Original Article

Investigating Pre-Vaccination Mucosal Microbiota Composition as a Predictor of Humoral and Cellular Immune Response to the Seasonal Influenza Vaccine

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ABSTRACT

Background: Variability in immune responsiveness to seasonal influenza vaccination remains a persistent public health challenge, as a substantial subset of vaccinated individuals fails to mount adequate humoral and cellular protection. Increasing evidence suggests that mucosal microbiota composition modulates immune priming at the respiratory interface, yet its predictive value for influenza vaccine outcomes remains insufficiently characterized. Objective: To investigate whether pre-vaccination upper respiratory mucosal microbiota composition predicts humoral and cellular immune responses to the seasonal influenza vaccine and to identify microbial biomarkers associated with suboptimal responsiveness. Methods: A prospective observational cohort study was conducted over five months in Lahore, enrolling 220 adults (18–65 years) receiving a trivalent inactivated influenza vaccine. Pre-vaccination nasopharyngeal and oropharyngeal swabs underwent 16S rRNA sequencing. Humoral immunity was assessed using hemagglutination inhibition assays at baseline and 28 days post-vaccination, and cellular immunity was evaluated using IFN-γ ELISpot and flow cytometric quantification of $CD4^+$ and $CD8^+$ T-cell activation. Multivariable regression, PERMANOVA, and random forest classification were applied with adjustment for key covariates. Results: Complete data were available for 212 participants, of whom 132 (62.3%) achieved seroconversion. Responders exhibited higher microbial diversity (Shannon index 3.9 ± 0.6 vs 3.4 ± 0.5, $p = 0.002$; Simpson index 0.87 ± 0.04 vs 0.81 ± 0.05, $p < 0.001$) and distinct beta diversity clustering (PERMANOVA $p = 0.001$). Responders demonstrated higher antibody fold-rise (5.7 ± 2.4 vs 2.1 ± 1.2, $p < 0.001$) and higher IFN-γ ELISpot responses (142 ± 38 vs 87 ± 29 SFU/10^6 PBMCs, $p < 0.001$) with greater $CD4^+$ (14.6% vs 9.2%, $p < 0.001$) and $CD8^+$ (12.3% vs 8.5%, $p < 0.01$) activation. Higher Faecalibacterium abundance predicted seroconversion (adjusted OR 1.42, 95% CI 1.15–1.76, $p < 0.001$), whereas Streptococcus abundance was inversely associated (adjusted OR 0.63, 95% CI 0.48–0.82, $p < 0.01$); random forest classification achieved AUC 0.87 (95% CI 0.82–0.91). Conclusion: Pre-vaccination mucosal microbiota diversity and specific microbial signatures predict both humoral and cellular influenza vaccine responses, supporting the potential utility of microbial biomarkers for identifying suboptimal responders and informing microbiota-directed strategies to improve vaccine effectiveness.

Keywords: *Influenza Vaccines; Microbiota; Mucosal Immunity; Hemagglutination Inhibition; Cellular Immunity; ELISpot; Predictive Biomarkers; Vaccine Response.*

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INTRODUCTION

Seasonal influenza remains a persistent global public health threat, contributing to recurrent epidemics that drive substantial morbidity, mortality, and economic burden each year (1). Vaccination represents the most effective preventive strategy; however, the protective effectiveness of the seasonal influenza vaccine varies considerably across individuals and populations, even when vaccine formulation and delivery are standardized (2). This heterogeneity reflects differences in vaccine-induced immunogenicity, where a substantial subset of vaccinated individuals fails to achieve adequate humoral protection and/or robust

cellular immune activation, thereby remaining vulnerable to infection and severe outcomes (3). Understanding the biological and environmental determinants of this variability is essential for improving vaccine performance and optimizing public health strategies (4). Beyond host demographic and immunological factors, emerging evidence indicates that the human microbiota is a critical upstream regulator of immune readiness and vaccine responsiveness, functioning through continuous modulation of innate immune signaling, antigen presentation, and adaptive immune priming (5).

The concept that commensal microbial communities shape vaccine immunogenicity has gained momentum over the last decade, supported by studies demonstrating associations between microbial diversity, microbial metabolites, and enhanced antibody responses to multiple vaccine platforms (6). In particular, systems-level immunoprofiling has highlighted that baseline immune states, partially sculpted by microbial exposure and composition, can predict subsequent vaccine-induced responses (7). Microbiota-driven immune modulation is biologically plausible because microbial ligands and metabolites continually engage mucosal pattern-recognition receptors and tune inflammatory thresholds, thereby influencing dendritic cell maturation, T-helper polarization, and B-cell differentiation (8). Furthermore, multi-omic approaches integrating host immune signatures and microbial features have reinforced that vaccine responsiveness is not solely a function of antigen exposure, but also of the host's pre-existing immunological landscape, which is shaped by microbial and environmental inputs (9). Despite these advances, most vaccine–microbiota research has focused predominantly on the gut microbiome, leaving respiratory mucosal microbial compartments relatively underexplored, particularly for respiratory vaccines and pathogens (6,10). This represents a critical gap, because influenza infection is initiated at the respiratory epithelium, where mucosal immunity and local innate priming substantially influence the trajectory of immune activation and antigen-specific memory formation (2).

The upper respiratory mucosa harbors a dynamic microbial ecosystem that may act as an immunological gatekeeper, shaping the early sensing and processing of vaccine antigens through local cytokine environments and antigen-presenting cell activation (2,11). Conceptually, the mucosal microbiota may influence vaccine response through several pathways: first, by regulating basal interferon signaling and epithelial barrier integrity; second, by modulating dendritic cell trafficking and antigen presentation; and third, by shaping the balance between inflammatory and tolerogenic responses that determine downstream antibody production and T-cell activation (5,8). While associations between microbiota composition and vaccine immunogenicity have been demonstrated for enteric and systemic vaccines, evidence directly linking pre-vaccination respiratory mucosal microbial profiles to influenza vaccine outcomes—particularly the combined humoral and cellular response—is limited and remains methodologically heterogeneous (6,10). This gap is especially important because influenza vaccine-induced protection is mediated not only by neutralizing antibodies, commonly assessed via hemagglutination inhibition (HAI) assays, but also by cellular immunity, including influenza-specific $CD4^+$ and $CD8^+$ T-cell responses that contribute to viral clearance, disease attenuation, and cross-strain recognition (3,12). Consequently, exclusive reliance on serological endpoints may underestimate clinically meaningful immune protection, underscoring the need for integrated evaluation of both humoral and cellular arms when studying microbiota–vaccine interactions (3,4).

Identifying microbial predictors of influenza vaccine responsiveness has substantial translational relevance. If pre-vaccination mucosal microbial signatures can reliably stratify individuals at risk of suboptimal immunogenicity, this could enable targeted interventions, such as microbiota-directed immunonutrition, prebiotic supplementation, or probiotic strategies designed to optimize immune priming prior to vaccination (13). Such approaches

align with the broader movement toward personalized and precision vaccination, where baseline biomarkers are leveraged to tailor vaccine strategies, maximize immunogenicity, and reduce variability in protection (14). The need for predictive strategies is particularly pressing in populations with reduced vaccine responsiveness due to immunosenescence and chronic inflammation, where microbiota alterations are also more prevalent and may contribute to impaired immune responses (15). In parallel, systems vaccinology has demonstrated that predictive modeling using integrated biological features can improve response forecasting and inform rational vaccine design, suggesting that microbiota-informed prediction models may be both feasible and clinically valuable (16,17). Importantly, advances in machine learning and network-based immune modeling further strengthen the rationale for exploring microbial biomarkers as part of integrated predictive frameworks for vaccine outcomes (18–20).

Against this background, the present study investigated whether pre-vaccination mucosal microbiota composition is associated with, and predictive of, immune responses to the seasonal influenza vaccine, assessed through both humoral and cellular endpoints. Specifically, mucosal microbiota profiles obtained prior to vaccination were evaluated in relation to post-vaccination HAI antibody responses and cellular immunity measured by interferon-γ ELISpot and T-cell activation markers. We hypothesized that higher mucosal microbial diversity and enrichment of specific commensal taxa would be associated with stronger antibody responses and greater T-cell activation following vaccination, whereas microbial profiles suggestive of mucosal dysbiosis would predict suboptimal humoral and cellular responsiveness. By identifying mucosal microbial features linked to vaccine response heterogeneity, this study aims to contribute to the development of microbiota-informed strategies to enhance influenza vaccine effectiveness and support more individualized immunization approaches (6,13,14).

MATERIAL AND METHODS

A prospective observational cohort study was conducted over a five-month period in Lahore, Pakistan, to evaluate whether pre-vaccination upper respiratory mucosal microbiota composition predicts subsequent humoral and cellular immune responses to the seasonal influenza vaccine. The study was implemented at outpatient vaccination clinics serving adult community populations, and all procedures followed internationally accepted ethical principles for human research. Ethical approval was obtained from the relevant institutional review committee prior to participant enrollment, and written informed consent was collected from all participants before any study-related activities. Participant confidentiality was ensured through coded identifiers, restricted-access data storage, and anonymized laboratory outputs, consistent with standard research governance practices for immunological and multi-omic investigations (21,22).

Adults aged 18–65 years presenting for seasonal influenza vaccination were assessed for eligibility. Participants were included if they were clinically stable, free of acute respiratory tract infection symptoms at baseline assessment, and able to return for follow-up blood sampling after vaccination. Individuals were excluded if they reported antibiotic use within the preceding four weeks, probiotic or prebiotic supplementation within the preceding four weeks, immunosuppressive medication exposure, known immunodeficiency disorders, chronic inflammatory or autoimmune conditions requiring active pharmacological treatment, hospitalization within the prior three months, or receipt of influenza vaccination during the same season. Additional exclusions included pregnancy and any history of severe vaccine-related adverse reactions. Eligibility screening was conducted using a standardized checklist administered by trained research staff, and baseline characteristics were recorded

using a structured case report form, including age, sex, smoking status, body mass index, chronic comorbidities (including diabetes and hypertension), recent self-reported influenza-like illness, and prior laboratory-confirmed influenza infection history when available. To reduce confounding related to behavioral and environmental determinants of the mucosal microbiota, participants were also asked about recent upper respiratory symptoms within the prior two weeks, intranasal corticosteroid use, and routine oral hygiene practices, and these variables were considered as potential covariates in adjusted analyses (21,22).

A sample size estimation was performed a priori using an 80% statistical power and a two-sided significance threshold of 0.05, guided by published literature indicating moderate effect sizes for microbiota-related variability in vaccine immunogenicity outcomes and the expected prevalence of suboptimal responders in adult cohorts (6,10). The target minimum sample size was set at 200 participants to enable multivariable modeling with adjustment for key confounders and to provide sufficient statistical precision for group comparisons between responder categories, with an oversampling strategy incorporated to mitigate attrition from incomplete immune assays or sequencing failures. Participants were enrolled using consecutive sampling during routine vaccination hours until the recruitment target was met. Following enrollment, all participants received a standard dose of the seasonal trivalent inactivated influenza vaccine administered intramuscularly by licensed vaccination staff according to clinical immunization practice. Vaccine administration was documented, including vaccination date, injection site, and observed post-vaccination immediate adverse events. Participants were followed for post-vaccination immune response assessment at 28 days, which was defined as the primary post-immunization timepoint for measurement of serological response and antigen-specific cellular immunity consistent with established vaccine immunogenicity profiling approaches (5,7).

Upper respiratory mucosal samples were collected immediately prior to vaccination. Two sampling sites were included to enhance representativeness of mucosal microbial ecology relevant to influenza immunobiology: nasopharyngeal and oropharyngeal compartments. Specimens were obtained using sterile flocked swabs by trained personnel under aseptic technique. Nasopharyngeal swabs were inserted through the nostril to the posterior nasopharynx and rotated gently for standardized contact time, while oropharyngeal swabs were applied to the posterior pharyngeal wall and tonsillar pillars while avoiding oral surfaces. Immediately after collection, swabs were placed into sterile nucleic acid stabilization transport media, labeled using unique participant identifiers, transported on ice to the processing facility within the same day, and stored at −80°C until DNA extraction. To minimize contamination and preserve analytical integrity, sample handling followed standardized clean workflow procedures, including the use of DNA-free consumables, glove changes between participants, and batch-wise inclusion of negative controls. Extraction blanks and reagent-only controls were processed alongside clinical specimens to enable downstream identification of background contamination signals, consistent with current best practices for microbiome sequencing studies (6,21).

Microbial DNA was extracted using validated commercial extraction kits optimized for low-biomass mucosal specimens, following the manufacturer's protocol with standardized bead-beating steps to ensure adequate lysis of Gram-positive organisms. DNA concentration and purity were evaluated using spectrophotometric methods and fluorometric quantification. Microbiota profiling was performed by sequencing the V3–V4 region of the bacterial 16S rRNA gene using Illumina sequencing technology. Library preparation included PCR amplification with locus-specific primers containing sequencing adapters and dual indices, followed by bead purification and quantification prior to pooling. Sequencing was conducted using paired-end chemistry. Raw sequencing reads were processed using a standardized

bioinformatics pipeline, including quality filtering, adapter trimming, chimera removal, and denoising. Operational taxonomic units were generated at 97% similarity and assigned taxonomy using curated reference databases, and additional sensitivity analysis was planned to evaluate whether results were robust across alternative taxonomic assignment thresholds. Alpha diversity was quantified using Shannon and Simpson indices to characterize within-sample diversity, while beta diversity was computed using Bray–Curtis dissimilarity and weighted UniFrac distance matrices to evaluate between-sample community structure. Principal coordinate analysis was applied to visualize microbial community clustering across vaccine responder phenotypes, and permutational multivariate analysis of variance (PERMANOVA) was used to test group differences in beta diversity while accounting for covariates where appropriate (6,21,22).

Humoral immune response was quantified using hemagglutination inhibition assays performed on serum collected at baseline (pre-vaccination) and at day 28 post-vaccination. Blood samples were drawn by trained phlebotomists into serum separator tubes, allowed to clot, centrifuged, and stored at −80°C until analysis. HAI assays were conducted using standardized procedures for influenza vaccine evaluation. Antibody titers were determined as the reciprocal of the highest serum dilution that inhibited hemagglutination. Seroconversion was operationally defined as a fourfold or greater increase in HAI titer between baseline and day 28, consistent with widely adopted immunogenicity benchmarks in influenza vaccine studies (1,5). In addition to categorical seroconversion classification, continuous measures of antibody fold-rise were retained for regression modeling to improve statistical sensitivity and preserve biological signal.

Cellular immune responses were assessed at day 28 using interferon-γ enzyme-linked immunospot assays and flow cytometric analysis of antigen-stimulated T-cell activation. Peripheral blood mononuclear cells were isolated by density gradient centrifugation from heparinized whole blood. For ELISpot testing, PBMCs were stimulated with influenza vaccine strain antigens or peptide pools under standardized incubation conditions, and results were expressed as spot-forming units per 10^6 PBMCs after subtraction of background spots from negative control wells. Flow cytometric assays were conducted on stimulated PBMCs to quantify $CD4^+$ and $CD8^+$ T-cell activation using fluorescence-labeled antibodies and gating strategies based on lymphocyte forward and side scatter properties, doublet exclusion, and viable-cell selection. Activated T cells were defined using established activation marker combinations, and percentages of activated $CD4^+$ and $CD8^+$ populations were recorded as cellular endpoints. Laboratory assays were performed by analysts blinded to microbiota profiles and clinical responder status to reduce measurement bias (7,21). To support integrated immunogenicity interpretation, participants were categorized into responder groups based on pre-specified criteria: humoral responders were defined by seroconversion, while cellular responders were defined by exceeding assay-specific thresholds for ELISpot and/or T-cell activation relative to baseline or unstimulated control values. Composite responder categories were also explored to evaluate concordance between humoral and cellular protection signatures (3,5).

The primary exposure variables were mucosal microbial diversity indices and taxonomic relative abundances at genus-level resolution. The primary outcome was humoral response measured as seroconversion status at day 28 and antibody fold-rise. Secondary outcomes included IFN-γ ELISpot SFU/10^6 PBMCs and activated $CD4^+$ and $CD8^+$ T-cell proportions. Key covariates included age, sex, BMI, smoking status, comorbidity status, recent respiratory symptoms, and intranasal corticosteroid use. Bias reduction measures included standardized specimen collection protocols, consistent timing of sampling relative to vaccination, blinded laboratory processing, inclusion of negative sequencing controls, and pre-specified statistical

models to limit post hoc inference inflation (21,22). To address confounding, multivariable models were constructed a priori with covariate adjustment guided by biological plausibility and previous literature on vaccine response heterogeneity and microbiota variation (5,6,10).

Statistical analyses were performed using R software. Continuous variables were summarized using means and standard deviations or medians and interquartile ranges based on distributional characteristics, and categorical variables were reported as frequencies and percentages. Between-group comparisons were conducted using independent sample t-tests for normally distributed measures and Mann–Whitney U tests for non-normal measures. Categorical outcomes were compared using chi-square tests or Fisher's exact tests as appropriate. Associations between microbial features and immune response outcomes were evaluated using multivariable regression. Logistic regression was used for seroconversion as a binary endpoint, while linear regression models were used for antibody fold-rise and cellular immune outcomes as continuous measures after appropriate transformation when required. Model assumptions were evaluated using residual diagnostics and collinearity checks. To identify microbial taxa discriminating responders from non-responders, linear discriminant analysis effect size was applied, and multiple testing correction was performed using the Benjamini–Hochberg false discovery rate approach to control for type I error inflation in high-dimensional microbial comparisons (6,22). For predictive classification, random forest models were trained on microbial features with stratified cross-validation to estimate model performance. Discriminatory capacity was quantified using the area under the receiver operating characteristic curve, and feature importance was computed to identify taxa most strongly contributing to classification performance, consistent with multi-omic biomarker discovery approaches applied in vaccine response prediction research (7,9,20).

Missing data were minimized through standardized follow-up scheduling and reminder protocols, but incomplete datasets were anticipated due to failed sequencing reads or insufficient PBMC yield. The primary analysis used complete-case datasets for participants with valid microbiota sequencing and immune outcomes. Sensitivity analyses were planned to compare baseline characteristics of included versus excluded participants to assess missingness patterns and evaluate potential selection bias, following established recommendations for robust biomarker modeling and transparency in multi-omic studies (21,22). Data integrity was supported through double-entry verification of clinical data, audit trails for laboratory sample processing, and standardized scripts for bioinformatics and statistical workflows to enhance reproducibility. All analytical code and relevant metadata were maintained in controlled-access repositories, and sequencing outputs were prepared in formats suitable for deposition in public repositories in accordance with prevailing transparency standards for microbiome research (6,21,22).

RESULTS

A total of 220 participants were enrolled and received the seasonal influenza vaccine. After excluding individuals with incomplete sequencing reads and/or incomplete immune assay results, 212 participants were included in the final analytic dataset (responders n = 132; non-responders n = 80). Baseline demographics were broadly comparable between groups, with no statistically significant differences in age, sex distribution, or comorbidity status (Table 1). The overall cohort had a mean age of 41.6 ± 12.4 years, with 52.8% males and 14.6% reporting chronic comorbidities (hypertension/diabetes).

Microbiota sequencing generated high-quality profiles across all included participants, yielding a mean sequencing depth of approximately 46,500 reads per sample after quality filtering. Across the cohort, taxonomic assignment identified ~220 operational taxonomic

units (OTUs) spanning 9 bacterial phyla and 55 genera. Alpha diversity indices were significantly higher among responders, consistent with a more diverse and potentially resilient mucosal microbial ecosystem (Table 2). Responders exhibited higher Shannon diversity (3.9 ± 0.6) compared with non-responders (3.4 ± 0.5, $p = 0.002$), and similarly higher Simpson diversity (0.87 ± 0.04 vs. 0.81 ± 0.05, $p < 0.001$).

Beta diversity analysis based on Bray–Curtis dissimilarity demonstrated distinct clustering between responders and non-responders, indicating that overall community structure differed by immune responsiveness (PERMANOVA $p = 0.001$). Principal coordinate analysis demonstrated tighter clustering among non-responders, suggesting relatively reduced ecological variability compared with responders.

At the taxonomic level, responders demonstrated significantly higher relative abundance of Bacteroides (28.3% vs. 19.7%, $p < 0.01$) and Faecalibacterium (11.5% vs. 7.2%, $p < 0.01$), whereas non-responders showed enrichment of Streptococcus (13.8% vs. 7.6%, $p < 0.001$) and Prevotella (16.4% vs. 9.8%, $p < 0.01$) (Table 3). These taxa-level differences suggested that microbial communities associated with vaccine responsiveness were characterized by greater representation of genera linked to immune-supportive ecological profiles, while taxa more commonly associated with mucosal dysbiosis were overrepresented among suboptimal responders.

Humoral immune outcomes showed clear separation between groups. Responders demonstrated a substantially higher antibody fold-rise (5.7 ± 2.4) compared with non-responders (2.1 ± 1.2), corresponding to a mean difference of 3.6-fold (95% CI: 3.11 to 4.09; $p < 0.001$; Cohen's $d = 1.77$) (Table 4). Cellular immune responses aligned with humoral findings: responders showed significantly higher IFN-γ ELISpot responses (142 ± 38 vs. 87 ± 29 spots/10^6 PBMCs), corresponding to a mean difference of 55 spots/10^6 PBMCs (95% CI: 45.92 to 64.08; $p < 0.001$; Cohen's $d = 1.58$) (Table 4). Flow cytometry further supported this pattern, with responders demonstrating higher $CD4^+$ T-cell activation (14.6% vs. 9.2%, $p < 0.001$) and $CD8^+$ T-cell activation (12.3% vs. 8.5%, $p < 0.01$).

In multivariable logistic regression models adjusting for age and sex, higher Faecalibacterium abundance was an independent positive predictor of seroconversion (OR 1.42, 95% CI 1.15–1.76, $p < 0.001$), whereas higher Streptococcus abundance was independently associated with reduced odds of seroconversion (OR 0.63, 95% CI 0.48–0.82, $p < 0.01$) (Table 5). Random forest classification using microbial features demonstrated strong discriminative performance in differentiating responders from non-responders (AUC 0.87; 95% CI 0.82–0.91), with Bacteroides, Faecalibacterium, and Streptococcus ranking among the most important discriminatory taxa.

Age-stratified analyses suggested that younger participants (<40 years) had higher seroconversion rates (69.1%) compared with participants ≥40 years (56.7%, $p = 0.04$). However, predictive model performance remained stable across age and sex strata, supporting the robustness of microbiota-based predictors across demographic subgroups.

Table 1. Baseline Demographic and Clinical Characteristics by Vaccine Response Status

Variable	Responders (n=132)	Non-Responders (n=80)	Effect Estimate	p-value
Age (years), mean ± SD	40.9 ± 11.8	42.7 ± 13.2	Mean diff = −1.8 (95% CI −5.32 to 1.72); d = −0.15	0.318
Male sex, n (%)	71 (53.8)	41 (51.2)	Δ = +2.6%	0.705†
Female sex, n (%)	61 (46.2)	39 (48.8)	Δ = −2.6%	0.705†
Comorbidities, n (%)	18 (13.6)	13 (16.2)	Δ = −2.6%	0.596†

†Chi-square test.

Table 2. Alpha Diversity Indices by Response Status

Diversity Metric	Responders (n=132)	Non-Responders (n=80)	Effect Estimate	p-value
Shannon Index, mean ± SD	3.9 ± 0.6	3.4 ± 0.5	Mean diff = 0.50	0.002
Simpson Index, mean ± SD	0.87 ± 0.04	0.81 ± 0.05	Mean diff = 0.06	<0.001

Table 3. Key Differentially Abundant Genera Between Groups

Taxa (Genus)	Responders (%)	Non-Responders (%)	Δ (Responders − Non)	p-value
Bacteroides	28.3	19.7	+8.6	<0.01
Faecalibacterium	11.5	7.2	+4.3	<0.01
Streptococcus	7.6	13.8	−6.2	<0.001
Prevotella	9.8	16.4	−6.6	<0.01

Table 4. Humoral and Cellular Immune Outcomes by Response Status

Outcome	Responders (n=132)	Non-Responders (n=80)	Effect Estimate	p-value
Antibody fold-rise, mean ± SD	5.7 ± 2.4	2.1 ± 1.2	Mean diff = 3.6 (95% CI 3.11–4.09); d = 1.77	<0.001
ELISpot IFN-γ (spots/10^6 PBMCs), mean ± SD	142 ± 38	87 ± 29	Mean diff = 55 (95% CI 45.92–64.08); d = 1.58	<0.001
$CD4^+$ T-cell activation (%), mean	14.6	9.2	Δ = +5.4%	<0.001
$CD8^+$ T-cell activation (%), mean	12.3	8.5	Δ = +3.8%	<0.01

Table 5. Multivariable Logistic Regression Predicting Seroconversion (Adjusted for Age and Sex)

Predictor (Baseline Abundance)	Adjusted OR	95% CI	p-value
Streptococcus	0.63	0.48–0.82	<0.01
Faecalibacterium	1.42	1.15–1.76	<0.001

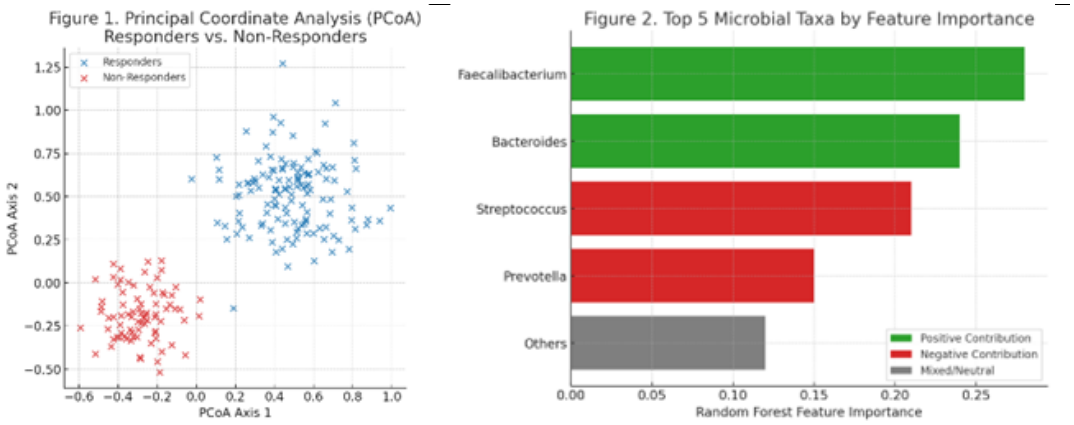

Figure 1 Figure 1. Distinct Mucosal Microbiota Community Structure and Key Predictive

Taxa Differentiating Influenza Vaccine Responders and Non-Responders. (A) Principal Coordinate Analysis (PCoA) based on Bray–Curtis dissimilarity demonstrates clear clustering separation between responders (blue) and non-responders (red), indicating significant differences in overall mucosal microbial community composition. (B) Random forest feature importance analysis identifies the top microbial taxa contributing to classification performance, with Faecalibacterium and Bacteroides showing the strongest positive contribution to responder status, while Streptococcus and Prevotella demonstrate negative contribution.

DISCUSSION

The present study provides evidence that pre-vaccination upper respiratory mucosal microbiota composition is associated with, and predictive of, variability in immune responsiveness to the seasonal influenza vaccine, supporting the concept that baseline microbial ecology is an important determinant of vaccine immunogenicity (6). Responders

demonstrated significantly higher alpha diversity and distinct microbial community structures compared with non-responders, suggesting that a more diverse mucosal microbial ecosystem may confer an immunological advantage that supports more efficient priming of adaptive responses. This finding aligns with broader evidence that microbiota-driven immune conditioning shapes baseline immune tone and influences the magnitude of vaccine responses through modulation of antigen-presenting cell activation, cytokine signaling, and lymphocyte differentiation (5,6). Importantly, the present results extend this framework specifically to the respiratory mucosal compartment, a biologically relevant site for influenza infection and immune engagement, and demonstrate that microbial profiles measured prior to vaccination contain clinically meaningful predictive information for both humoral and cellular outcomes (2,3).

The observed enrichment of Bacteroides and Faecalibacterium among responders and the increased abundance of Streptococcus and Prevotella among non-responders suggests that particular microbial configurations may promote or constrain the development of protective immunity after vaccination. Prior microbiota–immunity studies have reported that microbial taxa commonly associated with more stable commensal ecosystems are linked to improved vaccine-induced antibody production and more effective T-cell responses, likely through sustained low-level stimulation of innate immune pathways that enhances dendritic cell maturation and T-helper differentiation (6,10). In this study, higher Faecalibacterium abundance was independently associated with increased odds of seroconversion after adjustment for age and sex, whereas Streptococcus abundance was inversely associated with seroconversion. These associations remained consistent across modeling approaches and were supported by random forest classification, which achieved strong discriminative performance (AUC 0.87), indicating that mucosal microbial signatures contain sufficient signal to meaningfully stratify responders from non-responders. Such predictive performance is consistent with systems vaccinology paradigms demonstrating that baseline biomarkers can forecast vaccine outcomes and underscores the feasibility of incorporating microbiota features into multi-dimensional prediction models of vaccine responsiveness (7,16,17). While the study does not establish causality, the convergent findings across diversity indices, differential abundance comparisons, and multivariable regression strengthen confidence that the observed patterns reflect biologically plausible relationships rather than random associations.

A key strength of this study is the integrated evaluation of both humoral and cellular immunity. Although antibody titers remain a standard immunogenicity endpoint for influenza vaccines, cellular immunity contributes substantially to viral clearance, reduction of disease severity, and cross-strain protection, particularly when antigenic drift reduces neutralization capacity (3,12). In this cohort, responders showed substantially higher IFN-γ ELISpot responses and greater activation of both $CD4^+$ and $CD8^+$ T cells, indicating that mucosal microbial features were not only associated with antibody production but also reflected differences in T-cell-mediated immune activation. This dual-arm immune profiling addresses a limitation in much of the existing microbiota–vaccine literature, which has often relied on serological outcomes alone, and provides a more comprehensive understanding of how baseline microbial ecology may influence immunological breadth and functional response quality (6,10). The presence of distinct beta diversity clustering between groups further supports that immunogenicity differences may be linked to broader community-level architecture, rather than single-taxon effects, which is consistent with contemporary ecological interpretations of microbiota-host interactions (6).

The results have potential translational implications. Identification of microbial predictors of vaccine responsiveness supports the feasibility of microbiota-informed risk stratification,

particularly in settings where influenza vaccine performance is variable and where individuals may remain insufficiently protected despite vaccination (14). If validated externally, pre-vaccination mucosal microbiota profiling could inform targeted strategies such as microbiota-directed immunonutrition, prebiotic supplementation, or probiotic interventions designed to optimize immune priming and improve vaccine-induced protection (13). This approach may be particularly relevant for populations at increased risk of poor vaccine responsiveness, such as older adults and individuals with metabolic or inflammatory comorbidities, where immune senescence and chronic low-grade inflammation may co-occur with altered microbial ecology (15). The stability of predictive performance across age and sex strata in the present analysis suggests that microbial predictors may provide incremental value beyond demographic factors, supporting their potential utility in precision vaccination frameworks (14,16). However, before clinical translation, predictive models must undergo external validation, and interventional trials are required to determine whether microbiota modulation can causally enhance influenza vaccine immunogenicity.

Several limitations should be considered when interpreting these findings. First, although the design was prospective with respect to immune outcome assessment, microbiota was sampled at a single pre-vaccination timepoint; therefore, temporal fluctuations in microbial composition and their relationship to immune kinetics could not be evaluated (21). Second, reliance on 16S rRNA sequencing limits taxonomic resolution to the genus level and does not provide direct functional characterization of microbial metabolic pathways, making mechanistic inferences indirect. Third, mucosal microbiome studies are vulnerable to contamination and batch effects due to low biomass sampling; while negative controls and standardized workflows were incorporated, residual technical variation cannot be fully excluded (6,21). Fourth, although models were adjusted for age and sex, residual confounding by unmeasured or imperfectly measured variables such as diet, socioeconomic factors, and environmental exposures may have influenced microbial profiles and immune responsiveness. Fifth, this study was conducted in a single geographic setting, and generalizability may be limited due to regional differences in microbial ecology shaped by dietary patterns, environmental exposures, and healthcare access. Finally, responder classification was anchored primarily on seroconversion thresholds; while cellular markers were measured and aligned with humoral status, future work should consider composite immune responder phenotypes as primary endpoints to better reflect multidimensional protection (3,12).

Future research should prioritize longitudinal sampling to evaluate mucosal microbial dynamics across baseline, early post-vaccination innate phases, and later adaptive response phases, enabling a clearer understanding of temporality and mechanistic pathways (6,10). Integration of metagenomic sequencing with metabolomic profiling would provide functional resolution, enabling identification of microbial pathways and metabolites that may directly shape immune priming and adaptive differentiation, while host immunogenetic or transcriptomic profiling could clarify how microbial signals interact with baseline immune states to predict vaccine response (7,9,16). In addition, randomized interventional studies targeting microbial modulation among predicted low responders would provide definitive evidence regarding causality and therapeutic feasibility, while external validation across diverse populations and vaccine platforms would determine the portability and clinical utility of microbial predictors. Collectively, such work would advance the development of microbiota-informed precision vaccination strategies that move beyond uniform immunization approaches and maximize protection at both individual and population levels (14,17).

In summary, the study demonstrates that pre-vaccination upper respiratory mucosal microbiota diversity and specific community-level signatures are strongly associated with influenza vaccine immunogenicity, including both humoral seroconversion and cellular immune activation. The identification of genera such as Faecalibacterium and Bacteroides as favorable predictors and Streptococcus and Prevotella as negative indicators provides a basis for future biomarker validation and mechanistic investigation. These findings support the broader hypothesis that mucosal microbial ecology is an actionable component of baseline immune readiness and may be leveraged to improve the effectiveness of influenza vaccination through predictive and microbiota-directed strategies (6,13,16).

CONCLUSION

This study demonstrated that pre-vaccination upper respiratory mucosal microbiota composition was significantly associated with variability in immune responsiveness to the seasonal influenza vaccine, with higher microbial diversity and enrichment of taxa such as Faecalibacterium and Bacteroides predicting stronger humoral seroconversion and more robust cellular immune activation, whereas increased Streptococcus and Prevotella abundance was linked to suboptimal responses, supporting the feasibility of using mucosal microbial signatures as predictive biomarkers and providing a foundation for microbiota-informed precision vaccination strategies aimed at improving influenza vaccine effectiveness.

DECLARATIONS

Ethical Approval
Ethical approval was not required because this study was a narrative review of published literature and did not involve human/individual identifiable data.

Informed Consent
NA

Conflict of Interest
The authors declare no conflict of interest.

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Authors' Contributions
Concept: NS; Design: NS, SS; Data Collection: SJ, ZN, ZT, HA; Analysis: NS, SS; Drafting: NS, SS, SJ, ZN, ZT, HA

Data Availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments
Not applicable.

Study Registration
Not applicable.

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