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Systems Biology of Gut Microbiome Dysbiosis in Major Depressive Disorder: Functional Microbial Networks Linked to Inflammation, Illness Severity, Recurrence, and Suicidal Behavior

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Abstract

Background

Major depressive disorder (MDD) is characterized by disruptions in neuroimmune, metabolic, and oxidative stress (NIMETOX) pathways, which are influenced by leaky gut. Most microbiome studies in MDD focused on taxonomic differences rather than systems biology analyses of functional microbial pathways.

Methods

This cross-sectional study included 102 MDD inpatients and 38 healthy controls. Gut microbiome composition was assessed using 16S rRNA amplicon sequencing. Taxonomic differences across hierarchical levels were examined using ANCOM-BC and machine learning approaches. Functional metabolic pathways were inferred using Tax4Fun and PICRUSt. Microbial genera were grouped into functionally coherent modules.

Results

Beta diversity revealed significant differences in microbial community composition. Taxonomic analyses showed selective shifts at the genus and species levels. Feature selection identified 9 functional modules that discriminated MDD from controls. Functional module analysis demonstrated increased pro-inflammatory dysbiosis, lipopolysaccharide-related taxa, oral pathobionts, and Actinobacteria host–metabolite transformation, alongside reduced lactobacilli, and lactate cross-feeding modules. Specific and combined genera accounted for a substantial part of the variance in overall severity of depressive (75.7%) and physiosomatic symptoms (64.8%), suicidal ideation (27.2%), and recurrence of illness (36.7%). The acute phase inflammatory response was associated with dysbiotic genera promoting endotoxin exposure and mucosal immune activation. MDD microbiomes exhibit enhanced microbial stress responses, host–microbe interactions, and biofilm formation pathways, supporting a systems biology model linking gut dysbiosis with the clinical phenome and NIMETOX pathways.

Conclusions

MDD is associated with functional restructuring of the gut microbiome, characterized by inflammatory and barrier-disrupting microbial networks that define systems biology-informed personalized dysbiotic profiles.

Introduction

Accumulating evidence suggests that major depressive disorder (MDD) is strongly correlated with disruptions in the neuro-immune, metabolic, and oxidative stress (NIMETOX) pathways (Maes, Almulla et al. 2025, Maes, Jirakran et al. 2025; Maes et al., 2026 = CMI). Ultimately, the activation of these pathways may result in neurotoxic effects in affective brain networks and contribute to the phenotypic expression of depressive symptoms (Maes, Almulla et al. 2025; Maes et al., 2026). The NIMETOX framework's neuroimmune component encompasses the activation of the acute-phase inflammatory response, which can be evaluated through composite biomarkers that detect elevated inflammatory activity. This response can be captured by a composite index that combines elevated monomeric C-reactive protein (mCRP) with reduced levels of negative acute phase proteins, such as albumin and transferrin, indicating systemic inflammatory activation (Maes 1993, Maes 1995, Almulla, Niu et al. 2025). The NIMETOX pathways may be activated by a variety of environmental and biological stressors, such as chronic psychosocial stress, adverse childhood experiences (ACEs), and an increased translocation of lipopolysaccharide (LPS) from the gut into the circulation, indicating leaky gut or increased intestinal permeability (Maes, Almulla et al. 2025, Maes, Jirakran et al. 2025).

Leaky gut may be associated with gut dysbiosis, which facilitates gut barrier inflammation and the translocation of LPS (Maes, Kubera et al. 2008, Rudzki and Maes 2020, Tilg, Zmora et al. 2020, Rudzki and Maes 2021). This process has the potential to activate innate immune signaling pathways and induce systemic inflammation (Maes, Kubera et al. 2013, Tilg, Zmora et al. 2020, Zheng, Liwinski et al. 2020). Gut dysbiosis in MDD is frequently characterized by an increase in the abundance of pathobionts that disrupt epithelial barrier integrity and enhance inflammatory signaling, and a decrease in the number of beneficial microbial taxa that produce short-chain fatty acids (SCFAs) (Louis and Flint 2017). Some SCFAs are essential for preserving intestinal barrier function by enhancing tight junctions, modulating mucosal immunity, and generating anti-inflammatory effects (Louis and Flint 2017, Cheung, Goldenthal et al. 2019, Rudzki and Maes 2021).

The gut microbiome composition in MDD has been compared to that of healthy controls in studies that employed 16S ribosomal RNA (rRNA) amplicon sequencing. These studies typically document modest but heterogeneous compositional changes in the gut microbiome (Cheung, Goldenthal et al. 2019, Sanada, Nakajima et al. 2020, Gao, Wang et al. 2023, Maes, Vasupanrajit et al. 2023). Changes in alpha diversity are inconsistent across studies and frequently small (Sanada, Nakajima et al. 2020, Nikolova, Smith et al. 2021, Maes, Vasupanrajit et al. 2023). Beta diversity differences, which are indicative of community composition, are more frequently reported; however, they also differ among cohorts (Cheung, Goldenthal et al. 2019, Sanada, Nakajima et al. 2020, Maes, Vasupanrajit et al. 2023). Recurring taxonomic patterns have emerged, despite these inconsistencies. Numerous investigations have documented diminished populations of SCFA-producing genera, such as *Ruminococcus*, *Faecalibacterium*, and *Coprococcus* (Nikolova, Smith et al. 2021, Gao, Wang et al. 2023, Maes, Vasupanrajit et al. 2023). In contrast, MDD has been associated with an elevated prevalence of mucin-degrading pathobionts and pro-inflammatory taxa, including *Hungatella*, *Clostridium bolteae*, *Hungatella hathewayi*, and *Eggerthella* (Sanada, Nakajima et al. 2020, Radjabzadeh, Bosch et al. 2022, Maes,

Vasupanrajit et al. 2023). These findings bolster the notion that leaky gut and dysbiosis may significantly contribute to the pathophysiology of MDD by interacting with NIMETOX pathways.

The majority of microbiome studies in MDD have predominantly relied on classical comparative methods to identify taxa that differ between patients and controls. These methods encompass multivariate discrimination techniques, such as PLS-DA, LEfSe analyses, and univariate statistical tests (e.g., Wilcoxon tests, t-tests). One constraint of this approach is that the gut microbiome is significantly influenced by dietary patterns, lifestyle factors, geographic location, and cultural differences, which differ significantly among populations (Maes, Vasupanrajit et al. 2023). Consequently, taxa deemed significant in one study may not be replicated in another cohort (Maes, Vasupanrajit et al. 2023, Maes, Almulla et al. 2026). Additionally, these methodologies offer inadequate mechanistic insights into the biological significance of microbial modifications.

Tax4Fun (Aßhauer, Wemheuer et al. 2015) and PICRUSt (Douglas, Maffei et al. 2020) offer a practicable method for inferring the functional potential of 16S rRNA amplicon datasets beyond taxonomic profiling. This is achieved by mapping microbial profiles to KEGG orthologs and pathways. This process is especially pertinent to NIMETOX, as inferred functions can be associated with immune-inflammatory activation, barrier dysfunction/leaky gut, endotoxin burden, altered energy metabolism, and oxidative-redox adaptation (Maes, Almulla et al. 2026). PICRUSt/KEGG-style functional inference has already been implemented in a few 16S-based studies of MDD. Shen et al. reported abnormalities in glycan biosynthesis and metabolism, transport and catabolism, membrane transport, and nervous-system related functions in first-episode MDD (Shen, Yang et al. 2021). Ling et al. investigated childhood depression and identified PICRUSt-predicted changes in pathways associated with fatty acid metabolism, bile-acid biosynthesis, and other immunometabolic functions (Ling, Cheng et al. 2022). Dong et al. identified 69 substantially different KEGG orthologs in an MDD/GAD/healthy-control design using PICRUSt (Dong, Shen et al. 2021).

A more comprehensive approach is to generate functional composite scores based on genera that reflect functional entities, including SCFA/butyrate producers, lactobacilli, lactate cross-feeders, proteolytic fermenters, and inflammatory dysbiosis guilds (Romero, Wagg et al. 2005, Flint, Scott et al. 2012, Magnúsdóttir, Ravcheev et al. 2015, Clavel, Lagkouvardos et al. 2016, Schmidt, Hayward et al. 2019, Tilg, Zmora et al. 2020, Zheng, Liwinski et al. 2020). The most informative approach is to investigate the relationship between those functional modules and the key phenome features of MDD, including overall severity of depressive symptoms (OSOD), physiosomatic symptoms, suicidal ideation, and recurrence of illness (ROI) (Niu, Zhang et al. 2025). To the best of our knowledge, no previous MDD study has systematically constructed and tested a broader panel of functionally defined genus-level modules in parallel with PICRUSt/Tax4Fun outputs, despite the introduction of microbiome enterotypes/composite dysbiosis scores in MDD (Maes, Vasupanrajit et al. 2023).

The present study adopts a mechanistic systems biology approach to the gut microbiome in MDD. Rather than considering individual microbial taxa as isolated biomarkers, systems biology integrates

taxonomic composition, microbial metabolic pathways, functionally related microbial modules, host inflammatory responses, and clinical phenome features into a single biological framework. This approach enables identification of interacting microbial networks that influence NIMETOX pathways and allows delineation of the mechanisms through which gut dysbiosis may contribute to the clinical expression of MDD. Consequently, the emphasis is placed on biological mechanisms and network interactions rather than on simple taxonomic differences or diagnostic classification. Hence, the current 16S rRNA amplicon sequencing study was developed to examine microbiome alterations in MDD through the use of both taxonomic and functional approaches. The specific objectives were to delineate (a) the compositional differences in the gut microbiome between MDD patients and controls; (b) the functional alterations in microbial metabolic pathways using Tax4Fun and PICRUSt predictions at KEGG levels 2 and 3; (c) the degree to which functionally defined microbiome modules derived from genus-level data predict key components of the depression phenome; and (d) the associations between the acute inflammatory response in MDD and the gut microbiome.

Methods

Participants

This study enrolled 140 participants, including 102 inpatients with MDD and 38 healthy controls (HCs). The investigation employed a case–control cross-sectional design conducted at the International NIMETOX Center, Mental Health Center of Sichuan Provincial People's Hospital, Chengdu, China. Participants were aged 18–65 years, with an approximately balanced sex distribution. Patients and controls were matched for age, sex, body mass index (BMI), and years of education.

The control group consisted of hospital staff, their relatives, and acquaintances of patients, selected to match the case group in age, sex, education, and BMI. Controls were excluded if they had a current or past diagnosis of MDD, dysthymia, or DSM-5 anxiety disorders, or a family history of mood disorders, substance use disorders (except nicotine dependence), or suicide. MDD inpatients were diagnosed according to DSM-5 criteria, were in the acute phase of illness, and had a Hamilton Depression Rating Scale-21 (HAMD-21) score greater than 18 (Niu, Zhang et al. 2025). Patients with comorbid psychiatric disorders were excluded, including bipolar disorder, schizophrenia, schizoaffective disorder, substance use disorders (except nicotine dependence), autism spectrum disorder, eating disorders, and antisocial or borderline personality disorder.

Participants were also excluded if they had: (a) severe allergic reactions within the previous month, infections within the past three months, or surgery during the past three months; (b) immune or autoimmune disorders, including chronic obstructive pulmonary disease, type 1 diabetes, systemic lupus erythematosus, psoriasis, rheumatoid arthritis, cancer, or inflammatory bowel disease; (c) recent use of immunosuppressants, corticosteroids, immune-modulating medications, frequent analgesic use, or therapeutic doses of antioxidants or omega-3 supplements within the previous three months; (d)

neurological disorders, including stroke, epilepsy, Alzheimer's disease, Parkinson's disease, or multiple sclerosis; or (e) pregnancy or breastfeeding.

All participants or their legal representatives provided written informed consent prior to participation. The study protocol was approved by the Ethics Committee of Sichuan Provincial People's Hospital [Ethics (Research) 2024 – 203].

Clinical Assessment

A qualified physician conducted a structured clinical interview to collect demographic information as well as medical, psychiatric, and family histories. Psychiatric diagnoses were validated using the Mini International Neuropsychiatric Interview (M.I.N.I.), which evaluates both lifetime and current psychiatric disorders. On the same day, participants completed a series of standardized questionnaires assessing the severity of depressive, anxiety, and somatic symptoms, as previously described (Niu, Zhang et al. 2025). The severity of depressive symptoms was assessed with the Hamilton Depression Rating Scale (HAMD), while anxiety severity was evaluated using the Hamilton Anxiety Rating Scale (HAMA) (Hamilton 1959, Hamilton 1960); Self-reported depressive symptoms were measured with the Beck Depression Inventory, and current anxiety levels were assessed using the State-Trait Anxiety Inventory (state subscale) (Spielberger, Gorsuch et al. 1983, Beck, Steer et al. 1996). The Somatic Symptom Scale-8 (SSS-8) was used to quantify the intensity of somatic symptoms experienced during the preceding seven days (Gierk, Kohlmann et al. 2014). The FibroFatigue Scale (FFS), a 12-item clinical interview instrument, was employed to evaluate symptoms related to chronic fatigue syndrome and fibromyalgia (Zachrisson, Regland et al. 2002). Suicidal behaviors were assessed using the Columbia Suicide Severity Rating Scale (C-SSRS), which evaluates both lifetime and current suicidal ideation and suicide attempts (Posner, Brown et al. 2011).

These rating scales were used to compute composite indices of overall severity of depressive symptoms (OSOD), physiosomatic symptoms, and current suicidal ideation, as described previously (Niu, Zhang et al. 2025). In addition, two C-SSRS items (lifetime number of suicide attempts and suicidal ideation prior to the index episode), together with the number of depressive episodes were used to compute the recurrence of illness (ROI) index. ROI is a z-unit-based composite score derived from the sum of standardized scores for the number of depressive episodes, lifetime suicidal attempts, and lifetime suicidal ideation (Maes, Jirakran et al. 2024).

The study also evaluated components of metabolic syndrome (MetS), including height, weight, body mass index (BMI), and waist circumference (WC). BMI was calculated as body weight (kg) divided by height squared (m^2). Waist circumference was measured midway between the iliac crest and the lower rib margin. MetS was defined according to the 2009 joint statement of the American Heart Association and the National Heart, Lung, and Blood Institute (Alberti, Eckel et al. 2009). MetS was diagnosed when three or more of the following criteria were present: (a) waist circumference ≥ 90 cm in men or ≥ 80 cm in women; (b) triglycerides ≥ 150 mg/dL; (c) HDL cholesterol < 40 mg/dL in men or < 50 mg/dL in women;

(d) systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg, or current antihypertensive treatment; and (e) fasting plasma glucose ≥ 100 mg/dL or previously diagnosed diabetes mellitus.

Assays

Fasting venous blood samples (30 mL) were collected between 06:30 and 08:00 h using sterile syringes and transferred into serum collection tubes. The samples were then centrifuged at 3500 rpm, after which the serum fraction was carefully separated. The serum was aliquoted into Eppendorf tubes and stored at − 80°C until subsequent biochemical analyses.

mCRP concentrations were quantified using a BioVendor ELISA kit (Brno, Czech Republic), with a sensitivity of 0.63 ng/mL, an intra-assay coefficient of variation (CV) of less than 10%, and an inter-assay CV of less than 15%. Serum albumin was quantified using a Bromocresol Green kit (Beijing Strong Biotechnologies, Inc.) on the ADVIA 2400, with an intra-assay CV of 1.20% and an inter-assay of 2.10%. Serum transferrin was quantified using immunoturbidimetry (DIAYS DIAGNOSTIC SYSTEM) also on the ADVIA 2400, with a sensitivity of 0.03 g/L, an intra-assay CV of 1.96%, and an inter-assay CV of 0.67%. An acute-phase index (API) was determined by computing a z unit-based composite score as z mCRP (after regression on age and BMI) - z albumin - z transferrin. As such, increased scores are an index of the severity of the API response.

16S amplicon sequencing: The 16S sequencing was performed in Beijing Junfeix Biotech Company. Microbial DNA was extracted from fecal samples using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Norcross, GA, USA). The V4-V5 region of the bacterial 16S rRNA gene was amplified with primers 515F and 907R. PCR amplicons were purified, quantified, and pooled to construct an Illumina paired-end library, followed by paired-end sequencing (2×300). Raw reads were quality-filtered, demultiplexed, and assembled using Trimmomatic. OTUs were clustered at 97% similarity by UPARSE, and chimeras were removed using UCHIME. Taxonomic assignment was performed via the Uclust algorithm against the SILVA (SSU138.1) database with a confidence threshold of 80% (see further below).

Statistics

Classical statistical tests

The necessary sample size was determined based on prior research by Maes et al., indicating that multivariable regression models incorporating metabolic or NIMETOX-related factors can account for about 25% of the variance in overall severity of depressive symptoms (OSOD). Power calculations for the primary statistical analysis, namely multiple regression models predicting OSOD, were conducted utilizing G*Power version 3.1.9.4. The analysis presumed an effect size of 0.33 (equating to around 25% explained variance), a two-tailed alpha of 0.05, a statistical power of 0.80, and a maximum of seven

predictor variables. The minimum requisite sample size was 51 people. The sample size in the current study surpassed this criteria and was also increased to provide distinct training and testing samples and improve the accuracy of the regression coefficients.

Analysis of variance or the Wilcoxon test were employed to evaluate the differences across the various research groups for continuous variables, encompassing demographic and clinical data. Contingency table analysis was utilized to examine correlations across variables through categorical data, employing Chi-square testing. False discovery rate (FDR)-adjusted P values were used to account for multiple comparisons. Statistical studies were conducted utilizing IBM SPSS version 30 and Statistica version 14, with a two-tailed significance threshold established at $p < 0.05$.

Analytical strategy and systems biology framework

The primary objective of the statistical analyses was not to develop or optimize diagnostic classification models for MDD, but rather to implement a mechanistic systems biology approach aimed at elucidating the biological organization of gut microbiome dysbiosis in depression. Accordingly, multivariate analyses and machine-learning methods were employed to identify biologically meaningful microbial taxa, functional pathways, and microbiome modules, and to examine their relationships with the NIMETOX pathways and the clinical phenome of MDD. Classification indices were used solely to assess the robustness of the identified microbial signatures and not to establish clinically applicable diagnostic algorithms. A multistage analytical pipeline that included principal component analysis (PCA), linear discriminant analysis (LDA), partial least squares discriminant analysis (PLS-DA), PLS regression, and Random Forest was employed to conduct biomarker discovery. Statistical analyses were primarily conducted using R (v4.0), Statistica version 14.0, the R package metaX, and the R package ropls.

Alpha and beta diversity analyses

To evaluate the richness and evenness of the microbial community within each sample, alpha diversity was assessed. The Shannon index was implemented to evaluate microbial evenness and richness, while the Chao1 index was used to estimate species richness. The Simpson index measured diversity based on dominance patterns, whereas Faith's phylogenetic diversity (PD) index quantified evolutionary diversity by considering the total branch length of the phylogenetic tree.

To evaluate differences in microbial community composition between samples, beta diversity was assessed. Bray–Curtis distance matrices were calculated and group differences were statistically evaluated using permutational multivariate analysis of variance (PERMANOVA; ADONIS) with 999 permutations. The multi-response permutation procedure (MRPP) was employed as an additional validation method. The delta statistic, representing within-group agreement, and the chance-corrected within-group agreement statistic (A), which quantifies within-group similarity, were computed. Principal coordinate analysis (PCoA) was used to visualize clustering patterns between MDD patients and healthy controls.

Taxonomic composition analyses

Two complementary taxonomic approaches were employed to characterize microbial composition. First, broad organizational patterns of the gut microbiome were assessed across hierarchical taxonomic levels, including phylum, class, order, family, and genus. Stacked bar plots were used to visualize the relative abundances of dominant taxa. Second, the relative abundances of the most prevalent taxa in MDD patients and healthy controls were visualized using heatmaps, and differences between groups were evaluated using the Wilcoxon rank-sum test.

Linear Discriminant Analysis Effect Size (LEfSe) was additionally employed to identify microbial taxa that differed between groups. LEfSe was applied across hierarchical taxonomic levels ranging from phylum to species. The resulting cladogram visualized the phylogenetic relationships among taxa and highlighted taxa enriched in either MDD patients or healthy controls.

Differential abundance analysis

The Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC) was employed to evaluate differential abundance of microbial taxa between groups. This method corrects for biases resulting from unequal sampling fractions across samples while accounting for the compositional nature of microbiome data. ANCOM-BC estimates bias-corrected log-fold changes while controlling for multiple testing using false discovery rate (FDR) correction. The model allowed adjustment for age, sex, BMI, metabolic syndrome, smoking, and medication use. Taxa were considered significantly different when they fulfilled the following criteria: FDR-adjusted $p < 0.05$, $|\text{log-fold change}| > 0.6$, and the ANCOM-BC detection filter ("passed" = TRUE).

Functional microbiome pathway analyses

Tax4Fun and PICRUSt were employed to predict the functional potential of the gut microbiome. These tools infer functional pathways from 16S rRNA gene sequencing data by mapping microbial taxa onto Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. KEGG hierarchical pathway levels 2 and 3 were used to characterize intermediate and detailed metabolic pathway categories, respectively. Functional pathway scores were calculated for each sample and compared between MDD patients and healthy controls using the Wilcoxon rank-sum test. Mean pathway proportions together with confidence intervals were visualized using bar plots.

Data preprocessing for multivariate analyses

Genus-level data were employed in all subsequent multivariate analyses because genus-level assignments are taxonomically more reliable than species-level assignments, which are often ambiguous owing to the limited taxonomic resolution of 16S rRNA sequencing, while providing greater biological specificity than higher taxonomic levels. The selected genus abundances were transformed using the centered log-ratio (CLR) transformation to account for the compositional nature of microbiome data and to reduce spurious statistical relationships that may arise from raw relative abundances.

Selection of the microbiome enterotype

The study sample was randomly stratified within each diagnostic group and divided into training (60%) and testing (40%) subsets using a rank-based randomization procedure. Candidate genera were initially selected based on significant Wilcoxon rank-sum tests, differential abundance identified by ANCOM-BC, their contribution to multivariate discrimination as reflected by high Variable Importance in Projection (VIP) scores in PLS-DA models, and their biological relevance based on current knowledge of microbial ecology, host–microbe interactions, intestinal barrier function, immune activation, and NIMETOX-related pathways. Genera showing inconsistent statistical support across these complementary approaches or representing likely transient non-resident microbiota were excluded.

Subsequently, within the training dataset, Random Forest was combined with recursive feature elimination to iteratively rank the remaining genera according to their variable importance and progressively eliminate the least informative taxa. Feature selection, model training, z-score transformations, and model interpretation were performed exclusively within the training dataset, whereas the independent testing set was reserved solely for final model evaluation. This integrative feature-selection strategy yielded the smallest biologically meaningful and statistically robust subset of genera that maintained stable discriminatory performance while minimizing redundant and collinear features. The resulting parsimonious genus set constituted the final microbiome enterotype that was employed in all subsequent systems biology analyses.

Although the primary objective of the present study was mechanistic systems biology rather than diagnostic classification, Random Forest analysis was additionally performed as a secondary robustness analysis to evaluate whether the selected microbiome features could reliably discriminate MDD patients from healthy controls. This analysis served to assess the stability and generalizability of the identified microbial signatures rather than to develop a clinically applicable classifier. To account for the unequal group sizes, higher misclassification costs were assigned to the minority control class, and model performance was assessed using balanced accuracy, AUC, and additional robustness analyses. This analytical design minimized data leakage, reduced overfitting, and provided an unbiased assessment of the reproducibility of the identified microbial biomarkers (Maes, Niu et al. 2026).

Functional microbiome module analyses

Functional microbiome modules were constructed by grouping genera according to their shared biological functions. Module scores were computed as z-unit composite scores by summing the standardized abundances of the genera comprising each functional module. These module scores were subsequently incorporated into PLS-DA and PLS regression analyses to investigate their multivariate organization and associations with the MDD phenome.

Automatic linear discriminant analysis (LDA; F-to-enter $p < 0.01$, F-to-remove $p = 0.10$) was performed as an exploratory multivariate procedure to evaluate the discriminatory information contained within the

functional microbiome modules. A leave-one-out cross-validation procedure was used to estimate the robustness of the discriminant functions rather than to develop a diagnostic classifier.

Exploratory multivariate systems biology analyses

After the microbiome enterotype had been established and its robustness confirmed using the independent training and testing datasets, these datasets were merged for the subsequent explanatory analyses. Combining both datasets increased the effective sample size and statistical power, thereby providing more precise estimates of the associations between the microbiome enterotype, MDD, the phenome scores, and the API index. Because feature selection and validation had already been completed before merging the datasets, this procedure did not introduce information leakage or selection bias into the subsequent mechanistic systems biology analyses. These procedures were implemented solely to ensure the robustness of the exploratory multivariate analyses and biomarker identification. They were not intended for predictive model development, optimization, or external validation, as the primary objective of the present study was mechanistic systems biology rather than diagnostic classification.

Three complementary multivariate approaches were employed. PLS-DA identified the multivariate microbial signature (microbiome enterotype) distinguishing MDD from healthy controls using either the selected genera or the constructed functional modules. PLS regression and multiple regression were subsequently used to identify the microbial signatures that best explained the major phenome domains.

PLS-DA was employed to investigate multivariate differentiation between MDD patients and healthy controls and to examine associations between microbial taxa and clinical phenome scores. Model performance was evaluated using Q^2, which assessed cross-validated predictive performance, R^2Y, which quantified the proportion of variance explained in the outcome variable, and R^2X, which represented the proportion of variance explained in the predictor variables. Statistical significance of class separation was assessed using cross-validated ANOVA (CV-ANOVA). Variable importance in projection (VIP) scores were computed to identify microbial taxa contributing most strongly to multivariate discrimination.

Finally, the functional module scores were entered as explanatory variables in multiple regression analyses with phenome scores and the API index as dependent variables to quantify their contribution to the biological and clinical manifestations of MDD. Simultaneously with manual multiple regression analyses, automated regression techniques were utilized to determine microbiome predictors of the phenome scores. Initially, ridge regression was executed utilizing Statistica (version 14) with a regularization value of $\lambda = 0.1$ and a tolerance level of 0.4 to mitigate the effects of multicollinearity. Subsequently, forward stepwise automatic linear modeling was performed in SPSS (version 30) employing an overfitting control criterion and a maximum retention of five effects in the model. A best-subset regression analysis was conducted to determine the most significant predictors among the explanatory variables. The results of these complementary statistical procedures were integrated with prior systems biology knowledge of gut microbial functions and host–microbiome interactions to

identify the most biologically coherent and explainable predictor set. Thus, the objective was not to maximize predictive performance, but to derive a parsimonious microbiome model with mechanistic interpretability and biological relevance.

The resulting models were evaluated by manual multiple regression analyses (forced entry) in SPSS30, with a thorough review of model statistics, including F values, degrees of freedom, p-values, and explained variance (R^2). Standardized beta coefficients, t statistics, and p-values were analyzed to assess the relative importance of each predictor. Diagnostic examinations encompassed the evaluation of residual distributions and P–P plots to confirm multivariate normality. The White test and the modified Breusch–Pagan test were employed to assess homoscedasticity, and partial regression plots were created to illustrate the relationships between metabolites and phenome scores. The final regression models were evaluated for multicollinearity using variance inflation factors (VIF), with all retained predictors showing VIF values < 2.0, indicating negligible multicollinearity. Bootstrap resampling (1,000 iterations) was performed to assess the stability and robustness of the regression coefficients. When the bootstrapped standardized coefficients are virtually identical to the conventional estimates, only the conventional regression coefficients are presented for simplicity and ease of interpretation.

Results

Demographic and clinical data

Table 1 of the Electronic Supplementary File (ESF) indicates that there were no substantial differences in age, sex distribution, BMI, MetS prevalence, and years of education of MDD inpatients and controls. In comparison to controls, MDD patients exhibited elevated ROI, physiosomatic, suicidal ideation, and OSOD scores. The API index was significantly higher in MDD subjects than in controls.

Table 1

Significantly differentially abundant genera associated with major depression and effects of clinical covariates: results of analysis of compositions of microbiomes with bias correction (ANCOM-BC)

Results	Taxon	LFC	*q*
Major Depressive Disorder (MDD)	*Hungatella*	1.240	8.23×10^{-7}
	Clostridioides	0.987	1.25×10^{-7}
	Eisenbergiella	0.789	0.002
	Aggregatibacter	0.783	4.29×10^{-5}
	Slackia	0.716	0.001
	Sutterella	0.679	0.010
	Anaerotruncus	0.638	0.022
	Flavonifractor	0.620	0.036
	Fusobacterium	0.618	0.033
	Unclassified Clostridia	-0.637	0.012
	Limosilactobacillus	-0.671	0.042
	Anaerosporobacter	-0.694	0.001
	Paeniclostridium	-0.872	2.87×10^{-6}
	Romboutsia	-0.961	0.001
	Cronobacter	-1.354	4.21×10^{-5}
	Haemophilus	-1.933	2.65×10^{-8}
SSRIs	*Haemophilus*	-0.745	3.89×10^{-4}
	Klebsiella	-0.911	0.007
Mood Stabilizers	*Ligilactobacillus*	-2.293	0.004
Atypical Antipsychotics	*Methanosphaera*	1.510	4.14×10^{-4}
	Peptococcus	-0.876	0.025
	Clostridioides	-1.063	0.003
	Ligilactobacillus	-1.322	0.009
Smoking	*Muribaculum*	-1.055	0.023

Note: Differentially abundant taxa at the genus level were identified using ANCOM-BC. LFC: Log Fold Change. Positive LFC indicates enrichment, negative indicates depletion. q-value: FDR-adjusted p-value. SSRIs: Selective Serotonin Reuptake Inhibitors.

Gut microbiota diversity

The full-length bacterial 16S rDNA was sequenced using high-throughput long-read nanopore sequencing. The operational taxonomic units were assigned to the retained readings following quality filtering. Alpha diversities (Chao1, Shannon and Simpson index and PD faith) were employed to illustrate the richness and evenness of bacterial comparisons between MDD patients and controls, as determined by their relative abundances. Figure 1A-D demonstrate that the Mann–Whitney U tests did not reveal any statistically significant differences between the two study groups (e.g., $p = 0.617$ for Chao1 and $p = 0.985$ for Shannon).

The bacterial communities of MDD and controls are compared using the Bray–Curtis beta diversity index, as shown in Fig. 1E. The Bray–Curtis beta diversity analysis revealed significant differences in the constitution of the gut microbial community among the groups (PERMANOVA: sum of squares = 0.5907, $R^2 = 0.023$, $F = 3.27$, df = 1, $p < 0.001$; 999 permutations). The first two PCoA axes accounted for 10% and 8% of the total variance, respectively (principal coordinate analysis). The groups exhibit significant overlaps, although MDD samples exhibit a subtle shift along the ordination space, suggesting a modest but systematic difference in microbial community structure. The MRPP analysis indicated a significant difference in microbial community composition between MDD patients and controls. The observed delta (δ) was 0.5922, which was slightly lower than the expected delta of 0.5978, indicating that samples within groups were more similar to each other than expected by chance. The chance-corrected within-group agreement (A) was 0.0093, suggesting a small but measurable group structure. These findings support the presence of subtle but statistically significant differences in microbial community composition between MDD and the control group.

Gut microbiota composition

The differences in class, order, family, and genera major compositional patterns in MDD patients and controls are shown in (Fig. 2A-E**)**. The microbiome is primarily dominated by Clostridia in both groups, suggesting that Firmicutes-related fermenters remain the primary component of the gastrointestinal ecosystem (Fig. 2B). In MDD, minor changes are observed in other classes, including Bacteroidia, Actinobacteria, and Gammaproteobacteria, which indicates a modest restructuring of the microbial composition in MDD.

At the order level (Fig. 2C), Lachnospirales and Oscillospirales dominate the microbial community in both groups, reflecting the central role of Firmicutes-related anaerobes in the gut ecosystem. However, several orders—including Enterobacterales, Coriobacteriales, Bacteroidales, and Bifidobacteriales show differences between HC and MDD.

At the family level, Lachnospiraceae and Ruminococcaceae (primary anaerobic fermenters involved in the production of short-chain fatty acids) dominate the intestinal microbiota (Fig. 2D). In MDD, families such as Enterobacteriaceae, Bacteroidaceae, and Peptostreptococcaceae exhibit modest differences.

The genus-level composition is illustrated in Fig. 2E, which demonstrates that *Blautia, Faecalibacterium, Subdoligranulum*, and *Ruminococcus* account for a significant portion of the microbiome in both groups. The differences between HC and MDD are evident in the relative shifts among several genera, including *Bacteroides, Roseburia, Bifidobacterium*, and *Agathobacter*.

ESF, Fig. 1 illustrates the relative abundances of the dominant bacterial classes, orders, families, genera, and species in MDD inpatients and controls. The composition heatmaps shown in ESF, Fig. 1, are limited to the top 30 most abundant taxa at each taxonomic level in order to facilitate the visualization of key compositional patterns. In order to prevent overcrowding and to emphasize biologically meaningful differences between groups, lower-abundance taxa were excluded from visualization.

The LEfSe phylogenetic cladogram, which identifies bacterial taxa that differs between MDD and controls, is displayed in the **ESF**, Fig. 2. The cladogram illustrates the microbiome's hierarchical taxonomic structure, transitioning from phylum/class (inner circles) to genus and species (outer circles).

AMCOM-BC results and gut genera in MDD

The initial microbiota dataset contained data on 216 genera. The results of ANCOM-BC, which included the diagnosis, as well as age, sex, BMI, MetS, and smoking as covariates, are presented in Table 1. Table 1 illustrates that 16 genera were substantially different (in terms of q values, LFC > 0.6, and the “passed or TRUE” criterion) between MDD and controls. The top three genera were *Haemophilus, Cronobacter* (both decreased), and *Hungatella* (increased). There were no significant effects of age, sex, BMI, or MetS. The only genus that was significantly impacted by smoking was *Muribaculum*. As a result, we have conducted distinct ANCOM-BC analyses for each psychotropic class to investigate the impact of psychotropic medications on the gut microbiota. The results of Table 1 indicate that SSRIs had an effect on two genera (*Haemophilus* and *Klebsiella*), mood stabilizers on one (*Ligilactobacillus*), and antipsychotics on four (*Clostridioides, Ligilactobacillus, Methanosphaera, Peptococcus*).

Microbial metabolic pathways

Functional predictions for Tax4Fun at KEGG level 2 and 3 and PICRUSt at KEGG level 2 and 3 are illustrated in ESF, Figs. 3A/B and 4A/B. Overall, the Tax4Fun results indicate that the MDD-associated microbiota is characterized by increased environmental responsiveness and microbial interaction pathways, while the control microbiota exhibits greater core metabolic and biosynthetic capacity. This suggests a shift from core metabolic and biosynthetic microbial functions to stress-response and host-interaction pathways in MDD. The data from PICRUSt indicate that the MDD-associated microbiome is distinguished by two key characteristics: a) increased signaling, interaction, and catabolic activity; and b) enhanced carbohydrate metabolism and host-interaction pathways, whereas the control microbiota shows greater activity in fundamental biosynthetic and genetic information processing functions.

Table 2
Functional gut *m*icrobiome modules

Module	Members	Function
LPS-TLR inflammatory load (LPS-TLR)	*Bacteroides + Parabacteroides + Sutterella + Odoribacter*	Gram-negative, proteolytic microbiota linked to gut barrier dysfunction and systemic immune activation.
Protective SCFA module (PROTECTSCFA)	*Faecalibacterium + Coprococcus + Eubacterium + Christensenella + butyrocimonas*	Protective, SCFA-producing signature supporting gut–immune–brain axis stability.
Oral pathobiont enrichment (OPE)	*Fusobacterium + Porphyromonas + Phocea*	Inflammatory pathobionts promoting mucosal inflammation, barrier disruption, and microbial translocation.
Ecological filtered (ECOFILTER)	*Oribacterium + Haemophilus + Parvimonas + Sneathia*	Low-abundance mucosal taxa; reduction reflects ecological filtering and microbiome restructuring.
Proteolytic / Putrefactive Dysbiosis module (PPD)	*Anaerotruncus + Clostridioides + Hungatella + Intestinibacter + Eisenbergiella + Paeniclostridium*	Indicates a shift toward proteolytic metabolism, intestinal barrier dysfunction, and oxidative stress.
Functional polarization of Actinobacteria (FPA)	*Eggerthella + Slackia - Adlercreutzia*	Reflects a shift from protective polyphenol metabolism toward inflammatory host-metabolite transformation.
Lactobacilli Protective Barrier Module (LACTOBAR)	*Lactiplantibacillus + Limosilactobacillus + Loigolactobacillus*	Protective lactate producers supporting microbial cross-feeding, antimicrobial defense, and immune regulation.
Lactate cross-feeding module (LACTATECF)	*Megasphaera + Mitsuokella + Veillonella*	Lactate-utilizing anaerobes that prevent lactate accumulation and stabilize microbial fermentation via cross-feeding.
Proinflammatory dysbiosis module (INFLAMMDYS)	*Fusobacterium + Porphyromonas + Clostridioides + Eggerthella + Bacteroides*	Dysbiotic taxa promoting endotoxin exposure, proteolytic metabolism, and mucosal immune activation.
inflammatory-dysbiosis or IDI index	LPSTLR + FPA + INFLAMMYS - PROTECTSCFA - LACTOBAR	Overall index of gut-derived inflammation and dysbiosis.

Note: SCFA, short-chain fatty acids; Module scores represent the sum of z-transformed relative abundances of their constituent genera. FPA was calculated as *Eggerthella + Slackia − Adlercreutzia* to reflect opposing biological roles. Residualized values of *Haemophilus* and *Clostridioides* were used to adjust for SSRI and antipsychotic use, respectively.

Results of PLS-DA and Random Forest

The CLR transformation was applied to the genus-level abundance data (n = 216 taxa), prior to the ML and multivariate analysis. By expressing each taxon relative to the geometric mean of all taxa within a sample, the CLR transformation eliminates this compositional constraint. Taxa that are unlikely to be associated with the human gastrointestinal microbiome, as well as those with low taxonomic confidence, were removed from the genus-level dataset. Features were selected in the training sample as described above resulting in the identification of 50 selected high-confidence genera. The multivariate structure that was observed in the PLS-DA model, which was constructed using the overall genus dataset, was preserved in the reduced dataset.

Two significant latent components were extracted by PLS-DA on these 50 genera, which collectively accounted for 75.7% of the variance in the dependent variables (R^2Y). The initial component exhibited a high degree of predictive ability ($Q^2 = 0.537$) and accounted for the greatest percentage of group separation ($R^2Y = 0.667$). The second component contributed a smaller but additional explanatory variance ($R^2Y = 0.090$) with limited predictive value ($Q^2 = 0.178$). The 2D-PC plot of the PLS-DA model is illustrated in Fig. 3A. The PLS-DA model exhibits two discrete clusters, with MDD and control patients occupying separate, non-overlapping regions of the score plot. This PLS-DA model was well validated (Fig. 3B). The VIP graph derived from PLS-DA is depicted in Fig. 3C and shows that the most influential genera were *Haemophilus, Cronobacter*, and *Howardella*.

Seventy trees were employed to construct a Random Forest on the same 50 variables, with a maximal tree size of 1000. The misclassification rate decreased as the number of trees increased, eventually stabilizing at approximately 9% for the training set and ~ 7% for the test set, as evidenced by the decent model convergence. The final classification matrix in the testing set demonstrates an overall accuracy of 92.8%. In general, the Random Forest model exhibits satisfactory discriminative performance in distinguishing MDD from controls using microbiome features.

Construction of 9 functional microbiota modules and models

Table 2 summarizes the nine biologically meaningful modules that were constructed based on the 50 selected genera. We used the residualized values of *Haemophilus* (7.0%) and *Clostroides* in these modules although only a small part of the variance in these taxa was explained by use of SSRIs (7.0%) and antipsychotics (6.2%), respectively. The standardized abundances of the genera included in each module were summed to compute module scores using z-unit composite scores. With the exception of the Functional Polarization of Actinobacteria (FPA) module, which was calculated as z(*Eggerthella*) + z(*Slackia*) − z(*Adlercreutzia*) (to reflect the opposing biological roles of these taxa), all modules were computed as the sum of the respective z-transformed genera.

The discrepancies between MDD patients and controls in these nine modules are illustrated in Table 3. In MDD, the scores of Proinflammatory dysbiosis (INFLAMMDYS), FPA, LPS-TLR inflammatory pressure (LPSTLR), and Oral Pathobiont Enrichment (OPE) modules were significantly increased (in descending order of importance). Conversely, the Ecological Filtered (ECOFILTER), Lactobacilli Protective Barrier

Module (LACTOBAR), and Lactate Cross-Feeding (LactateCF) modules were significantly decreased in MDD. Furthermore, we developed a new index composed of the three most elevated functional inflammatory-dysbiosis modules, minus the protective modules, as z LPSTLR + z FPA + z INFLAMMDYS – z LACTOBAR – z LactateCF, referred to as the inflammatory-dysbiosis or IDI index. All intergroup differences remained significant after DFR p correction (at $p = 0.0075$).

Table 3
Discrepancies in the functional microbiome modules between major depression *(*MDD*)* patients and healthy controls (HC).

Microbiome Module	HC (Mean (SE))	MDD (Mean (SE))	*F*	*p*
INFLAMMDYS	-0.616 (0.142)	0.247 (0.087)	26.636	**< .001**
FPA	-0.543 (0.156)	0.202 (0.095)	16.494	**< .001**
LPSTLR	-0.532 (0.155)	0.199 (0.095)	16.004	**< .001**
OPE	-0.387 (0.160)	0.144 (0.097)	7.985	**.005**
ECOFILTER	0.584 (0.155)	-0.219 (0.095)	19.360	**< .001**
LACTOBAC	0.425 (0.154)	-0.184 (0.094)	11.303	**.001**
LACTATECF	0.398 (0.158)	-0.126 (0.096)	7.950	**.006**
PPD	0.181 (0.164)	-0.070 (0.100)	1.700	.195
PROTECTSCFA	0.116 (0.140)	-0.010 (0.085)	0.580	.448
IDI index	-2.282 (0.327)	0.872 (0.199)	67.049	**< .001**

Note: Data are presented as Estimated Marginal Means (SE) derived from z-transformed module scores. F and P values were calculated using General Linear Models, adjusting for metabolic syndrome, age, gender, and body mass index. Significant P values (< .05) are bold. INFLAMMDYS, Proinflammatory dysbiosis module; FPA, Functional polarization of Actinobacteria; LPSTLR, LPS-TLR inflammatory pressure; OPE, Oral pathobiont enrichment; ECOFILTER, Ecological filtered; LACTOBAC, Lactobacilli Protective Barrier Module; LactateCF, Lactate cross-feeding module; PPD, Proteolytic / Putrefactive Dysbiosis module; PROTECTSCFA, Protective SCFA module; IDI, inflammatory-dysbiosis index.

The PLS-DA analysis conducted using these 9 module scores demonstrated a substantial separation of MDD from controls, with two latent components. This separation accounted for 45.6% of the variance in the predictor variables and 39.9% of the variance in the dependent variable. The first component was responsible for the majority of the explanatory and predictive information ($R^2Y = 0.397$; $Q^2 = 0.227$), while the second component provided only a small amount of additional predictive power ($Q^2 = 0.009$). Permutation testing confirmed that the reported model performance was not due to random structure, as all permuted Q^2 and R^2 values were substantially inferior to those of the empirical model. Additionally, the CV-ANOVA demonstrated a significant degree of class differentiation, which supported the PLS-DA model's robustness and non-randomness ($p < 0.001$). Figure 3D shows the rank importance of order of those 9 modules in discriminating MDD inpatients from controls.

Stepwise automatic LDA with the MDD diagnosis as the dependent variable demonstrated that 7 of the 9 modules, namely LPSTLR, PROTECTSCFA, ECOFILTER, PPD, FPA, LACTOBAR, and INFLAMMDYS, significantly predicted MDD with a cross-validated accuracy of 85.7% (sensitivity: 87.3% and specificity = 81.6%). The AUC ROC curve was 0.914 (SE = 0.030, 95% confidence intervals: 0.854; 0.973). The GINI index was 0.827, and the overall model quality was 0.85.

Associations between microbiota and phenome scores

The results of PLS-regression with the microbiota as predictors of OSOS, physiosomatic symptoms, suicidal ideation, and ROI are illustrated in Fig. 4. These regressions accounted for up to 75.7% and 64.8% of the variance in OSOD and physiosomatic symptoms, respectively (cumulative Q^2 values of 0.437 and 0.321, respectively, and R^2X values of approximately 14.5%). The VIP scores of the 50 genera are depicted in Figs. 4A and 4B. We discovered that a single latent variable derived from the 50 genera accounted for 27.2% of the variance in suicidal ideation (Fig. 4C; $Q^2 = 0.103$ and $R^2X = 0.094$) and that a single latent variable accounted for 36.7% of the variance in ROI (Fig. 4D; $Q^2 = 0.228$ and $R^2X = 0.094$).

The heatmap in Fig. 5 illustrates Pearson's correlation between the 9 module scores, demographic and clinical data. No significant correlations were observed between any of the functional scores and age, sex, BMI, or MetS (even without p-correction). The ACE score and sexual abuse, as well as the ECOFILTER score, were substantially correlated with the IDI index. The physiosomatic score and OSOD were significantly correlated with the scores of nearly all modules, with the exception of PROTECTSCFA and PPD. Suicidal ideation was significantly correlated with the IDI score, INFLAMMDYS, and FPA module scores (all positively) and inversely with the ECOFILTER and LACTOBAR scores. The scores of all modules, with the exception of PROTECTSCFA, OPE, PPD, and LactateCF, were significantly correlated with ROI.

We conducted multiple regression analyses with the phenome scores as dependent variables and the 9 functional modules with demographic data with and without the API index, ACEs, and sexual abuse as dependent variables to investigate the functional predictors of the phenome score. Table 4 regression #1 demonstrates that INFLAMMDYS, FPA (both positively), and LACTOBAR (inversely) accounted for 35.7% of the variance in OSOD. The same module scores could account for 28.5% of the variance in physiosomatic symptoms, as demonstrated by regression #2. We discovered that the regression on FPA (positively), LACTOBAR, and BMI (both inversely), accounted for 14.2% of the variance in suicidal behavior (regression #3). FPA (positively) and LACTOBAR (inversely) accounted for a portion of the variance in ROI (15.2% in regression #4).

Table 4
Stepwise multiple regression analysis investigating the functional predictors of the key clinical symptom domains of major depression.

Dependent Variables	Explanatory Variables	Coefficient statistics			Model statistics			
		β	t	*p*	R^2	*F*	*df*	*p*
#1. Severity of Illness (OSOD)	**Model**				.357	22.769	3/123	< .001
	INFLAMMDYS	.317	3.916	< .001				
	LACTOBAC	− .354	-4.888	< .001				
	FPA	.239	2.962	.004				
#2. Physiosomatic Symptoms	**Model**				.285	16.325	3/123	< .001
	INFLAMMDYS	.289	3.385	.001				
	LACTOBAC	− .275	-3.607	< .001				
	FPA	.242	2.843	.005				
#3. Current Suicidal Ideation	**Model**				.142	6.944	3/126	< .001
	LACTOBAC	− .264	-3.193	.002				
	FPA	.192	2.295	.023				
	BMI (Z-score)	− .170	-2.033	.044				
#4. Recurrence of Illness (ROI)	**Model**				.152	11.346	2/127	< .001
	FPA	.310	3.788	< .001				
	LACTOBAC	− .240	-2.931	.004				

Note: Coefficient statistics reflect the individual contribution of each explanatory variable, while model statistics reflect the overall fit. β, standardized coefficient; BMI, body mass index; OSOD, overall severity of depression; INFLAMMDYS, Proinflammatory dysbiosis module; LACTOBAC, Lactobacilli Protective Barrier Module; FPA, Functional polarization of Actinobacteria.

Table 5 shows the results of regression analysis after allowing for the effects of the API index, ACEs, and sexual abuse. The purpose of these analyses was to determine whether the effects of functional genera modules were still significant after the aforementioned established predictors of MDD were incorporated. Table 5 regression #1 demonstrates that API index, ACEs, FPA, LPSTLR (all positively), LACTOBAR and PROTECTSCFA (inversely) accounted for 57.1% of the variance in OSOD. The same predictors accounted for up to 48.5% of the variance in physiosomatic symptoms, while ACEs, sexual abuse, FPA (positively), and LACTOBAR (inversely) explained 31.2% of the variance in suicidal ideation (regression #3). The ROI variance was explained by ACEs, FPA, and API index (all positively) in regression #4, which displayed a 37.5% explanation.

Table 5

Stepwise multiple regression analysis predicting clinical symptoms after allowing for the effects of the acute phase inflammatory (API) index, adverse childhood experiences (ACEs) including sexual abuse.

Dependent Variables	Explanatory Variables	Coefficient statistics			Model statistics			
		β	*t*	*p*	R^2	*F*	*df*	*p*
#1. Overall severity of Illness (OSOD)	**Model**				.571	26.582	6/120	< .001
	API index	.216	3.186	.002				
	ACEs	.329	5.131	< .001				
	FPA	.315	5.081	< .001				
	LPSTLR	.295	4.052	< .001				
	LACTOBAC	− .234	-3.793	< .001				
	PROTECTSCFA	− .165	-2.341	.021				
#2. Physiosomatic Symptoms	**Model**				.485	18.825	6/120	< .001
	API index	.191	2.567	.011				
	ACEs	.329	4.692	< .001				
	FPA	.315	4.640	< .001				
	LPSTLR	.272	3.417	.001				
	LACTOBAC	− .162	-2.394	.018				
	PROTECTSCFA	− .176	-2.273	.025				
#3. Current Suicidal Ideation	**Model**				.312	14.165	4/125	< .001
	ACEs	.366	4.565	< .001				
	FPA	.189	2.544	.012				
	Sexual abuse	.172	2.180	.031				
	LACTOBAC	− .179	-2.370	.019				
#4. Recurrence of Illness (ROI)	**Model**				.375	25.216	3/126	< .001
	ACEs	.438	5.891	< .001				
	FPA	.255	3.566	.001				
	API index	.194	2.578	.011				

Note: Significant p values (< .05) are bolded. Coefficient statistics reflect the individual contribution of each explanatory variable, while model statistics reflect the overall fit. β, standardized coefficient; API, atherogenic plasma index; ACEs, adverse childhood experiences; FPA, Functional polarization of Actinobacteria; LPSTLR, LPS-TLR inflammatory pressure; LACTOBAC, Lactobacilli Protective Barrier Module; PROTECTSCFA, Protective SCFA module.

After accounting for the effects of ACEs and the API index, Fig. 6 illustrates the partial regressions (Figs. 6A-C) of the phenome scores (excluding suicidal ideation) on the IDI index. This suggests that the IDI index significantly correlates with phenome scores, with the exception of suicidal ideation (which has a 6.7% explained variance). The API index was significantly correlated with IMFLAMMDYS ($\beta = 0.377$, $t = 5.00$, $p < 0.001$) and ACEs ($\beta = 0.272$, $t = 3.62$, $p < 0.001$) ($F = 21.32$, $df = 2/136$, $p < 0.001$; $R^2 = 0.239$). The partial regression of the API index on INFLAMMDYS is illustrated in Fig. 6D.

Personalized gut dysbiosis profiles

We calculated the score contribution profile scores using the PLS-DA model, which is described in "Construction of 9 functional microbiota modules and models" (based on the 9 functional modules). Sample profiles from six individuals, including one control and five MDD inpatients, are depicted in Fig. 7. The control profile is indicative of the typical control profile, while the patients exhibited a significantly different profile. Nevertheless, the 5 patients exhibited substantial heterogeneity: some exhibited substantial positive contributions from LPSTLP + INFLAMMDYS, while others exhibited contributions from FPA; FPA + INFLAMMDYS; or OPE + LPSTLR + INFLAMMDYS.

Discussion

Gut microbiome diversity in MDD

This research indicates that the gut microbial ecosystem in MDD is not characterized by a global loss of microbial diversity. Recent microbiome investigations in depression have reported comparable findings, including the preservation of alpha diversity in the face of some changes in microbial composition (Sanada, Nakajima et al. 2020, Maes, Vasupanrajit et al. 2023). Systematic reviews have shown that the alpha-diversity results are inconsistent and the pooled effect sizes revealed minimal differences. The heterogeneity is significantly influenced by the sequencing region, methodologies, geography, diet, body mass index, and psychotropic medication (Cheung, Goldenthal et al. 2019, Sanada, Nakajima et al. 2020, Gao, Wang et al. 2023).

Conversely, our beta diversity analyses revealed significant differences in community composition among the groups, suggesting that the microbial communities of MDD patients are structured differently than those of healthy individuals. The majority of studies (Jiang, Ling et al. 2015, Chung, Chen et al. 2019, Nikolova, Smith et al. 2021, Zhang, Yun et al. 2021)have identified beta-diversity differences between MDD and controls, although not all (Naseribafrouei, Hestad et al. 2014, Maes, Vasupanrajit et al.

2023). Kim et al. (Kim, Woo et al. 2025) have recently conducted re-analyses that have confirmed the existence of significant β-diversity differentiation.

This pattern of stable alpha diversity in conjunction with altered beta diversity indicates a reorganization of the gut microbial community, whereby the total number of microbial taxa remains relatively constant, but the relative abundances and ecological interactions among taxa have changed.

Compositional patterns

Another important finding of this investigation is that MDD is distinguished by selective microbial reorganization. Major fermentative lineages, including Lachnospirales and Oscillospirales, were relatively stable across groups and remained highly abundant at the order level, indicating the preservation of the primary anaerobic microbiota of the gut ecosystem. At the family level, a comparable pattern was observed, with dominant families such as Lachnospiraceae remaining prominent, while only a few families exhibited modest but significant changes. This is in accordance with recent reviews and meta-analyses that demonstrate that MDD is frequently linked to the preservation of higher-order community structure, despite the fact that there are measurable differences in specific taxa (Gao, Wang et al. 2023, Maes, Vasupanrajit et al. 2023, Cao, Cheng et al. 2025, Maes, Almulla et al. 2026). While the dominant microbiome framework remained essentially unaltered, specific genera exhibited significant changes, suggesting restructuring with internal composition shifts. This is consistent with previous MDD studies that have reported reductions in certain SCFA-associated genera and enrichment of inflammatory, proteolytic, or mucosa-associated taxa, when global richness is maintained (Kumar, Pramanik et al. 2023, Maes, Vasupanrajit et al. 2023, Zhao, Baranova et al. 2024, Maes, Almulla et al. 2026). The species-level heatmap further refined this signal by demonstrating that a limited number of individual species differed between MDD and controls. Consequently, the most informative MDD signals emerged at the genus level.

Functional microbiome pathways and modules in MDD

The third significant discovery of this study is the identification of functional microbiome pathways and guilds networks that connect microbial metabolism with intestinal barrier integrity, immune-inflammatory, and metabolic processes in MDD. To achieve this objective, we implemented two distinct methodologies: a) the computation of microbial metabolic pathways using the Tax4Fun and PICRUSt functional prediction at KEGG levels 2 and 3; and b) the development of functional genera modules based on their shared functions, which encompassed the regulation of fermentation, host–microbe metabolic interactions, and immune signaling.

The first method showed that the MDD microbiota was more abundant in signal transduction, transport and catabolism, lipid metabolism, environmental stress, glyoxylate metabolism, carbohydrate utilization, lipopolysaccharide biosynthesis, and glycan degradation, suggesting an increased microbial metabolic adaptation and inflammatory potential. Conversely, we detected that healthy controls have increased activity in pathways associated with nucleotide metabolism, translation, transcription, and

replication/repair, suggesting that microbial biosynthetic and cellular maintenance functions were more robust.

These results are consistent with previous microbiome studies in MDD that utilized KEGG-based functional prediction approaches. These studies reported an enrichment of inflammatory and associated neurotoxic pathways, biosynthesis and metabolism of long-chain unsaturated fatty acids; lipopolysaccharide biosynthesis, carbohydrate metabolism, and microbial signaling pathways (Dong, Shen et al. 2021, Shen, Yang et al. 2021, Lin, Li et al. 2023, Cao, Lu et al. 2025, Kozhakhmetov, Kossumov et al. 2025, Du, Qin et al. 2026, Mora-Martínez, Molina-Mendoza et al. 2026). Combined, these studies indicate that gut dysbiosis in MDD might be characterized by a functional reprogramming of microbial metabolism toward adaptive, stress-related, and host-interaction pathways.

Functional modules and features of MDD

Another significant discovery is that we were able to develop functional modules based on microbial genera categorization, such as fermentative guilds, oral pathobionts, metabolic converters, inflammatory or proteolytic taxa, and SCFA producers. Consequently, it was possible to determine which functional microbial modules predict specific components of the MDD phenome and the percentage of variance they account for.

MDD exhibited a substantial increase in four functional modules. (a) the LPSTLR module, which is characterized by an elevated abundance of *Bacteroides, Parabacteroides, Sutterella*, and *Odoribacter*, Gram-negative anaerobes that are frequently linked to proteolytic metabolism. Intestinal barrier disruptions, endotoxin exposure, and systemic immune activation have been associated with an increase in the abundance of these genera. These mechanisms are responsible for inflammatory dysbiosis and host–microbe immune signaling (Maes, Kubera et al. 2008, Simeonova, Ivanovska et al. 2018, Rudzki and Maes 2020, Yu, Meng et al. 2021). b). Module for proinflammatory dysbiosis (INFLAMMDYS). Activation of mucosal immune pathways, endotoxin exposure, proteolytic metabolism, and epithelial stress are all associated with an increased abundance of *Fusobacterium, Porphyromonas, Clostridioides, Eggerthella*, and *Bacteroides*, which is a defining feature of inflammatory gut dysbiosis (Tilg, Zmora et al. 2020). c) The Actinobacteria host-metabolite transformation module (z*Eggerthella* + z*Slackia* − z*Adlercreutzia*). This module is indicative of changes within the Eggerthellaceae family that relate to the metabolism of polyphenols, steroids, and bile acids. *Adlercreutzia* is linked to the production of equol, an anti-inflammatory metabolite that is derived from dietary isoflavones, whereas *Eggerthella* and *Slackia* are frequently enriched in inflammatory states (Clavel, Lagkouvardos et al. 2016). d) The oral–gut translocation module (OPE) (*Fusobacterium, Porphyromonas, Phocea*) is indicative of oral-derived anaerobes that have the capacity to colonize the gut during mucosal inflammation and activate innate immune pathways (Schmidt, Hayward et al. 2019). Two modules were significantly reduced in MDD: a) The Lactobacilli Protective Barrier Module (LACTOBAR)—comprising *Lactiplantibacillus, Limosilactobacillus*, and *Loigolactobacillus*. This module represents lactate-producing commensals that contribute to host defense and microbial cross-feeding in the gut ecosystem by reinforcing epithelial

barrier integrity, producing antimicrobial metabolites, and modulating mucosal immune responses (Zheng, Liwinski et al. 2020). B) The lactate cross-feeding module (*Megasphaera, Mitsuokella, Veillonella*) converts lactate into propionate and other SCFAs, stabilizing microbial fermentation networks and preventing lactate accumulation (Flint, Scott et al. 2012).

We found that the key phenome features of MDD, including OSOD, physiosomatic symptoms, suicidal ideation, and ROI, were strongly associated with these functional modules. Furthermore, these modules (with the exception of the OPE module) could be consolidated into a single composite that is closely linked to MDD and its characteristics. As a result, we constructed an enterotype of MDD that exhibits a pro-inflammatory and leaky gut profile, as well as reduced protection against activated NIMETOX pathways. Importantly, the objective of constructing this enterotype was not to maximize diagnostic prediction, but to derive a robust, biologically interpretable systems biology model that integrates complementary statistical approaches with current knowledge of microbial ecology and host–microbe interactions. The enterotype therefore represents a mechanistically meaningful microbial network rather than a purely predictive classifier. The high discriminatory performance obtained during feature selection primarily supports the robustness and reproducibility of this biological construct, which subsequently allowed mechanistic analyses linking the enterotype to NIMETOX activation, the acute phase inflammatory response, and the depression phenome.

A previous amplicon study also delineated a dysbiotic enterotype of MDD that could be associated with clinical features of MDD and NIMETOX pathways (Maes, Vasupanrajit et al. 2023). A recent shotgun metagenomic study found that the differential abundance of gene families and pathway scores in MDD is indicative of a substantial proportion of the variance in OSOD, ROI, suicidal ideation, neurocognitive impairment, and immune functions (Maes, Almulla et al. 2026).

The functional findings are corroborated by the current observation that the acute phase inflammatory response index was significantly and independently correlated (after adjusting for ACEs) with the INFLAMM module, which includes genera often associated with inflammatory or dysbiotic gut conditions. These taxa are recognized for their role in enhancing immune activation, inducing epithelial stress and mucosal inflammatory responses, which collectively lead to leaky gut (Magnúsdóttir, Ravcheev et al. 2015, Tilg, Zmora et al. 2020, Zheng, Liwinski et al. 2020, Wishart, Guo et al. 2022). Importantly, the enterotype of inflammatory dysbiosis identified in this study was positively linked with the ACE score, indicating that ACEs may affect the formation of this particular dysbiotic microbial composition. Maes et al. (2023) previously identified a microbiome-derived enterotype linked to ACE exposure in MDD inpatients.

Significantly, none of the analyzed taxa at any taxonomic level (order, family, phylum, genus, or species), nor any of the developed functional microbiome modules, exhibited substantial associations with metabolic syndrome, BMI, age, or sex, suggesting that the microbial changes observed are improbable to be attributable to these demographic or metabolic factors. Furthermore, ANCOM-BC analyses indicated negligible drug effects. Selective serotonin reuptake inhibitors exhibited minor inverse correlations with

Haemophilus and *Klebsiella*, accounting for less than 8% of the variation. This finding may indicate a restricted protective impact of SSRIs by inhibiting potentially harmful taxa like *Klebsiella*, which has been previously associated with inflammatory processes in MDD (Maes, Kubera et al. 2008). Overall, the modifications in the gut microbiome and functional modules identified herein seem to be directly linked to MDD, rather than being influenced by concomitant metabolic syndrome, elevated BMI, demographic factors, or the use of psychotropic medications.

The high discriminative performance obtained with the selected genera and functional microbiome modules should not be interpreted as evidence for a clinically applicable diagnostic classifier. Rather, these findings demonstrate that the identified taxa and functionally coherent microbial modules constitute robust, biologically integrated microbial networks that consistently distinguish the gut ecosystems of MDD patients from those of healthy controls. Within a mechanistic systems biology framework, the primary objective was not to maximize diagnostic prediction but to construct a biologically interpretable microbiome enterotype that integrates complementary statistical approaches with current knowledge of microbial ecology and host–microbe interactions. The observed discriminatory performance therefore provides evidence for the biological coherence, robustness, and reproducibility of this systems biology construct, which was subsequently used to elucidate the relationships between gut dysbiosis, NIMETOX activation, the acute phase inflammatory response, and the depression phenome. Thus, the emphasis of the present study is on developing an explainable mechanistic model of gut dysbiosis in MDD rather than a diagnostic prediction or classification algorithm.

Limitations

The present study has limitations that should be considered. First, the cross-sectional design prohibits the inference of causality regarding whether microbiome alterations are secondary consequences of the disorder, lifestyle factors, or whether they contribute to the pathogenesis of MDD. In order to ascertain the temporal relationships between microbiome alterations and the progression or recurrence of depressive episodes, longitudinal studies are necessary. Second, the microbiome analysis was conducted using 16S rRNA amplicon sequencing, which predominantly enables reliable interpretation at the genus level and provides more limited taxonomic resolution. As a result, the functional heterogeneity within genera at the species level cannot be completely resolved. In addition, the functional predictions that are derived from PICRUSt and Tax4Fun are indirect inferences that are based on reference genomes. Consequently, they do not accurately measure metabolic activity or gene expression. Shotgun metagenomic, metatranscriptomic, or metabolomic approaches are needed to obtain more precise insights into microbial metabolic functions. Third, the intestinal microbiome is significantly impacted by a variety of environmental factors, including diet, geography, lifestyle, medication exposure, and other environmental factors. Despite the fact that we have accounted for a variety of covariates, such as smoking status, BMI, metabolic syndrome, and medication status, residual confounding cannot be entirely ruled out. Fourth, the functional microbial modules that were developed in this study were derived from genus-level guilds. Despite their biological plausibility, these modules necessitate

independent validation in other cohorts and countries. The mechanistic pathways that connect gastrointestinal dysbiosis with NIMETOX activation and the phenome of depression may be further clarified by future studies that integrate multi-omics approaches, such as microbiome, metabolomics, and immune biomarkers.

Conclusions

The current study illustrates that MDD is distinguished by a selective restructuring of the gut microbiome, rather than a global loss of microbial diversity. Although the overall richness and evenness of the gut microbiota were comparable between MDD and controls, beta diversity analyses revealed significant differences in the composition of the microbial community, suggesting a reorganization of the gut microbiota. The core microbial backbone remained essentially stable, as evidenced by taxonomic analyses conducted at multiple phylogenetic levels. However, specific genera and species exhibited disease-associated shifts. Depressive microbiomes are enriched in stress-adaptation, environmental sensing, inflammatory signaling, and carbohydrate metabolism pathways, as indicated by functional analyses using Tax4Fun and PICRUSt KEGG pathway predictions.

The principal contribution of the present study is the construction of a robust and biologically interpretable microbiome enterotype that provides mechanistic insight into the interactions between gut dysbiosis, NIMETOX pathways, inflammation, and the depression phenome, rather than the development of a diagnostic prediction algorithm. The development of functionally defined microbial modules demonstrated that pro-inflammatory and proteolytic modules were elevated in MDD, while protective lactobacilli and lactate cross-feeding modules were diminished. Importantly, these functional modules substantially predict key components of the depression phenome, such as OSOD, physiosomatic symptoms, suicidal ideation, and recurrence of illness. We found a significant mechanistic link between gut microbial functions and the acute phase inflammatory response in MDD. The functional microbiome-derived modules establish a framework for personalized medicine therapy, as their profiles significantly differ among MDD patients, indicating unique gut dysbiotic patterns. These differences suggest that MDD individuals demonstrate particular functional abnormalities, like less lactobacilli-mediated barrier protection, or heightened inflammatory and proteolytic microbial activity. Consequently, these module-based profiles may inform targeted, mechanism-driven interventions, including microbiome-directed treatments personalized to the predominant dysbiotic signature in each patient.

IRB approval and consent to participate

This study received permission from the ethics committee of Sichuan Provincial People's Hospital under "Ethics (Research) 2024 – 203" and was conducted in strict adherence to ethical standards and privacy rules. All subjects granted informed written consent.

Declarations

Declaration of interest

The authors have no conflicts of interest.

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Author Contribution

Changyang Meng: writing and review; Jing Li: recruitment of participants and supervision; Mengqi Niu: project coordination and editing; Abbas F. Almulla: editing and visualization, Yiping Luo: patient recruitment; Andre F Carvalho: editing; Annabel Maes: data analysis; Yingqian Zhang: conceptualization, sample processing, data analysis, and review; Michael Maes: supervision, data analysis, writing, review, and editing.

Availability of data

The data underpinning the findings of this investigation is available upon request from the corresponding author (MM).

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Figures

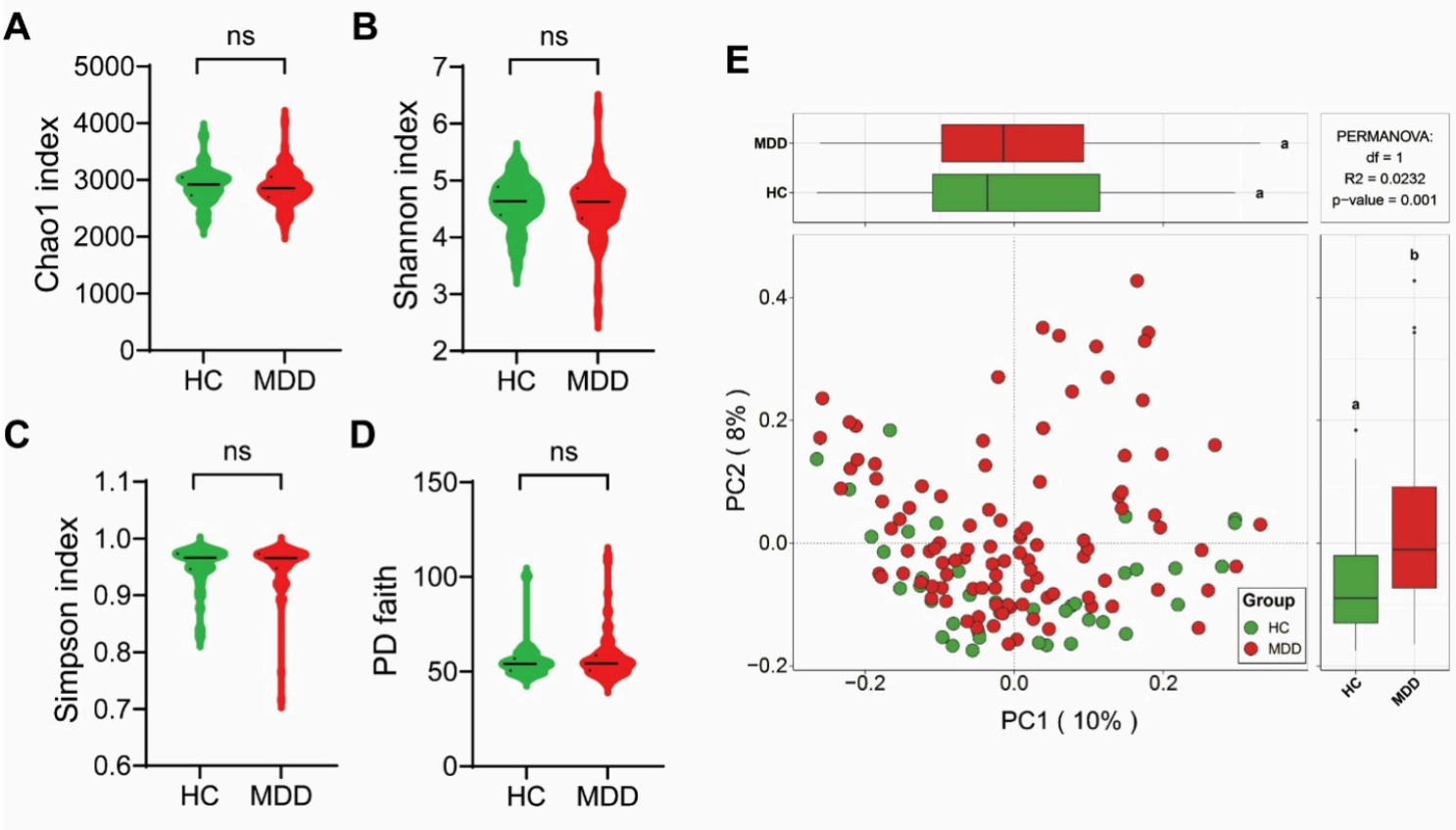


Figure 1

Diversity data. Figure 1A-D: alpha diversities (Chao1, Shannon and Simpson index and PD faith) between major depression (MDD) patients and healthy controls (HC), as determined by their relative abundances. All Mann–Whitney U tests were non-significant (ns). Figure 1E: Bray–Curtis beta diversity index and principal coordinate analysis. The PERMANOVA test was significant.

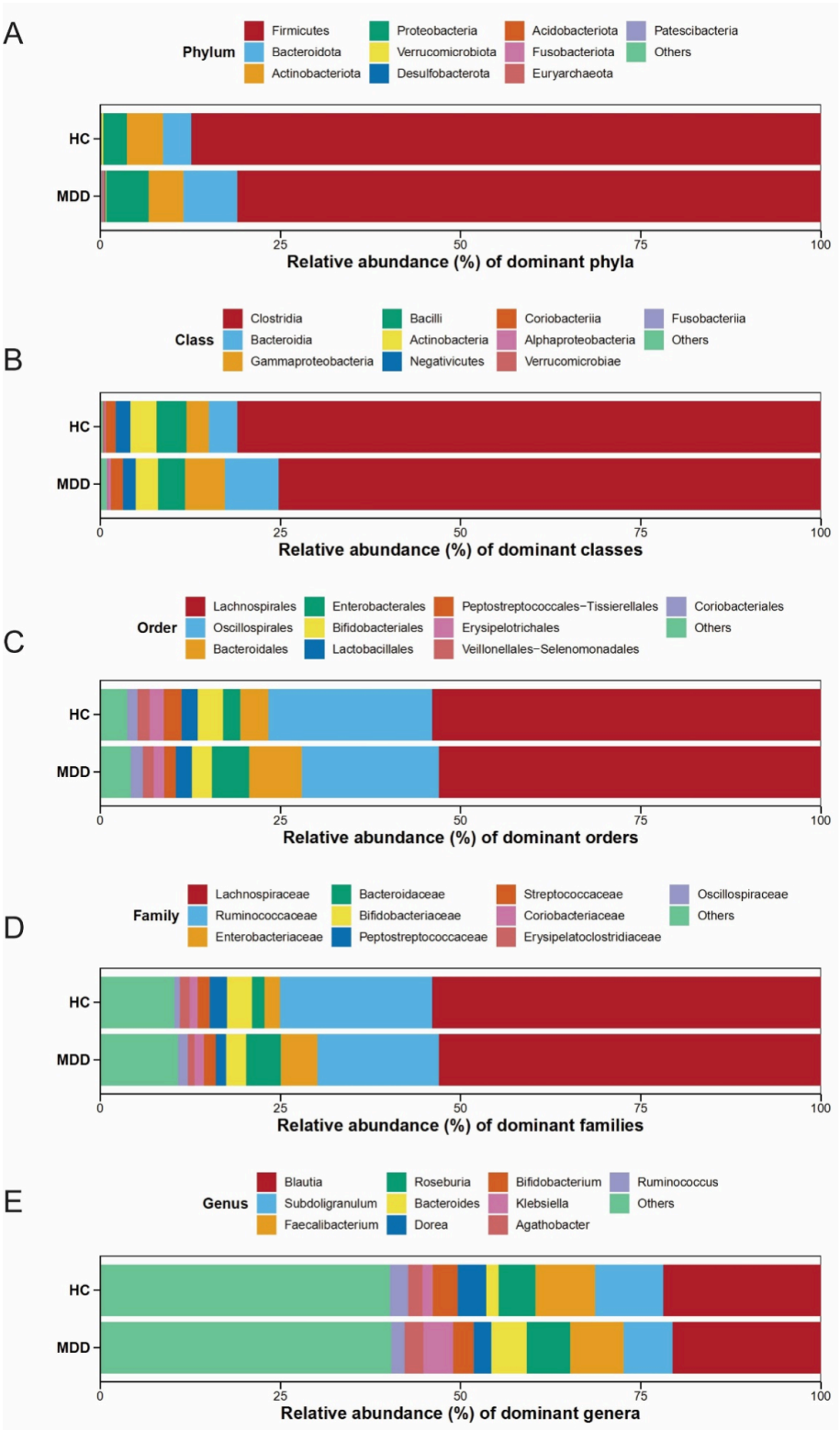


Figure 2

Stacked bar graphs of relative abundance (compositional stacked bar charts) across taxonomic levels. This plot depicts the relative abundance (%) of dominant microbial taxa across taxonomic levels (phylum, class, order, family, and genus) in major depression (MDD) and healthy controls (HC), illustrating compositional differences in gut microbiota structure.

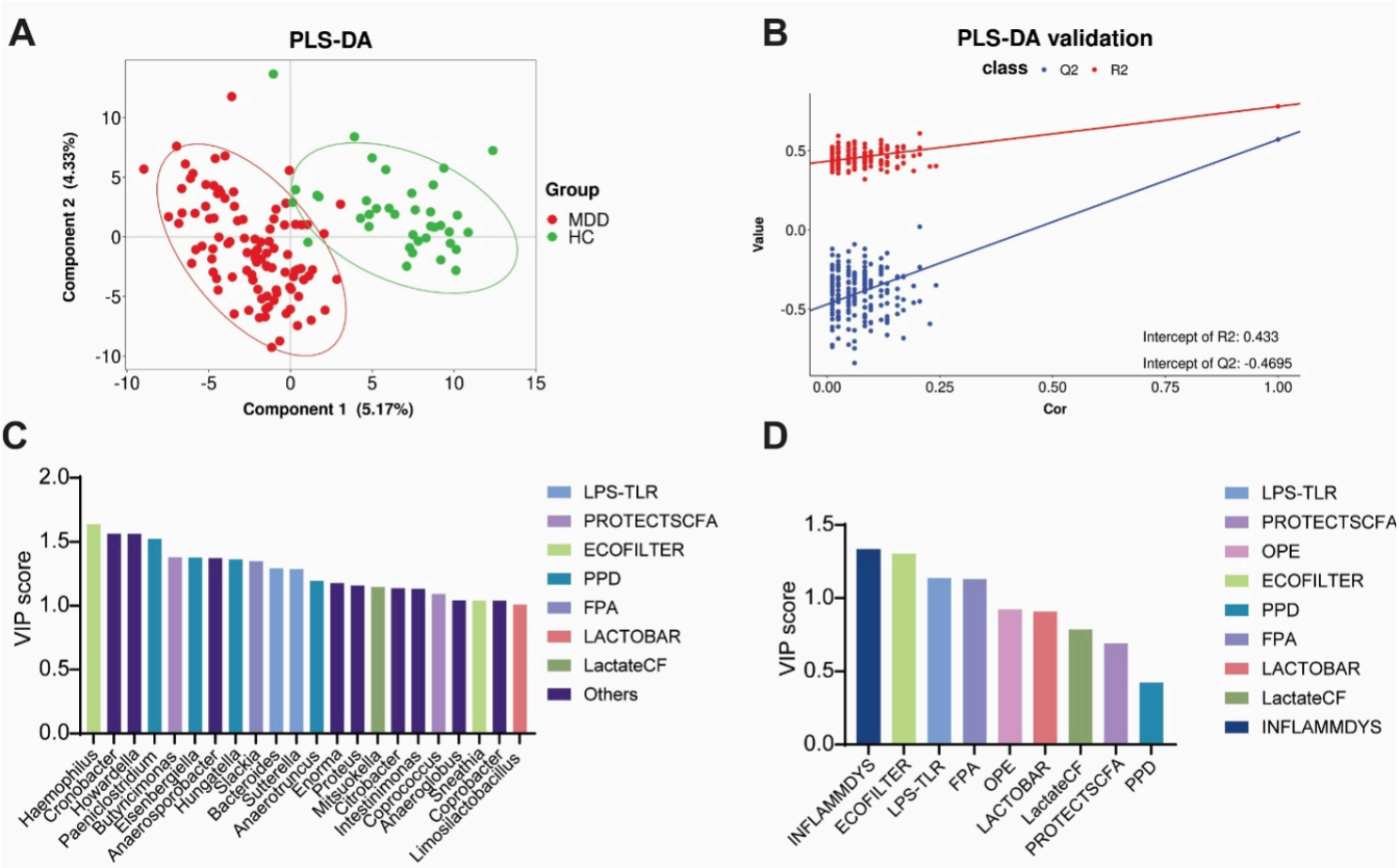


Figure 3

Results of partial least squares discriminant analysis (PLS-DA) separating patients with major depression (MDD) from healthy controls (HC). Figures 3A and 3B show the PLS-DA model and its validation. Figures 3C and 3D show the variable importance in projection (VIP) scores which quantify how much the taxa contribute to the PLS-DA model discriminating MDD for HC. Toward this end we used the 50 curated and selected genera (Figure 3C) and the 9 functional models (Figure 3D, see Table 2 for explanation).

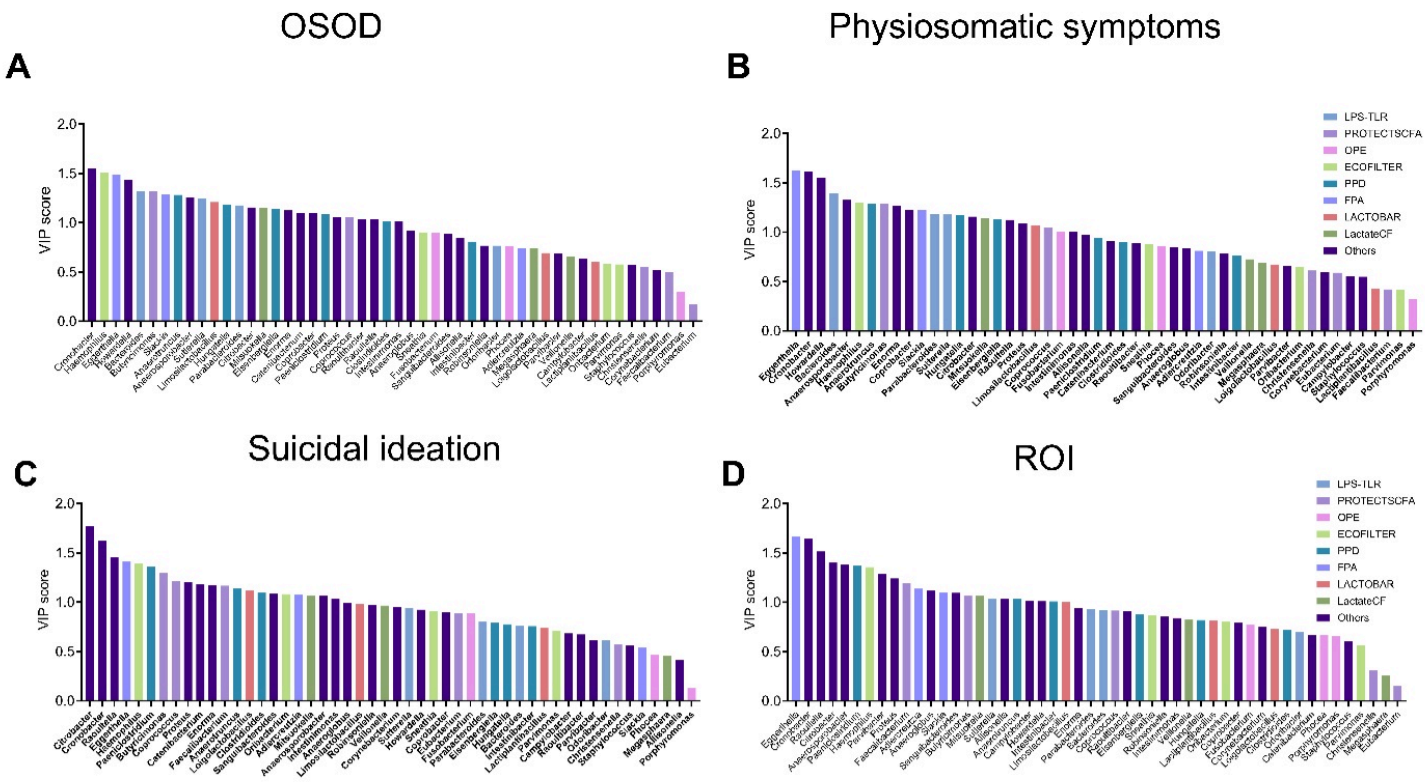


Figure 4

Results of partial least squares regression analysis with the key symptom domains of major depression as dependent variables and gut microbiome genera as explanatory variables. Figures 4A (overall severity of depression, OSOD), 4B, 4C and 4D (recurrence of illness or ROI) show the variable importance in projection (VIP) scores of the top genera significantly predicting the clinical scores.

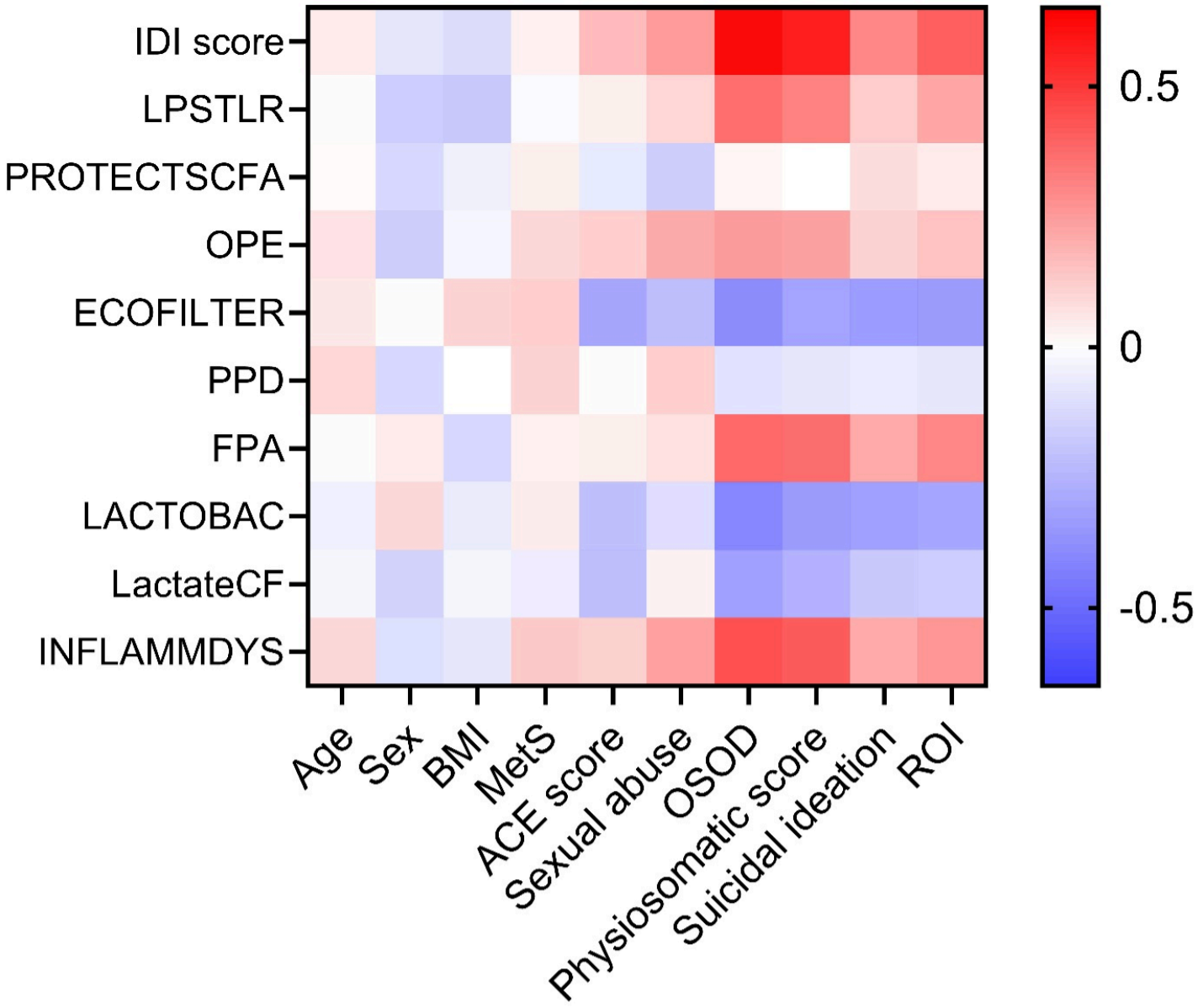


Figure 5

Heatmap showing the correlations between genera-derived functional modules (rows) and clinical, demographic, and phenome variables (columns), including age, sex, body mass index (BMI), metabolic syndrome (MetS), adverse childhood experiences (ACE score), sexual abuse, overall severity of depression (OSOD), physiosomatic symptoms, suicidal ideation, and recurrence of illness (ROI). Color intensity represents the strength and direction of the associations (red = positive; blue = negative), with values corresponding to correlation coefficients. Table 2 lists the different functional modules used here.

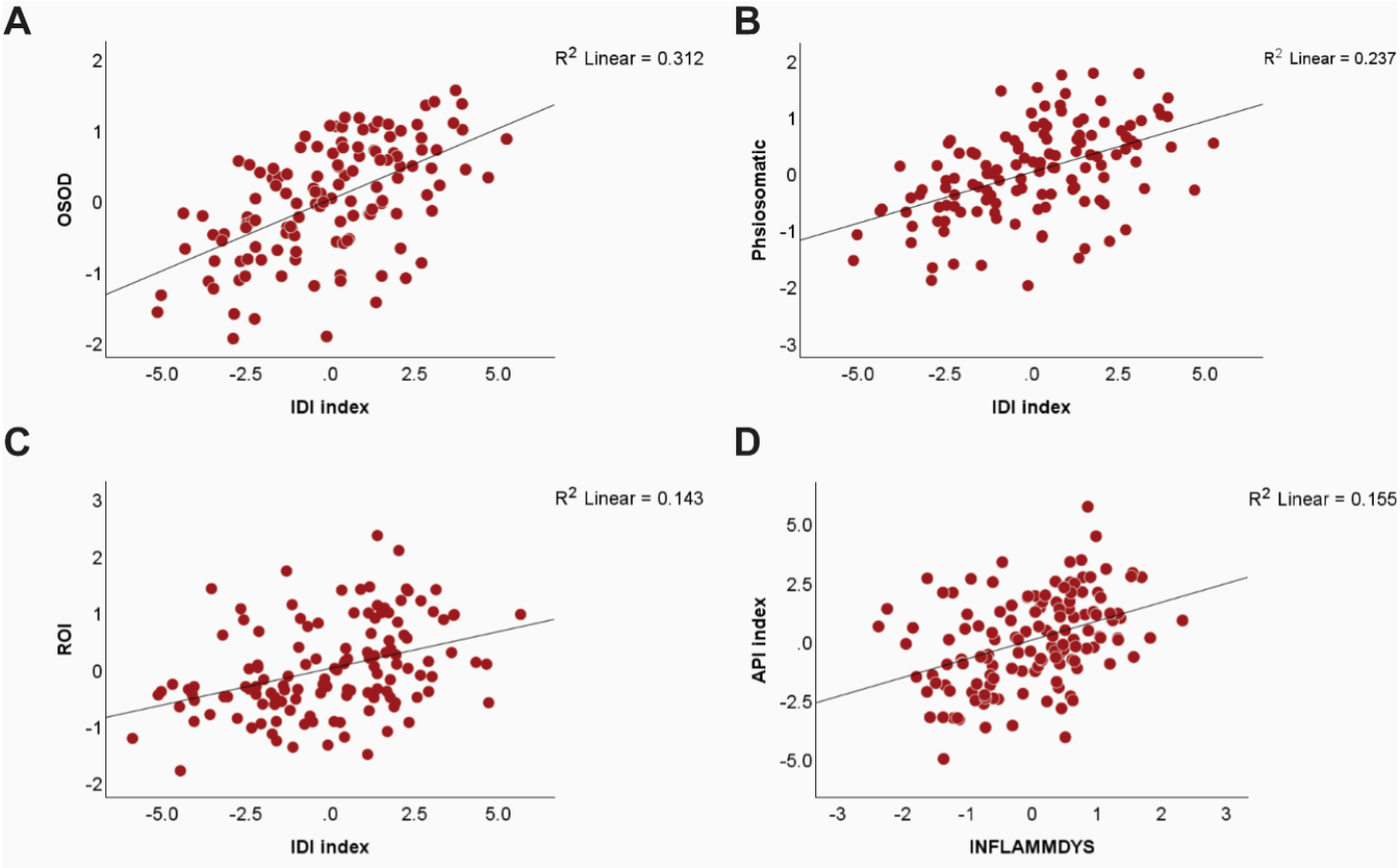


Figure 6

Results of partial regression analysis showing the regressions of overall severity of illness (OSOD) and the inflammatory-dysbiosis (IDI) index (Figure 6A), physiosomatic symptoms on the IDI index (Figure 6B), recurrence of illness (ROI) on the IDI index (Figure 6C), and the acute phase-inflammatory (API) index on the proinflammatory dysbiosis module (INFLAMMDYS) score (Figure 6D).

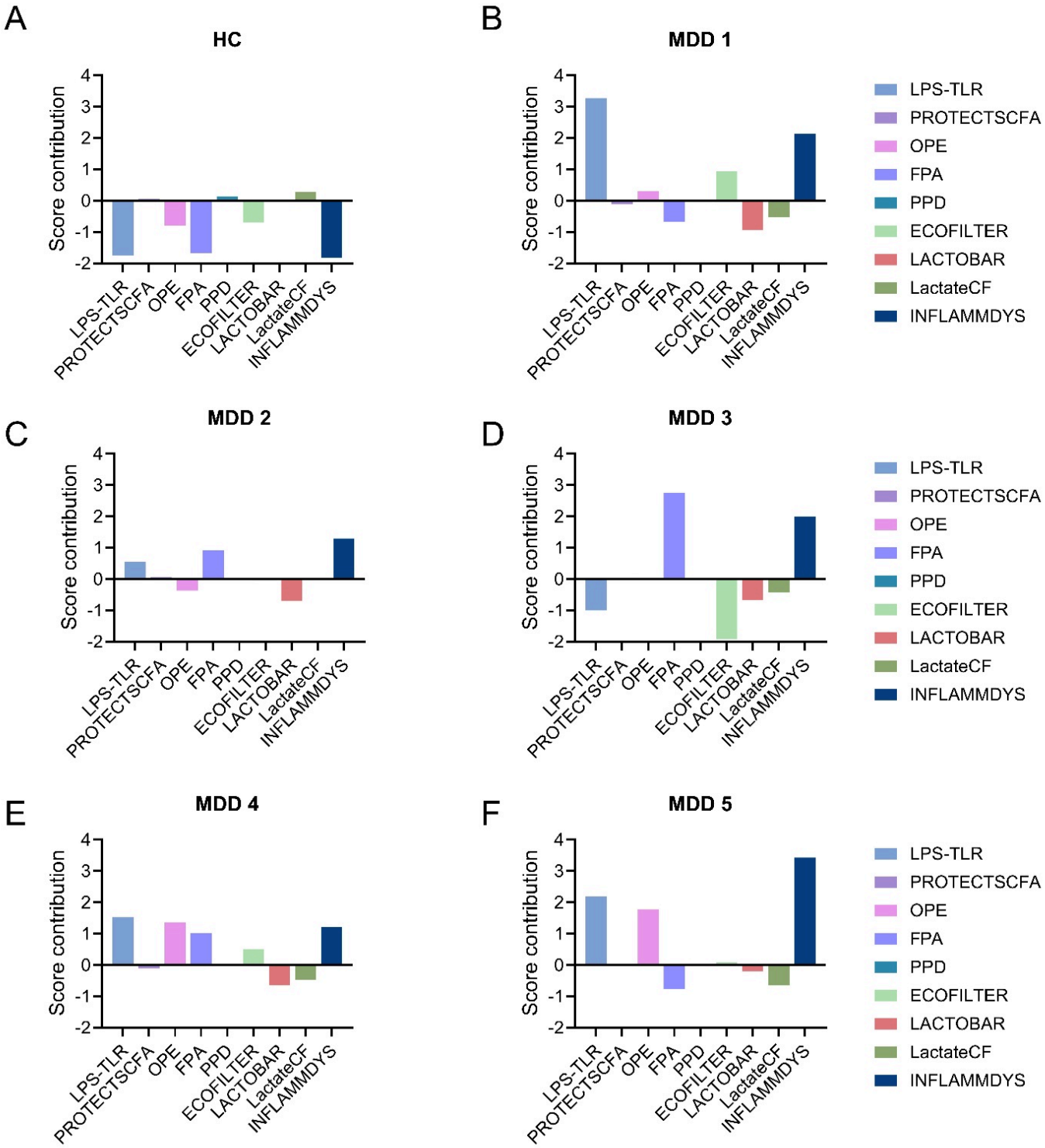


Figure 7

Contribution profile scores using the partial least squares discriminant analysis model shown in Figure 3D. We show the personalized profiles using the 9 functional genera modules (see table 2) from six individuals, namely one healthy control (HC, Figure 7A) and five inpatients with major depression (MDD) (MDD1-MDD5, Figures 7B-7F).

Supplementary Files

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