulicatella* و *Rothia* ، في حين أن الأفراد ذوي الاستجابة الضعيفة كانوا غالبًا ما يحملون بكتيريا لاهوائية سالبة الجرام مثل *Prevotella* و*Leptotrichia*. توضح هذه النتائج أن الميكروبيوم الفموي يلعب دورًا محوريًا في تشكيل الاستجابة المناعية، مما قد يساعد في تحسين استراتيجيات التطعيم المستقبلية.

تناولت الدراسة الرابعة التفاعل بين الإفرازات الميكروبية والخلايا التائية المرتبطة بالغشاء المخاطي (MAIT cells) ، وهي مكون رئيسي في الجهاز المناعي. كشفت النتائج أن مصدر الإفرازات الميكروبية – سواء من الفم أو مجرى الدم أو أورام البنكرياس – يؤثر على تنشيط خلايا MAIT وإنتاج السيتوكينات. يسلط هذا البحث الضوء على الدور المناعي للمنتجات الأيضية الميكروبية، ويشير إلى تأثيرها المحتمل على الالتهابات الفموية المناعية والسرطانات ذات الصلة، مما يفتح المجال أمام علاجات مناعية قائمة على الميكروبات.

Abstract

This thesis investigates the diversity of oral microbial signatures, and the profound clinical and immunological significance they have in human pathologies, from localized infections to systemic immunological interactions.

Study 1 was an 11-year epidemiological analysis of severe oral infections in Stockholm County. Characterization of 1,014 samples using MALDI-TOF mass spectrometry revealed highly dynamic microbial populations associated with different oral infections. *Streptococcus* spp., *Prevotella* spp., and *Staphylococcus* spp. predominated, while the evolving populations of *Streptococcus anginosus* and *Actinomyces* spp. had potential systemic implications.

Study 2 was a 6-year retrospective analysis exploring the mycobiota profiles of head and neck cancer (HNC) patients with oral fungal infections (OFIs) and undergoing radiotherapy. *Candida albicans* was the most prevalent pathogen, co-occurring with emerging populations of *non-albicans Candida* (NAC) species and novel isolates, including *Fusarium solani* and *Candida jadinii*. Network analyses revealed significant multispecies co-occurrence, particularly during radiotherapy, underscoring the need for tailored antifungal strategies in this vulnerable population.

Study 3 evaluated the oral microbiome's influence on coronavirus disease 2019 (COVID-19) vaccine immunogenicity in 115 participants, including healthy controls and people living with HIV (PLHIV). Comparative analyses revealed distinct microbial profiles in high- and low-responders. Gram-negative anaerobes, such as *Campylobacter* and *Prevotella*, predominated in low-responders, and Gram-positive saccharolytic species in high-responders, implicating the distribution of oral microbial populations in modulating mucosal immunity and possibly in long-term vaccine outcomes.

Study 4 focused on MAIT cell responses to bacterial secretomes derived from blood, oral, and pancreatic tumor isolates. Activation patterns were strongly influenced by bacterial origin, irrespective of species, with tumor-derived strains inducing unique polyfunctional cytokine profiles. This highlights the pivotal role of microbial metabolites in shaping immune responses, offering novel insights into host-microbe interactions in oral infections and cancer.

Collectively, this work advances our understanding of oral microbial signatures in health and disease, revealing their critical role in shaping systemic immunity and providing a foundation for microbiome-targeted therapeutic interventions.

List of Scientific Papers

I. **Al-Manei K**, Ghorbani M, Naud S, Al-Manei KK, Sobkowiak MJ, Lund B, Hazirolan G, Sällberg Chen M,, Özenci V. Clinical microbial identification of severe oral infections by MALDI-TOF mass spectrometry in Stockholm County: an 11-Year (2010 to 2020) epidemiological investigation. Microbiology Spectrum. 2022 Dec 21;10(6):e02487-22.

II. **Al-Manei K**, Sobkowiak MJ, Nagadia RH, Heymann R, Sällberg Chen M, Özenci V. Mycobiota profile of oral fungal infections in head and neck cancer patients receiving radiotherapy: A 6-year retrospective MALDI-TOF mass spectrometry study. Oral Oncology. 2023 Nov 1;146:106556.

III. Ghorbani M*, **Al-Manei K***, Naud S, Healy K, Gabarrini G, Sobkowiak MJ, Chen P, Ray S, Akber M, Muschiol S, Bogdanovic G, Bergman P, Ljungman P, Buggert M, Ljunggren HG, Pin E, Nowak P, Aleman S, Sällberg Chen M. Persistence of salivary antibody responses after COVID-19 vaccination is associated with oral microbiome variation in both healthy and people living with HIV. Frontiers in Immunology. 2023 Jan 10;13:1079995.

IV. **Al-Manei K**, Unalan-Altintop T, Johnsson A, Santini R, Khan ZA, Halimi A, Özenci V, Sällberg Chen M, Sobkowiak MJ. MAIT cell polyfunctional response to bacterial secretome is dependent on site of microbial origin. Manuscript. 2025.

*Contributed equally

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List of Abbreviations

16S rRNA – 16S Ribosomal RNA
18S rRNA – 18S Ribosomal RNA
ACE2 – Angiotensin-Converting Enzyme 2
APC – Antigen-Presenting Cell
ARDS – Acute Respiratory Distress Syndrome
BFA – Brefeldin A
CCL20 – Chemokine (C-C motif) Ligand 20 (Immune/ signaling marker)
CD28 – Cluster of Differentiation 28
CLIA – Chemiluminescence Immunoassay
COVID-19 – Coronavirus Disease 2019
CXCL16 – Chemokine (C-X-C motif) Ligand 16 (Immune/ signaling marker)
CyTOF – Cytometry by Time-of-Flight
ELISA – Enzyme-Linked Immunosorbent Assay
FAB – Fastidious Anaerobic Broth
FACS – Fluorescence-Activated Cell Sorting
FBS – Fetal Bovine Serum
FISH – Fluorescence *In Situ* Hybridization
GF – Germ-Free
GzB – Granzyme B
HIV – Human Immunodeficiency Virus
HNC – Head and Neck Cancer
IFN-γ – Interferon-γ (Immune/ signaling marker)
IL-7, IL-12, IL-15, IL-17, IL-18, IL-23 – (Types of Interleukin)
ITS – Internal Transcribed Spacer
LFA – Lateral Flow Assay
mAb – Monoclonal Antibody
MAIT – Mucosal-Associated Invariant T (cell)
MALDI-TOF MS – Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MFU – McFarland Unit
MLST – Multilocus Sequence Typing
MR1 – MHC class I-related protein 1 (Immune/ signaling marker)
mRNA – Messenger RNA
NGS – Next-Generation Sequencing
NK – Natural Killer (cell)
OFIs – Oral Fungal Infections
PBMC – Peripheral Blood Mononuclear Cell
PBS – Phosphate-Buffered Saline
PCR – Polymerase Chain Reaction

PD-1 – Programmed Cell Death Protein 1 (Immune/ signaling marker)
PLHIV – People Living with HIV
PRRs – Pattern Recognition Receptors
RPMI 1640 – Roswell Park Memorial Institute Medium 1640 (A cell culture medium)
RT – Radiotherapy
SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2
SCFA – Short-Chain Fatty Acid
TCRVα7.2 – T Cell Receptor Variable Alpha-chain 7.2 (specific to MAIT cells)
TLR – Toll-Like Receptor
TNF – Tumor Necrosis Factor (Immune/ signaling marker)
V(D)J – Variable, Diversity, and Joining (gene segments in immune receptor rearrangement)
WHO – World Health Organization

Introduction

The human microbiota is a vast and intricate ecosystem of microbial communities, including bacteria, fungi, and viruses, that coexist in a delicate balance [1]. Recent advances in molecular analysis have unveiled the profound influence that the composition and functional characteristics of these communities (their signatures) have on human health and disease [2]. This is particularly evident in the oral and gut microbiota, which play crucial roles in both local and systemic processes, including infections and immune responses [3-5]. Understanding the composition, diversity, and functional roles of the oral microbiota is essential for developing effective treatments and reducing disease risks.

The oral cavity hosts an extraordinary diversity of over 700 microbial species [6] that coexist in a delicate balance, acting as a protective barrier against harmful pathogens. The oral microbiota supports the host by aiding metabolism, regulating the immune system, and defending against external threats [7]. However, immunosuppression, antimicrobial use, or environmental changes, can disrupt this balance, leading to dysbiosis—a state where microbial composition becomes suboptimal or even detrimental to health. Dysbiosis allows pathogenic microbes to proliferate, potentially leading to dental caries, periodontitis, endodontic infections, and oral fungal diseases [8]. The continuous interaction of oral microorganisms with the immune system and epithelial cells can also increase the host's susceptibility to infection by pathogenic microbes within the oral cavity [9-11]. Moreover, emerging evidence links oral dysbiosis to systemic disorders, including cardiovascular disease, diabetes, and certain malignancies [9-11].

Oral infections often result from complex interactions within polymicrobial communities. Anaerobic bacteria and opportunistic fungi, for example, can amplify disease severity and complicate treatment [12]. High-throughput technologies, such as next-generation sequencing (NGS) and MALDI-TOF mass spectrometry have transformed our understanding of these microbial ecosystems, uncovered novel pathogens, and illuminated the microbial diversity associated with severe oral infections and malignancies [13, 14]. Despite these advances, epidemiological data on oral microbial signatures (which refer to the unique composition and functional characteristics of the (oral) microbial community) in severe infections remain limited. Addressing this gap is essential for improving diagnostic and therapeutic strategies.

Another critical area of research is the relationship between oral microbiota and systemic immunity. While the gut microbiota is well-known to influence immune

responses and vaccine efficacy (e.g., for COVID-19), emerging evidence suggests that its species composition also affects mucosal and systemic immunity, thereby impacting vaccine-induced immunogenicity [16, 17]. Less is known about the oral microbiota, although its imbalance has been shown to provoke abnormal immune responses, with chronic inflammation and increased susceptibility to diseases [15], its specific influence on vaccine-induced immunity requires further investigation.

The oral microbiota is also increasingly implicated in carcinogenesis. Specific microbes, such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, have been linked to pro-inflammatory and immunosuppressive environments that promote tumor growth and metastasis [18]. The interactions between microbial metabolites and immune cells, such as mucosal-associated invariant T (MAIT) cells, represent a promising yet understudied area. It remains unclear whether metabolites from the same microbial species, when isolated from different body sites, influence MAIT cell functionality in similar ways. Understanding such interactions is crucial for unraveling the complex dynamics between microbiota, immunity, and cancer.

It is crucial, therefore, to examine in greater depth the involvement of the composition, diversity, and functional activities of the oral microbiota in health and disease. Advances in microbial profiling and immune sensing technologies hold promises for identifying novel biomarkers and therapeutic targets. This doctoral thesis addresses this gap by exploring the intricate relationships between oral microbial signatures and their clinical and immunological implications, focusing on severe infections, vaccine responses, and immune modulation. By deepening our understanding of these relationships, this work aims to provide a solid foundation for innovative diagnostic and therapeutic approaches that harness the power of oral microbiota to improve health and combat disease.

1 Literature Review

1.1 The Global Burden of Oral Infections

Worldwide, acute and chronic oral infections affect over 3.5 billion individuals annually, and include 514 million primary caries in children, 2 billion adults with permanent tooth decay, periodontal pathologies in 1 billion [19], and apical periodontitis of one or more teeth per person [20]. Odontogenic maxillary sinusitis [21] affects 50% of adults with sinusitis. Oropharyngeal and oral malignancies are the thirteenth most frequently occurring tumors, with a yearly incidence of 377,713 and a mortality rate of 177,757 in 2020 (Global Cancer Observatory), with an expected increase of 40% by 2040 [22]. Oral pathologies contribute to pain, reduced quality of life, decreased productivity, higher mortality rates, and increased financial burdens on both individuals and healthcare systems [23]. Risk factors include high sugar intake, tobacco and alcohol use, and socioeconomic disparities. Contemporary research also suggests a bidirectional relationship between oral infections and systemic non-communicable diseases [24] (Figure 1). Despite their major impact on both individuals and health systems, the lack of large-scale microbiome studies makes comprehensive epidemiological data on the microbial underpinnings of oral infections scarce, limiting our understanding of pathogen diversity, transmission dynamics, and interactions with systemic diseases. Improving clinical outcomes requires the integration of longitudinal microbial surveillance and evidence-based public health strategies that prioritize oral health on a global scale.

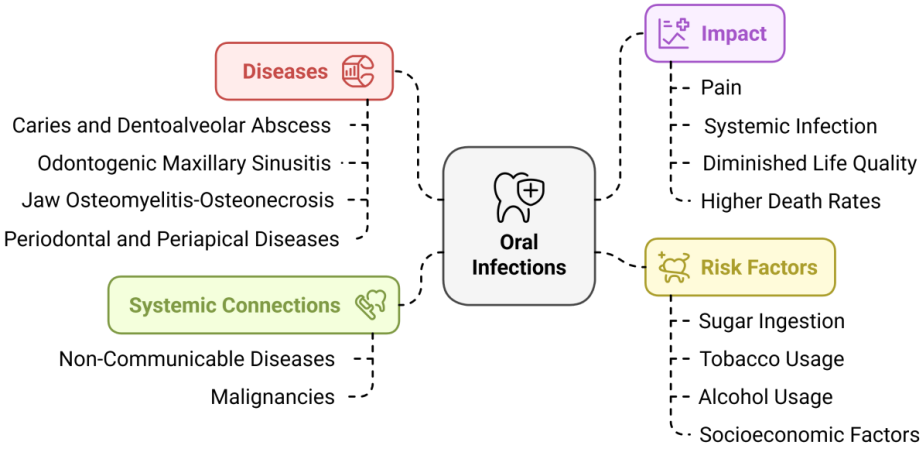


Figure 1 Oral Infections: impact, risk factors, diseases, and systemic connections. This diagram illustrates the multifaceted nature of oral infections, highlighting their disease burden, risk factors, systemic links, and overall impact. Oral infections contribute to significant health,

economic, and quality-of-life challenges, with growing evidence of their connection to systemic diseases. Understanding these relationships is crucial for effective prevention and public health strategies.

1.2 Oral Infections and the Oral Microbiome

1.2.1 Endodontic Infections

Microorganisms that infect dental structures or injuries to dental pulp, stimulate inflammatory cell accrual by macrophages, lymphocytes, and plasma cells which leads to periapical pathosis, and ultimately, tissue necrosis [25]. Infection severity is influenced by factors such as microbial virulence and the effectiveness of the host immune response [26]. Untreated infection may result in abscesses and severe pain [27], and potential spread into intracranial sinuses or deep cervical tissue [27].

Conventional techniques for studying endodontic infections and identifying the infectious agents include culture-based and closed-ended genomic methods, e.g. fluorescent *in situ* hybridization, DNA-DNA hybridization checkerboards, and PCR [28]. Primary endodontics infection is predominantly by anaerobic gram-negative organisms (e.g. *Fusobacterium* and *Prevotella*) [28, 29], while secondary is predominantly by gram-positive facultative anaerobic species (e.g. *E. faecalis*) [28].

High-throughput DNA sequencing has established *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria* [30, 31] as among the dominant phyla in endodontic infections, with common genera including *Fusobacterium, Pseudoramibacter* and *Porphyromonoas* genera [31-34]. The *Synergistetes* species, *Streptococcus,* and *Prevotella* have been well-documented in persistent and secondary infections [31-34], but there is little consensus about the spectrum of microorganisms in these two infection patterns [31, 33], or their association with clinical symptomatology [35-37]. *Fusobacterium* and *Parvimonas* are commonly involved in abscess development, but de novo microbes such as *Spirochaetes* and *Synergistetes*, have recently also been identified as possible contributors, as well as currently uncultivated pathogens [28, 31, 38]. The difference between infection types tends to be more about the relative population sizes of the species comprising the biota than about which species are present. Species identification in apical abscesses typically relies on genomic techniques, while proteomic approaches, such as MALDI-TOF MS, remain largely unexplored. However, the roles of emerging and uncultured pathogens, their functional interactions within polymicrobial communities, and their virulence mechanisms in endodontic infections remain poorly understood. Furthermore, no attempts have yet been made to understand species-specific

contributions using the combination of metagenomic, transcriptomic, and metabolomic data, to identify potential targets for intervention.

1.2.2 Odontogenic Maxillary Sinusitis

As the roots of upper posterior teeth are close to the maxillary floor, their infection may alter the sinus mucosal lining and facilitate the spread of infection [39]. Around 75% of odontogenic sinusitis cases are attributed to endodontic infections, with the remainder linked to periodontal or implant-related issues, respectively [40]. Multiple organisms, particularly anaerobic Gram-negative bacilli, e.g. *Peptostreptococcus* and *Fusobacteria* [41-48], potentially alongside *Streptococcus* or *Staphylococcus* [49], as well as HACEK organisms [50], have been shown to contribute to odontogenic sinusitis issues. 16S rRNA sequencing has shown that non-odontogenic rhinosinusitis has a less variable microbiota with a larger anerobic population than odontogenic [51]. Limited data exist on the viral and fungal components of the odontogenic sinusitis microbiome, and its bacterial diversity remains insufficiently characterized by advanced techniques such as MALDI-TOF MS. Additionally, metagenomic and metatranscriptomic studies have yet to be conducted to elucidate functional microbial interactions, the role of biofilms in persistent infections, and the potential involvement of uncultured or antimicrobial-resistant species in the pathogenesis of odontogenic sinusitis.

1.2.3 Jaw Osteomyelitis and Osteonecrosis

Chronic bacterial infection can lead to cortical bone and bone marrow inflammation, as well as necrosis of the jaw. Infecting microorganisms can spread from blood-localized injury sites, prostheses, or odontogenic infections, or as a consequence of ischemia [52, 53]. Jaw osteomyelitis remains prevalent despite advances in antimicrobials and dental care [54]. Another serious oral condition associated with immunocompromise is jaw osteonecrosis, characterized by persistent bone exposure within the oral cavity [55, 56].

Research on the microbial etiologies of these conditions has primarily has relied on case reports. While jaw osteomyelitis has been largely attributed to *Staphylococcus* infection, many other microorganisms, such as *Streptococcus* and multiple anaerobes have been identified through culture-independent techniques [57, 58]. The specific microorganisms contributing to jaw osteomyelitis or necrosis vary depending on the infection source, and 16S rRNA sequencing has indicated the presence of *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria,* and *Actinobacteria* phyla [59]. Oxygen-tolerating microbes (e.g., *Enterobacteriaceae* and *Staphylococci)* are associated with

hematogenous infection, and anaerobes with local infection spread [60]. The precise microbial dynamics driving bone necrosis and infection persistence remain poorly understood, and the severity of jaw osteomyelitis has only been tentatively linked to red complex bacteria. The lack of multi-omics studies that combine metagenomics, transcriptomics, and metabolomics, limits the current understanding of functional microbial interactions, biofilm formation, and antimicrobial resistance mechanisms in osteomyelitis and jaw osteonecrosis. Furthermore, no longitudinal studies incorporating host immune response profiling have been undertaken to explicate the interrelations between microbial communities and bone homeostasis, which are essential for developing targeted therapeutic strategies.

1.2.4 Periodontal Diseases

Periodontal disease affects the supporting dental tissues, and includes gums, alveolar bone, cementum, and periodontal ligament. Mild reactive gingivitis or peri-implantitis mucositis results from dental plaque, which if unresolved by hygiene measures can develop into a chronic infection of the deep tissues and periodontium [61, 62]. Modifiable risk factors include tobacco use, suboptimal mouth hygiene, diabetes mellitus, and pregnancy, while age, keratinized mucosal tissue, and certain inherited conditions serve as predisposing factors [63, 64].

More aggressive periodontitis has been linked to species such as *Filifactor alocis* and *Porphyromonas* [157-160], and disease progression seems associated with specific patterns of microbial dysbiosis. Until recently, culture techniques primarily implicated *Prevotella* in periodontal abscesses [69, 70, 161, 162], but 16S rRNA sequencing has shown the microbiome's greater complexity, including *F. alocis* and *A. rimae* [71, 163], while no MALDI-TOF MS analysis has yet been conducted.

The transformation from health to periodontal disease is associated with a significant microbiome shift. The advent of genomic DNA probes and checkerboard DNA–DNA hybridization methods have enabled researchers to understand and recognize the importance of interspecies interactions in periodontitis, including those within the red complex—a frequently observed co-existence of *Porphyromonas gingivalis*, *Tannerella forsythia,* and *Treponema denticola* [65]. More aggressive periodontal diseases have been linked with *F. alocis*, *Porphyromonas*, and *Aggregatibacter actinomycetemcomitans*, among others [66-68].

The microbiome of periodontitis is distinguished from dental caries and other infections by greater microorganism heterogeneity [77]. Periodontal health is associated with an abundance of *Veillonella*, *Neisseria*, *Rothia*, and

Corynebacterium, whereas periodontal disease is linked to a reduction in the *Bacteroidetes* and *Firmicutes* phyla, along with an increase in *Actinobacteria* and *Proteobacteria*, including *Actinomyces*, *Eubacterium*, *Porphyromonas*, and *Peptostreptococcus* [67, 72-76]. The periodontal microbiome's transition from health to disease and the microbiota's dysbiosis is marked by increased heterogeneity, and the emergence of key pathogens, but the functional mechanisms underlying these changes and their effects on immune modulation remain poorly understood. Gaining deeper insights into the host-microbe interactions that drive disease progression requires clinical proteomic data and multi-omics analyses. Additionally, longitudinal studies are required to understand the microbial dynamics of disease progression and identify biomarkers for early diagnosis and intervention.

1.2.5 Oral Fungal Infections and Head and Neck Cancer

Oral fungal infections (OFIs) resulting from excessive fungal growth are common and more likely to occur with suboptimal oral hygiene, impaired immunity, or the use of antimicrobials or immunosuppressants [78, 79]. Reduced host resistance allows opportunistic fungal colonization of the oral cavity, increasing the risk of disease progression [80]. Early infections are often asymptomatic, but as they progress, they can affect the pharynx, leading to lingual and gingival plaque proliferation, possible ulceration, and if not treated can be life-threatening [80].

Selective chromogenic agar or biochemical investigations can initially identify OFIs [81, 82], with subsequent confirmation by laboratory 18S rDNA, ITS [83, 84], or MALDI-TOF MS techniques [85]. Genomic/proteomic techniques have re-confirmed *Candida* as the most prolific genus in OFIs [86, 87]. Of the 200+ *Candida* species, 20 are common in OFIs, with *C. albicans* dominant, while non-*albicans Candida* (NAC) species appear to be increasing. Additional fungal genera may be present, such as *Aspergillus, Malassezia,* and *Cladosporium*, amongst many others [86-88]. Though rare, pathogenic fungi, such as *Aspergillus* and *Cryptococcus*, can also cause oral infection.

Candida and *Malassezia* have been proposed as the dominant mycotypes in the oral cavity [88], with *Candida* associated with lower bacterial and fungal heterogeneity, malignancy, and dental caries, while *Malassezia* is linked to higher heterogeneity [88]. OFIs occur commonly in patients with head, neck, and hematopoietic neoplasms, and after chemotherapy or radiotherapy [89, 90]. *C. albicans* is nearly twice as prevalent as other species in oral squamous cell carcinoma [91]. Additionally, *Candida* infection has been associated with the malignant transformation of oral leukoplakia [92]. Proteomic approaches, such as MALDI-TOF MS, have not yet been applied to the study of OFIs in malignancy,

and the precise role of oral fungi in tumor progression or suppression remains poorly understood. Furthermore, the functional contributions of the oral mycobiota—including its metabolic interactions with bacteria, effects on immune modulation, and potential protective or pathogenic roles—remain largely unexplored.

1.3 The Oral Microbiome and COVID-19

1.3.1 Pathogenesis of COVID-19-SARS-CoV-2 Infection

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) responsible for the COVID-19 pandemic, generated a huge global research effort to understand and combat the virus [93, 94]. The virus targets epithelial cells mainly in the respiratory tract by binding to angiotensin-converting enzyme 2 (ACE2) receptors—a process facilitated by the spike (S) protein. Once bound the virus penetrates the host cell, and releases its RNA genome, hijacking the cell's machinery to replicate and produce new viral particles [96]. This triggers an inflammatory immune response, which, in severe cases, can lead to acute respiratory distress syndrome (ARDS) and systemic complications [96]. The risk of developing severe COVID-19 increases with age (particularly in individuals over 60) and is exacerbated by underlying health conditions, blood type A, and low vitamin D levels [97].

1.3.2 Oral Microbiome and COVID-19 Severity

COVID-19 progression and severity are strongly associated with the health of the oral microbiome, with dysbiosis linked to immune dysregulation and inflammation. Patients with COVID-19, or those recovering from infection, often have persistent fungal imbalances, including increased *Candida albicans* and *Aspergillus* [98], suggesting long-term disruption to the microbial balance [98]. Severely affected patients may also have increased *Prevotella* and *Fusobacterium,* the levels of which have been shown to correlate with the severity of inflammation and systemic disease [99]. Higher SARS-CoV-2 viral loads have additionally been linked to microbial imbalances of the oral and gut microbiomes, highlighting the importance of microbial eubiosis for optimal immune responses.

Oral microbiome signatures can also reflect disease severity and outcomes. Increases in *Porphyromonas* and *Streptococcus mitis* bacteria, for example, have been linked to COVID-19 severity and altered cytokine profiles [100]. Similar microbial effects extend to the oral-lung axis, where pulmonary symptoms appear to be exacerbated by increased *Rothia mucilaginosa* and mitigated by

increased *Actinomyces* [101]. In severe COVID-19 cases, the diversity of the oral microbiome tends to be reduced, with elevated levels of *Veillonella* and *Leptotrichia* [102] particularly linked to local inflammation. These findings underscore the oral microbiome's potential as both a biomarker and a therapeutic target in managing COVID-19 severity. However, the mechanistic pathways linking microbial dysbiosis to immune modulation and systemic inflammation remain poorly defined.

1.3.3 Development of COVID-19 Vaccine

The rapid development of COVID-19 vaccines since the initial detection of SARS-CoV-2 has made enormous contributions to immunology. As of February 2025, 64 vaccines have been licensed by national regulatory agencies [104], with vaccine platforms including inactivated viruses, mRNA, DNA, recombinant proteins, and non-replicating and replicating viral vectors [105]. Immunogens include spike proteins, receptor binding domains, and spike protein ectodomains [105]. Of all vaccines, 13 tested in phase III clinical trials produced neutralizing antibodies, with mRNA vaccines generating the highest antibody titers [106, 107]. Despite a dramatic 95% reduction in COVID-19-related mortality since the pandemic began, the disease remains a serious public health concern, with up to 2 million new infections reported annually [108].

The development of mRNA vaccines by Pfizer-BioNTech and Moderna marked a groundbreaking moment in viral immunology. These vaccines are encapsulated in unique lipid nanoparticles and utilize messenger RNA to instruct cells to produce harmless spike proteins stimulating robust immune responses [106], including the activation of antigen-specific T-follicular helper cells and germinal center B cells [106]. These vaccines revealed key structural vulnerabilities of the SARS-CoV-2 virus, particularly in the spike protein's receptor-binding domain [109]. Both Pfizer and Moderna vaccines demonstrate similar clinical efficacy in preventing moderate SARS-CoV-2 infections [110]. Parallel research by AstraZeneca, Johnson & Johnson, and others on viral vector vaccines—which deliver genetic material via modified viruses—also represented a major advancement, as they provided alternative approaches to achieving immunity and expanded vaccine research [110].

1.4 Oral Microbiota and Pancreatic Cancer

One theory of pancreatic cancer (PC) carcinogenesis proposes that local and remote neoplasms may be induced following the circulatory transportation of oral pathobionts or oncopathogens, e.g. *F. nucleatum*, *P. heparinolytica*, *P.*

gingivalis, and *P. anaerobius* to remote sites where they initiate carcinogenesis [111–117].

Many studies, including meta-analyses, have shown that changes in the oral microbiota are associated with pancreatic tumorigenesis and therapy [118, 119]. High-throughput genome sequencing now allows for a more in-depth characterization of the relationship between the microbiome and PC. The Cancer Prevention Study II and Prostate, Lung, Colorectal and Ovarian prospective databases [120] stored oral microbiota samples from patients ten years prior to their presenting with PC, and were subsequently compared to matched controls. *P. gingivalis* and *A. actinomycetemcomitans* were associated with heightened PC risk, while the presence of *Fusobacterium* and *Leptotrichia* with a lower risk [120]. Similarly, two European studies also found elevated *P. gingivalis* and *F. nucleatum* antibody titers to be associated with greater PC risk and tumor severity [121, 122], with *P. gingivalis* and *A. actinomycetemcomitans* linked to a higher PC rate [123].

The oral microbiota of PC patients also appears to be characterized by a higher prevalence of *Enterobacteriaceae* and *Lachnospiraceae G7*, whereas healthy controls exhibit higher frequencies of *Hemophilus* [124]. Symbiotic microbes from the intracapsular pancreas, like *G. adiacens* and *F. nucleatum*, also colonize the mouth [125]. Another study identified a lower prevalence of *N. elongata* and *S. mitis*, alongside a higher prevalence of *G. adiacens*, as biomarkers to distinguish PC patients from controls, and achieved a sensitivity of 96.4% and specificity of 82.1% [126]. Interestingly, increased frequencies of these two bacteria have also been documented in chronic pancreatitis [126]. Saliva testing of patients with PC and controls revealed a greater *Leptotrichia to Porphyromonas* ratio, but no changes in *S. mitis* or *G. adiacens* [127]. In a Chinese study, *Viellonella* and *Neisseria* were prevalent in controls whereas patients with PC demonstrated high rates of *Streptococcus* and *Leptotrichina* [128]. Microbiome disparities may also have diagnostic potential for differentiating precancerous from cancerous tissue. The intraductal papillary mucinous neoplasms (IPMN) of subjects with PC exhibited increased populations of *Firmicutes* compared to controls, who had higher numbers of *Proteobacteria* [129].

Currently, the role of opportunistic oral microorganisms in PC remains poorly understood, and causal relationships between the oral microbiome and the disease have yet to be clearly demonstrated. Some microbial species, while not directly carcinogenic, may facilitate the activity of tumor-inducing agents or suppress host defense mechanisms. Additional research is required to further elucidate the role of the oral microbiome in this context.

1.5 The Human Immune System: A Sophisticated Defense Network

Over millions of years, mammals have developed intricate defense mechanisms that integrate innate and adaptive immune responses that provide a remarkable level of protection [130]. The immune system has evolved into a complex network for protecting against both external threats, such as microorganisms (bacteria, viruses, fungi, parasites), toxins, and internal threats such as cancer [130].

1.5.1 Innate Immunity: The First Line of Defense

The innate immune system provides a rapid, non-specific response to threats that serves as the body's first line of defense. This ancient mechanism relies on processes such as phagocytosis and the secretion of antimicrobial peptides (AMPs) [131]. AMPs, together with reactive oxygen species and nitric oxide (NO), effectively combat microbial invaders [132]. In humans, pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), NOD-like receptors, and retinoic acid-inducible gene I (RIG-I)-like receptors, detect microbial components, such as microbe-associated molecular patterns (MAMPs), including lipopolysaccharides, CpG DNA, and flagellin [131, 133]. PRR activation initiates host defense mechanisms, triggering inflammation and immune cell recruitment [133]. Despite the low precision of the innate immune system in recognizing specific pathogen strains [133], it remains crucial for pathogen clearance, and serves as a critical bridge to adaptive immunity. However, the extent to which oral microbiota-derived signals modulate PRR activation, influence inflammatory homeostasis, or contribute to immune tolerance, remains incompletely understood.

1.5.2 Innate–Adaptive Crosstalk: Precision and Memory

The innate and adaptive branches work in tandem to provide a dynamic and effective defense against pathogens. While anatomical and chemical barriers, such as skin and mucosal surfaces, typically prevent microbial entry [134], if they fail, innate immune cells, including neutrophils, macrophages, and dendritic cells, respond swiftly by phagocytosing pathogens and releasing cytokines and chemokines [134]. Natural killer (NK) cells and innate lymphoid cells (ILCs) further amplify inflammation and eliminate infected cells [134].

In contrast to the innate system, the adaptive immune system has high precision and provides long-lasting immunity. Adaptive immunity is mediated by B and T lymphocytes, which undergo V(D)J recombination to generate a diverse repertoire of antigen-specific receptors. Upon infection, selected B and T cell clones expand and differentiate into effector cells to combat the pathogen [135].

Differentiated B cells (antibody-secreting plasma cells), produce antibodies that confer long-term protection [135], with a subset of these cells persisting as memory cells, enabling a rapid and robust response during reinfection.

1.5.3 Innate-Like T Cells: Bridging Innate and Adaptive Immunity

Innate-like T cells, such as γδ T cells, invariant natural killer T cells, and mucosa-associated invariant T (MAIT) cells, exhibit features of both innate and adaptive immunity [136]. Unlike conventional T cells, these cells recognize non-peptide antigens and respond rapidly in large numbers to protect the host while adaptive responses mature [136]. Their unique positioning underscores their importance in bridging the innate and adaptive systems.

1.5.4 The Crosstalk Between Microbiota and the Immune System

The microbiota plays an indispensable role in the development and regulation of the host immune system. This intricate relationship ensures immune homeostasis, promotes tolerance to commensal microbes, and defends against pathogens. Emerging evidence highlights the dynamic interplay between microbial signals and immune components, which together maintain a delicate balance essential for health (Figure 2).

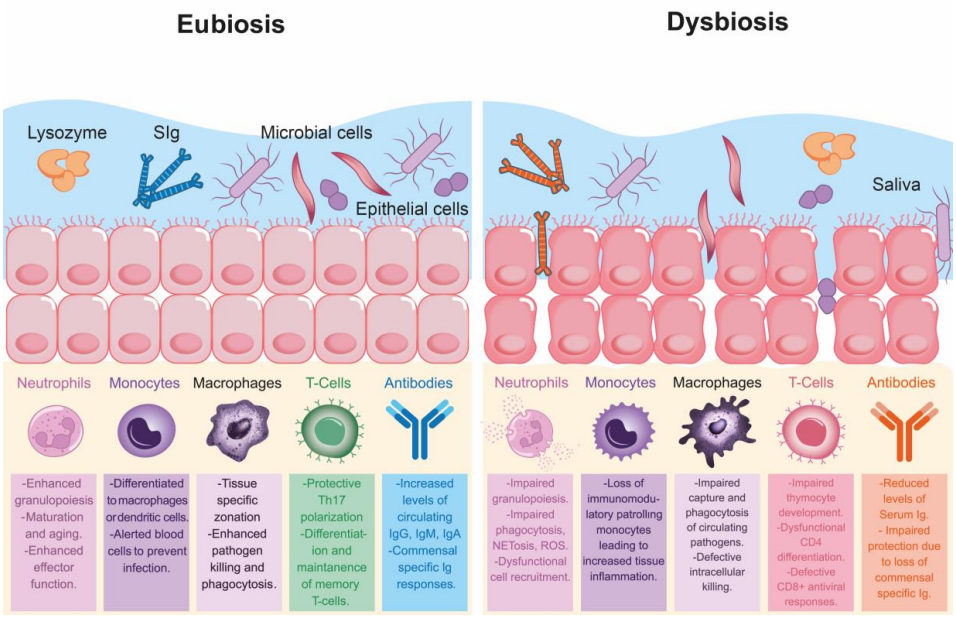


Figure 2 Immune responses in eubiosis and dysbiosis of the oral environment. The left panel depicts eubiosis, where lysozyme, secretory Ig (SIg), and commensal microbes maintain immune balance. Neutrophils, monocytes, macrophages, T cells, and antibodies function efficiently for pathogen clearance. The right panel shows dysbiosis, marked by microbial imbalance, reduced SIg, and immune dysfunction, leading to impaired neutrophil recruitment, monocyte and

macrophage activity, disrupted T-cell differentiation, and weakened antibody responses against incoming pathogens.

1.5.5 Balancing Tolerance and Immunity

A hallmark of the microbiota-immune interaction is balancing immune tolerance to commensal microbes with inflammatory responses against pathogens when required. Regulatory T cells play a central role in maintaining this balance by suppressing excessive immune activation and preventing tissue damage [137]. Similarly, ILCs, which are enriched at mucosal surfaces, produce cytokines such as IL-22 and IL-13 to enhance epithelial barrier function and promote antimicrobial peptide production [138]. However, dysbiosis can lead to immune dysregulation and chronic inflammatory conditions. Dysbiosis is characterized by a loss of microbial diversity and overgrowth of pathogenic species, which can drive inflammation in many diseases such as inflammatory bowel disease, metabolic syndrome, and autoimmune disorders [139].

1.6 The Role of the Microbiome in Modulating the Immune Response to Parenteral Vaccines

The human microbiota is increasingly being recognized as crucial in regulating systemic immunity, with emerging evidence demonstrating its role in shaping the immune response to vaccines. Much has been discovered about its impact on mucosal vaccines, but research into its effects on the immune response to parenteral vaccines (those delivered intramuscularly, subcutaneously, or intradermally) is still in its early stages.

The gut microbiota plays a fundamental role in training and priming the host immune system. Bacterial flagellin, for example, can stimulate TLR5, leading to the activation of antigen-presenting cells (APCs), enhanced cytokine production, and a more robust T and B cell response following vaccination [140]. The immunomodulatory effects of microbiota extend beyond direct microbial signaling, as gut bacteria also produce key metabolites, such as short-chain fatty acids (SCFAs) like butyrate, acetate, and propionate, which influence the differentiation and activation of immune cells. SCFAs have been shown to promote dendritic and macrophage cell activity, enhance T follicular helper (Tfh) cell responses, and increase the antibody production of plasma cells [141-145]. These mechanisms collectively facilitate the development of high-affinity, antigen-specific immune responses that are critical for effective vaccination.

Evidence from germ-free (GF) animal models highlights the importance of the microbiota in vaccine immunogenicity. GF mice, which lack microbial colonization, exhibit severely impaired immune responses to parenteral

vaccines, such as the influenza vaccines [140], with reduced antigen-specific antibody titers and weaker T-cell activation. Similarly, significantly reducing microbial diversity in non-GF mice through antibiotic treatment leads to suboptimal vaccine responses, further highlighting the importance of a healthy microbiota in supporting vaccine-induced immunity [146]. However, while this research has primarily focused on the gut microbiota, the potential contributions of oral microbiota—particularly its role in antigen presentation and systemic cytokine responses—remain underexplored, despite their proximity to mucosal immune interfaces.

1.6.1 Evolving Immunity: Vaccines and Adaption to SARS-CoV-2 Variants

Insights into the roles of humoral and cellular immunity in COVID-19 protection continue to evolve. Heinz and Stiansy explored how different COVID-19 vaccines present the spike (S) protein to the immune system, shaping the resulting immune response [147]. Circulating antibodies targeting the S-protein are known to be capable of neutralizing the virus, and their abundance correlates with protection. However, comparative data on vaccine efficacy remain scarce.

A significant advance has been the calibration of neutralizing antibody levels against the WHO International Standard (NIBSC code 20/136), enabling standardized comparisons to be made. These standards enabled Karbiener *et al.* to show that the levels of neutralizing antibodies generated by the AstraZeneca viral vector vaccine were significantly lower than those induced by the Pfizer-BioNTech mRNA vaccine or the adjuvanted S-protein nanoparticle vaccine from Novavax [148]. Notably, the antibody levels induced by the latter two vaccines were statistically indistinguishable from each other [148].

The emergence of SARS-CoV-2 variants, particularly the Wuhan-Hu-1 and Omicron lineage, has challenged immune protection from prior vaccinations and natural infections, raising concerns about the efficacy of existing vaccines. In response, there has been a surge in studies exploring immunological cross-reactivity to these evolving variants. The evidence shows that individuals, especially children, can develop immune responses that cross-react with newly emerging variants, which is encouraging, as it makes the development of universal vaccines a realistic possibility [149, 150]. Both hybrid immunity and rapid booster vaccinations have demonstrated potential for enhanced protection against variants [149, 150]. Successful vaccine development depends critically on global collaboration and the rigorous monitoring of viral mutations [151]. This global effort has driven updates in vaccine formulations, ensuring they remain effective against emerging threats and enable rapid responses to future pandemics. Continued monitoring of vaccine efficacy and safety across diverse

populations and against new SARS-CoV-2 variants, remains crucial to sustain progress in combating COVID-19.

1.6.2 Gut Microbiota and the Immune Response to COVID-19 Vaccines

The immune response to vaccines is influenced by many factors, including host characteristics and behavior, vaccine formulation, microbiota composition, and the environment (Figure 3). Gut microbiota are fundamental modulators of immune responses and significantly influence vaccine efficacy. These microbes shape both innate and adaptive immunity by regulating antigen presentation, cytokine production, and T and B cell activation, making them key determinants of interindividual variations in vaccine outcomes. Studies investigating their role in the response to SARS-CoV-2 vaccines have identified specific microbial taxa and metabolites associated with enhanced immune responses [152, 153], deepening our understanding of the microbiota's potential to optimize vaccine-induced immunity across populations, but knowledge of the causal mechanisms linking specific taxa and metabolites to immunity remains incomplete.

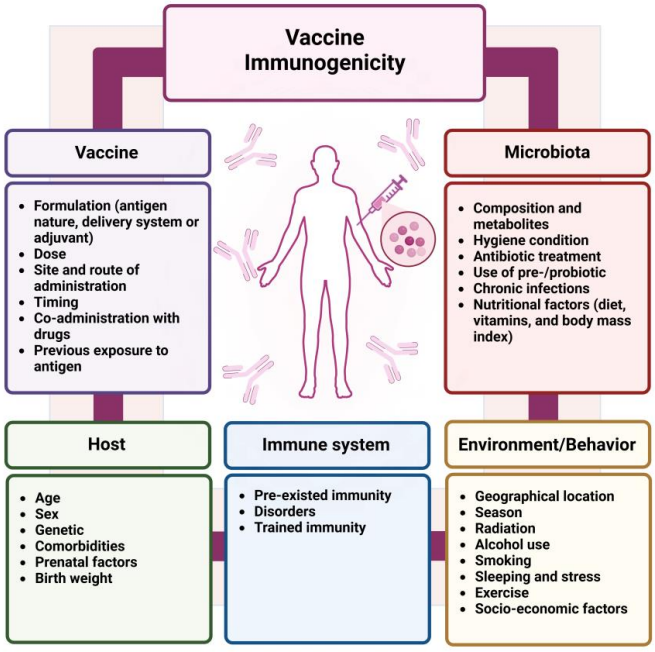


Figure 3 Factors influencing vaccine immunogenicity. Vaccine immunogenicity is shaped by multiple interconnected factors, including vaccine-specific attributes, host characteristics, immune system status, microbiota composition, and environmental/behavioral influences.

1.6.3 Gut Microbiota and Vaccine Immunogenicity

Research indicates that individuals with higher gut microbial diversity exhibit stronger immune responses to COVID-19 vaccines. Two bacteria, *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*, known for their anti-inflammatory properties and production of SCFAs, have also been associated with enhanced IgG titers and T-cell responses to the Pfizer-BioNTech vaccine [17]. In immune responses to the Moderna vaccine, *Roseburia inulinivorans* and *Akkermansia muciniphila,* have also been associated with sustained antibody levels [154] and are known to support long-term immune memory, gut epithelial health, and metabolic regulation.

The role of microbial metabolites, particularly SCFAs such as butyrate and acetate, in modulating immune responses is well established. SCFAs enhance dendritic cell activity, promote Tfh cell differentiation, regulate antigen-specific immunity, and support plasma cell function. As such they are all essential components of vaccine-induced immunity. In the study by Seong *et al.* [155], higher levels of microbes that produce these metabolites were associated with improved post-vaccination antibody production and T-cell activation. In contrast, gut dysbiosis induced by antibiotic treatment was characterized by a reduction in beneficial microbes such as *Bacteroides fragilis* and *Prevotella copri*.

1.6.4 Microbiota and Adverse Vaccine Outcomes

Gut microbiota composition has also been linked to the severity of post-vaccine adverse events. Microbes such as *Clostridium butyricum* and *Lactobacillus rhamnosus,* which promote the production of anti-inflammatory cytokines (e.g., IL-10 and TGF-β), help prevent excessive inflammation. Higher levels of these microbes have been associated with reduced inflammatory symptoms in older adults vaccinated with the AstraZeneca vaccine [156].

In immunocompromised populations, dysbiosis has been associated with increased adverse outcomes and reduced vaccine efficacy. For example, a study on patients with autoimmune diseases and organ transplants who received mRNA vaccines found that the loss of SCFA-producing bacteria, such as *Ruminococcus obeum*, correlated with more severe adverse events and lower antibody responses [157]. Their findings highlight the potential for microbiota-targeted therapies to mitigate vaccine-related side effects and improve outcomes in vulnerable populations.

1.6.5 Microbial Diversity in Vulnerable Populations

The microbiota's influence on vaccine responses varies significantly across life stages and health conditions. The early colonization of infants' microbiomes is crucial for their immune development and their effective response to vaccination. Infants harboring higher levels of *Bifidobacterium breve* and *Bifidobacterium longum* exhibit stronger IgG and IgA responses to mRNA vaccines [17]. Both these beneficial bacteria help establish robust vaccine responses by enhancing mucosal immunity and antigen presentation. In contrast, infants with dysbiosis, marked by elevated *Escherichia coli* levels and a reduction in beneficial taxa, showed weaker and delayed immune responses.

In older adults, gut dysbiosis and immunosenescence impair vaccine efficacy. Age-related dysbiosis has been characterized as a decline in SCFA-producing bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia hominis*, coupled with an increase in pro-inflammatory taxa like *Enterobacteriaceae* [153]. Such microbial shifts exacerbate chronic inflammation and weaken antigen presentation, leading to reduced immune responses following vaccination. Probiotic interventions aimed at restoring microbial balance, have shown promise for enhancing vaccine efficacy in older adults. In individuals living with HIV, the diversity of the microbiota, as well as the particular species it contains, also play a significant role in modulating vaccine-induced immunity. Lower levels of the crucial SCFA-producing *Bacteroides thetaiotaomicron,* in HIV-positive participants are associated with reduced antibody titers following vaccination [158]. This suggests that interventions targeting microbial health could improve vaccine efficacy in immunocompromised populations.

Despite growing evidence of the gut microbiota's role in COVID-19 vaccine responses, the contribution of oral microbiota remains largely unexplored. Oral microbes, including *Streptococcus mitis* and *Fusobacterium nucleatum*, are known to influence mucosal immunity and may play a significant role in systemic immune responses. However, to date, only one study has investigated this connection, reporting post-vaccination shifts in oral microbiota, such as increased levels of *Streptococcus salivarius* [159]. Further research is therefore needed to elucidate how oral microbiota influence vaccine-induced immunity.

1.7 Immune Sensing and Activation in MAIT Cells

MAIT cells are a specialized subset of innate-like T cells that play a critical role in mucosal immunity and infection defense. They express semi-invariant T cell receptors (TCRs), primarily Vα7.2-Jα33 in humans, which detect microbial-derived riboflavin (vitamin B2) metabolites presented by MR1 (MHC class I-

related protein). Unlike conventional T cells, MAIT cells mount a rapid immune response, contributing to antimicrobial defense, immune surveillance, and tissue homeostasis [160]. Conserved for over 150 million years, they act as a bridge between innate and adaptive immunity and are increasingly recognized for their roles in infection control, tumor immunity, and autoimmune diseases, highlighting their broad immunological significance [161, 162].

MAIT cell activation can occur via the major histocompatibility complex class I-related protein (MR1) pathway, following ligand presentation to TCRVα7.2 on the MAIT cells. The two most potent activating compounds are the riboflavin metabolites, 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil; 5-OE-RU and 5-OP-RU [163, 164]. These are synthesized via non-enzymatic fusion (e.g., with glyoxal and methylglyoxal), by microorganisms including *Mycobacterium*, *Escherichia coli*, *Lactobacillus*, *Staphylococcus*, *Shigella fowleri*, *Salmonella*, and *Clostridium* or fungi such as *Saccharomyces*, *Candida*, and *Aspergillus* [165-169].

Certain MAIT cell TCRs can also recognize MR1-bound tumor antigens, obviating the need for riboflavin metabolites [170]. MR1 occurs within the endoplasmic reticulum of APCs. For riboflavin metabolites, a functional element is generated following the binding of the MR1 and riboflavin metabolite conjugate with β2 microglobulin, which then engages with TCRVα7.2 [171]. Activated MAIT cells release inflammatory response cytokines, annihilate pathogens, and trigger an immune response to infected cells [172].

The MR1-independent pathway, associated with viral infection and reliant on cytokines and type I interferons, also enables MAIT cell activation [173]. This activation occurs by the direct effect of pro-inflammatory cytokines, such as IL-12 and IL-18, released by APCs [173]. Positive correlations have been reported between elevated serum IL-18 levels and amplified MAIT cell CD69 expression [174-176]. MAIT cell surface receptors for these interleukins can be triggered by PLZF, which promotes a swift cellular response [177]. The release of IL-12 and IL-18 is promoted by APC TLR-4 agonists, as well as IL-15 [178]. Following MAIT cell activation, IL-7 and IL-23 receptors encourage IL-17 synthesis [179] (Figure 4).

The two mechanisms of MAIT cell activation may overlap; early activation may be favored by MR1 [177], whereas late activation can require both pathways. Expression of various molecules and cytokines, (e.g., CD25, CD69), IFN-γ and IL-17, is amplified following MAIT cell activation, which promotes both innate and adaptive immune cell activity as well as infected cell apoptosis. MAIT cell hyperstimulation, however, may engender upregulated activation-induced programmed cell death.

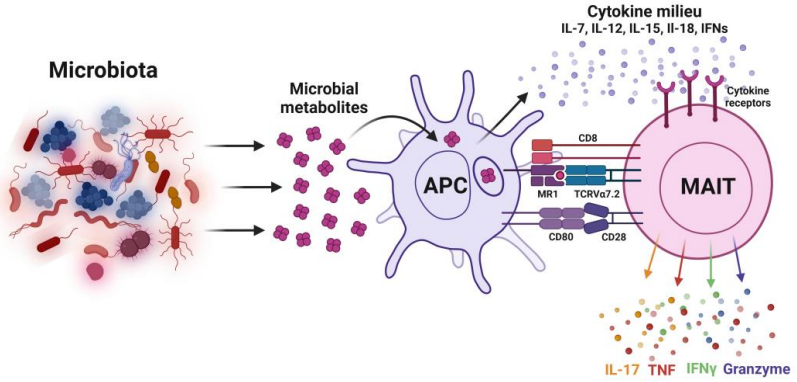


Figure 4 Microbiota-driven MAIT cell activation and the immune response. MAIT cells are activated through two distinct pathways: MR1-dependent APCs, forming a complex that engages the TCRVα7.2 receptor on MAIT cells, leading to activation. Additionally, cytokines such as IL-12, IL-18, IL-15, and IFNs can stimulate MAIT cells independently of MR1. Once activated, MAIT cells secrete pro-inflammatory cytokines (IL-17, TNF, IFN-γ) and cytotoxic molecules (granzymes), contributing to pathogen clearance and immune modulation.

1.7.1 Oral Microbiota and MAIT Cells

Human MAIT cells congregate between the connective and epithelial cell tissues within the buccal mucosa and gingiva [180]. These cells have amplified expression of CCL20 and CXCL16 in connective tissue, CCR6 and CXCR16 in gums, and CD69, CD103, HLA-DR, and PD-1 in the oral mucosa, which indicates an activated tissue resident phenotype [181]. Exposure to pathogens or cytokines initiates IL-17 and TNF release more readily from oral mucosal than circulatory MAIT cells [181-184].

MAIT cells are important contributors to infection control, in part because they can recognize byproducts from microbial synthesis of riboflavin. Chronic periodontitis, for example, is associated with microorganisms like *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans,* which produce riboflavin metabolites [114, 185-189], and porphyrin monocytosis and nuclear bacteria from the gum are also associated with more riboflavin synthesis than occurs in suspension [190]. MAIT cells are activated by the presence of riboflavin metabolites, and their uptake influences bone remodeling in the oral cavity [191]. In periodontitis, circulatory MAIT cell titers are reduced, and CD69+ MAIT proportions and IL-17 and TNF secretion are elevated with CCL20 and CXCL16 expression [181], implying MAIT cell recruitment into the inflamed tissue.

In apical periodontitis, a higher proportion of CD4+ MAIT cells are present, especially those with Vα7.2Jα33, Vα7.2Jα12, and Vα7.2Jα20 TCRs [192], as well as amplified IL-17, IFN-γ, and TNF-α expressions. Riboflavin pathway gene

enrichment promotes MAIT cell activation, thus enabling diseased tissue invasion and proinflammatory cytokine synthesis [192]. Fungal infections in oral tissues are associated with higher MAIT cell populations than in circulation [176], as well as increased percentages of CD4+, CD69+, and CD103+ MAIT cells which can manufacture GzB and PD-1 [176]. While these observations enhance the understanding of MAIT cell involvement in oral and systemic pathology, the precise mechanisms regulating their activation, tissue recruitment, and functional plasticity remain unclear.

1.8 Approaches to Microbial Characterization and Immune Profiling

1.8.1 Techniques for Oral Microbiome Characterization

First recognized by Antonie van Leeuwenhoek [193], the oral microbiome has been extensively studied using both culture-dependent and culture-independent techniques. Culture-dependent techniques, such as microscopy, require bacterial isolation and growth and have underpinned oral microbiology research. Characterization is typically based on microbe appearance, metabolism, and both physiological and pathogenic traits [194, 195]. Consequently, past microbiome species characterization has been biased toward organisms that are easier to investigate. With the advent of de novo culture methods, more sophisticated analyses have become possible, allowing microbes to be cultured under anaerobic or aerobic conditions, on specific media at controlled temperatures, and with co-colonization by one or more additional species [194-196]. However, these approaches remain limited to species amenable to cultivation, thereby overlooking a significant proportion of microbial diversity. In contrast, the development of high-throughput genomic sequencing technologies has revolutionized microbial research by enabling the identification of uncultured taxa and providing deeper insights into the dynamics of microbial communities.

Next-generation sequencing (NGS) methods, including 16S rRNA gene sequencing for bacteria and ITS gene sequencing for fungi, allow for taxonomic profiling with high resolution. Shotgun metagenomics further enhances these analyses by providing insights into the function of microbial genes and metabolic pathways. Additionally, proteomic techniques such as matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) have improved microbial identification by enabling rapid and accurate protein-based classification [197, 198].

1.8.2 Omics: Culturomics and Multiomics Integration

Despite the advances in sequencing-based techniques, culture-based methods remain indispensable for microbial characterization. Culturomics is a high-throughput method using diverse culture conditions to facilitate the growth of previously uncultivable microbes. [199]. Combined with MALDI-TOF MS, this approach has led to a significant expansion of bacterial and fungal reference databases, refining microbial taxonomy and phenotypic characterization, and overcoming some limitations of genomics tools [199]. However, culturomics alone lacks insights into microbial functionality, necessitating the integration of multiomics approaches. Metagenomics deciphers microbial composition, while metatranscriptomics and metaproteomics reveal gene expression and functional protein activity, respectively [200]. Metabolomics further elucidates microbial metabolic interactions and host responses [200]. The convergence of these omics technologies provides a holistic view of microbial communities, their ecological roles, and contributions to health and disease. Despite these advances, challenges remain in standardizing data integration and establishing functional correlations within complex microbiomes (Table 1, Figure 5).

Table 1 Comparison of microbial characterization approaches.

Approach	Advantages	Disadvantages
Amplicon Sequencing (16S rRNA, ITS sequencing)	- Cost-effective and widely used for taxonomic profiling - Requires minimal input DNA - Suitable for low-biomass or host DNA-contaminated samples	- Limited to known reference sequences - Provides only taxonomic, not functional insights - PCR biases and primer selection affect outcomes
Shotgun Metagenomics	- Provides strain-level resolution and whole-genome data - Enables functional potential analysis - Detects both known and novel organisms	- Higher cost and computational demands - Host DNA contamination can be an issue - Requires extensive bioinformatics expertise
Metatranscriptomics	- Captures actively transcribed genes, revealing functional activity - Provides insights into microbial responses to environmental conditions	- RNA is prone to degradation, requiring careful handling - High host RNA contamination - Computationally intensive and requires extensive reference databases

Metaproteomics	- Directly measures expressed proteins, reflecting microbial activity - Identifies functional microbial interactions - Can be used for taxonomic and functional classification	- Protein extraction is challenging and susceptible to biases - Poor coverage of low-abundance species - Requires high-quality reference protein databases
Metabolomics	- Identifies metabolic end-products, linking microbial activity to host interactions - Provides functional insights complementary to genomics and proteomics - Can reveal potential biomarkers	- Complex sample preparation and extraction methods - High variability and difficulty in standardization - Limited reference metabolite databases
MALDI-TOF MS	- Rapid and cost-effective microbial identification - Requires minimal sample preparation - Can differentiate closely related species	- Limited ability to detect unknown or uncultured microorganisms - Requires well-curated reference libraries - Provides taxonomic but limited functional information
Culturomics	- Allows the isolation of viable microbes, including rare and uncultured species - Provides direct access to microbial isolates for functional studies and antimicrobial susceptibility testing - Enables the discovery of new bacterial species	- Time-consuming and labor-intensive - Culture conditions may not support the growth of all microorganisms - Requires extensive optimization for different microbial groups

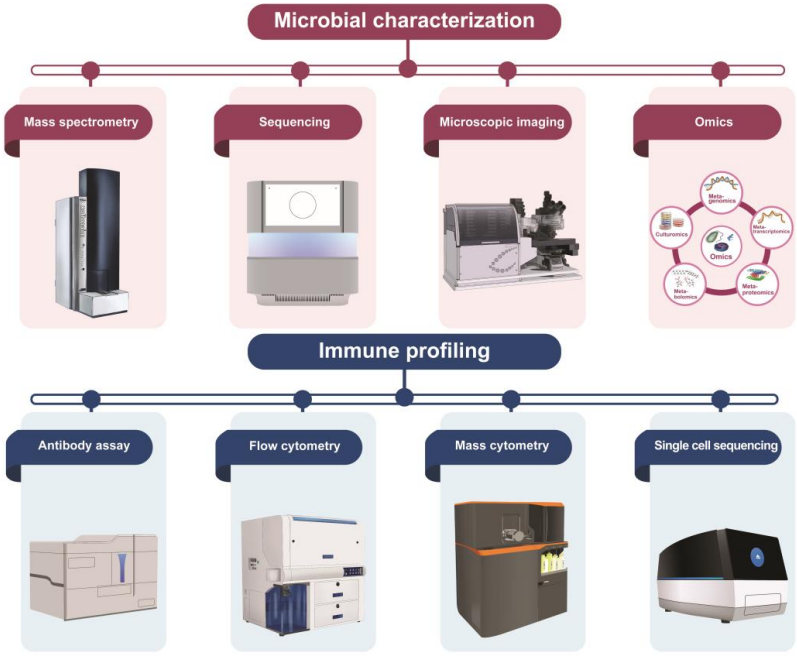


Figure 5 Microbial characterization and immune profiling techniques. The figure illustrates various tools used for microbial characterization (top) and immune profiling (bottom).

To better understand the ecosystem of oral microbiota, including their distribution, in situ abundance within biofilms, and their role in oral diseases, it is essential to monitor and compare microorganisms from various niches within the oral cavity. Fluorescence *in situ* hybridization (FISH) has emerged as a powerful tool for studying oral microorganisms in situ. This cytochemical technique uses fluorescently labeled nucleic acid probes to detect, identify, and localize targeted genetic sequences with high sensitivity and specificity [201]. FISH offers several advantages, including rapid and accurate detection of microorganisms, making it one of the most widely used methods in microbiological research. Furthermore, it enables the simultaneous identification and quantification of diverse oral microorganisms [201]. The integration of FISH with other molecular biology techniques, enables the visualization of microbial communities and their distribution within oral biofilm-associated diseases to be mapped, providing deeper insights into the oral microbiome and its link to health and disease.

1.8.2.1 Cultivation Approach - MALDI-TOF MS

Investigations of the human microbiome that rely on culture techniques are regaining popularity. These resource-intensive techniques examine phenotypic and biochemical characteristics of microbes, as well as colony morphology,

contributing to the identification of different species, particularly fungi [202]. Microbial analysis has been greatly advanced by MALDI-TOF MS [202] which has contributed to both the bacterial biomarker database (established in 2004) [203], and the characterization of the human GIT microbiome since 2012 [204]. Consequently, a combination of MALDI-TOF MS and NGS strategies has been utilized to extract and sequence previously uncultured microorganisms, reduce unmatched or undesignated sequences within the microbiome [205, 206], and explore microbe-specific protein configurations [207]. Until recently, MALDI-TOF characterization of microbiome organisms has concentrated on the GIT microbiome, leaving the oral microbiome relatively unexplored [208].

1.8.2.1.1 Principles of MALDI-TOF MS and Platforms

The identification of microorganisms utilizing MALDI-TOF MS is achieved through the comparison of either the peptide mass or the mass of the biomarkers of the microbe under investigation with the fingerprints stored in reference databases. Recognition of microorganisms at the level of the species generally requires a mass spectrum, m/z, of 2-20 kDa, largely reflecting ribosomal and housekeeping proteins [209], which make up between 60% and 70% of the microbe's dry cell weight. These form part of the unique mass fingerprint of the peptides for database comparison [210, 211]. If reference data are unavailable, NGS sequencing is required. MALDI-TOF MS characterization involves sample collection and processing, followed by isolation, culture, identification, and microbe preservation [199].

More recent MALDI-TOF MS-based systems for scientific and clinical analysis include MALDI Biotyper and the VITEK® MS [212, 213, 214]. Andromas from bioMérieux later introduced a distinct database and software for routine microbiological identification, compatible with Bruker and Shimadzu instruments [215].

1.8.2.2 Sequencing Approach – Next Generation Sequencing (NGS)

Since understanding the double helical structure of DNA, microorganism identification is now possible through sequencing methods, polymerase chain reaction (PCR), cloning techniques [216, 217], and the use of phylogenetic indicators, like 16S ribosomal RNA (rRNA) [218]. DNA NGS microorganism barcodes can be generated using information from 16S and 18S rRNA genes, internal transcribed spacers (ITS), and metagenomic sequencing [219-221]. Over 770 oral cavity taxa have defined, full-length 16S rRNA gene sequences in the Human Oral Microbiome Database, a resource that greatly facilitates genome

resolution and encompasses much of the oral metagenome [222, 223], although 30% of the oral taxa data still remain absent or incomplete [224].

1.8.2.2.1 Principles of NGS and Platforms

Rapid advances in molecular diagnostics have significantly reduced identification times for non-cultivatable microbes, encouraging their clinical application. One such approach, Sanger sequencing, is used for microorganism identification, establishing inter-microbe associations, and identifying mutations underlying therapeutic resistance (although issues occur in the presence of more than one species) [225]. Typically, specific primers are used beforehand to amplify individual genes or genomic loci.

NGS is another widely used method, particularly for studying and genotyping genotyping high-resistance organisms. Unlike Sanger sequencing, NGS requires only a single sequencing run to generate comprehensive data on microbial identity, resistance genes, and virulence factors. Additionally, NGS employs a universal protocol for all microorganisms, and whole genome sequencing obviates targeting with primers [225]. Prior to sequencing, genomic DNA is fragmented, and the resulting fragments are ligated with adapters and barcodes for identification. These fragments are then clonally amplified, normalized, and sequenced. Post-sequencing, the adapters and barcodes facilitate the accurate reconstruction of DNA sequences.

NGS methods vary based on the platform used. Illumina sequencing employs fluorescent reversible terminators to identify nucleotide incorporation. Thermo Fisher's semiconductor technology detects pH changes during nucleotide addition. Pacific Biosciences utilizes single-molecule real-time (SMRT) sequencing, leveraging fluorescently labeled nucleotides. Meanwhile, the Oxford Nanopore platform detects nucleotide-specific ionic current changes as DNA passes through a nanopore. [225]. Microbial identification is achieved either through de novo assembly of sequenced fragments (without a reference genome) or by comparison with a reference library. Different bioinformatics pipelines can expedite data analysis, and the results can be stored in a networked database.

1.8.2.2.2 Targeted and Non-Targeted Microbiome Sequencing

16S/18S amplicon-targeted NGS and non-targeted metagenomic sequencing (shotgun or whole-genome sequencing) are the primary strategies for studying the human microbiome. Both approaches require interpretation of the relative percentages of DNA sequences present in a sample and comparison to a

reference sequence database of known microorganisms [226-228]. The technique requires generating multiple random DNA fragments which are read in parallel (simultaneously) and then joined at their overlapping termini to create full or partial sequences [226-228]. A widely used method is 16s rRNA (ribosomal RNA) gene sequencing, which has steadily increased in precision. All bacteria and archaea express this marker gene at a highly conserved locus within which are nine hypervariable regions (~ 1500 base pairs long) that facilitate taxonomic identification through database comparison [227, 228]. Advances in sequencing now allow differentiation at the species level and, in some cases, strain-level resolution, while also enabling functional microbiota profiling [219, 228]. For fungi, a marker locus with taxonomic accuracy similar to the bacterial 16S rRNA gene is the internal transcribed spacer (ITS) of the rRNA cistron, which is frequently used for the analysis of fungal elements within the microbiota [229]. Made up of two fragments (ITS-1 and ITS-2), primers aimed toward the adjacent 18s, 5.8S and 28S rRNA units can be used for capture [230].

Investigating the viral components of microbiota presents greater challenges as few marker loci are conserved [231], and the viral-to-non-viral nucleic acid ratio in samples from healthy individuals is low [232]. The sampling techniques, sample storage, nucleic acid retrieval, and sequencing approach to microbial analyses must be chosen assiduously to acquire accurate data.

1.8.2.3 MALDI-TOF MS versus NGS Sequencing

1.8.2.3.1 Advantages and Limitations

MALDI-TOF MS is highly sensitive, precise, and capable of analyzing coexisting microbes in a sample without their isolation [208, 233]. It is cost- and time-efficient, with rapid, non-destructive sample preparation, and analysis of multiple samples can be performed concurrently [233]. MALDI-TOF MS is based on one-step identification using protein marker quantification and can facilitate clinical diagnoses [234]. However, its limitations include reduced sensitivity at lower microbial densities, as detectable concentrations of proteins or peptides are required for identification [233, 234]. Additionally, MALDI-TOF MS does not provide information about the genetic structures, gene expression, or metabolic pathways of the microbes.

The benefits of NGS include in-depth analysis of a sample's genetic conformation, such as the existence of genes related to specific metabolic processes or other key pathways [235, 236]. Unlike MALDI-TOF MS, NGS can differentiate between closely related species and strains within a sample [235, 237, 238]. Its ability to detect antimicrobial resistance genes through

metagenomics has significant clinical relevance [239]. However, NGS remains time- and cost-intensive, limiting its routine clinical applications [209]. Additionally, NGS produces enormous amounts of data, making interpretation sometimes difficult, particularly in multi-faceted microorganism populations [240]. NGS also needs standardized protocols. Standardization remains a challenge, as variations in DNA extraction methods and primer selection can introduce biases [241]. In addition, no information is provided by NGS with respect to strain viability, adaptation, and availability for investigations performed *in vivo* [205]. The choice of method should be dictated by the reason for the analysis, the specific characteristics of the chosen technique, and its accessibility.

1.8.2.3.2 Reproducibility and Accuracy

The performance of MALDI-TOF MS has been compared to alternative methods of genome sequencing. It has performed comparably to MLST (multilocus sequence typing), with a 99.3% accuracy reported for the recognition of various species of *Staphylococcus* [242]. In comparison with 16S rRNA sequencing, oral *Prevotella* species were identified appropriately in 88.6% cases. Multi-center studies have demonstrated accuracy ranges of between 92.8% and 99.8%, and 91.7% and 98.5%, for MALDI-TOF MS with respect to genus and species, respectively [243-247]. Comparison of MALDI-TOF MS with 18S and ITS sequencing data revealed 95.4% and 98.8% accuracy for the diagnosis of genus and species of fungal isolates, respectively [248, 249]. In relation to the recognition of non-*Candida albicans* species, a concordance rate of 90% was exhibited between MALDI-TOF MS and ITS sequencing methodology [250]. MALDI-TOF MS is therefore a promising method for use either alone or alongside complementary techniques of genome sequencing for the identification of uncultivable microbial population segments or for infrequently arising disease-inducing microorganisms that present culture challenges.

1.8.3 Immune Profiling: Tools for Exploring Immune Responses

Immune profiling leverages advanced technological platforms to capture and analyze critical aspects of immune responses. Blood samples (typically peripheral) are used to monitor immune system changes through techniques like antibody assays, multiparameter flow cytometry, single-cell sequencing, and cytometry by time-of-flight (CyTOF)—tools that have become essential for understanding immune responses to infections and vaccinations.

1.8.3.1 Antibody Assays

Traditional single-analyte detection methods, such as Western blotting, lateral flow assays (LFAs), automated chemiluminescence assays, and enzyme-linked immunosorbent assays (ELISAs), have long been used to identify antibodies against specific infectious disease antigens [251]. However, bead-based multiplex platforms, such as Luminex and Mesoscale, now offer significant improvements. These systems can detect and quantify antibodies against multiple antigens simultaneously, allowing for a comprehensive analysis of an individual's serological/salivary profile across various pathogens [251, 252]. Multiplex assays are not only efficient and cost-effective but also provide a practical approach for analyzing multiple cytokines and chemokines [252]. They are invaluable for understanding epidemiological patterns and co-endemic disease burdens in specific regions [252, 253]. By generating more data in a single assay, these platforms enhance the ability to study the interplay between pathogens and host immunity.

1.8.3.2 Multiparametric Flow Cytometry

Multiparametric flow cytometry is widely used in immunology research, employing fluorophore-tagged antibodies to detect proteins expressed on the surface or inside cells. This technique has been instrumental for decades in monitoring immune responses to infections and vaccines [137]. It enables the tracking of phenotypic and functional characteristics of antigen-specific cells within bulk populations [137]. Beyond research, the technique has broad clinical applications, including disease diagnosis, staging, prognosis, and therapeutic monitoring [254], its versatility makes it a cornerstone of immunological studies and routine clinical workflows [254]. Recent advances in computational techniques have revolutionized the analysis and interpretation of complex flow cytometry data [255]. These tools enhance the visualization of polyfunctional T-cells, offering deeper insights into their roles in immune responses to pathogens and vaccines.

1.8.3.3 Single-Cell Sequencing

Single-cell RNA sequencing (scRNA-seq) and single-cell multiomics have transformed immune cell analysis by enabling transcriptomic profiling at single-cell resolution. Unlike bulk RNA sequencing, scRNA-seq captures heterogeneity within immune populations, identifying rare cell subsets, lineage trajectories, and activation states [256]. It has revealed novel immune interactions and functional diversity, particularly in oral and systemic diseases. However, transcriptomic data alone does not fully capture immune regulation, prompting the

development of single-cell multiomics, which combines genomic, epigenomic, proteomic, and metabolomic data from individual cells [257]. This approach provides deeper insights than transcriptional profiling alone, offering a more comprehensive view of immune modulation, microbial interactions, and disease pathogenesis. Despite its potential, single-cell multiomics faces challenges related to data integration, sensitivity, and standardization. Addressing these limitations will refine precision immunology and expand its applications in microbiome research and immune-related diseases.

1.8.3.4 Cytometry by Time-of-Flight (CyTOF)

CyTOF represents a major advancement in single-cell analysis. This mass cytometry technology combines the precision of flow cytometry with the ability to analyze approximately 45 metal probes simultaneously, thus avoiding signal overlap [258]. By detecting signals as the discrete line spectra of mass spectrometry, CyTOF offers unparalleled resolution, revealing previously unknown complexities in lymphoid populations. This platform is particularly suited for studying immune responses when sample material is limited [252]. With only a few million peripheral blood mononuclear cells (PBMCs), CyTOF can generate a comprehensive dataset, making it an ideal tool for investigating cellular functions and markers related to infection and vaccination [252]. Its ability to uncover new insights into immune cell dynamics underscores its potential to revolutionize immunological research.

CyTOF has several limitations, including lower sensitivity compared to fluorophore-based flow cytometry due to restricted metal ion conjugation, which limits signal intensity [258]. Its slower acquisition flow rate and susceptibility to clogging further reduce efficiency, though newer models like CyTOF XT® help mitigate these issues. Additional challenges are the high costs of metal-tagged reagents and the inability to sort cells (as they are vaporized during analysis), and the need for meticulous sample preparation to avoid heavy metal contamination [258]. Continuing advances in technology and reagents may address these limitations in future.

2 Research Aims

This doctoral thesis aims to explore the oral microbial signatures associated with severe infections and immune modulation. It focuses on oral infections, COVID-19 vaccination, and mucosal immune responses, highlighting their clinical significance, diagnostic potential, and relationship to host immunity.

Research Aims of the Studies

Study 1: Clinical Microbial Identification of Severe Oral Infections

This is a longitudinal study analyzing the trends in composition and diversity of the bacterial populations involved in severe oral infections, such as dentoalveolar abscesses and jaw osteomyelitis, over an 11-year period using MALDI-TOF mass spectrometry for species identification. The goal is to provide epidemiological insights into emerging microbial patterns and their potential impact on systemic health.

Study 2: The Mycobiota Profile of Oral Fungal Infections in Head and Neck Cancer Patients

This study focuses on characterizing the mycobiota associated with oral fungal infections in head and neck cancer patients undergoing radiotherapy. Using MALDI-TOF mass spectrometry, it aims to identify fungal species diversity, co-occurrence patterns, and novel pathogens to inform potential targeted therapeutic strategies for managing these infections.

Study 3: The Role of the Oral Microbiome in Vaccine-Induced Salivary Antibody Responses

This study investigates the baseline oral microbiome and its influence on the durability of salivary antibody responses following COVID-19 vaccination. By comparing microbiome features in high- and low responders, it seeks to uncover microbial signatures that contribute to long-term mucosal immunity in both healthy individuals and those living with HIV.

Study 4: MAIT Cell Responses to Microbial Secretomes

This study aims to explore the functional and phenotypic responses of MAIT cells to bacterial secretomes derived from diverse anatomical sites, including the oral cavity, blood, and pancreatic tumors. It examines how microbial origin influences MAIT cell polyfunctionality and emphasizes the role of microbial metabolites in modulating immune responses.

3 Materials and Methods

3.1 Ethical Considerations

The studies included in this thesis relied on clinical samples to investigate oral microbial signatures and immune responses. All procedures adhered to ethical standards and received approval from the relevant Regional Ethics Review Boards in Sweden.

For Study 1, previously collected microbiological data from clinical samples of severe oral infections, obtained as part of routine diagnostics in Stockholm County between 2010 and 2020, were utilized in a retrospective epidemiological investigation of oral-associated bacterial infections. Data collection, processing, and analysis were performed in accordance with ethical guidelines, under registration number Dnr 2021-03974.

Study 2 utilized retrospective data from oral fungal infection samples collected from head and neck cancer patients undergoing radiotherapy. Ethics approval was granted under the same registration number as Study 1. All analyses were conducted with ethical clearance, ensuring no additional burden or risk to participants.

For Study 3, saliva and blood samples were collected from participants in a COVID-19 vaccine immunogenicity study. Ethical approval was granted under registration number Dnr 2022-04468-02, with no direct interventions affecting participants.

Study 4 was a functional analysis of immune responses to bacterial secretomes, using clinical microbial isolates collected from the Medical Unit of Clinical Microbiology (ME Clinical Microbiology) at Karolinska University Hospital. The study was processed under ethical approval Dnr 2024-04297-02, and adhered to strict biosafety protocols.

All data were pseudonymized to ensure participant privacy, and personal information was kept confidential in accordance with data protection regulations. Sample storage followed Karolinska Institutet's biobanking standards. These measures ensured compliance with ethical and legal frameworks throughout the research.

3.2 Sample and Data Collection

Study 1

This retrospective study analyzed the microbiological data of clinical samples collected from patients with severe oral infections in Stockholm County over a 11-year period (2010–2020). A total of 1,014 samples, including swabs, abscess aspirates, and tissue biopsies, were submitted to the Clinical Microbiology Laboratory at Karolinska University Hospital as part of routine diagnostic procedures. Clinical diagnoses, such as dentoalveolar abscesses, jaw osteomyelitis, jaw osteonecrosis, odontogenic maxillary sinusitis, periapical pathosis, and periodontal diseases, were recorded (Figure 6).

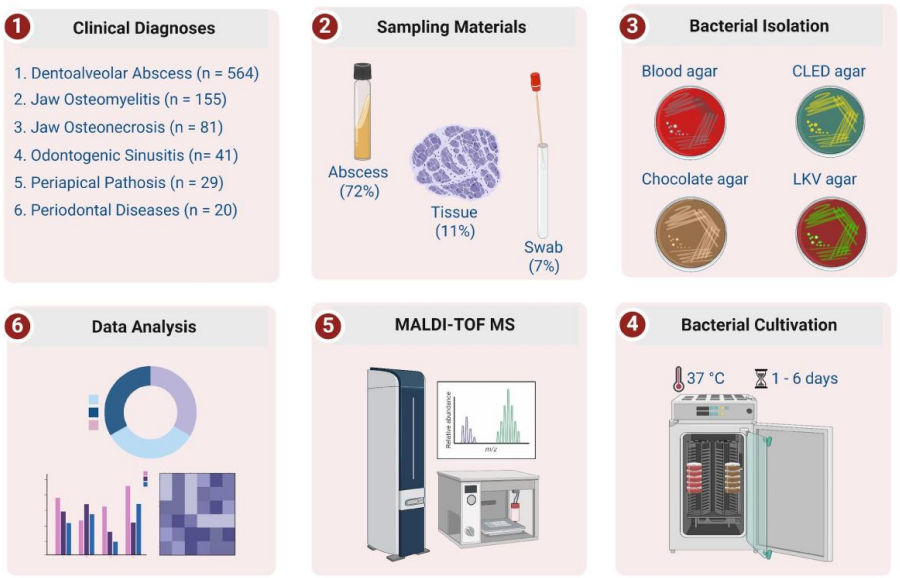


Figure 6 Workflow for Study 1. Stepwise representation of Study 1, showcasing the identification of bacterial pathogens in oral infections. Clinical diagnoses included dentoalveolar abscesses, osteomyelitis, and jaw osteonecrosis. Samples were collected from abscesses (72%), tissue biopsies (11%), and swabs (7%). Bacteria were cultured on selective agar, incubated at 37°C for 1–6 days, and identified via MALDI-TOF MS. The data analyses provide insights into microbial diversity and emerging pathogenic trends.

Study 2

Sample data were collected retrospectively from 162 head and neck cancer (HNC) patients receiving radiotherapy between 2014 and 2019. Oral mucosal swabs were obtained from HNC patients and cultured at the Clinical Microbiology Laboratory at Karolinska University Hospital. In addition, oral mucosal samples from 833 non-cancer individuals were collected and utilized

as controls. Patients were categorized into two groups: those undergoing radiotherapy (ongoing-RT) and those who had completed radiotherapy (post-RT) (Figure 7).

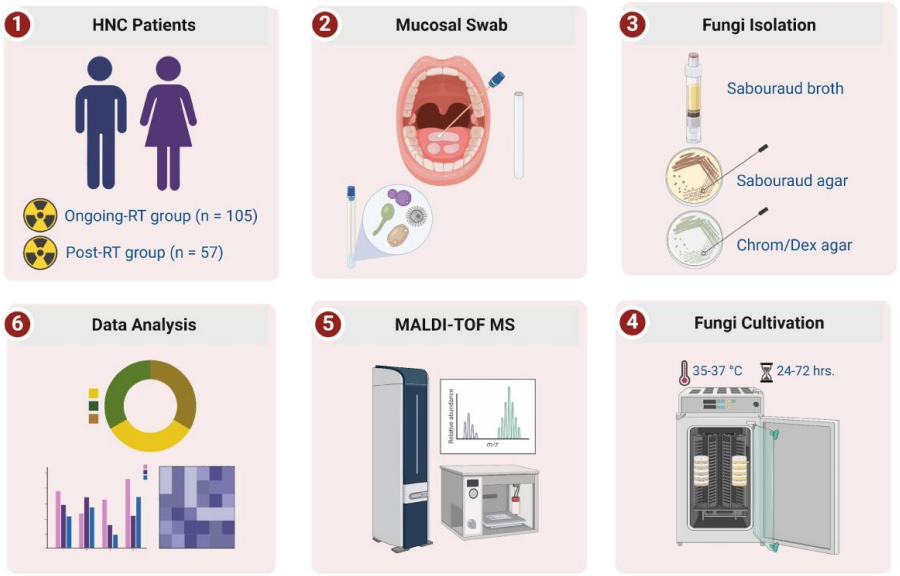


Figure 7 Workflow for Study 2. Overview of the methodology for Study 2, on fungal colonization in HNC patients undergoing radiotherapy. Mucosal swabs were collected and processed for fungal isolation using Sabouraud agar, Sabouraud broth, and chromogenic agar. Cultivation was conducted at 35-37°C for 24-72 hours, followed by MALDI-TOF MS identification. Data analyses included species distribution profiling and correlation with clinical parameters.

Study 3

Self-collected unstimulated saliva and blood samples were obtained from 115 participants enrolled in the COVAXID clinical trial. The cohort included healthy controls (n=57) and people living with HIV (n=58). All participants tested negative for SARS-CoV-2 via nasopharyngeal PCR and were seronegative for anti-SARS-CoV-2 antibodies using the Elecsys® assay. None had recent or ongoing antibiotic treatment. Saliva and blood samples were collected at five-time points: baseline, 10 days, 21 days, 35 days, and six months post-vaccination. Baseline saliva samples were used to analyze oral microbiome profiles and assess their correlation with vaccine-induced immune responses over time (Figure 8).

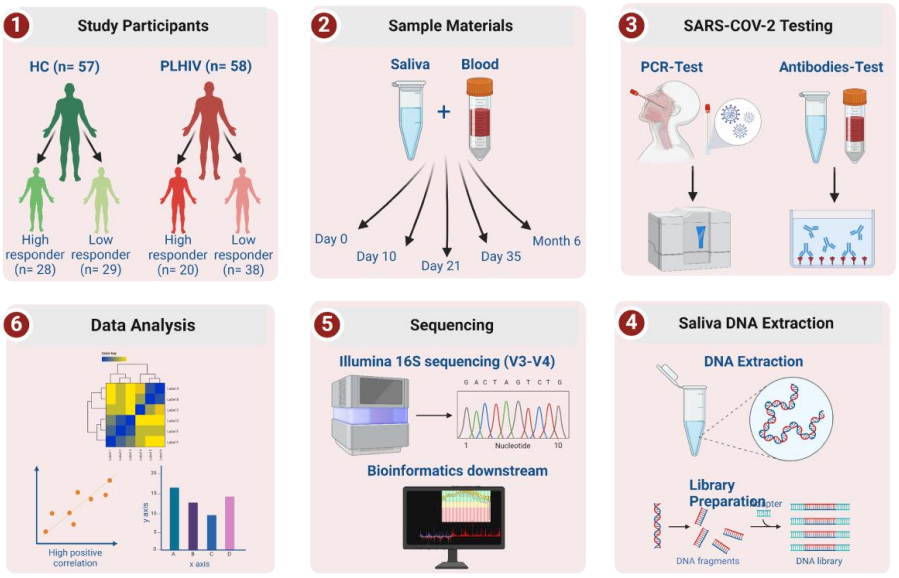


Figure 8 Workflow for Study 3. Experimental strategy for Study 3, investigating the dynamic interplay between the oral microbiome and COVID-19 vaccine immunogenicity. Participants were grouped as high- or low responders based on antibody titers after vaccination. Saliva and blood samples were collected at multiple time points (Day 0, 10, 21, 35, and Month 6). DNA was extracted and prepared for Illumina 16S sequencing, enabling analysis of microbial composition and its correlation with vaccine response variability.

Study 4

Peripheral blood mononuclear cells (PBMCs) were obtained from the buffy coat of four healthy donors recruited from the Blood Transfusion Clinic of Huddinge Karolinska University Hospital. PBMCs were isolated using Ficoll-Paque density gradient centrifugation. After centrifugation at 2000 RPM for 20 minutes at room temperature, the PBMC layer was collected, washed twice with PBS, stained with trypan blue, and counted using an automated cell counter. Cells were preserved in Cryovials and placed in a Corning® CoolCell™ LX freezing container and stored at -80°C. After 24–48 hours, vials were transferred to a liquid nitrogen storage system until further use (Figure 9).

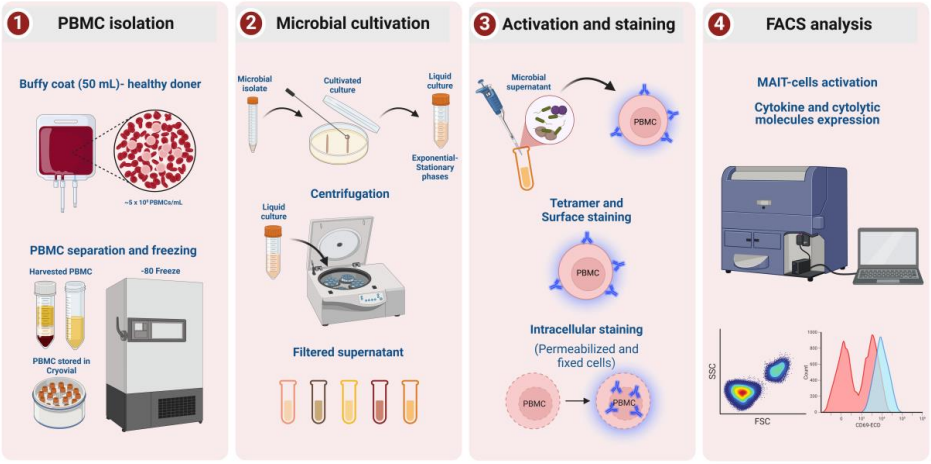


Figure 9 Workflow for Study 4. Experimental overview of Study 4, illustrating immune responses to microbial secretomes. PBMC isolation was performed from healthy donor blood samples, followed by microbial cultivation and stimulation assays. PBMCs were exposed to microbial supernatants, followed by tetramer, surface, and intracellular staining to analyze cytokine and cytolytic molecule expression. FACS analysis was used to quantify MAIT cell activation and immune modulation by microbial metabolites. [FACS: Fluorescence-Activated Cell Sorting]

On the day of activation, frozen PBMCs were thawed to room temperature, washed, and resuspended in RPMI-1640 supplemented with 10% FBS and 100 µg/mL Normocin™ antimicrobial solution (referred to as complete medium). Cell viability was assessed using trypan blue staining, with >80% of thawed cells being viable. For activation assays, 50 µL of complete medium was added to each well of a U-bottom 96-well plate. A total of 100 µL of PBMC suspension (1×10^6 cells per well) was seeded and stimulated with 40 µL of bacterial cell-free supernatants derived from oral, blood, and pancreatic clinical isolates. Cells were incubated for one hour at 37°C in a humidified 5% CO_2 incubator before adding anti-CD28 mAb to give a final concentration of 1.5 mg/mL. The plate was then incubated for an additional 18 hours under the same conditions.

To assess cytokine production, monensin (GolgiStop) at a concentration of 1:1000 and brefeldin A (BFA, GolgiPlug) at 1:500 were added, making up a final volume of 10 µL, six hours before the end of incubation. For negative controls, PBMCs were cultured with fastidious anaerobic broth (FAB) alone or in complete medium. As a positive control, PBMCs were stimulated for six hours with lymphocyte activation cocktail (LAC) at 1 mg/m L in the presence of BFA and monensin.

3.3 Culturing Techniques

Bacterial Cultures

Samples from Study 1 were cultured on standard aerobic and anaerobic media, including blood agar, chocolate agar, and anaerobic sheep blood agar. Cultures were incubated at 35°C under appropriate atmospheric conditions for 48–72 hours (Figure 6). Colony growth was monitored daily, and isolates were prepared for downstream analysis.

Fungal Cultures

Swabs from Study 2 were cultured on Chromagar and Sabouraud dextrose agar. Plates were incubated at 35°C and 37°C for 24 hours, with additional incubation for up to 72 hours as needed. Liquid Sabouraud broth was also used to enhance fungal growth (Figure 7). Colonies were subsequently analyzed for species identification.

3.4 Microbial Secretome Preparation

In Study 4, pure oral isolates from severe oral infections (Study 1) and blood infections, including *Klebsiella aerogenes, Klebsiella oxytoca, Klebsiella pneumoniae, Enterobacter cloacae, Stenotrophomonas maltophilia, Streptococcus anginosus, Enterococcus faecalis,* and *Fusobacterium nucleatum,* were collected and preserved in glycerol stocks at –80°C. The same bacterial species, except *F. nucleatum*, were also isolated from pancreatic cancer tumors and stored under identical conditions.

Bacterial growth was monitored using the same culturing protocols as in Study 1, with species confirmation performed via MALDI-TOF MS. To standardize bacterial density, a DENSICheck instrument was used with a glass saline tube. The instrument was first calibrated with a blank saline tube containing 2–3 mL of saline solution. Then, 3–4 bacterial colonies were suspended in saline and diluted to obtain a 1 McFarland unit (MFU).

A 100 µL aliquot of the standardized bacterial suspension was inoculated into 8 mL of FAB and incubated in a shaker at 37°C for two growth phases: exponential and stationary. Following incubation, the cultures were centrifuged at 4000 RPM for 20 minutes at 4°C. The supernatant was carefully collected and subjected to a second centrifugation. The final supernatant was filtered twice through a 0.2 µm filter, yielding a bacterial secretome suspension for use in functional assays. The same preparation procedure was applied to FAB alone, which served as a negative control.

3.5 Microbial Identification Methods

MALDI-TOF MS

For both bacterial and fungal samples (Studies 1 and 2), microbial identification was performed using MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany) (Figures 6 & 7). Samples were prepared according to the manufacturer's protocol, spotted onto a target plate, treated with formic acid, and overlaid with a matrix solution. The spectra were then compared to a reference library for species-level identification.

DNA Extraction and Next-Generation Sequencing (NGS)

In Study 3, microbial DNA was extracted from saliva samples using the ZymoBIOMICS™ DNA Mini Kit. DNA concentration and quality were measured, and samples were normalized to a standard concentration of 1 ng/µL in a volume of 15 µL. The 16S rRNA gene (V3-V4 region) was amplified and sequenced using the Illumina platform (Figure 8). Library preparation was conducted with dual-index barcoding to enable multiplexing. Raw sequencing reads were generated and processed for bioinformatics analysis, ensuring high-quality microbial profiling.

3.6 Immunoassays for Antibody Detection

In Study 3, salivary antibodies against SARS-CoV-2 were quantified using a multiplex bead-based assay. Saliva samples were diluted and analyzed for IgG levels specific to the full-length Spike protein and the S1 subunit of SARS-CoV-2. Readouts were obtained using the Luminex™ FLEXMAP 3D™ system. For serum samples, SARS-CoV-2-specific antibodies were detected using the Elecsys® anti-SARS-CoV-2 assay.

3.7 Flow Cytometry and Functional Analysis

In Study 4, following microbial stimulation, PBMCs were transferred to a V-bottom 96-well plate and centrifuged at 1700 RPM for two minutes at 3–4°C. The supernatant was discarded, and cells were stained with an antibody panel including CD3, CD161, CD4, CD8, CD69, IFN-γ, TNF-α, IL-17A, and GzB, along with the MR1:5-OP-RU tetramer, and a dead/live cell marker. After staining, cells were washed, centrifuged at 1900 RPM for two minutes at 3–4°C, and resuspended in FACS buffer before acquisition.

Fluorescence data were acquired using a BD LSRFortessa flow cytometer (Figure 9), and analysis was conducted using FlowJo software. To evaluate MAIT cell

polyfunctionality, the SPICE (Simplified Presentation of Incredibly Complex Evaluations) software was utilized to generate combinatorial analysis plots of cytokine-producing cell subsets. The frequency and distribution of single, double, and triple cytokine-producing MAIT cells were quantified.

3.8 Bioinformatics and Statistical Analyses

Bioinformatics Pipeline

For Study 3, raw sequencing data were processed using QIIME2. Reads were quality-filtered, denoised, and clustered into amplicon sequence variants (ASVs). Taxonomic classification was performed using the SILVA reference database. Alpha and beta diversity metrics were calculated to assess microbial richness and community structure. Functional capabilities of the microbial community were predicted using the Tax4Fun software package.

Statistical Analyses

In Study 1, descriptive statistics summarized the prevalence of microbial species. Temporal trends in microbial composition were analyzed using linear regression models. Microbial diversity across different sample types and clinical diagnoses was illustrated with heatmaps and Venn diagrams.

For Study 2, descriptive statistics summarized patient demographics and fungal species distributions. Comparisons of fungal diversity and co-occurrence patterns between ongoing- and post-radio therapy groups were analyzed using chi-square tests. Network analysis was used to visualize multi-species co-occurrence, while temporal changes in fungal species prevalence were evaluated using linear regression.

In Study 3, alpha diversity metrics, including Observed, Chao1, Shannon, and Simpson indices, were compared between high- and low responders using Mann-Whitney U tests. Beta diversity was assessed using Bray-Curtis dissimilarity and Jaccard indices, and visualized through non-metric multidimensional scaling (NMDS). Differential abundance analysis to identify taxa enriched in each group was performed using Linear Discriminant Analysis Effect Size (LEfSe). Receiver operating characteristic (ROC) curve analysis evaluated the predictive accuracy of microbial signatures for vaccine responses. Correlations between microbial composition and salivary antibody levels were analyzed using Spearman's rank correlation.

In Study 4, MAIT cell activation and cytokine production were analyzed using FlowJo software. Differences in cytokine expression between stimulation conditions and controls were tested using paired and independent t-tests. Spearman correlation was used to assess relationships between variables. Data were visualized using GraphPad Prism, R, and SPICE software.

4 Results and Discussion

4.1 Study 1

4.1.1 Oral Microbial Signatures in Severe Infections: A Longitudinal Perspective

The microbial landscape of severe oral infections exhibited distinct infection-related signatures that changed over the 11-year study period. These shifts in the relative species composition of the oral microbiota may in part have been driven by changes in antibiotic usage, host immune responses, and environmental factors. Most identified bacteria belonged to four dominant phyla: *Firmicutes* (51%), *Bacteroidetes* (19%), *Proteobacteria* (12%), and *Actinobacteria* (5%), while 13% remained unclassified (Figure 10). At the genus level, *Streptococcus* spp. and *Prevotella* spp. accounted for 50% of all identified species, followed by *Staphylococcus* spp. (11%).

These findings highlight the significant role of *Firmicutes* and *Bacteroidetes* in oral infections, reinforcing their critical involvement in disease severity and progression [2, 7]. Their widespread presence across different infections also reflects their adaptability to diverse oral niches [1]. Moreover, the microbial diversity observed in severe oral infections suggests varying pathogenic potential, with certain bacteria exhibiting commensal-like behavior in healthy microbiomes but adopting pathogenic traits under dysbiotic conditions.

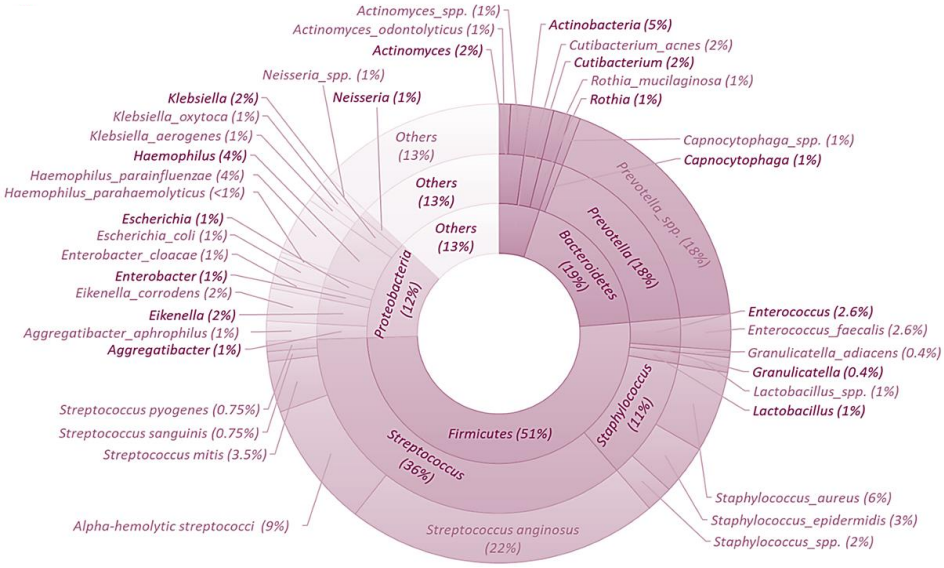


Figure 10 Microbial signatures in severe oral infections. An overview of the bacterial diversity identified in oral infections over the 11-year study period. The donut chart illustrates microbial classification at the phylum, genus, and species levels, providing a proportional representation of each taxonomic group and highlighting the predominant bacterial taxa associated with severe oral infections.

4.1.2 Temporal Trends in Microbial Composition

The proportion of Gram-positive bacteria significantly increased over time, which may reflect a shift in antibiotic resistance patterns, the natural adaptation of oral pathogens, or the increased persistence of biofilm-forming species. ($R = 0.68$, $P < 0.05$), replacing Gram-negative species. In particular, there was a notable rise in *S. anginosus*, *S. mitis*, and *S. sanguinis* ($R = 0.79$, 0.86, and 0.66, respectively; ($Ps < 0.05$) (Figure 11), with other species showing significant upward trends including *S. epidermidis* ($R = 0.69$), *Eikenella corrodens* ($R = 0.77$), *Actinomyces* spp. ($R = 0.73$), and *Aggregatibacter aphrophilus* ($R = 0.89$). Moderate but non-significant increases were also observed in *Enterococcus faecalis*, *Klebsiella* spp., *Capnocytophaga* spp., *Lactobacillus* spp., and *Neisseria* spp.

The observed increase in Gram-positive bacteria suggests that this microbial shift may have been driven by host immune responses, antibiotic interventions, or evolving microbial interactions [259]. The increasing prevalence of Gram-positive species is of particular interest, as it reflects the broader epidemiological trends observed in systemic infections [260]. The rise in *S.*

anginosus and other *Streptococcus* spp. suggests their increasing role in deep-seated infections, including abscess formation [261, 262]. Concurrently, the emergence of Gram-negative opportunists, such as *Klebsiella* spp., raises concerns about their evolving pathogenicity and potential antimicrobial resistance [263, 264], especially given the potential for horizontal gene transfer within polymicrobial environments. This makes early detection and targeted therapeutic approaches all the more important in the management of severe oral infections.

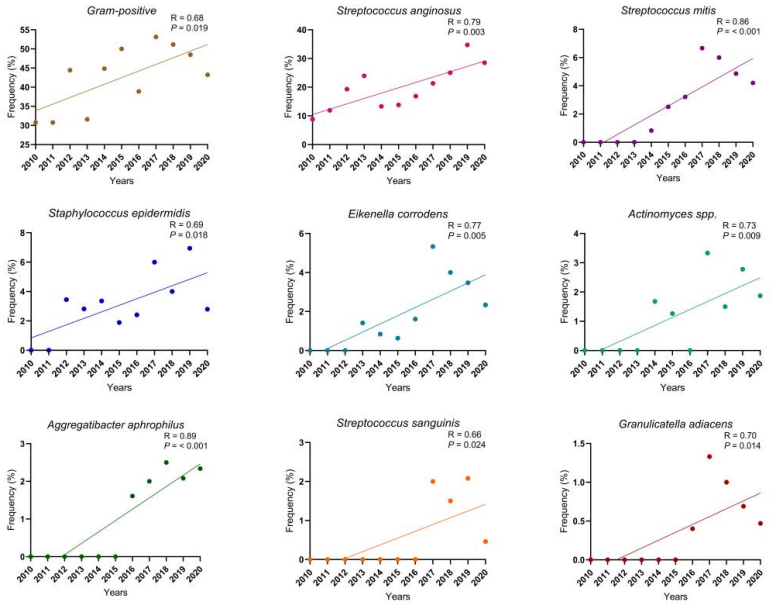


Figure 11 Trends in microbial prevalence in oral infections from 2010 to 2020. Simple linear regression analyses revealed significant increases in the prevalence of key bacterial taxa associated with oral infections. Notable trends include the rising frequencies of Gram-positive spp., *Streptococcus anginosus*, *Streptococcus mitis*, *Streptococcus sanguinis, Eikenella corrodens, Actinomyces spp., Aggregatibacter aphrophilus, Staphylococcus epidermidis,* and *Granulicatella adiacens* across clinical samples. P-values <0.05 indicate statistically significant changes in relative microbial populations over time.

4.1.3 Microbial Profiles Across Clinical Diagnoses and Sample Materials

Among the 1,014 clinical samples, 890 cases were categorized into six distinct clinical conditions: dentoalveolar abscesses, jaw osteomyelitis, jaw osteonecrosis, odontogenic maxillary sinusitis, periradicular pathosis, and periodontal diseases, while 124 samples remained undiagnosed. The submandibular (43%) and buccal (38%) spaces were the most frequently affected anatomical regions.

We identified 21 core bacterial species that were present in all infections (Figure 10), reinforcing the notion that a subset of microbial taxa is consistently implicated in severe infections [6, 28]. Emerging bacteria, including *Klebsiella* spp. and *Enterobacter* spp., were also detected across multiple conditions, suggesting their increasing role in oral pathogenesis (Figure 12).

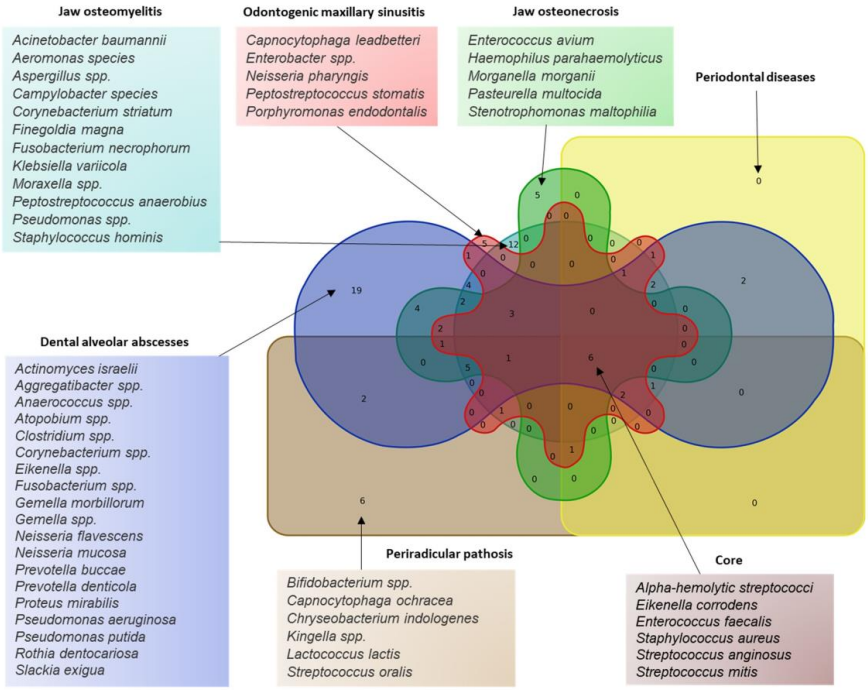


Figure 12 Unique, shared, and core microbial signatures in oral infections. The Venn diagram shows the distribution microbial taxa in patients diagnosed with different infections. Intersections represent microbes common to two or more types of infection, while non-overlapping areas, taxa unique to that infection. The central region where all infection types intersect represents the core microbial taxa present in all infections.

Dentoalveolar abscesses accounted for 55.6% of all cases (Figure 13). The most frequently identified bacteria were *S. anginosus*, *Prevotella* spp., and *Haemophilus parainfluenzae*. The abscesses exhibited a Gram-positive dominance (57%) and largely comprised facultative anaerobes (68%). Jaw osteomyelitis exhibited high prevalences of *S. anginosus*, *S. epidermidis*, *S. aureus*, and *E. faecalis*, with Gram-positive bacteria (67%) outnumbering Gram-negative species (33%), and 81% of the microbiota being facultative anaerobes. Jaw osteonecrosis predominantly featured *S. anginosus*, *S. mitis*, and *Actinomyces* spp. The microbial composition was 56% Gram-positive and 44% Gram-negative, with 80% of the detected species being facultative anaerobes.

Odontogenic maxillary sinusitis showed a strong presence of *S. anginosus*, *H. parainfluenzae*, and *Prevotella* spp. and comprised 70% Gram-positive and 30% Gram-negative, with facultative anaerobes making up 77% of the total. Periradicular pathosis frequently harbored *E. faecalis*, *E. coli*, *Prevotella* spp., and *Actinomyces* spp. and overall, 65% were Gram-positive and 35% Gram-negative, with 84% facultative anaerobes. Periodontal diseases had a distinct microbial pattern, with *Prevotella* spp. and *Streptococcus* spp. detected in over 50% of cases. This condition showed a shift toward Gram-negative bacteria (66%) and a higher anaerobic species prevalence (52%).

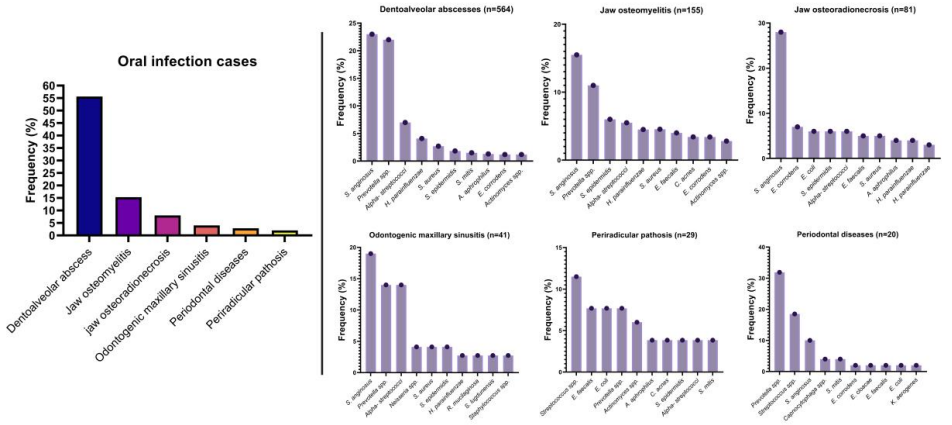


Figure 13 Oral microbial signatures in severe infections: frequency of cases and bacterial distribution. The left panel illustrates the frequency of different oral infection cases referred for clinical microbiological testing. The right panel presents bar charts displaying the frequency of the top ten detected bacterial species associated with specific infection types.

These results confirm the polymicrobial nature of oral infections, where opportunistic bacteria, particularly *S. anginosus*, play a dominant role [261, 262]. The presence of HACEK organisms (*H. haemolyticus*, *H. parainfluenzae, A. aphrophilus, E. corrodens*) in jaw osteonecrosis and periradicular pathosis suggests a potential link between these infections and systemic dissemination [265]. Moreover, the high prevalence of Gram-negative bacteria in periodontal diseases underscores their unique inflammatory nature [266]. The high proportion of facultative anaerobes in odontogenic maxillary sinusitis suggests their role in microbial persistence and chronicity [267], as these are known to invade beyond the oral cavity, posing a risk for bacterial translocation and systemic complications [267]. In periradicular pathosis, the presence of resilient bacteria such as *E. faecalis* and *Actinomyces* spp. reflects their persistence in endodontic infections and resistance to treatment [268]. Likewise, the abundance of anaerobes in periodontal disease reflects their ability to thrive in

deep pockets and inflamed tissues, fueling chronic inflammation and progressive tissue damage [266].

A heatmap analysis revealed substantial microbial overlap across clinical conditions, suggesting a core group of opportunistic pathogens present in multiple infection types, and possibly a core microbiome itself, thereby complicating treatment approaches (Figure 14). Interestingly, *F. nucleatum* and *Aggregatibacter actinomycetemcomitans* were frequently identified in jaw osteomyelitis and dentoalveolar abscesses, while fastidious HACEK organisms were mainly found in jaw osteonecrosis and periradicular pathosis.

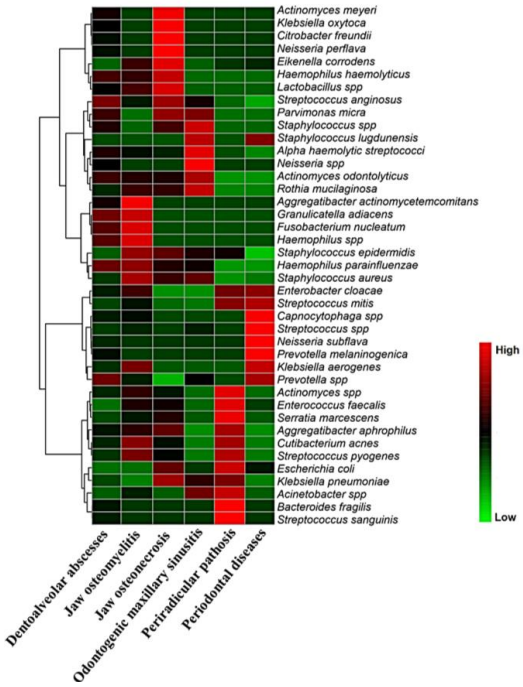


Figure 14 Hierarchical clustering of bacterial species across oral infections. A heat map representation illustrates the occurrence of bacterial species across different oral infections. The clustering analysis groups infections based on microbial composition, with color intensity denoting species prevalence—ranging from low (light green) to high (light red). The clustering is based on Euclidean distance, highlighting variations in bacterial distribution among diagnosed groups and revealing distinct microbial patterns associated with specific infection types.

4.2 Study 2

4.2.1 Oral Fungal Signatures in Radiotherapy Patients: A Shifting Landscape

Over the six-year study period, a total of 190 fungal isolates were identified from oral mucosal samples of 162 head and neck cancer (HNC) patients undergoing radiotherapy (RT). *Candida* was the dominant genus (88%), with *Pichia* accounting for only 5%. Notably, *Candida albicans* remained the most prevalent species in all patients, although non-*albicans Candida* (NAC) species, including *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *P. kudriavzevii*, were also frequently

detected (Figure 15). Longitudinal analysis of fungal profiles revealed subtle yet significant shifts in species distribution over time. While *C. albicans* and *P. kudriavzevii* remained stable (R = 0.12 and R = 0.15, P > 0.05), the prevalence of *C. tropicalis* and *C. parapsilosis* increased gradually (R = 0.31 and R = 0.60, P > 0.05), and *C. glabrata* exhibited a marked decline (R = -0.82, P < 0.05) (Figure 15).

The predominance of *C. albicans* in both ongoing-RT and post-RT groups aligns with previous studies that note its ability to switch opportunistically from commensal to pathogenic in immunocompromised individuals [269]. The presence of NAC species, particularly *C. glabrata*, is clinically significant, as these species exhibit increased resistance to antifungal therapy, particularly azole-based drugs, which are commonly used in treating oral fungal infections [269]. The detection of *Pichia* species, which have been suggested to play a protective role by inhibiting excessive *Candida* proliferation through competitive interactions, is also noteworthy [270], as it suggests a dynamic shift in fungal ecology, where NAC species may be gaining a foothold in response to radiotherapy-induced dysbiosis and selective pressures from antifungal treatments. The decline in *C. glabrata* is unexpected, given its known antifungal resistance, may indicate interspecies competition, differences in treatment protocols, or variations in patient immune responses. Understanding the reasons underlying this surprising finding only emphasizes the need for further investigation into host-microbe interactions [271].

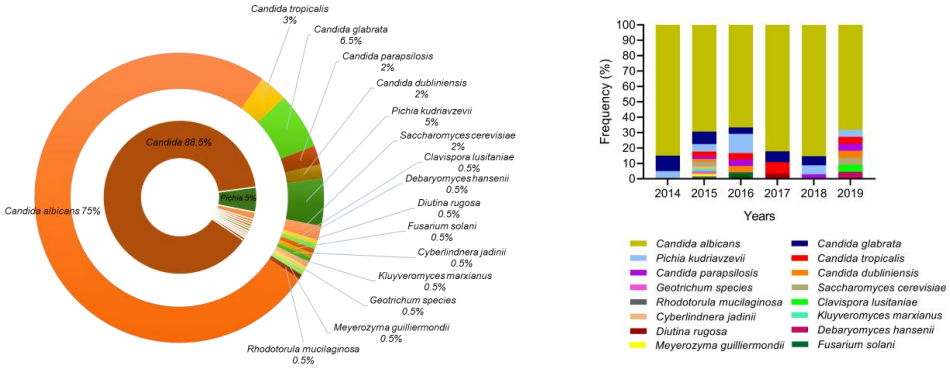


Figure 15 Oral fungal signatures in radiotherapy patients. A donut chart illustrates the distribution of fungal taxa identified in oral fungal infection (OFI) samples from head and neck cancer (HNC) patients undergoing radiotherapy over a six-year study period. The chart categorizes the oral mycobiota at the genus and species levels, providing a comprehensive visualization of fungal diversity and relative prevalence in OFIs. The stacked bar charts depict the yearly percentage frequencies of fungal species in oral swab samples.

4.2.2 Interplay of Fungal Communities: Multi-Species Colonization and Co-Occurrence

C. albicans, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *P. kudriavzevii* were the dominant fungi in both ongoing-RT and post-RT groups (Figure 16). Interestingly, network analysis revealed greater fungal diversity and multi-species co-occurrence in the ongoing-RT group compared to post-RT patients, with *C. albicans* co-occurring with *C. glabrata* in 21% and 30% of cases respectively (Figure 16).

The increased fungal diversity in ongoing-RT patients suggests that radiotherapy creates a disrupted oral microbiome conducive to fungal colonization [272]. The high rate of *C. albicans*-*C. glabrata* co-occurrence also aligns with evidence showing that these species synergize to enhance biofilm formation and antifungal resistance, making infections more persistent and difficult to treat [273]. These findings highlight the need for antifungal strategies that go beyond targeting individual species and instead consider polymicrobial interactions and the potential pathogenic synergies within the mycobiota.

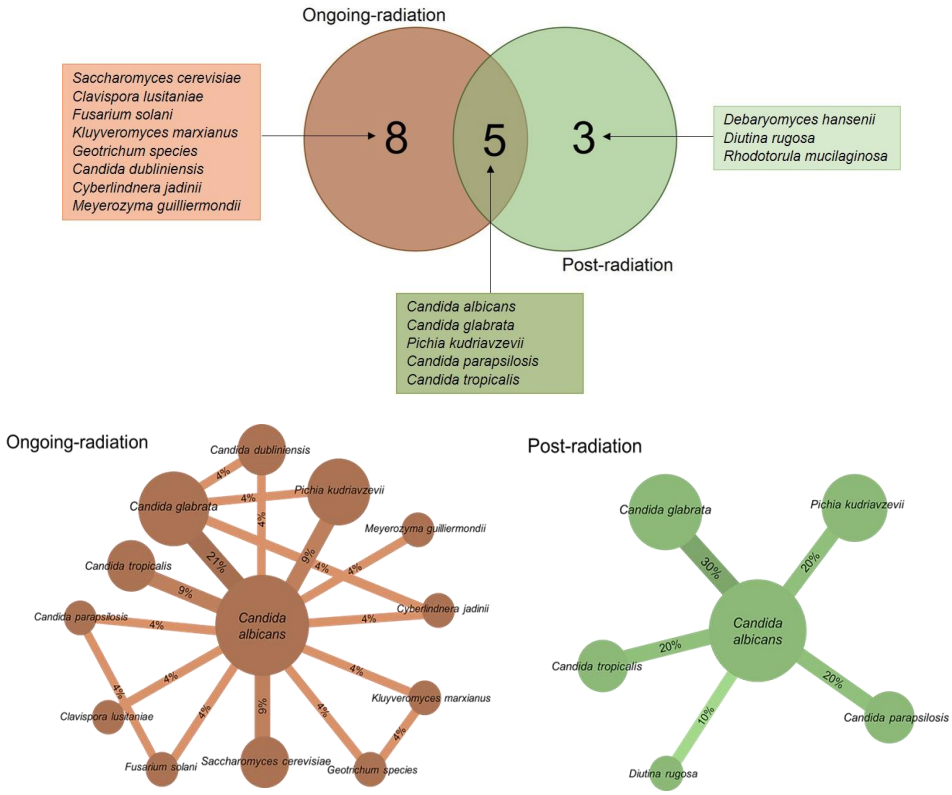


Figure 16 Multi-species colonization and co-occurrence in oral fungal infections. Unique and core fungal species detected in oral swab samples from HNC patients in the ongoing-RT and Post-RT groups. Overlapping regions represent core mycobiota shared between the two groups,

while non-overlapping areas highlight group-specific fungal species. The co-occurrence network analysis depicts the frequency of co-occurring fungal species within each patient group, providing insights into multi-species colonization and interspecies interactions in oral fungal infections.

4.2.3 Emerging Fungal Pathogens in Radiotherapy Patients: A Hidden Threat

For the first time, *Fusarium solani* and *Cyberlindnera jadinii* were detected in HNC patients undergoing radiotherapy (Figures 16 and 17). Notably, both species were only found in these patients.

The detection of *F. solani* is of clinical importance, as this filamentous fungus is known for its high virulence and innate resistance to conventional antifungal agents, particularly azoles and echinocandins [274]. Similarly, while *C. jadinii* is rarely implicated in oral infections, it has been associated with fungemia in immunocompromised hosts, raising concerns about its potential for systemic dissemination [275]. The findings thus stress the critical importance of routine fungal surveillance and the use of broad-spectrum antifungal diagnostics to detect all clinically relevant pathogens, including those less common.

4.2.4 Impact of Radiotherapy on Oral Mycobiota: A Disrupted Equilibrium

Comparison of the oral mycobiota between the two patient groups showed *C. albicans* clearly dominant (~75%) in both groups, yet the overall fungal diversity was higher in the group still undergoing RT. NAC species, particularly *C. glabrata*, *C. parapsilosis*, and *P. kudriavzevii*, also occurred less frequently in post-RT patients (Figure 16).

The reduction in *NAC* species post-RT suggests that radiation-induced immunosuppression creates an early window of susceptibility to fungal colonization, particularly by opportunistic and azole-resistant species [276]. However, as patients and their immune systems recover from RT, the microbiome may move away from dysbiosis and reduce the fungal burden, thus favoring the survival of more adaptable species, such as *C. albicans* [277]. Understanding these temporal shifts in fungal dynamics could aid in developing stage-specific antifungal interventions for HNC patients.

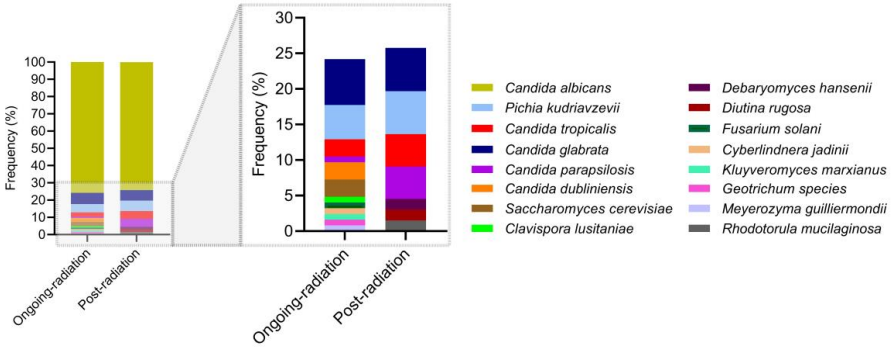


Figure 17 The Impact of radiotherapy on oral mycobiota composition. Stacked bar chart display the relative percentage frequency of fungal species detected in oral swab samples from HNC patients undergoing radiotherapy (Ongoing-RT) and post-radiotherapy (Post-RT). This comparative view of the oral mycobiota of the two groups highlights the disruptive effects of radiotherapy on fungal diversity and species prevalence.

4.3 Study 3

4.3.1 Oral Microbial Signatures in Vaccine Response: A Comparative Analysis

Eligible participants included 57 healthy controls (HCs) and 58 people living with HIV (PLHIV), all of whom received two vaccine doses and met the inclusion criteria of baseline and six-month screenings for SARS-CoV-2 exposure negativity. By day 35—one month after the second dose, all participants had seroconverted, confirming robust short-term immune responses. However, individual variations in the persistence of salivary spike IgG required classifying participants into high- and low responders based on their expected convalescent IgG levels at six months.

Low responders in both groups exhibited a greater decline in salivary IgG against the full-length spike (S.f) and S1 antigen, indicating variability in the mucosal immune response. Among PLHIV, the reduced magnitude and duration of salivary and serum spike IgG throughout follow-up suggests a weaker immunological memory. Importantly, no significant differences were observed between high- and low responders in age, gender, BMI, diet, ethnicity, medication use, or clinical immune parameters, implicating other intrinsic parameters, including the oral microbiota, in the immune response variation.

These findings emphasize the variability of antibody persistence between individuals, especially when immunocompromised, and align with recent reports linking HIV-related immune dysregulation to altered vaccine responses [278]. The reduced salivary IgG persistence in low responders may reflect differences

in mucosal B-cell function or systemic immune priming deficits [279], making it important to implement tailored vaccination strategies for immunocompromised individuals.

4.3.2 Microbial Diversity in Relation to Vaccine Responses

The salivary microbiome's bacterial community was dominated by *Prevotella*, *Veillonella*, and *Neisseria* in all participants (Figure 18). No significant differences between high- and low responders were detected in microbial richness or evenness in baseline samples (alpha diversity: Observed, Chao1, Shannon, and Simpson's indices) (Figure 19). However, HCs displayed higher overall microbial diversity compared to PLHIV.

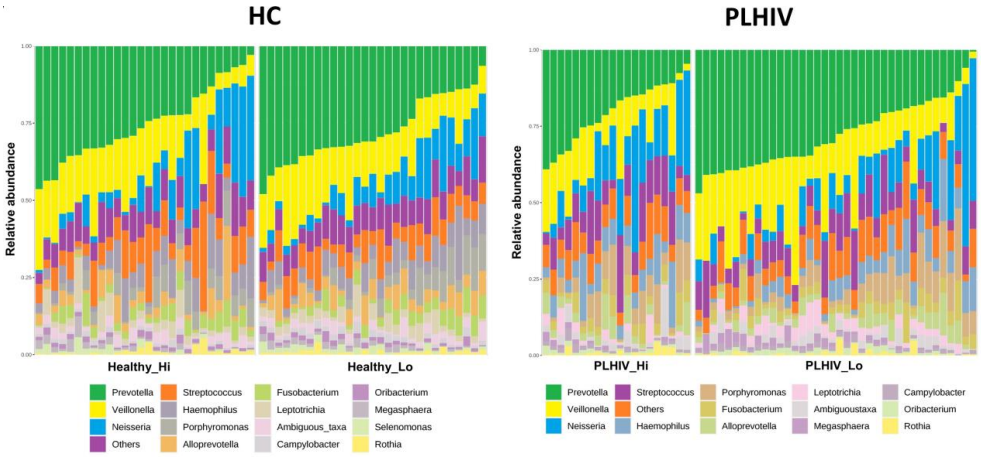


Figure 18 Oral microbiome diversity and composition in high- and low responders to vaccination. Microbiome composition profiles at the genus level in high- and low responders among healthy controls (HC) and people living with HIV (PLHIV). The stacked bar charts illustrate the 15 most frequently occurring genera by subgroups. Vertical bars represent individual samples. The analysis highlights the dominance of *Prevotella* (green), *Veillonella* (yellow), and *Neisseria* (blue), and individual variations in oral microbiome diversity and richness.

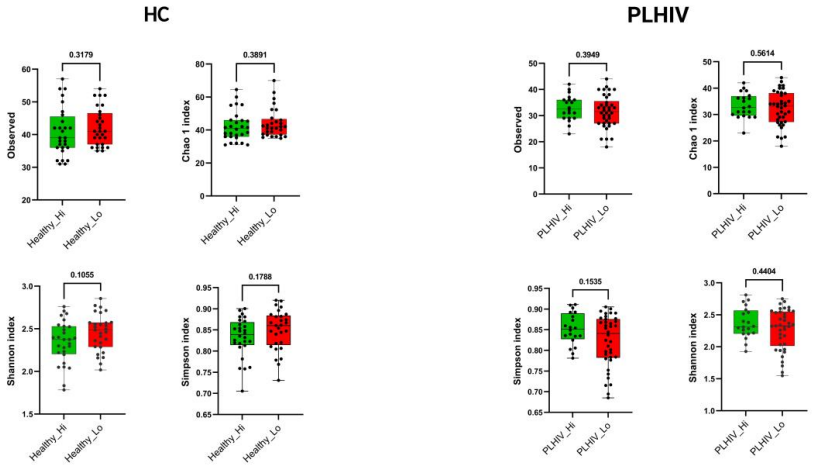


Figure 19 Alpha diversity of the oral microbiome. Box plots illustrate the alpha diversity of the amplicon sequence variant (ASV) abundance among participants, measured by Observed and Chao1 indices for richness, and Shannon and Simpson's indices for diversity. Lines and error bars represent geometric means and standard deviations, providing a comparative view of microbiome diversity across study groups.

In contrast to HCs (Bray Curtis and PERMANOVA: P = 0.371; Jaccard index PERMANOVA: P = 0.252), the beta diversity of the oral microbiomes of PLHIV varied significantly between high- and low responders (Bray Curtis and PERMANOVA: P = 0.019; Jaccard index and PERMANOVA: P = 0.022) (Figure 20).

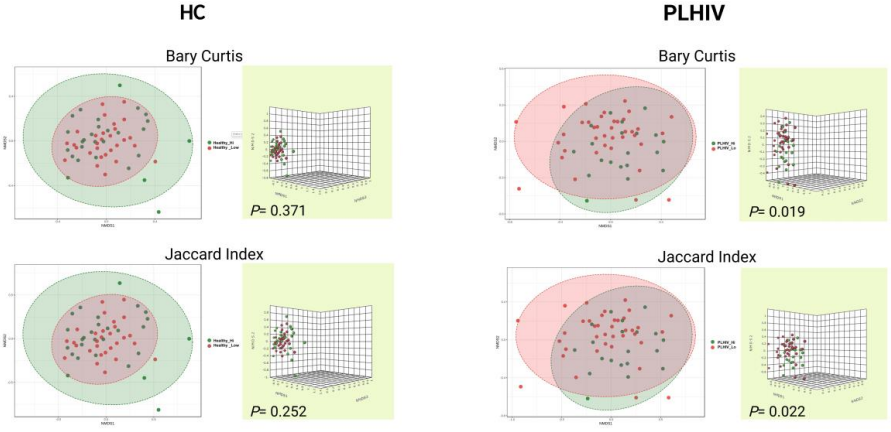


Figure 20 Beta diversity of the oral microbiome across study groups. Non-metric multidimensional scaling (NMDS) plots illustrate beta diversity, represented by Bray Curtis dissimilarity distances and the Jaccard index. The clustering patterns reflect variations in microbial community composition among study groups. Statistical validation was performed using the PERMANOVA test.

Previous studies have also demonstrated that microbial composition influences vaccine efficacy [17]. The distinct microbial variations observed in PLHIV

compared to HC participants could reflect immune dysfunction or increased microbial translocation, both of which have been linked to systemic inflammation [158]. The reduced microbial diversity in low-responding PLHIV is suggestive of dysbiosis, which has been associated with impaired mucosal immunity [280]. Differences in the immune responses of PLHIV may reflect variations in immune restoration, influencing the oral microbiota-immune axis [159]. From a broader perspective, these findings further reinforce the association between higher oral microbial diversity and immunological resilience [2, 159].

4.3.3 Taxonomic Differences Between High and Low Responders

LEfSe analysis identified taxonomic differences in the oral microbiota between high- and low responders. In low-responding HCs, *Campylobacter*, *Selenomonas*, and *Butyrivibrio 2* were significantly enriched, while *Abiotrophia* and *Corynebacterium* were reduced. In low-responding PLHIV, *Prevotella*, *Stomatobaculum*, *Lachnoanaerobaculum*, *Megasphaera*, and *Leptotrichia* abundances were increased, whereas *Rothia*, *Granulicatella*, *Haemophilus*, and *Gemella* were depleted (Figure 21).

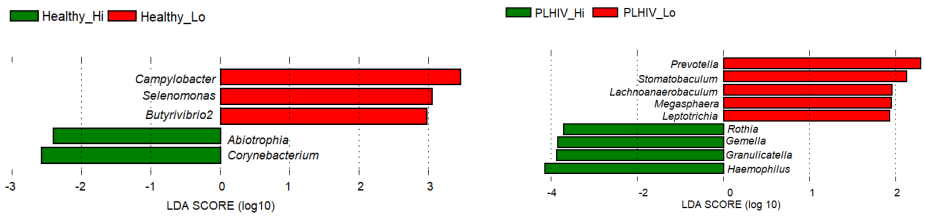


Figure 21 Differentially abundant genera in high- vs. low responders. LEfSe analysis was used to identify the most differentially abundant genera between high and low responders among HCs and PLHIV. Genera associated with high responders are represented by negative LDA scores (green), while those associated with low responders are indicated by positive LDA scores (red). Only significantly ($P < 0.05$) discriminatory genera with LDA scores > 2 are displayed.

Predictive modeling using AUROC (Area Under the Receiver Operating Characteristic) analysis was conducted to assess genera or groups of genera predictive of high- and low responders in both HCs and PLHIV. In HCs, *Campylobacter* and *Butyrivibrio 2* were predictive individually (AUROC scores 0.810, $P < 0.0001$; 0.703, $P < 0.005$), with their combined predictive accuracy increasing to 0.879 ($P < 0.0001$), suggesting a strong association between bacterial composition and vaccine responses. In PLHIV, *Stomatobaculum*, *Granulicatella*, and *Prevotella* were individually predictive, with accuracies ranging from 0.703 to 0.710, increasing to 0.82 when combined ($P < 0.05$) (Figure 22).

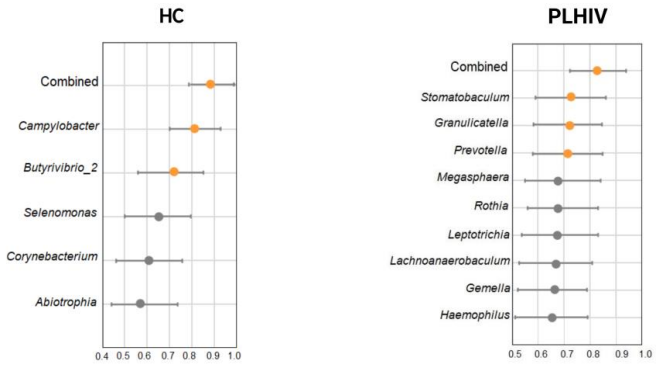


Figure 22 Predictive power of microbial genera in vaccine responses. AUROC scores illustrate the predictive accuracy of individual and combined genera in distinguishing high- and low responders among HCs and PLHIV. Orange dots indicate predictive genera with accuracies > 0.70, while gray dots indicate non-predictive genera with scores < 0.70.

The enrichment of Gram-negative anaerobic taxa in low responders underscores a possible link between oral dysbiosis and weaker immune responses, as these microbes are known to modulate inflammation via lipopolysaccharide (LPS) signaling [281]. In contrast, high responders tended to harbor Gram-positive, facultative taxa, which may characterize healthier mucosal environments and enhanced antigen presentation [7]. These microbial signatures have significant similarities with those observed in models of mucosal vaccine failure, reinforcing their relevance in vaccine immunogenicity.

4.3.4 Functional Microbial Signatures Associated with Vaccine Responses

Microbial functional profiling revealed distinct metabolic pathways between low- and high responders. In low-responders, primarily anaerobic, Gram-negative species with proteolytic activity, associated with amino acid and nucleotide metabolism, and biosynthesis of secondary metabolites, were enriched ($P < 0.05$, FDR (false discovery rate) < 0.05). Conversely, in high-responders facultative Gram-positive linked to carbohydrate and vitamin metabolism, and xenobiotic degradation, were enriched ($P < 0.05$, FDR < 0.05) (Figure 23).

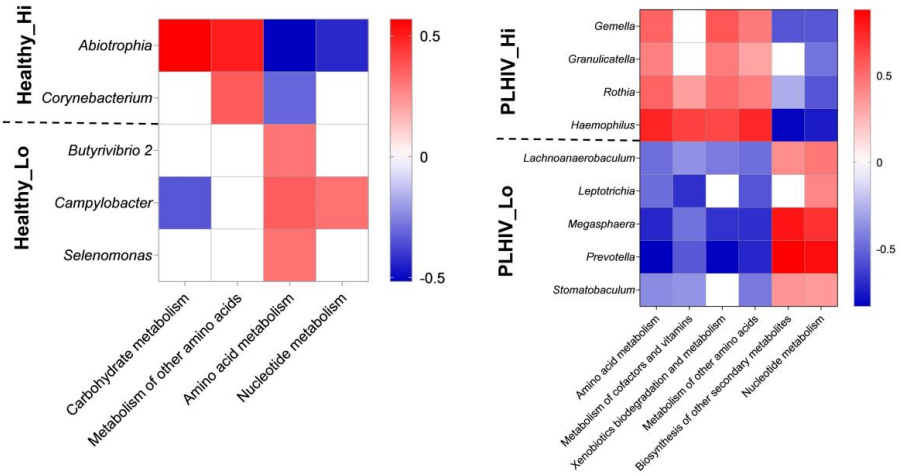


Figure 23 Microbial metabolic pathway associations in vaccine responders. A heatmap representing KEGG metabolic pathway enrichment in the microbial signatures of high and low responders, identified using LEfSe at the genus level (LDA score > 2, P < 0.05). Data are shown for both healthy individuals and people living with PLHIV. Red shading represents positive Spearman correlations, while blue shading indicates negative correlations (P < 0.05 and FDR < 0.05).

Metabolically distinct microbial communities may differentially shape the immune response, with anaerobic, LPS-rich bacteria potentially contributing to chronic low-grade inflammation that impairs mucosal IgG persistence in low responders [281]. In contrast, the microbiota of high responders may support enhanced energy metabolism and mucosal integrity, thereby promoting sustained immune activation.

4.4 Study 4

4.4.1 Broad-Spectrum MAIT Cell Activation by Bacterial Secretomes

To evaluate the activation potential of bacterial metabolites on human MAIT cells, PBMCs from healthy donors were stimulated with secretomes from various bacterial species. Flow cytometry revealed a consistent upregulation of CD69 across all strains, indicating MAIT cell activation (Figure 24). This activation was observed in both the total MAIT cell population and its CD4/CD8-defined subsets, with CD69 induction 1 to 2 times that of unstimulated cells. Despite variations in activation levels, no substantial differences were observed between bacterial species, species from different anatomical sites of origin (Figure 25), or between the exponential and stationary phases of bacterial secretomes.

The finding that species or tissue origin had no significant effect on MAIT cell activation, supports the view that these cells are sensitive to generic, rather than

species-specific, microbial stimuli, including riboflavin metabolites—consistent with their being innate-like immune responders that use a conserved detection and effector mechanism [162]. However, many aspects of MAIT cell activation remain obscure. In particular, how MAIT cell activation is affected by pathogenic bacteria that modulate the immune response by altering their metabolites, MR1 antigen presentation, or cytokine release. How MAIT cells respond under such circumstances requires more in-depth metabolic profiling of bacterial secretomes.

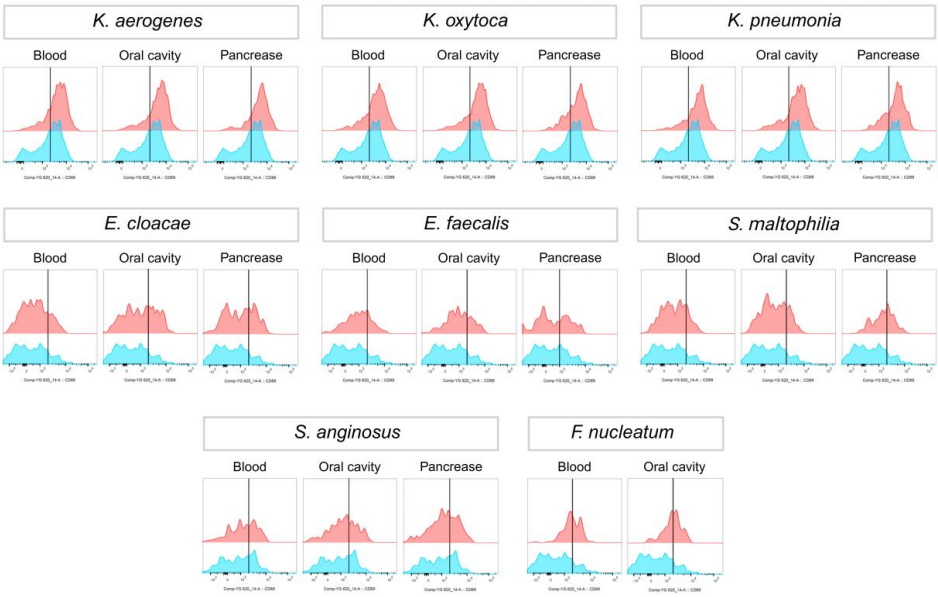


Figure 24 CD69 expression in MAIT-cells across bacterial species and anatomical sites. Representative histogram overlays display the geometric mean fluorescence intensity (gMFI) of CD69 expression in MAIT cells following stimulation with bacterial secretomes. Each panel represents a different bacterial species, with data categorized by anatomical site (blood, oral cavity, pancreas). Red histograms correspond to stimulated samples, while blue histograms represent unstimulated controls.

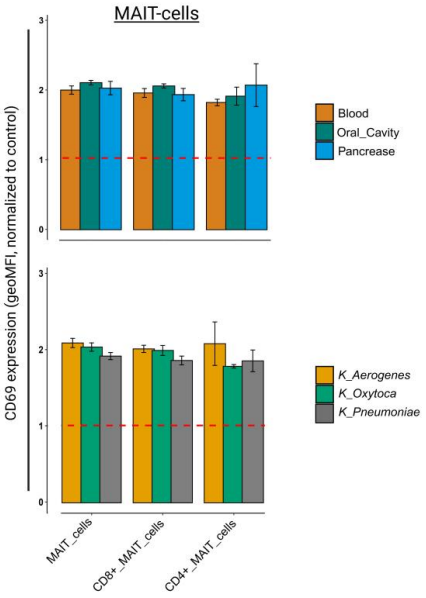


Figure 25 CD69 expression (in fold change) by anatomical site and bacterial species. Representative bar plots show CD69 expression fold change in MAIT cells and their $CD8^+$ and $CD4^+$ subpopulations after stimulation with *Klebsiella* spp. secretomes. The top panel groups data by anatomical site (blood, oral cavity, pancreas), while the bottom groups by bacterial species (*K. aerogenes, K. oxytoca, K. pneumoniae*). Bars are color-coded by site or species. The red dashed line at 1.0 represents the unstimulated baseline.

4.4.2 Preferential MAIT Cell Enrichment within the Activated T Cell Population

Having confirmed MAIT cells' activation to the secretomes, we then examined their relative enrichment within the activated $CD3^+CD69^+$ T cell population. These cells were significantly enriched by all bacterial strains except those derived from *K. pneumoniae* blood. MAIT cell enrichment was highest in response to *F. nucleatum* secretomes, particularly from blood and oral strains, where nearly 90% of the activated T cell population consisted of MAIT cells. While tissue origin had no significant impact on enrichment, the pancreatic-derived strains of *S. maltophilia*, *K. oxytoca*, and *K. pneumoniae*, triggered the highest enrichment (Figure 26).

The substantial enrichment of MAIT cells within the activated T cell population suggests they respond more robustly than conventional T cells to bacterial secretomes—consistent with their role as rapid innate-like immune responders [282]. The pronounced response to *F. nucleatum* secretomes is particularly intriguing, as this species has been implicated in periodontitis, head and neck-, and colorectal cancer, and systemic infections, suggesting its potential role in shaping immune activation [114].

Interestingly, *K. pneumoniae* blood strains did not significantly stimulate MAIT cell enrichment, suggesting potential strain-specific variations in riboflavin

metabolism or the presence of inhibitory immune-modulatory factors. Previous reports indicate that certain bacterial species can evade MR1-mediated antigen presentation, suppressing MAIT cell activation through metabolic interference or immune evasion mechanisms [283]. The stronger MAIT cell enrichment in pancreatic-derived strains of *S. maltophilia*, *K. oxytoca*, and *K. pneumoniae* suggests bacterial metabolism could be significantly shaped by tissue microenvironments, thereby influencing immune activation. Given that pancreatic infections are often associated with opportunistic and multidrug-resistant bacteria, this may have important implications for the role of MAIT cells in pancreatic immune surveillance and infection control [284].

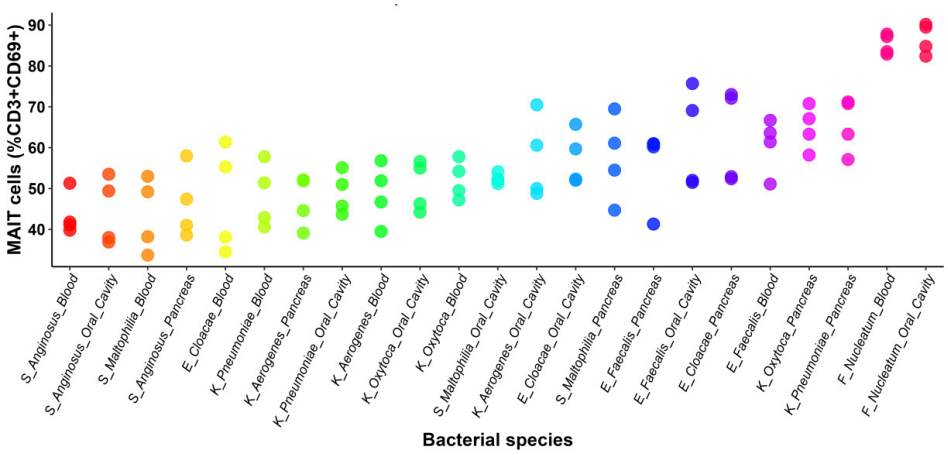


Figure 26 MAIT cell enrichment in CD3+CD69+ T-cell population across bacterial species. The scatter plot illustrates the percentage of $CD3^{+}CD69^{+}$ MAIT cells following stimulation with bacterial secretomes from different bacterial species. Each point represents an individual sample, with different colors indicating the response of each bacterial species in terms of MAIT cell enrichment.

4.4.3 Granzyme B Dominates MAIT Cell Cytokine Response to Bacterial Secretomes

We next analyzed MAIT cells' cytokine expression in response to bacterial secretomes. We observed a dominant monofunctional GzB response, which was largely absent in mitogenically activated control cells (Figure 27). Interestingly, blood-derived bacterial strains induced a more pronounced IL-17A+IFN-γ+ dual-function response, while pancreatic strains elicited the lowest levels of IFN-γ. Despite overall similarities between bacterial species, notable differences emerged at the tissue-of-origin level, particularly in *S. maltophilia*, *K. aerogenes*, and *E. faecalis*, which showed tissue-dependent variations in IL-17A and IFN-γ production.

The bias towards GzB production suggests a cytotoxic phenotype upon MAIT cell activation, which is consistent with previous studies describing MAIT cell-mediated antimicrobial functions [172]. The influence of bacterial origin on cytokine profiles raises intriguing questions about how MAIT cell responses are fine-tuned in different infections. The environment surrounding pancreatic infections is known to be immunosuppressive, which suggests the lower IFN-γ levels in response to pancreatic strains may indicate a modulated immune response aimed at preventing excessive inflammation [285]. Conversely, the heightened IL-17A response to blood-derived strains suggests a more inflammatory phenotype, which may be important in bloodstream infections where rapid immune activation is crucial [285].

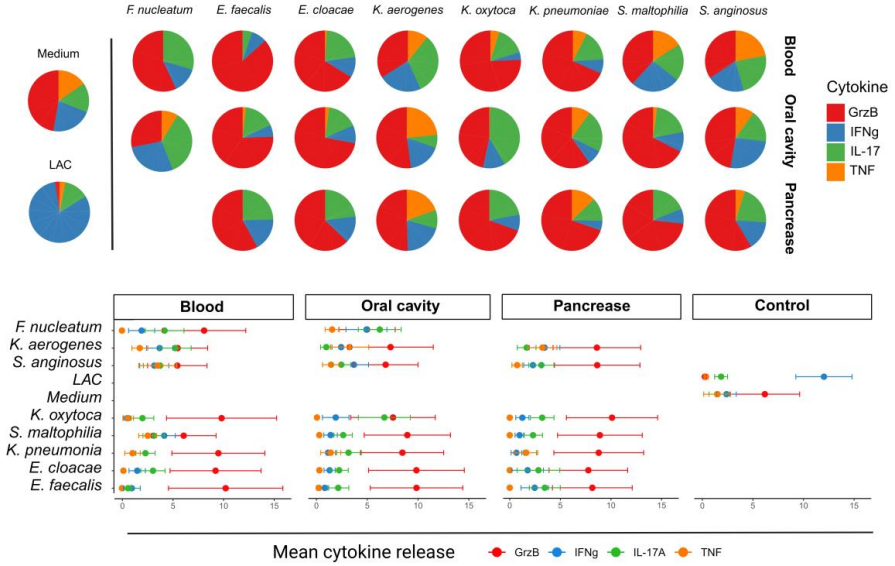


Figure 27 Cytokine release profile across bacterial species and sites. The top panel presents pie charts, each representing a bacterial species at a specific site or control condition (LAC; Mitogenic Activator and Medium: Growth Medium), illustrating the proportion of cytokines released. Colors denote different cytokines. The bottom panel displays a forest plot showing the mean cytokine release across bacterial species, categorized by anatomical site and compared to controls. Error bars represent confidence intervals.

4.4.4 Bacterial Secretomes and Site of Origin Influence MAIT Cell Functionality: Selective Correlations with Granzyme B Release

We then examined how CD69 expression correlates with GzB production in MAIT cells, and found distinct patterns across anatomical sites and bacterial species. While CD69 is a key activation marker, its link to cytotoxic function remains context-dependent. In terms of anatomical origin, only pancreatic isolates showed a significant correlation ($R = 0.41$, $P = 0.037$), while bacteria from the oral

cavity (R = 0.15, P = 0.415) and blood (R = 0.10, P = 0.574) did not (Figure 28, A). This suggests that pancreatic-derived bacterial secretomes create an environment that enhances the connection between MAIT cell activation and cytotoxic function. In terms of species, only three exhibited a strong correlation—*E. faecalis* (R = 0.80, P = 0.001), *S. anginosus* (R = 0.69, P = 0.015), and *K. aerogenes* (R = 0.60, P = 0.048). No other species, including *K. oxytoca, K. pneumoniae, F. nucleatum,* and *S. maltophilia*, showed significant correlations, highlighting that high CD69 expression does not always equate to strong cytotoxicity (Figure 28, B).

These findings align with previous studies demonstrating MAIT cells' diverse functional responses to microbial-derived stimuli. While CD69 upregulation is a hallmark of activation, its relationship with effector function is inconsistent. Research suggests that CD69 expression can increase independently of GzB release, indicating a complex regulatory mechanism [177,286,287]. In individuals exposed to mycobacterium tuberculosis, infection resistance correlates with higher CD25 and GzB levels but lower CD69 and IFN-γ expression [286]. Similarly, in adults with diabetes and autoimmune diseases, MAIT cells show increased CD69 expression but reduced GzB release [288]. These studies suggest that MAIT cell activation does not always lead to cytotoxicity. In addition, the tissue microenvironment plays a critical role in shaping MAIT cell function, which may explain the stronger correlation observed in pancreatic isolates [162]. An *ex vivo* study of tumor-infiltrating MAIT cells in colon adenocarcinomas demonstrated their cytotoxic potential, showing that tumor-derived MAIT cells produce GzB upon activation [289].

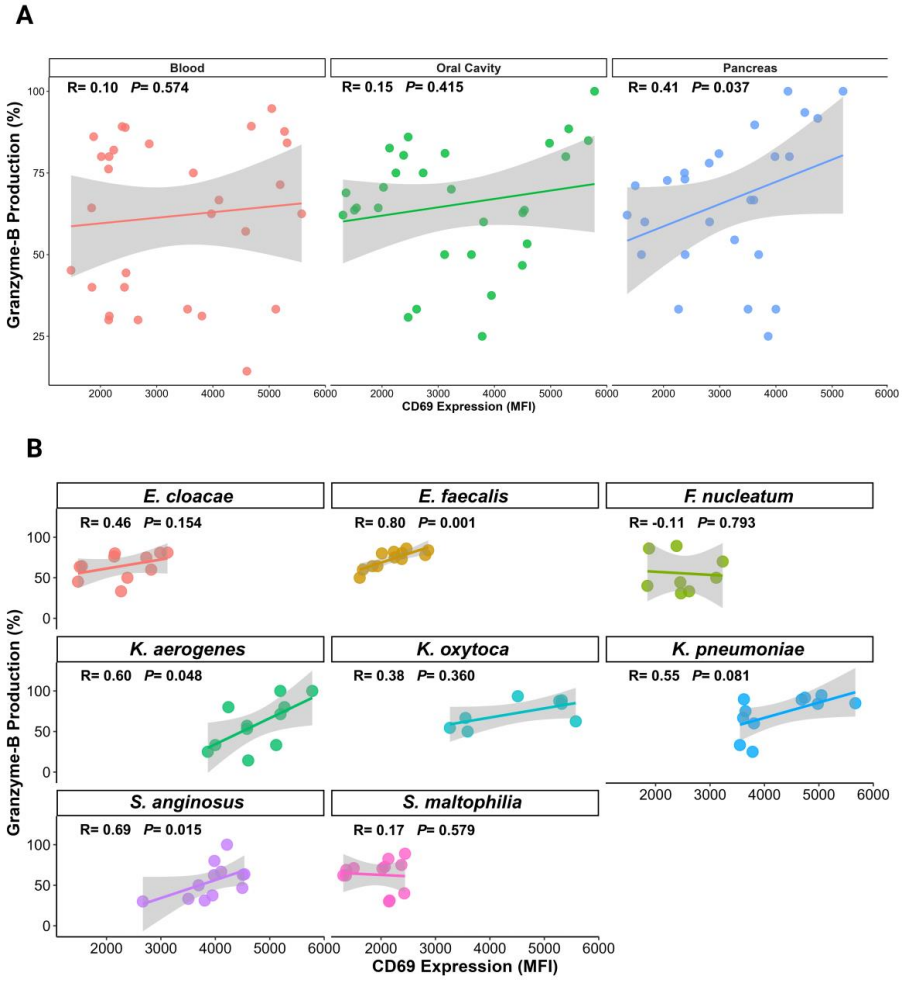


Figure 28 Correlation between CD69 expression and granzyme B production by anatomical site and bacterial species. Regression lines and confidence intervals show the correlation between CD69 expression and GzB production in MAIT cells stimulated with: (A) bacterial secretomes derived from various anatomical sites (blood, oral cavity, and pancreas) and (B) secretomes from different bacterial species. Each dot represents an individual sample, with displayed. A p-value < 0.05 indicates a significant association between CD69 expression and GzB production.

5 Concluding Remarks and Future Perspectives

5.1 Concluding Remarks

Oral microbial signatures play a critical role in shaping local and systemic immune responses, influencing both vaccine efficacy and disease susceptibility. This thesis explored the intricate relationships between oral microbiota, severe infections, and immune modulation, offering novel insights into their clinical and immunological implications. The findings highlight the evolving nature of microbial communities and their profound impact on host health, underscoring the necessity for innovative diagnostic and therapeutic strategies.

Study 1 revealed the dynamic microbial landscape of severe oral infections, with shifts in bacterial composition that may be influenced by antibiotic exposure, host immune responses, and environmental factors. The predominance of *S. anginosus*, *Prevotella* spp., and *Staphylococcus* spp. underscores the polymicrobial nature of these infections, posing significant diagnostic and therapeutic challenges. The emergence of antibiotic-resistant and opportunistic pathogens over time, highlights the urgent need for adaptive antimicrobial strategies and continual microbial surveillance to mitigate the burden of persistent and recurrent infections.

Study 2 provided a detailed characterization of oral fungal infections in head and neck cancer patients undergoing radiotherapy, identifying *Candida* spp., as the major contributors. The co-existence of diverse fungal communities and radiotherapy-induced dysbiosis complicates infection control, emphasizing the need for precision-based antifungal strategies and improved diagnostic tools. The identification of novel fungal associations suggests that conventional antifungal treatments may require re-evaluation, particularly in immunocompromised patients where opportunistic pathogens continue to emerge.

Study 3 demonstrated a connection between oral microbiota composition and the persistence of vaccine-induced salivary antibody responses to COVID-19 vaccination. High responders exhibited a distinct microbial profile enriched in Gram-positive commensals, whereas low responders had an increased prevalence of Gram-negative anaerobes, commonly associated with immune dysregulation and chronic inflammation. These findings suggest that oral microbial signatures may serve as predictive biomarkers for vaccine efficacy, paving the way for microbiota-based interventions to enhance vaccine durability, particularly in immunocompromised populations such as PLHIV.

Study 4 highlighted the immunomodulatory potential of microbial metabolites, showing that bacterial secretomes from different compartments (oral cavity, bloodstream, and pancreatic tumors) elicited distinct MAIT cell activation patterns. This suggests that microbial metabolic products play a crucial role in shaping immune responses and may serve as potential targets for microbiome-based immunotherapies. Understanding how microbial-derived metabolites influence immune regulation and disease progression may open new avenues for infection-associated cancer immunotherapy and immune modulation strategies.

5.2 Strengths and Limitations

This thesis involved many disciplines and methodologies within different patient contexts, giving it a wide clinical relevance. It emphasizes the importance of interdisciplinary collaboration and the continuation of innovation in infection biology and microbiome-targeted interventions. The inclusion of longitudinal data, high-throughput sequencing, and immunological profiling provides a comprehensive understanding of oral microbial signatures and their systemic impact, shedding light on microbial evolution and emerging pathogens. Advanced microbial and immunological profiling using MALDI-TOF MS, 16S rRNA sequencing, and flow cytometry, ensured high-resolution microbial identification and immune phenotyping. Our findings contribute to the growing body of evidence linking oral microbiota to immune function, infection susceptibility, and vaccine responses, reinforcing the importance of oral health in systemic disease management.

Despite its strengths, this work has certain limitations. The observational/correlational design limits causal inferences, necessitating future longitudinal and experimental studies to validate microbial associations. Sample size and geographic constraints may impact the representativeness of the observed microbial diversity, emphasizing the need for expanded cohorts and multi-center collaborations. While our research extensively characterizes bacterial and fungal communities, oral viromes and archaeomes remain unexplored, highlighting an avenue for future investigations. Additionally, functional metabolic interactions between host immunity and microbial communities require deeper, multiomic exploration to unravel key mechanisms driving immune modulation and disease progression.

5.3 Perspectives and Future Research Directions

Understanding oral microbial signatures in infection dynamics, immune modulation, and vaccine responses presents a unique opportunity to bridge oral and systemic health. Microbiota-driven interventions hold promising therapeutic potential for managing oral and systemic infections. Future research should therefore explore microbiome-targeted interventions, such as probiotics, engineered bacteria, and bacteriophage therapy, to restore microbial balance and reinforce host defense mechanisms. In addition, future research should explore how microbial composition influences vaccine durability and whether modulating oral microbiota through probiotics, prebiotics, or dietary interventions could enhance immune resilience. Investigating microbiome-based strategies to optimize vaccine responses is particularly relevant for immunocompromised populations, where immune responses often wane prematurely.

Oral microbiome transplantation (OMT), much like fecal microbiota transplantation (FMT), is emerging as a potential strategy to enhance host immunity and restore eubiosis [290]. This approach could be particularly valuable for patients suffering from recurrent oral infections, immunosuppression, or post-radiotherapy dysbiosis. Investigating the impact of OMT on immune homeostasis and vaccine response may also open new possibilities for personalized medicine and immunomodulatory therapies. Furthermore, OMT may play a role in mitigating dysbiosis-associated immune dysfunction, particularly in immunocompromised patients. Evaluating its therapeutic potential in restoring immune balance and preventing infection-related complications remains an important future direction.

To fully harness the potential of these interventions, longitudinal multiomics studies are needed to track microbial dynamics and assess how microbiome alterations influence disease progression. Integrating omics data can provide a deeper understanding of microbial community functions and their interactions with host immunity. Single-cell immune profiling could further elucidate how microbial secretions influence immune cell activation, particularly in relation to unconventional T cell responses. More extensive fractionation and metabolic profiling of microbial secretomes could provide deeper insights into their impact on unconventional T cell functionality and response mechanisms, including those involving MAIT cells. Moreover, conducting studies on purified and isolated MAIT cells could help determine whether activation is primarily driven by microbial metabolites or the cytokine milieu of ACPs.

Advances in AI-driven microbiome analytics have the potential to revolutionize microbial diagnostics and precision medicine. AI-driven predictive modeling, when combined with multiomics data, can enhance our understanding of microbiome-driven disease pathways and enable early detection of pathogenic shifts. Real-time microbial profiling in clinical workflows may support precision antimicrobial therapy, improve pathogen surveillance, and prevent multidrug-resistant infections. These integrated approaches will be key to translating microbiome research into clinical applications, ultimately paving the way for precision microbiome-targeted medicine in oral and systemic health.

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7 Declaration About the Use of Generative AI

I take full responsibility for the writing and content of this thesis summary. Generative AI was not used in the writing process or for reference management. All the work presented reflects my own analysis, interpretation, and synthesis of the conducted research

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