

Integrative Multiomics Analysis of Gut Microbial Dysbiosis Associated with Immune Dysfunction in Human Populations

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Abstract

Gut microbial dysbiosis is an essential factor dependent on immune regulation; however, complex interactions among microbial communities, metabolites and immune responses remain inefficiently characterized. This research develops an integrative multi-omics analysis of gut microbial dysbiosis associated with immune dysfunction in human populations. Prospective cohort research involving 132 participants, aged 18–65, comprising 66 immune dysfunction patients and 66 healthy controls, was conducted. Clinical characteristics, gut microbial profiles, transcriptomic and immune-related biomarkers included in this research were systematically analyzed. Microbial diversity and community composition were characterized using alpha- and beta-diversity analyses, while differential abundance analysis identified dysbiosis-associated microbial taxa and functional alterations. Differential metabolites, immune-related genes, proteins, cytokines, and chemokines were identified across the population groups using statistical comparisons. Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (DIABLO), Multi-omics factor analysis (MOFA) and correlation-based integration were employed to characterize relationships among microbial, metabolic, transcriptomic, and immune features. Statistical association analyses identified significant microbe–metabolite–gene–immune relationships, and network analysis highlights hub features. Functional and pathway enrichment analyses characterized biological mechanisms involving toll-like receptor, oxidative-stress, bile-acid, cytokine, T-cell receptor, tryptophan-related, short-chain-fatty-acid and Janus Kinase–Signal Transducer and Activator of Transcription (JAK–STAT) pathways. Multivariate statistical analyses, Support Vector Machine (SVM) modeling, Extreme Gradient Boosting (XGBoost) based imputation, and Partial Least Squares Discriminant Analysis (PLS-DA), were further used to evaluate group distinctions. MOFA identified microbial-metabolic-immune dysregulation provides 28.4% variance, with Metabolite A exhibited Variable Importance in Projection (VIP) of 0.42. The integrated analysis provides a comprehensive characterization of dysbiosis-associated microbial, metabolic, genetic, proteomic, and immune signatures, as well as potential biomarkers underlying immune dysfunction across human populations.

Keywords: *Gut Microbiome, Multi-omics Analysis, Immune Dysfunction, Microbial Dysbiosis, Gut–Immune Interactions*

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Introduction

The human gut contains a complicated community that plays an essential role, metabolism, intestinal barrier maintenance, and immune regulation [1]. These microorganisms interact with host cells and help in regulating physiological homeostasis and proper functioning of the gastrointestinal system [2]. Gut microbiota is responsible for utilizing nutrients and producing metabolites while protecting against pathogens, thus aiding the well-being of humans [3]. Gut microbial communities provide many factors like diet, age, and environment [4]. Changes in microbial population can interfere with the microbial balance and cause dysbiosis of the gut microbes [5]. Dysbiosis could affect intestinal permeability, microbial metabolism, inflammatory response, and microbial-immune cell interactions [6]. The gut microbes have an intimate relationship with the immune system due to the effect of microbial components and products on immune-cell functions [7]. Gut microorganisms provides metabolites like short-chain fatty acids that can participate in signaling and immune regulation [8].

Changes in these microbial metabolites affect inflammatory responses, cellular communication, intestinal barrier function, and immune-system regulation [9]. Altered microbes are linked to immune-related gene expression and protein activity that regulates inflammation and immunity [10]. Despite the information provided by microbiome profiling on bacterial populations, an individual omics-based approach among microorganisms and host immune responses [11]. Combining information on microbiome, metabolomics, transcriptomics, proteomics, and immunology can provide extended knowledge on the connections among microbes and molecular alterations [12]. Statistical correlation, multivariate analyses, network analysis, and pathway enrichment analysis can help to detect correlated microbes, metabolites, genetics, proteins, and immune components [13]. But the analysis of each biological layer separately might hinder the identification of molecular networks connected to immune Dysfunction in the human population [14]. Hence, it combines the

multi-omics data to define gut microbial dysbiosis and identify corresponding microbes, metabolites, genetics, proteins, and immune signatures in the human population [15].

The recent literature was reviewed to identify existing findings, methodological limitations, and research gaps related to microbiome, metabolomic, molecular, and immune alterations.

The gut microbiota, lipid metabolism, and inflammation in gestational hypertension were established [16]. Using ribosomal deoxyribonucleic acid (16S-rDNA) sequencing, lipid metabolomics, and correlation analysis, the results showed reduced microbial richness, increased inflammatory cytokines, and altered lipid metabolites, with specific associations between Oxalobacteraceae and TG (54:5/FA22:5). However, it was limited by its disease-specific population and reliance on correlation analysis, which constrained generalization. The multi-omics gene signature for immune regulation and predicting treatment efficacy in patients with inflammatory bowel disease (IBD) was established [17]. A four-gene signature was defined using GEO datasets, immune infiltration analysis, and single-cell Ribonucleic Acid (RNA) sequencing. This signature showed an Area under the Curve (AUC) = 0.86–0.97, whereas the nomogram provided an AUC = 0.952 and discriminated between immune-related molecular subtypes and treatment response. Nevertheless, this analysis used primarily public datasets and computational validation, and the results were specific only to inflammatory bowel disease.

The esophageal microbiotadysbiosis and immune-related molecular changes in eosinophilic esophagitis (EoE) were developed [18]. Multi-omics integration of transcriptomic, meta transcriptomic, and single-cell data using Multi-Omics Factor Analysis (MOFA) identified disease-specific molecular signatures and microbiotadysbiosis as a predominant feature of EOE, with specific microbial groups potentially contributing to antigen-mediated immune responses. However, it was based largely on publicly available datasets, provide batch and population differences and limit direct generalization to broader human populations. The cesarean delivery, gut microbiota and immune responses in neonates were investigated. Metagenomic sequencing, untargeted metabolomics, RNA sequencing (RNA-Seq), flow cytometry (FC) and multi-omics integration were used in 38 neonates [19]. The results showed altered gut microbiota and metabolites, a T helper 2 (Th2)-biased immune response, and reduced Bacteroides abundance associated with dysregulation. However, the small sample size limited generalizability and causal interpretation.

Gut microbiota dysbiosis and impaired immune function are associated with systemic inflammation in Patients in serious condition colonized or infected

multidrug-resistant organisms (MDROs) [20]. The results show low microbial diversity, lower levels of beneficial bacteria, high levels of pathobionts, a changed cytokine profile, and disturbed carbohydrate and lipid metabolism in MDRO-infected/colonized cases, with Enterococcus and Klebsiella being the potential microbial markers for discrimination. The Functional predictions limited direct confirmation of microbial metabolic functions. The gut microbiome composition and Microbial Dysbiosis Index (MDI) with disease activity in IBD were developed [21]. The methods of metabolic dysbiosis profiling and mucosal immune activation test were conducted on 35 patients. As a result, it was found that an increased amount of Gemellaceae and Veillonella was observed in patients with elevated MDI; the small sample size and IBD patients' specific group restrict generalization.

The Intestinal Microbiome on Radiotherapy-Induced Antitumor Immune Responses in Patients with Hepatocellular Carcinoma (HCC) was established [22]. The intestinal 16S RNA sequencing, fecal microbiome transplantation (FMT) and transcriptional sequencing were utilized to analyze the interaction between microbiome and immunity. The microbiotadysbiosis in the gut is linked with poor response to RT, impaired antigen presentation, and poor effector function of T-cells. This research was limited by a small sample size of only 50 HCC patients. The cytotoxic T-cells (CD8⁺) in the tumor microenvironment (TME) were established [23]. Monocarboxylate transporter 11 (MCT11) expression was examined, and conditional MCT11 deletion and antibody-based targeting were tested in T cells and tumor-bearing mice. The results showed that MCT11 promoted lactate in exhausted T cells, while its deletion or inhibition improved T-cell effector function and reduced tumor growth. However, this limits their direct applicability to human immune dysfunction and gut microbiota-related mechanisms.

The indole metabolites from gut microbiota control of fat metabolism and inflammation in steatotic liver disease due to metabolic dysfunction were developed [24]. The fecalindole-3-propionic acid (IPA) and indole-3-acetic acid (IAA) levels in patients were evaluated, and their effects were assessed in a Western diet (WD)-induced model. Low concentrations of IPA and IAA in subjects with hepatic steatosis, and indoles decreased hepatic steatosis. However, the sample size is limited. The gut microbiome, metabolism, immunity, and brain molecular profiles associated with gut-brain axis (GBA) were established [25]. Bayesian ranking analysis was performed on 10 microbiome datasets, metabolomics, cytokines, and brain gene expression. ASD-associated amino acid and lipid profiles were found to be related to *Prevotella* and *Bacteroides*, in addition to pro-inflammatory cytokine profiles.

Nevertheless, the cross-sectional characteristics limited the causal inferences and generalizability. Gut dysbiosis, metabolic dysregulation, and aortic inflammation in patients with abdominal aortic aneurysm (AAA) were established. Utilizing techniques such as microbiome profiling, metabolomics, transcriptomics and DIABLO, differences in microbial diversity, bile acid, amino acid, and inflammatory pathways have been identified. Nevertheless, the use of a mouse-specific AAA model limited the generalizability [26]. The Diabetic kidney disease (DKD) associated microbiota, metabolites, and biomarkers have been established. By using Mendelian randomization (MR) and microbiome and metabolomics profiling, tongue image features (TIFs), dysbiosis, fatty acids, and microbiota-metabolite interactions have been identified. Nevertheless, the sample size is limited [27].

The Biomarkers related to Ulcerative Colitis (UC) were characterized. 16S ribosomal RNA (rRNA) and gas chromatography-mass spectrometry solid-phase microextraction(GC-MS/SPME), and network analysis techniques were employed to characterize microbial and metabolic signatures as well as interactions. The result of the model showed improving metabolites biomarkers. However, the study was limited by fecal sampling [28]. Existing studies have mainly examined gut microbial composition, metabolites, or immune responses separately. Limited work has integrated microbiome, metabolomics, transcriptomics, proteomics, and immune-related profiles to characterize the coordinated molecular alterations associated with immune dysfunction. In addition, the relationships among microbial dysbiosis, metabolic changes, and immune signaling pathways remain insufficiently characterized using a unified multi-omics framework.

1.1 Research Objective

The main objective is to characterize gut microbiota, metabolomic, transcriptomic, proteomic, and immune profiles in aged 18 years or older with immune dysfunction and healthy controls. The multi-omics profiles between the two participant groups. Cross-omics associations among microbial, metabolic, molecular, and immune features were analyzed. The objective was to identify integrated biological patterns associated with immune dysfunction. These findings were used to improve the analysis of the interconnected microbial, metabolic, molecular, and immune alterations underlying immune dysfunction.

1.2 Research Organization

Section 1 provides the background. Section 2 provides materials and methods. Section 3 describes results, and Section 4 presents findings. Section 5 summarizes conclusions, limitations, and future scope.

1. Methods

The proposed workflow integrates microbial profiling, immune-cell analysis, metabolomics, transcriptomics, and proteomics to characterize gut microbial dysbiosis associated with immune dysfunction. Figure 1 outlines an integrated multi-omics analysis workflow for 132 participants, using advanced machine learning (XGBoost) and integration methods (MOFA, DIABLO, SVM, PLS-DA) to link gut microbial dysbiosis with immune dysfunction.

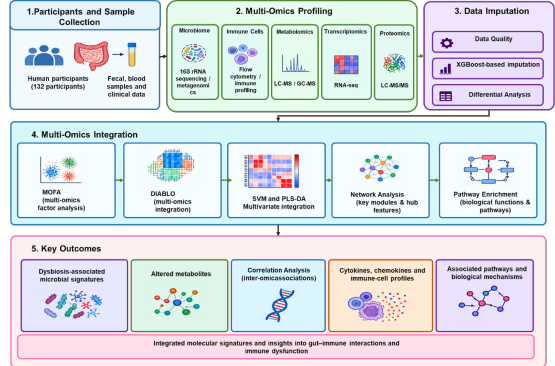


Figure 1. Overall Proposed Architecture

2.1 Participants Information

A prospective observational cohort research was conducted to provide clinical characteristics and outcomes of the enrolled participants. Prior to enrollment, every participant provided written informed consent. The relevant ethical principles were followed while conducting the research procedures. All participants provided their informed consent prior to enrollment in research. A total of 132 participants participated in this study; these included 66 immune dysfunction participants had immune dysfunction as well as 66 healthy subjects. The participants age ranged between 18 and 65 years, while the enrollment of participants in the study was conducted from December 2024 to August 2025. Table 1 summarizes demographic profile of 132 participants, including age range and sex distribution. The immune dysfunction and healthy control groups consisted of 66 participants each. Microbial data include microbial taxa, relative abundance, microbial diversity, and gut microbiome composition; metabolomic data include metabolites such as glucose, amino acids, lipids, and short-chain fatty acids and their concentrations; transcriptomic data include RNA transcripts, gene-expression levels, and differentially expressed genes; proteomic data include protein types, protein abundance, protein-expression levels, and differentially expressed proteins; and immune data include immune-cell populations, cytokines, chemokines, immunoglobulins, inflammatory markers, and other immune-related biomarkers.

Table 1. Demographic Profile of the participants

Characteristics	Immune Dysfunction (n = 66)	Healthy Controls (n = 66)	Total (n = 132)
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Age range (years)	18–65	18–65	18–65
Male, n	33	30	63
Female, n	33	36	69
Total participants, n	66	66	132

2.1.1 Criteria for Inclusion and Exclusion

The criteria for inclusion and exclusion were established to provide appropriate participant selection and to minimize factors that could influence gut microbiota and immune-related measurements. The criteria were applied to both groups of participants equally before samples and data collection.

Criteria for Inclusion

- Adults aged 18 years or older who are voluntary informed consent
- For the immune dysfunction group, participants had documented evidence of altered immune-related status based on available clinical or immune-related biomarker information.
- Availability of appropriate biological samples for gut microbiota and immune-related analyses
- Participants had available biological or molecular data relevant to the study, including gut microbial profiles and metabolomic, transcriptomic, proteomic, or immune-related biomarker measurements.
- Availability of sufficient clinical, demographic, and molecular information for integration with metabolomic, transcriptomic, proteomic, and immune-related datasets.

Exclusion Criteria

- Participants with severe or uncontrolled systemic diseases that could substantially affect immune function or gut microbiota.
- Participants with active gastrointestinal disorders or gastrointestinal infections that could independently alter gut microbial composition.
- Participants who had recently used antibiotics, probiotics, prebiotics, corticosteroids, immunosuppressive agents, or immunomodulatory medications that could influence microbiome or immune-related measurements
- Participants who had undergone major dietary or medical interventions immediately before biological sample collection that could substantially modify gut microbiota or metabolomic profiles.
- Pregnant or breastfeeding women, because pregnancy and lactation may produce substantial physiological and immune-related changes that could influence the measured molecular profile.

Each of the participants completed the structured questionnaire to obtain demographic information and medical history. The items on the questionnaire covered immune-related symptoms, medical history, medications, and any other information that would be considered important for health. The Immune Status Questionnaire with 55 items (<https://eternalhealth.org/immune-system-assessment-questionnaire/>) is applied for assessment of the participants' immune-related status. The information from the questionnaire, along with clinical and laboratory data, is used for the classification of the participants in the study groups.

2.2 16S rRNA Sequencing and Metagenomics

Fecal samples gathered from all participants, including 66 (33 Males and 33 Females) individuals with immune dysfunction and 66 (30 Males and 36 Females) healthy controls, using sterile sample collection tubes providing RNA later and transported to the laboratory under 24-hour controlled conditions. Upon receipt, the samples are stored at -70°C until to maintain integrity of Microbial DNA and minimize changes in microbial composition.

DNA isolation and Polymerase Chain Reaction(PCR) amplification were conducted. In particular, genomic DNA was isolated 300 mg of feces samples through the use of a DNA Isolation Kit. The isolated quality of genomic DNA was provided by 1.2% agarose gel electrophoresis. The 16S rRNA gene amplicon for gut microbial dysbiosis and its association with immune dysfunction characterization, the primers amplified the 16S rRNA genes' V3–V4 hypervariable regions. The amplicons were sterilized by Agencourt AMPure XP magnetic beads and pooled to equal concentration. The purified amplicons with sequencing adapters, pooled at equal concentrations, and subjected to 2×400 bp paired-end sequencing.

The PCR reactions generated in a volume of 35 μL contains 13.5 μL of $2 \times$ KAPA HiFiHotStartReadyMix, 6 μL of each primer (2 μM), and 2.0 μL (~ 10 ng) DNA.

Metagenomic sequencing are used to characterize gut microbial composition and functional potential associated with immune dysfunction. The DNA concentration and quality were measured by TBS-380 and Nanodrop2000. The DNA isolation were performed based on agarose gel electrophoresis analysis.

DNA isolated were divided into fragments of 400 bp average size using a Covaris M220 machine for paired-end library preparation. Adapter molecules containing all possible primer sequences to be hybridized were joined onto the blunt ends of the DNA molecules.

Metagenomic sequencing data generated through this process were analyzed for research of microbial taxonomy and functionality as well as microbial variations related to immune dysfunction. The variations found in the microbes among the groups with immune dysfunction and healthy ones were then combined with metabolomics, transcriptomics, proteomics, and immune-related datasets to explore cross-omics interactions.

2.2.1 Analysis of Alpha and Beta Diversity

The alpha-diversity analyses (Shannon and Simpson indices) and beta-diversity analysis based on Bray–Curtis dissimilarity were calculated to analyses distinctions in microbial diversity and composition among samples, respectively. The generated microbiota profiles were then used for characterization of alpha- and beta-diversity, detection of differentially abundant microbial taxa and elucidation of gut microbial dysbiosis in the immune dysfunction versus healthy group. The detected microbial taxa were further integrated with the metabolomics, transcriptomics, proteomics, and immune-related datasets for investigations of cross-omics associations regarding the underlying mechanisms for the gut microbial dysbiosis and immune dysfunction. Alpha diversity showed reduced microbial richness and evenness, while Bray–Curtis PCoA demonstrated group separation, confirmed by significant PERMANOVA results.

1.3 Mass Cytometric Profiling of Immune Cells from Blood

Blood from humans was gathered in K2-EDTA tubes and centrifuged at 1000 rpm for 7 min. RBC Lysis was used twice to lyse blood cells for 7 minutes at room temperature. Next, cells were washed two times in staining buffer (D-PBS), filtered through 70µm strainer. The antibodies are provided as staining of immunophenotype properties using manufacturer's recommendations. Viability staining was performed via cisplatin staining at concentration of 0.5 µM. The cells were stained with blocking antibody to block Fc receptors 7 minutes at room temperature before the surface staining.

The Intracellular Staining Steps before intracellular staining included washing of the cells with staining buffer followed by fixation in 1.6% paraformaldehyde for 7 minutes at room temperature, and then washing of the cells in Perm-S buffer. Thereafter, intracellular antibody cocktail was used, followed by staining of the cells for 40 minutes at 3°C. After that, the cells were again washed in Perm-S buffer and were incubated at 2–8°C for entire overnight after being rinsed again in 1 mL of Ir-Interchelator.

Additionally, a total of 3×10^5 cells per sample were obtained using the Helios Mass Cytometer at rate of 300–400 events/second. These acquired datasets were analyzed using the analysis of software known as Cytobank (<https://www.cytobank.org/>). In this

way, mass cytometry data were employed to study the immune cell populations as well as the immune-associated cellular changes among the immune dysfunction and control groups. Subsequently, these identified immune-related markers were analysed.

2.4 MxP® Quant 500 Kit-Based Targeted Metabolite Profiling

Metabolomic profiling was conducted on plasma samples gathered from participant groups to characterize metabolic differences and their associations with clinical data. The plasma samples are processed and analyzed according to the protocol. Plasma samples from the 132 participants, and each test plate on four quality-control (QC) pool duplicates produced from ten samples of human plasma. The metabolites are measured using a focused metabolomics method coupled for ultra-performance liquid chromatography. This method allows for the quantitative and semi-quantitative measurement of up to 730 metabolites produced from the microbiome and endogenous.

Metabolites with $P < 0.05$ were provided statistically significant significantly altered metabolites were subsequently used for correlation and multi-omics integration analyses. The Quant 500 kit was designed and identifies following the bioanalytical method guidelines laid down of the FDA and EMA for application of the kit in analyzing human plasma samples. The kit is comprised of various internal standards. The combination of three distinction concentrations of quality controls, were applied to facilitate metabolite quantitation and reproducibility of the analysis. The derived metabolite profiles were used to identify changes in the metabolic pathways linked with gut dysbiosis and immune malfunctioning and further analyzed in the context of microbiome, transcriptomics, proteomics, and immune profiling data.

2.5 The Integrative Multi-Omics Analysis

DIABLO analysis was used on individuals with measurements available for immune-cell profiles, plasma cytokines, metabolites, and gut microbial characteristics to find significantly correlated multi-omics features linked to immunological dysfunction. Immune-cell and metabolite data were log-transformed before integration analysis with DIABLO, and The centered log-ratio (CLR) transformation was applied to microbial abundance data utilizing the R microbiome package's `transform` function to the compositional nature of microbial abundance data.

DIABLO was used to analyse a correlated multi-omics signature which simultaneously maximized covariance among the different omics datasets while discriminating between the immune dysfunction and healthy control groups. The method enabled the identification of complementary microbial, metabolic, and immune-related features that contributed to the distinction between the two participant groups. The final DIABLO model was

fitted using the chosen features after the ideal parameters were established, and its capacity to distinguish immunological dysfunction from healthy controls was assessed. The mixOmics R package's functions were used to show and explain the final model's results.

In this research, Multi-Omics Factor Analysis (MOFA) was applied to determine the major sources of variation within the microbe, metabolite, transcript, protein, and immune features. The appropriate normalization and grouping of the omics dataset as distinct data views were done for analysis. The MOFA method decomposes the composite omics data into latent factors that define correlated molecular patterns in omics layers. The factors with high variance explained were investigated to determine the features causing immune dysfunction and dysbiosis of gut microbiota. The relationship among identified factors and groups of participants was further determined to reveal the multi-omics patterns associated with immune dysfunction. A correlation network of multi-omics was generated to investigate the relationship between the altered microbes, metabolites, genes, proteins, cytokines, chemokines, and immune cell features. Significant pairwise associations obtained from the correlation analysis were utilized to develop the network where edges represent the significant association between the molecular features while nodes represent the unique microbial or host molecular features. Topology measurements of the network were carried out to identify the highly connected features that could be considered potential hubs in the dysbiosis associated molecular network analysis.

2.6.1 Microbe–Metabolite–Gene–Immune Association Analysis

Association between omics was conducted to establish associations among microbial features, metabolites, immunity-related genes, proteins, cytokines, chemokines, and immune cell populations. Significant associations among microbial and molecular features that were obtained from the individual omics data were combined by means of Spearman correlation and regularized canonical correlation analysis (rCCA). Significant associations were established based on a cutoff correlation value and a p-value less than 0.05. These associations were used to determine coordinated associations among microbes, metabolites, genes, and immunity.

2.6.2 Functional Characterization and Pathway Enrichment Analysis

The biological processes and molecular pathways connected to observed dysbiosis-associated characteristics, functional and pathway enrichment analyzes were conducted. Differentially expressed immune-related genes and other significant molecular features were mapped to relevant biological pathways. The pathways linked to inflammatory reactions were found using

enrichment analysis., cytokine signaling, Toll-like receptor signaling, NF- κ B signaling, JAK–STAT signaling, T-cell receptor signaling, oxidative stress, short-chain fatty acid metabolism and tryptophan metabolism. Pathways that were significantly enriched were found using statistical significance and interpreted in relation to gut microbial dysbiosis and immune dysfunction.

2.5 Statistical Analysis

Metabolites with $\geq 30\%$ missing values were eliminated prior to further analysis, and metabolites measured in $\geq 50\%$ of samples were provided. Then, XGBoost-based imputation was performed to estimate missing values using the available molecular features and sample characteristics. Support Vector Machine (SVM) analysis, adjusting for body mass index (BMI), smoking status, age and sex where applicable, was employed to determine features that discriminated between immune dysfunction and healthy control samples. Significance level also considered to be $p < 0.05$. The Shannon/Simpson diversity index and PERMANOVA were applied in the diversity analysis, and the Spearman correlation and rCCA were used in the analysis of omics data correlations. The imputations were performed using XGBoost, while SVM and PLS-DA were utilized in discriminations. In these analyzes, correlations with $p < 0.05$ were regarded as statistically significant. The data preprocessing were performed using R 4.1.0 and Python 3.9.1. XGBoost-based imputation was used to estimate the missing values. The process involved identifying missing entries, training the XGBoost model using the available observed values, and predicting the corresponding missing values based on the learned relationships among the available features. The analysis of the differential immune molecules was performed to identify the genes, proteins, cytokines, and chemokine molecules that have different expression levels in the immune disorder and healthy controls. Group comparisons were done to identify any significant changes in these molecules based on a p-value of less than 0.05. After analyzing differential immune molecules, they were correlated and integrated into the gut microbes and other molecular profiles.

The PLS-DA approach for multivariate data analysis using supervised latent-variable discrimination. In particular, significant features were provide as variables, and data were log-transformed. The PLS-DA methodology was analyzed using the statistical software R version 4.1.0 (<http://cran.r-project.org>). The mixOmics R package (<http://mixomics.org>) to analyze rCCA. The data were appropriately scaled and transposed, and the rCCA parameters were repeatedly adjusted until a higher cross-validation score was achieved. rCCA is a method that searches for correlation relationships through the maximization of the correlation between two datasets using regularization to reduce dimensions.

In rCCA, λ_1 and λ_2 are the parameter values for regularize the correlation matrices to obtain solutions to CCA. The optimal λ values were obtained via two-dimensional grid. It includes Regularized CCA includes Normalization of the regularization covariance matrices of W and Z :

$$Cov(W) + \lambda_1 J$$

(1)

$$Cov(Z) + \lambda_2 J$$

(2)

In Equations (1) and (2), $Cov(W)$ and $Cov(Z)$ represent the covariance matrices of the two datasets, while λ_1 and λ_2 are the regularization parameters controlling the degree of regularization applied to each covariance matrix. J denotes the identity matrix. The regularization terms $\lambda_1 J$ and $\lambda_2 J$ improve matrix stability and help reduce the effects of high dimensionality and multicollinearity. The mixOmics package's rcc function with shrinkage was used to conduct the analysis. The analysis was also represented through use of the cim function in mixOmics package. This type of analysis employed for analyze the relationship among gut microbe characteristics, metabolomics, transcriptomics and proteomics data, as well as immune biomarkers of immune dysregulation.

2. Result

The results present the distinctions in gut microbial diversity and composition among immune dysfunction and healthy groups, followed by alterations in metabolites and immune-related features. Multi-omics integration, association, network, and pathway analyses are then presented to identify coordinated molecular patterns associated with gut microbial dysbiosis and immune dysfunction.

The Shannon and Simpson diversity in immune dysfunction category compared to healthy group, implying low diversity of gut microflora, is presented in Figure 2. The PCoA analysis using the Bray-Curtis method reveals well-separated groups, where samples of the immune dysfunction group form a separate cluster from those of the healthy group. PERMANOVA results show a p-value < 0.001. In Figure 2(a), alpha diversity assessment using the values of both Shannon and Simpson indices, indicates a statistically substantial loss of the richness and microbes in the immune dysfunction group to healthy controls (p < 0.01). Figure 2(b) Beta diversity assessment, visualized through PCoA, clearly differentiates the composition of microbes in the two groups; this observation is statistically substantiated by PERMANOVA analysis (p < 0.001).

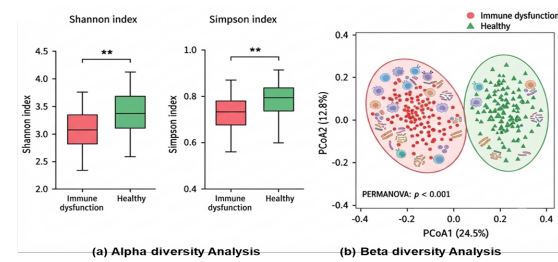


Figure 2. Microbial diversity analysis comparing immune dysfunction and healthy groups. (a) Alpha diversity analysis evaluated using the Simpson and Shannon indices (p < 0.01). (b) Beta diversity analysis illustrated via a Principal Coordinates Analysis (PCoA) plot based on microbial composition, with statistical significance determined by PERMANOVA (p < 0.001).

In the altered microbial taxa between immune dysfunction and healthy groups, red defines increased and blue defines decreased abundance in Figure 3. *Faecalibacterium*, *Bifidobacterium*, and *Lactobacillus* show decreased abundance, whereas *Escherichia*, *Klebsiella*, and *Enterococcus* show increased abundance. The right panel further illustrates distinct abundance patterns across groups, supporting differences in microbial composition associated with immune dysfunction. It provides hierarchical clusters of the differential expression of individual samples, demonstrating clustering in the immune dysfunction group compared to the healthy one.

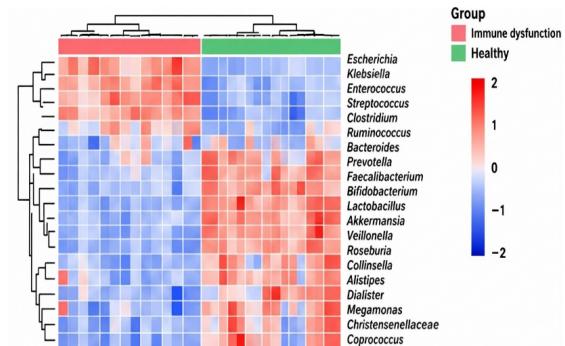


Figure 3. Hierarchical Cluster of Gut Microbiome Abundance between Immune Dysfunction and Healthy Groups

The metabolic changes and differences in immune functions between dysfunctional and healthy groups is depicted in Figure 4. There are some reduced metabolites such as tryptophan, citrate, and butyrate, while some other metabolites like lactate, glutamate, and acetate are higher. There are different cytokine and chemokine profiles between the two groups. The composition of immune cells is also different in both groups, including changes in T-cell, B-cell, NK-cell, monocytes, macrophages, dendritic cells, and neutrophils. Figure (4a) displayed differential metabolite abundance between immune dysfunction and control samples. Figure (4b) indicated differential metabolite 20 expression patterns.

Figure (4c) presented cytokines and chemokines (IL-6, TNF) and T cell subtypes (CD4⁺ T cells, CD8⁺ T cells). Figure (4d) indicated differential expression patterns of cytokines and chemokines. Figure (4e) identified percent relative abundance of major immune cell composition. Figure (4f) displayed percent relative expression of key host immune genes and proteins.

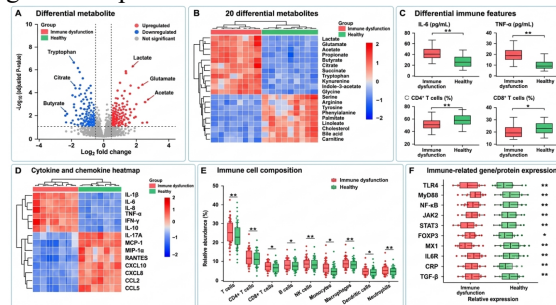


Figure 4. Metabolic and immune alterations. (a) the differentially abundant metabolites comparing immune dysfunction and healthy groups. (b) Expression patterns of 20 differential metabolites across samples. (c) Displaying key immune features. (d) Differential cytokine and chemokine expression profiles. (e) Relative abundance percentages across major immune cell populations. (f) Relative expression levels of key host immune-related genes and proteins ($p < 0.05$, $p < 0.01$).

Multi-Omics Factor Analysis (MOFA) demonstrates that Factor 1 is responsible for the highest percentage of variance (28.4%) and enables differentiation between the healthy controls and patients with immune dysfunction in Figure 5. The number 28.4% is the percentage of the variance explained by MOFA Microbial-metabolic-immune dysregulation. This calculation of the contribution of Microbial-metabolic-immune dysregulation to the total variance based on the variance explained output of the MOFA model that had been fitted. Therefore, the total variation was explained by Microbial-metabolic-immune dysregulation to the tune of 28.4%. (5a) Percent variance explained by the top five MOFA factors depicts that the first factor explains the highest percent of variance, which is 28.4%. (5b) Factor contribution from various omics views depicts the contribution of each omics dataset to latent factors. (5c) Individual sample distribution of samples along Factor 1 and Factor 2 clearly demonstrates the separation between healthy control samples and immune dysfunction samples. (5d) Important factors contributing to Factor 1 depict factors contributing positively or negatively to the variance. (5e) Multi-omics factor association matrix depicts the strength of association of each MOFA factor and individual omics view along with clinical status. From the manuscript, the first factor 1 identified by the MOFA could be characterized as microbial-metabolic-immune dysregulation, the factor 2 as metabolic-immune interaction, the factor 3 as

transcriptomic-metabolic variability, the factor 4 as proteomic-immune variability, and the fifth as multi-omics variability. Of these, microbial-metabolic-immune dysregulation was the major factor, which accounted for 28.4% of the total variation, and was related to features of microbial, metabolic, and immune function impairment. The MOFA analysis provide five latent factors, where Factor 1 accounted for the greatest variance (28.4%) followed by Factors 2 to 5 which explained 16.2%, 9.5%, 6.1%, and 4.3% variance, respectively. Factor 1 was also found to have a high correlation with immune dysfunction (0.92) and was composed of mainly microbe, metabolism, and immune-related features such as Faecalibacterium, butyrate, Treg cells, IL-10, TLR4, TNF- α , Enterococcus, LPS, and IL-6. In summary, it can be said that Factor 1 is the dominant factor related to immune dysfunction.

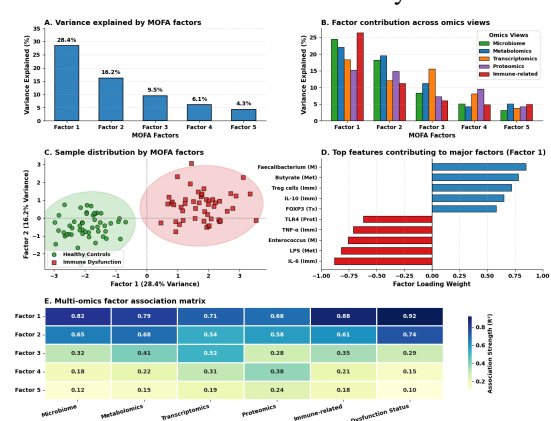


Figure 5. Multi-omics factor analysis (MOFA) of immune dysfunction: (a) displays the percentage of variance explained by each MOFA factor; (b) illustrates the factor contribution across different omics views; (c) shows the sample distribution separating healthy controls and immune dysfunction; (d) highlights the top features contributing to microbial-metabolic-immune dysregulation, and (e) presents the multi-omics factor association matrix displaying association strength across omics views and immune dysfunction status.

The interrelationships between microbial, metabolite, host gene/protein, and cytokine/immune cell properties are shown in Figure 6. The microbial nodes consist of pathogenic/pathobiont and probiotic or regulatory microorganisms, while metabolite nodes consist of SCFAs, butyrate, propionate, and bile acids. Host gene/protein nodes consist of TLR4 and NF- κ B signaling proteins, whereas cytokine/immune cell nodes consist of mediators like TNF- α and immune cells. Connected elements for network analysis were found to be the microbiome, metabolites, genes/proteins, cytokines, and immune cells. Connected elements such as butyrate, indoles, IL-6, and genes associated with NF- κ B pathway.

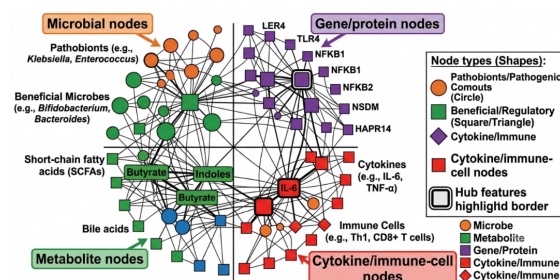


Figure 6. Integrated microbe-metabolite-host-immune network

The regulation of immune functions through the gut microorganisms' and their metabolites' actions is elucidated in Figure 7. Figure (7a) shows the pathways that are enriched in inflammatory and metabolic terms. Figure (7b) describes the inflammatory pathways of TLR/NF-κB through microbe action. However, in Figure (7c), the metabolites produced by microbes from the food ingredients are anti-inflammatory metabolites such as SCFAs and indoles. Figure (7d) summarizes all the relationships of microbe-metabolite-immune associations. The results found that inflammation and immune signaling were highly correlated with gut dysbiosis. The role of TLR, NF-κB, and JAK-STAT signaling was significant, whereas alterations in SCFA, bile acid, and tryptophan metabolism were related to gut microbe and immune changes. Another important network, the microbe-metabolite-immune network, discovered the relationship between *Faecalibacterium*, *Bacteroides*, *Ruminococcus*, *Enterobacteriaceae*, butyrate, bile acids, indole metabolites, Treg/Th17, IL-10, and TNF-α.

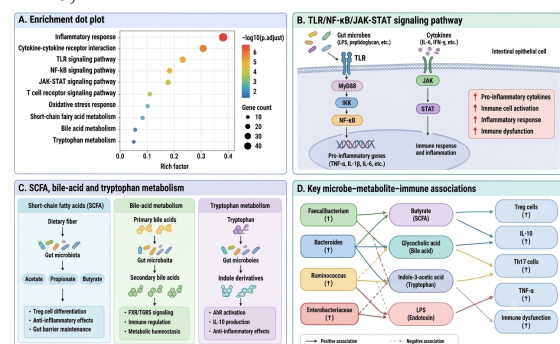


Figure 7. Multi-omics pathway enrichment and microbe-metabolite-immune associations: (a) pathway enrichment, (b) TLR/NF-κB/JAK-STAT signaling, (c) SCFA, bile-acid and tryptophan metabolism, and (d) key microbe-metabolite-immune interactions.

Multivariate analysis of multi-omics DIABLO provides unique segregation of Healthy Controls from Immune Dysfunction based on microbiome, metabolomics, transcriptomics, and proteomics data in Figure 8. The features chosen by the DIABLO model were those which contributed the most towards the discrimination of multi-omics data. The chosen features were *Enterococcus*, *Klebsiella*, butyrate, bile acids, TLR4, TNF, IL-6, and NF-κB-

related features. The contribution of these features was seen to be in a coordinated manner with respect to all the omics parts and helped in differentiating patients with immune malfunctioning from the controls. The microbial and metabolomic features denoted gut dysbiosis and changes in metabolites, whereas TLR4, TNF, IL-6, and NF-κB-related features indicated changes in immunity and inflammation. In the individual components and integrated blocks plot, clear segregation between cohorts is shown, whereas the loading weights plot reveals the most important features responsible for such segregation, including *Enterococcus*, Butyrate, TLR4, and TNF. The DIABLO analysis indicated better separation between the healthy controls and the immune dysfunction clusters in the joint component space. Of the highly correlated multi-omics variables, *Enterococcus*, butyrate, TLR4, TNF, *Klebsiella*, bile acids, IL-6, and NF-κB were among those making the most notable contributions. Figure (8a): Component Space of DIABLO module clearly distinguishing between healthy controls and those affected by immune system dysfunction. Figure (8b): Highly correlated multi-omics features and their importance for group discrimination in the DIABLO model.

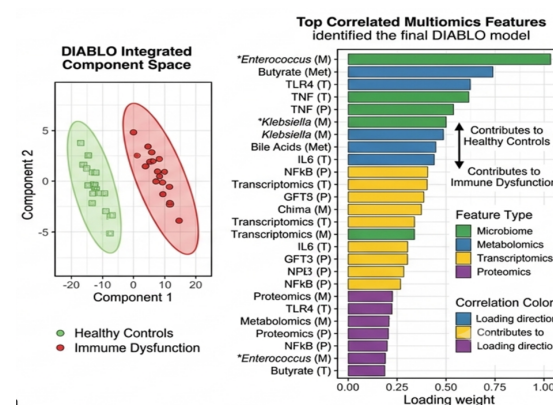


Figure 8. DIABLO-based multi-omics integration and feature contributions: (a) integrated component space showing group separation, and (b) top correlated multi-omics features identified by the final DIABLO model.

The robustness of the multivariate statistical models employed in discriminating Immune Dysfunction group from the Healthy Controls, and thus the credibility of the multi-omics biomarker signatures found through this process. Figure (9a) represents the Support Vector Machine (SVM) importance ranking of features where specific molecules like Metabolite A and Metabolite B are recognized as the major molecules leading to the distinction of the two categories. Figure (9b) shows the ability of Extreme Gradient Boosting (XGBoost) in handling missing values where its imputation method exhibits the least error. Hence, ensuring a complete and accurate dataset for analysis. Figure (9c) illustrates the PLS-DA score plot indicating the clear and statistically significant separation of samples into distinct

clusters based on their clinical status (Red = Immune Dysfunction; Blue = Healthy Control).

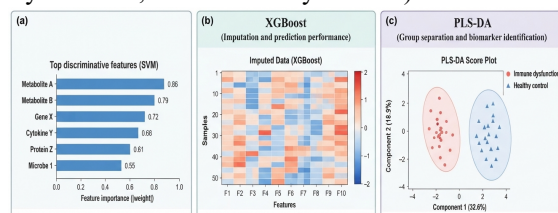


Figure 9. Multivariate Statistical Modeling and Validation of Multi-Omics Biomarker Signatures (a) Support Vector Machine (SVM) feature importance ranking identifying top discriminative molecules. (b) Evaluation of XGBoost imputation performance and prediction accuracy. (c) PLS-DA score plot demonstrating clear group separation.

3. Discussion

The biomarkers and mechanisms related to Crohn's disease were determined; however, limitations included unequal samples of omics, lack of diversity in the cohort, confounders, and lack of experimental validation of the causal mechanisms [29]. Colonic dysbiosis and disrupted homeostasis due to the high enteral starch diet were observed, but limitations, included the need for additional validation of the mechanism related to the microbial, fungal, and metabolite [30]. Gut microbiome dysbiosis and interaction between the microbes and metabolism during allergic rhinitis were identified using non-targeted metabolomics and 16S rRNA gene sequencing, but limitations included small sample size of 38 people and the requirement for validation of the causality between the microbiome and the metabolite [31]. Metabolites and immunoregulatory pathway mediated by the gut microbiota and development of osteoarthritis based on multi-omics, Mendelian randomization, and single-cell analysis techniques were observed, but limitations included the need for experimental validation of the microbial-metabolite-target relationship [32]. Microbiome and metabolomics data to determine mechanisms of bowel damage due to Crohn's disease and prediction model were investigated, its limitations were the need for more validations of the identified microbial, metabolomic, and imaging relationships [33]. Existing methods were characterized by unequal omics sample sizes, limited cohort diversity, potential confounding factors, small sample sizes, insufficient experimental validation, limited validation of microbial-metabolite causal relationships, and restricted validation of integrated microbiome-metabolome-imaging associations. The integrated multi-omics analysis revealed distinct microbial, metabolic, transcriptomic, proteomic, and immune profiles between the immune dysfunction and healthy groups. These findings indicated coordinated alterations across microbial composition, metabolic activity, molecular regulation, and immune responses. Cross-omics

associations provided complementary for interactions between microbial and metabolic changes and immune-related processes. Overall, the integrated findings provided a broader characterization of immune dysfunction than analysis based on a single omics layer.

4. Conclusion

In this research, Multi-omics analysis of microbes characterized gut microbial dysbiosis, and the association between immune function and microbes. Microbial diversity, abundance of microorganisms, metabolite changes, immune cell types, immune molecules such as cytokines and chemokines, and host molecules were investigated in this multi-omics analysis to identify the signatures associated with dysbiosis. This analysis revealed decreased microbial diversity and distinct microbiota community structure in subjects with immune dysfunction with low abundance of *Faecalibacterium*, *Bifidobacterium*, and *Lactobacillus* and high abundance of *Escherichia*, *Klebsiella*, and *Enterococcus*. Multi-omics integration using DIABLO, correlation analysis, network analysis, and MOFA showed a coordinated relationship among microbes, metabolites, host molecular features, and immune response, with *Enterococcus*, butyrate, TLR4, TNF, and immune regulatory features being involved in the molecular signature. Microbial-metabolic-immune dysregulation is the main factor that accounts for most of the variance in the multi-omics data using MOFA, explaining 28.4% of the variance. XGBoost provides least imputation error (RMSE = 0.082) and VIP as 0.42 in Metabolite A. These results successfully characterized the interrelationship between microbes, metabolism, host molecules, and immune functions in immune dysfunction and revealed molecular signatures and molecular pathways for further investigation.

Limitation and Future Scope

The main limitations includes small cohort size, observational design, and limited experimental validation. Future work may include larger and more diverse cohorts, longitudinal sampling, and experimental validation of the identified microbial-metabolic-immune associations.

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