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Integrated gut microbiome and cross-tissue metabolomic profiling of the gut–spleen–brain axis identifies tryptophan-related networks associated with depressive-like behaviors in a mouse model of chronic restraint stress

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Abstract

Background: Depression is associated with disruptions of gut microbiota and host metabolism. However, the coordination of metabolic changes across the gut, circulation, peripheral immune organs, and brain remains poorly understood. We investigated whether chronic restraint stress (CRS) is accompanied by linked microbial and metabolic alterations along the gut-spleen-brain axis in mice.

Methods: Male C57BL/6J mice were randomly assigned to control or three-week CRS groups. Depressive-like and anxiety-like behaviors were evaluated using a series of specialized behavioral tests: the sucrose preference, forced swim, tail suspension, and open-field tests. Fecal bacterial communities were profiled by 16S rRNA gene sequencing, and untargeted metabolomics were performed on feces, serum, spleen, and hippocampus. Differential-abundance analysis, pathway enrichment, weighted gene co-expression network analysis (WGCNA), and Spearman correlation analysis were used to integrate systematically microbial, metabolic, and behavioral indicators.

Results: CRS reduced sucrose preference and open-field activity and increased immobility time in the forced swim and tail suspension tests. CRS altered gut microbial community structure without affecting alpha diversity. We identified 1,719 fecal, 642 serum, 200 splenic, and 145 hippocampal differential metabolites. Tryptophan metabolism was recurrently enriched across fecal and host compartments. Cross-tissue WGCNA identified blue and turquoise metabolite modules associated with behavioral measures. Correlation networks further linked tryptophan-related metabolites to differential bacterial genera and depressive-like behaviors, with *Corynebacterium* and *Mucispirillum* among the most highly connected microbial nodes.

Conclusions: CRS was associated with gut microbial dysbiosis and coordinated, tissue-specific alterations in tryptophan-related metabolism along the gut-spleen-brain axis. These findings provide a cross-tissue framework for investigating metabolic communication in stress-related behavioral phenotypes. Targeted metabolite validation and causal experiments are required.

Keywords: depression; chronic restraint stress; gut microbiota; tryptophan metabolism; metabolomics; gut-spleen-brain axis

1. Background

Depressive disorders are among the leading global causes of disability and impose substantial functional impairment across the lifespan[1-3]. Clinically, depression encompasses persistent low mood, anhedonia, cognitive and somatic symptoms, yet its presentation, course, and treatment response vary markedly. Although available treatments benefit many patients, a substantial proportion do not achieve sustained remission, leaving a persistent treatment gap[3-8]. This heterogeneity reflects biological complexity involving interacting neural, endocrine, immune, epigenetic, and metabolic processes rather than a single neurotransmitter abnormality[9-12]. Defining how peripheral and central processes become coordinated during chronic stress may therefore reveal biologically coherent pathways for patient stratification and therapeutic development.

The microbiota-gut-brain axis provides a framework for understanding how intestinal ecology may influence stress responsivity and behavior[13-16]. Gut microorganisms can affect the host through microbial metabolites, immune signaling, endocrine pathways, vagal communication, and changes in intestinal barrier function[16]. Experimental evidence supports the biological relevance of this communication. Transfer of fecal microbiota from patients with depression to germ-free mice can reproduce behavioral and metabolic abnormalities[17]. Related rodent and nonhuman-primate studies link depression-associated microbiota to brain proteomic or region-specific metabolic changes[18, 19]. Interventions that alter microbial communities or microbial metabolites can also modify depressive-like behaviors in preclinical models[20-22]. Chronic stress itself reshapes gut microbial composition and fecal metabolic profiles [14, 15, 23, 24]. However, many studies assess microbiota, peripheral metabolism, or the brain separately, making it difficult to identify coordinated cross-compartment patterns.

The spleen may represent an important but comparatively understudied component of this systemic network. As the largest secondary lymphoid organ, it filters blood-borne antigens and supports leukocyte trafficking and coordinated innate and adaptive immune responses[25]. Its immune functions and autonomic connections place it at a potential interface between gut-derived signals and stress-sensitive neural processes. Neuroimmune dysregulation is increasingly implicated in depression, and chronic stress can alter both peripheral immune activity and central inflammatory signaling[26-28]. Moreover, broad-spectrum antibiotics have been shown to affect the gut-microbiota-spleen-brain axis in mice, supporting the possibility that splenic responses participate in microbiota-associated brain changes[29]. Nevertheless, direct evidence for coordinated metabolic remodeling across the gut, spleen, circulation, and brain remains limited.

Tryptophan metabolism is a strong candidate for such cross-tissue coordination. Host and microbial pathways convert tryptophan into serotonergic, kynurenine, and indole metabolites with neural, immune, oxidative, and intestinal-barrier functions[16, 30, 31]. Clinical and preclinical multi-omics studies further link depression to microbiota-associated disturbances in amino acid and tryptophan-related metabolism[32-34]. Because these pathways are distributed across microbial and host compartments, measurements from a single tissue may capture only one component of the response. Multi-compartment metabolomics can instead identify recurrent pathway changes and distinguish shared from tissue-specific signatures. Network approaches such as WGCNA can organize correlated metabolites into modules and relate those modules to behavioral phenotypes without assuming that individual metabolites act independently[35-37]. The unresolved question is whether chronic stress produces

a coordinated tryptophan-related metabolic pattern spanning the intestinal, circulating, immune, and central compartments.

In this study, we tested whether CRS is accompanied by coordinated microbial and metabolic alterations along the gut-spleen-brain axis. Male C57BL/6J mice underwent behavioral phenotyping, fecal 16S rRNA gene sequencing, and untargeted metabolomics of feces, serum, spleen, and hippocampus. We combined compartment-specific pathway enrichment with cross-tissue WGCNA and correlation-network analyses to identify metabolite modules and tryptophan-related associations linked to depressive-like behaviors. This design was intended to generate an integrated, hypothesis-generating map of cross-compartment relationships rather than to establish causal inter-organ signaling.

2. Materials and methods

2.1 Animals

Male C57BL/6J mice (7 weeks old, 22 ± 1 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (China). Mice were housed at 24 ± 1 °C and $50 \pm 10\%$ relative humidity under a 12-h light/dark cycle (lights on, 08:00-20:00), which conforms to the standard feeding environment requirements for experimental mice. Food and water were available ad libitum except during the specified deprivation procedures. All animal experimental procedures were approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (approval no. 22-306) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 CRS paradigm

After 1 week of acclimation, mice were randomly assigned to control (CON) and CRS groups matched for baseline body weight and sucrose preference[20]. CRS was performed as described previously[14, 15]. Briefly, mice were placed in ventilated 50-mL centrifuge tubes (NEST Biotechnology) for 6 h/day (11:00-17:00) for 3 consecutive weeks. The tubes allowed normal breathing and limited postural adjustment but prevented forward or backward movement. After each restraint session, mice were returned to their home cages. Control mice underwent fasting during the corresponding period without restraint.

2.3 Behavioral tests

Before behavioral testing, mice were transferred to a dedicated testing room for acclimation. Specifically, the mice should be acclimated in the behavioral testing laboratory for 30-60 minutes every day in the 3 days prior to the test. The test battery comprised the open-field test (OFT), tail suspension test (TST), forced swim test (FST), and sucrose preference test (SPT). Investigators were blinded to group allocation, and testing followed previously described protocols[14, 15, 21, 27, 38]. Detailed procedures are provided in the Supplementary Materials, and the experimental timeline is shown in Figure 1A. On the second day after behavioral testing, mice received deep anesthesia by intraperitoneal injection of 20% ethyl carbamate (A600448-0250; Sangon Biotech, Shanghai,

China). The dose was 100 μ L per 20 g body weight. Feces, spleen, blood, and hippocampus were collected and stored at -80 °C until analysis.

2.4 16S rRNA sequence analysis

Fecal 16S rRNA gene sequencing was performed by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) according to previously reported procedures [14, 15, 21, 37]. Briefly, the V3-V4 region was amplified using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Amplicons were sequenced on an Illumina NovaSeq 2000 platform. Reads were denoised using DADA2 (version 2024.10) to generate amplicon sequence variants (ASVs), which were taxonomically classified in QIIME 2 (version 2024.10). Alpha-diversity indices included Ace, Chao, Shannon, and Simpson. Bray Curtis beta diversity and LefSe (LDA >2.0 and P < 0.05) were analyzed on the Majorbio Cloud Platform.

2.5 Metabolome analysis based on UPLC-MS/MS

Metabolite extraction, untargeted UPLC-MS/MS, data preprocessing, and annotation were performed by Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) using published procedures[37, 39]. Briefly, peripheral (fecal, serum and spleen) and hippocampus tissue samples were prepared by homogenization, dissociation, and centrifugation, and the resulting supernatants were retained for analysis. The untargeted metabolomics analysis was conducted on a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY UPLC T3 column (100 mm $\times$ 2.1 mm i.d., 1.8 μ m; Waters, USA). Mobile phase A consisted of acetonitrile–water (5:95, v/v) and mobile phase B of 2-propanol–acetonitrile–water (47.5:47.5:5, v/v/v), both supplemented with 0.1% formic acid. The flow rate was 0.40 mL/min, and the column temperature was 40°C. Electrospray ionization (ESI) in positive (3.4 kV) and negative (–3.0 kV) modes. Full MS/dd-MS² acquisition over m/z 70–1050; S-lens RF level 70; capillary temperature 320°C; auxiliary heater 350°C; sheath, auxiliary, and sweep gases at 60, 20, and 1 arbitrary unit. Full MS resolution: 60,000 (AGC 1 $\times$ 10⁶, max injection 50 ms); MS² resolution: 15,000 (AGC 1 $\times$ 10⁵, max injection 22 ms). Top 5 precursors fragmented with 1.5 m/z isolation window and stepped NCE (20, 40, 60 eV) in data-dependent acquisition mode (for the detailed protocol, see the Supplementary material).

Raw LC-MS data were processed in Progenesis QI (Waters Corporation, Milford, USA) using baseline filtering, peak detection, alignment, retention-time correction, and normalization. Quality-control samples were used to monitor analytical stability. Metabolites were annotated against the Human Metabolome Database, METLIN, and the in-house Majorbio Database. The resulting matrices were processed on the Majorbio Cloud Platform (<https://cloud.majorbio.com>). Features detected in at least 80% of samples in either group were retained. Missing values were imputed with the minimum value in the corresponding matrix. Intensities were normalized to the total signal within each sample, and variables with a quality-control relative standard deviation >30% were excluded. The data was then log₁₀ transformed. Principal component analysis and OPLS-DA were performed using ropls R package (version 1.4.0) and ggplot2 (version 4.0.3), with sevenfold cross-validation. Metabolites with a VIP score >1.0 and P < 0.05 were considered significant differential and were included in pathway-enrichment analyses. The extraction of metabolites from biological samples, untargeted UPLC-MS/MS analysis, and data preprocessing and annotation were carried out by Major Bio-pharm Technology Co., Ltd (Shanghai, China), adhering to previously published protocols [37, 39].

2.6 Weighted gene co-expression network analysis

Weighted gene co-expression network analysis (WGCNA) was used to identify metabolite modules associated with depressive-like behavioral (DLB) phenotypes across the gut–spleen–brain axis. Analyses were performed in R using the WGCNA package (version 1.74), and comparable applications to multi-compartment metabolomic data have been reported previously[37, 40]. A signed co-abundance network was constructed from the cross-tissue differential-metabolite matrix using biweight midcorrelation(“bicor”). The soft-thresholding power was selected by jointly considering scale-free topology and mean connectivity. Adjacency values were converted to a signed topological overlap matrix, and modules were identified by dynamic tree cutting with a minimum module size of 30. Modules with highly correlated eigengenes were merged at a cut height of 0.25. Module eigengenes were then correlated with behavioral phenotypes using biweight midcorrelation. Modules associated with at least one depressive-like behavioral phenotype at $P < 0.05$ were designated phenotype-associated modules, and their metabolites were pooled for pathway analysis.

2.7 Statistical analysis

Statistical analyses were performed using SPSS 21.0 (SPSS, Chicago, IL, United States) and R software (V4.5.2). Distributional normality and variance homogeneity were assessed using the Shapiro-Wilk and Levene test, respectively. Between group comparisons used two-tailed Student’s T-tests for normally distributed data with homogeneous variances and Mann-Whitney U tests otherwise. Alpha-diversity indices and bacterial relative abundances were compared using Mann-Whitney U tests. Beta-diversity differences were evaluated by analysis of similarities (ANOSIM) on Bray-Curtis distances at the ASV level using the “vegan R package. Differential bacterial taxa were identified by LEfSe using an LDA threshold >2.0 and $P < 0.05$. Differential metabolites were defined by $VIP > 1.0$ and $P < 0.05$. Spearman rank correlations were used to evaluate associations among behavioral measures, bacterial taxa, and metabolites, and networks were visualized in Cytoscape version 3.10.4. Data is presented as the mean $\pm$ standard error of the mean. Unless otherwise stated, $P < 0.05$ was considered statistically significant.

3. Results

3.1 CRS induces depressive-like and anxiety-like behaviors

To minimize initial variability, mice were randomized into control and CRS groups according to baseline body weight and sucrose preference, ensuring no significant pre-existing differences. The experimental timeline is shown in Figure 1A. . After 1 week of acclimation and 3 weeks of CRS, behavioral tests were conducted over 4 days before tissue collection. In the OFT, CRS reduced total distance ($z = -2.656$, $P = 0.007$; Figure 1B), center entries ($t = 3.167$, $P = 0.004$; Figure 1C), and center time ($z = -2.829$, $P = 0.0041$; Figure 1D), while increasing corner time ($z = 2.714$, $P = 0.0061$; Figure 1E). CRS also increased immobility time in the TST ($t = -4.584$, $P < 0.001$; Figure 1F) and FST ($t = -4.914$, $P < 0.001$; Figure 1G). Baseline sucrose preference did not differ between groups ($t = -0.002$, $P = 0.999$; Figure 1H), whereas sucrose preference was lower after CRS ($t = 3.550$, $P = 0.003$; Figure 1I). Body-weight trajectories are shown in Figure 1J (week-specific comparisons: $t = 0.926$, $P = 0.368$; $t = 7.519$, $P < 0.001$; $t = 11.396$, $P < 0.001$; and $t = 7.652$, $P < 0.001$). Collectively, these results indicated that mice exposed to CRS exhibited depressive-like and anxiety-like behaviors.

3.2 CRS alters gut microbial community structure

Fecal 16S rRNA gene sequencing generated 794,125 high-quality reads from 16 samples, with a mean read length of 419.35 bp. Alpha-diversity indices did not differ between the CON and CRS groups (Figure S1A-D). In contrast, beta-diversity analyses indicated significant between-group differences ($P = 0.022$; Figure 2A). Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) further separated the groups (ANOSIM: $R = 0.2315$, $P = 0.009$ and $R = 0.2315$, $P = 0.008$, respectively; Figure 2B). Bacteroidota, Bacillota, and Actinomycetota were the dominant phyla in both groups. Relative abundances of Bacteroidota ($P = 0.014$) and Actinomycetota ($P = 0.031$) were higher after CRS, whereas Bacillota was lower ($P = 0.007$; Figure 2C and Figure S1E). LEfSe identified enrichment of Bacteroidota, Bacteroidia, Bacteroidales, Muribaculaceae, and norank_f_Muribaculaceae in CRS mice. Bacillota, Erysipelotrichales, Bacilli, norank_o_Clostridia_UCG-014, and Clostridia_UCG-014 were enriched in controls (Figure 2D and Table S1). Fifteen genera differed between groups. In particular, norank_f_Muribaculaceae was more abundant after CRS, whereas norank_o_Clostridia_UCG-014, Allobaculum, and norank_o_RF39 were less abundant (Figure 2E and Table S2). Thus, CRS altered gut microbial community composition without reducing alpha diversity.

Subsequently, we examined associations between differential genera and behavioral phenotype using Spearman correlations with nominal $P < 0.05$ and $|r| > 0.5$. Differential genera were most frequently associated with sucrose preference (SP) and immobility time. Nine, six, and ten genera were associated with SP, immobility time (IT) in the FST and TST, respectively (Figure S2). No retained associations involved center time (CT) or corner time (CoT). *Corynebacterium* correlated positively with IT in the FST ($r = 0.627$, $P = 0.009$) and TST ($r = 0.581$, $P = 0.018$). It correlated negatively with total distance ($r = -0.522$, $P = 0.038$), center entries ($r = -0.555$, $P = 0.026$), and sucrose preference ($r = -0.558$, $P = 0.025$). norank_o_Clostridia_UCG-014 ($r = -0.534$, $p = 0.033$), *Turicimonas* ($r = -0.726$, $p = 0.001$), *Psychrobacter* ($r = -0.522$, $p = 0.038$), and *Streptococcus* ($r = -0.578$, $p = 0.019$) were negatively associated with IT in the TST. These genera were also positively associated with SP.

3.3 CRS alters the fecal metabolome

A total of 5,109 fecal metabolites were annotated (Table S3). OPLS-DA separated CRS and CON samples without overlapping, indicating distinct fecal metabolic profiles (Figure 3A). A 200-permutation test did not indicate model overfitting (Figure 3B). Using $VIP > 1.0$ and $P < 0.05$, we identified 1,719 differential fecal metabolites, including 1,282 increased and 437 decreased features (Figure 3C and Table S4). Most differential metabolites were assigned to the KEGG metabolism category (Figure 3D). Hypergeometric testing identified 25 enriched KEGG level 3 pathways (Figure 3E). Ten of those pathways involved amino acid metabolism. These included alanine, aspartate, and glutamate metabolism ($P = 0.006$), cysteine and methionine metabolism ($P = 0.001$), and arginine biosynthesis ($P = 0.020$). Other pathways included lysine degradation ($P = 0.002$), tyrosine metabolism ($P = 0.004$), phenylalanine metabolism ($P = 0.001$), and tryptophan metabolism ($P = 0.047$). Beta-alanine metabolism ($P = 0.025$), glutathione metabolism ($P = 0.003$), and D-amino acid metabolism ($P = 0.0415$) were also enriched.

3.4 CRS alters metabolite profiles in the spleen, serum, and hippocampus

Untargeted metabolomics annotated 2,592 splenic, 2,354 serum, and 1,036 hippocampal metabolites (Tables S5, S7, and S9, respectively). OPLS-DA score plots showed clear separation between the CON and CRS groups in tissues and serum (Figure S3A-C). Two-hundred-round permutation tests did not indicate model overfitting (Figure S3D-F). Using VIP >1.0 and $P < 0.05$, we identified 200 splenic, 642 serum, and 145 hippocampal differential metabolites (Figure 4A-C and Tables S6, S8, and S10). The Venn diagram showed 27 metabolites shared between serum and spleen, 11 between hippocampus and serum, and 3 between hippocampus and spleen. One metabolite was shared across all three specimens. A further 171, 605, and 132 metabolites were unique to the spleen, serum, and hippocampus, respectively (Figure 4D).

3.5 KEGG pathway analysis of differential metabolites across host compartments

To evaluate the biological significance of the identified metabolites, we conducted KEGG topology-based enrichment analysis using Majorbio Cloud Platform and visualized via the ‘ImageGP’ R package[21]. KEGG topology-based enrichment analysis identified 41 altered pathways across the spleen, serum, and hippocampus, including 8 in the spleen, 18 in serum, and 15 in the hippocampus (Figure 4E and Table S11). Tryptophan metabolism was altered in all three compartments. Glycerophospholipid, glutathione, and arachidonic acid metabolism were shared between the spleen and hippocampus. Pantothenate and CoA biosynthesis, biosynthesis of cofactors, and phenylalanine, tyrosine and tryptophan biosynthesis were shared between serum and hippocampus. Linoleic acid metabolism and phenylalanine metabolism were shared between serum and spleen.

3.6 WGCNA identifies behavior-associated metabolic modules

To identify coordinated metabolic alterations associated with DLB, we constructed a cross-tissue weighted metabolite co-abundance network. The network included differential metabolites from feces, serum, spleen, and hippocampus. A soft-thresholding power of 14 was selected to construct a signed scale-free network (Figure S4A). Hierarchical clustering identified seven non-grey modules: turquoise, blue, brown, yellow, green, red, and black (Figure 5A). Module-trait analysis showed that all seven modules were associated with at least one DLB (Figure 5B). Notably, the seven-module showed strong associations with IT in the TST and FST; four modules were associated with SP, and whereas only the red module was associated with CE. The turquoise and blue modules were the largest, containing 1,447 and 860 metabolites, respectively (Figure 5C and Table S12). These findings identified the blue and turquoise modules as the principal behavioral phenotype-associated metabolic modules. Moreover, the blue module showed the broadest pattern of behavioral associations. The blue module eigengene correlated negatively with IT in the TST ($r = -0.874$, $P = 9.53 \times 10^{-6}$) and FST ($r = -0.795$, $P = 0.0002$). It correlated positively with SP ($r = 0.636$, $P = 0.0081$). The turquoise module was also positively associated with IT in the FST ($r = 0.813$, $P = 0.0001$). Therefore, we prioritized the blue and turquoise modules for pathway analysis. These modules and their union were enriched for linoleic acid, cysteine, and methionine metabolism, purine metabolism, and tryptophan metabolism (Figure 5D and Table S13).

3.7 Cross-tissue associations among gut microbiota, metabolites, and behaviors

Because tryptophan metabolism recurred across compartment-specific enrichment and network analyses, we examined correlations among tryptophan-related metabolites, differential bacterial genera, and behavioral measures. Using nominal $P < 0.05$ and $|r| > 0.5$, we retained 138 metabolite-behavior or metabolite-bacterial taxon

associations, including 69 positive and 69 negative correlations (Figure 6A). Of these, 53 involved behavioral measures and 85 involved bacterial taxa. Immobility time accounted for most behavioral associations, including 19 involving the FST and 17 involving the TST. Most were negative correlations (14/19 for the FST and 13/17 for the TST).

IT in the FST showed its strongest inverse correlation with serum indole pyruvate ($r = -0.794$, $P = 2.39 \times 10^{-4}$), whereas IT in the TST was most strongly inversely associated with hippocampal 5-(2'-carboxyethyl)-4,6-dihydroxypicolinate ($r = -0.779$, $P = 3.72 \times 10^{-4}$). Sucrose preference correlated positively with fecal 4-(2-aminophenyl)-2,4-dioxobutanoic acid ($r = 0.606$, $P = 0.0129$). UCG-005 exhibited the strongest positive correlation with hippocampal 5-hydroxyindoleacetate ($r = 0.898$, $P = 2.28 \times 10^{-6}$), while *norank_f_Muribaculaceae* showed the strongest negative correlation with fecal 4-(2-aminophenyl)-2,4-dioxobutanoic acid ($r = -0.812$, $P = 1.34 \times 10^{-4}$), including splenic 5-Hydroxyindoleacetic Acid ($r = -0.571$, $P = 0.0210$). Retained associations involved fecal ($n = 55$), serum ($n = 43$), hippocampal ($n = 27$), and splenic ($n = 13$) metabolites. These results indicate compartment-spanning relationships among tryptophan-related metabolites, bacterial taxa, and behavior.

An integrated network was constructed to characterize the topology of these associations (Figure 6B). *Corynebacterium* (degree = 0.359, closeness = 0.520) emerged as the most prominent hub node, suggesting its dual role as both a highly connected node and a critical bridge between distinct network modules. *Mucispirillum* was the second most connected microbial node (degree centrality = 0.308) and was linked to fecal, serum, and hippocampal metabolites. Among metabolites, 5-hydroxytryptophan (degree = 0.282, closeness = 0.520) and 4-(2-aminophenyl)-2,4-dioxobutanoic acid (degree = 0.256, closeness = 0.505) occupied central positions. UCG-005 (degree = 0.256; closeness = 0.459) and *norank_f_Muribaculaceae* (degree = 0.251; closeness = 0.459) were additional central microbial nodes. Behaviorally, IT in the TST (degree = 0.256, closeness = 0.520) showed the greatest network integration among behavioral measures (degree = 0.256; closeness = 0.520). These centrality measures prioritize candidate taxa and metabolites for mechanistic follow-up but do not establish directional signaling between compartments.

4. Discussion

This study integrated fecal 16S rRNA gene sequencing with untargeted metabolomics of feces, serum, spleen, and hippocampus in a CRS mouse model. CRS induced depressive-like and anxiety-like behavioral, altered gut microbial community structure, and produced extensive compartment-specific metabolic changes. Across pathway enrichment, WGCNA, and correlation analyses, tryptophan metabolism emerged as a recurrent feature linking bacterial taxa, host metabolites, and behavioral measures. These findings support coordinated metabolic remodeling along the gut-spleen-brain axis during chronic stress, while remaining associative rather than mechanistic.

CRS altered beta diversity and several bacterial taxa without changing alpha diversity, indicating community reorganization rather than a uniform loss of richness. *Muribaculaceae*, *Corynebacterium*, and several *Clostridia*-related taxa were also associated with behavioral measures. These observations are consistent with reports of community-level disruption but variable taxon-specific signatures across chronic stress and depression models [14, 15, 23, 24, 41, 42]. Differences in stress paradigms, housing, diet, sequencing workflows, and host age may

contribute to incomplete taxonomic concordance. Because this study did not manipulate individual taxa or transfer microbiota, the observed relationships do not establish microbial causality.

The metabolomic findings extend microbial observations by demonstrating altered profiles in feces, serum, spleen, and hippocampus. Tryptophan metabolism was the only pathway altered across all three host compartments and was also enriched among fecal differential metabolites. Tryptophan-derived compounds participate in serotonergic, kynurenine, and microbial indole pathways with neural, immune, and barrier-related functions[16, 30, 31]. Human multi-omics and metabolomic studies further associate depression with disturbances in amino acid and tryptophan-related metabolism[32-34]. Their recurrence across compartments therefore provides a plausible framework for stress-associated metabolic communication. However, untargeted annotation and pathway enrichment cannot establish metabolite identity, source, flux, or transport direction. Targeted quantification and isotope-tracing studies are needed to determine whether gut-derived metabolites reach the spleen, circulation, or brain.

WGCNA identified blue and turquoise metabolite modules associated with sucrose preference and immobility time, supporting coordinated rather than isolated metabolic changes. The correlation network further connected tryptophan-related metabolites to bacterial genera and behavioral phenotypes. *Corynebacterium* and *Mucispirillum* were highly connected microbial nodes, while 5-hydroxytryptophan and related metabolites occupied central network positions. Centrality analysis is useful for prioritizing candidates, but it does not demonstrate that these taxa or metabolites transmit signals between tissues. The absence of direct immune, endocrine, intestinal-barrier, and neural measurements also prevents assignment of a specific gut-spleen-brain route.

The study has several strengths, including multidimensional behavioral phenotyping, four-compartment metabolomics, and integration of microbial and metabolic data from the same experimental framework. Several limitations should temper interpretation. Only male mice and one stress paradigm were studied, and the relatively small omics groups may limit generalizability and network stability. Untargeted metabolite annotations require confirmation with authentic standards. In addition, nominal P-value thresholds without reported multiple-testing correction may increase false-positive findings in high-dimensional analyses. Cross-sectional correlations cannot establish temporal sequence or causality. Future studies should include both sexes, independent cohorts, multiple-testing control, targeted validation, and perturbation of prioritized taxa or metabolites. Taken together, the results position tryptophan metabolism as a candidate cross-tissue pathway associated with CRS-induced behavioral abnormalities. The principal contribution is an integrated map of testable relationships across the gut, spleen, circulation, and hippocampus, rather than proof of a microbiota-mediated inter-organ mechanism.

5. Conclusion

In conclusion, our integrative multi-omics approach provides a tissue-resolved landscape of host-microbiota crosstalk under chronic stress, revealing that tryptophan metabolism is consistently disrupted across fecal, serum, splenic, and hippocampal compartments despite unaltered microbial alpha diversity. Network analyses prioritized specific bacterial taxa (such as *Corynebacterium*, *Mucispirillum*, UCG-005, and norank_f__Muribaculacea) and metabolites (such as 5-hydroxytryptophan and 4-(2-aminophenyl)-2,4-dioxobutanoic acid) as behavior-associated hubs that warrant mechanistic follow-up. Beyond these biological insights, our analytical framework offers a

transferable strategy for reducing high-dimensional multi-omics data to experimentally tractable hypotheses. However, because all cross-compartment relationships are correlational, causal inter-organ signaling must be established through targeted metabolite tracing, microbiota perturbation, and independent validation in sex-balanced cohorts and alternative stress models.

List of abbreviations

ANOSIM, analysis of similarities; ASV, amplicon sequence variant; CON, control; CRS, chronic restraint stress; DLB, depressive-like behavior; IT, immobility time; FST, forced swim test; SPT, sucrose preference test; SP, sucrose preference; OFT, open-field test; CT, center time; CoT, corner time; TST, tail suspension test. KEGG, Kyoto Encyclopedia of Genes and Genomes; OPLS-DA, orthogonal partial least-squares discriminant analysis; PCoA, principal coordinate analysis; UPLC-MS/MS, ultra-high-performance liquid chromatography-tandem mass spectrometry; VIP, variable importance in projection; WGCNA, weighted gene co-expression network analysis.

Declarations

Availability of data and materials

Raw 16S rRNA gene sequence data are available in the NCBI Sequence Read Archive under BioProject accession PRJNA1497328. All other data is available from the corresponding authors upon reasonable request.

Authors' contributions

QM and JLP performed the formal analysis and drafted the manuscript. FLD and CJZ contributed to the method and initial drafting. ZJX, YL and CGL contributed to the method and visualization. HQW and YZZ supervised the study and contributed to the method. RRH reviewed and edited the manuscript. JXP and XG conceptualized and administered the project and reviewed and edited the manuscript. All authors read and approved of the final manuscript.

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Competing interests

The authors declare no competing interests.

Trial registration

Not applicable.

373 Ethics approval and consent to participate

374 All animal procedures were approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing
375 Medical University (approval no. 22-306) and were conducted in accordance with the National Institutes of Health
376 Guide for the Care and Use of Laboratory Animals.

377 Consent for publication

378 Not applicable.

379 Acknowledgements

380 Not applicable.

381 **Figure legends**

382 **Figure 1. CRS induces depression-like and anxiety-like behaviors in mice.** (A) Experimental timeline. After
383 1 week of acclimation, mice in the CRS group underwent restraint for 6 h/day for 3 weeks. Behavioral testing
384 comprised the OFT, TST, FST, and SPT, followed by tissue collection. (B–E) OFT outcomes: total distance (B),
385 center entries (C), center time (D), and corner time (E). (F, G) Immobility time in the TST (F) and FST (G). (H, I)
386 Sucrose preference at baseline (H) and after CRS (I). (J) Body weight during the modeling period. Data are
387 presented as mean $\pm$ SEM. A two-tailed Student's t-test was used for normally distributed data with homogeneous
388 variance; otherwise, the Mann-Whitney U test was used. n = 12 per group. *P < 0.05, **P < 0.01, ***P < 0.001;
389 ns, not significant. CON, control; CRS, chronic restraint stress; FST, forced swim test; OFT, open-field test; SPT,
390 sucrose preference test; TST, tail suspension test.

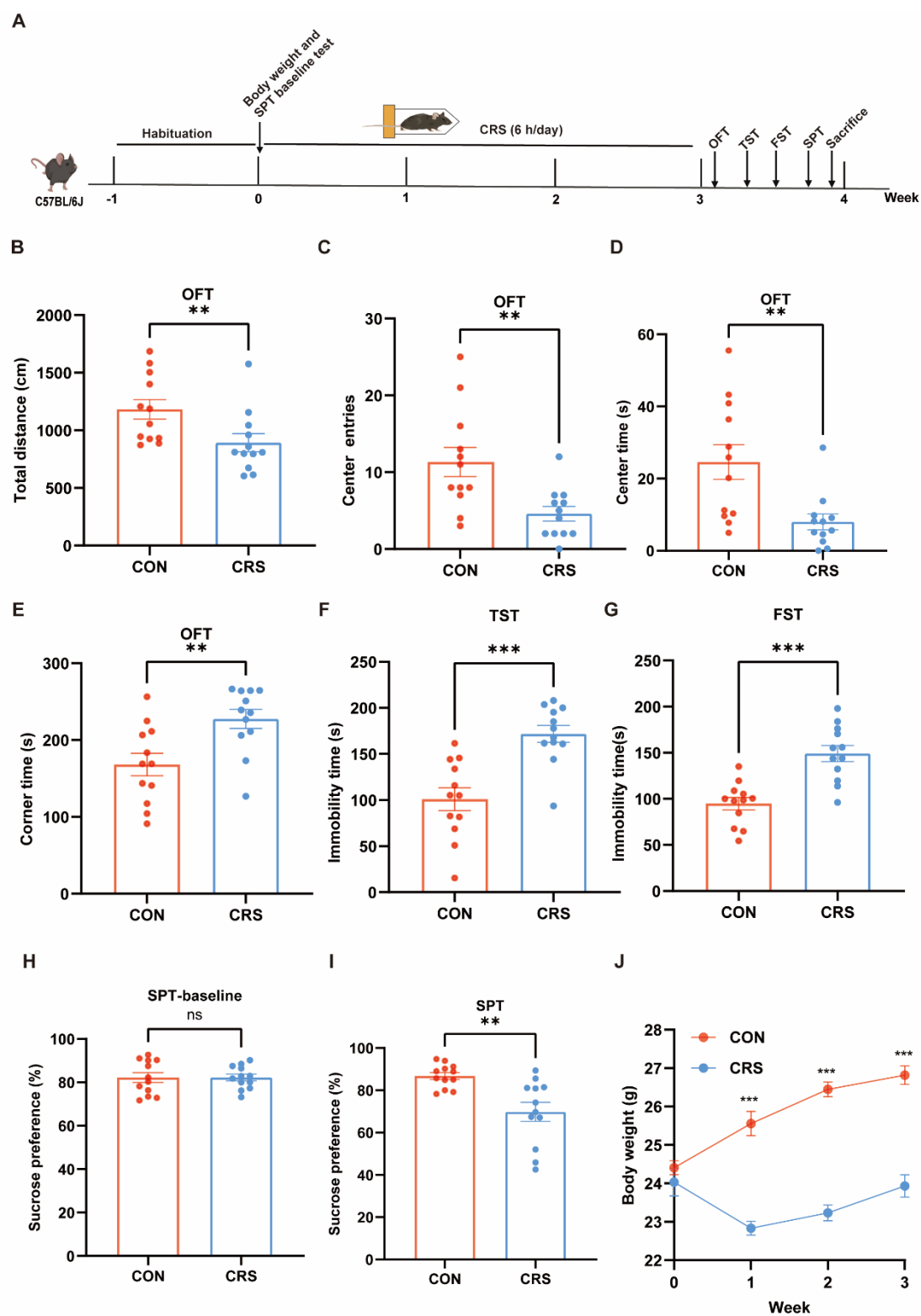


Figure 3. CRS alters the fecal metabolome in mice. (A) OPLS-DA score plot of fecal metabolomic profiles. (B) A 200-permutation test used to assess model over-fitting. (C) Volcano plot of differential fecal metabolites identified using VIP > 1.0 and P < 0.05. (D) KEGG classification of annotated differential metabolites. (E) KEGG level 3 pathway enrichment based on a hypergeometric test; bubble size represents pathway impact, and color represents the P value. n = 8 per group. KEGG, Kyoto Encyclopedia of Genes and Genomes; OPLS-DA, orthogonal partial least-squares discriminant analysis; VIP, variable importance in projection.

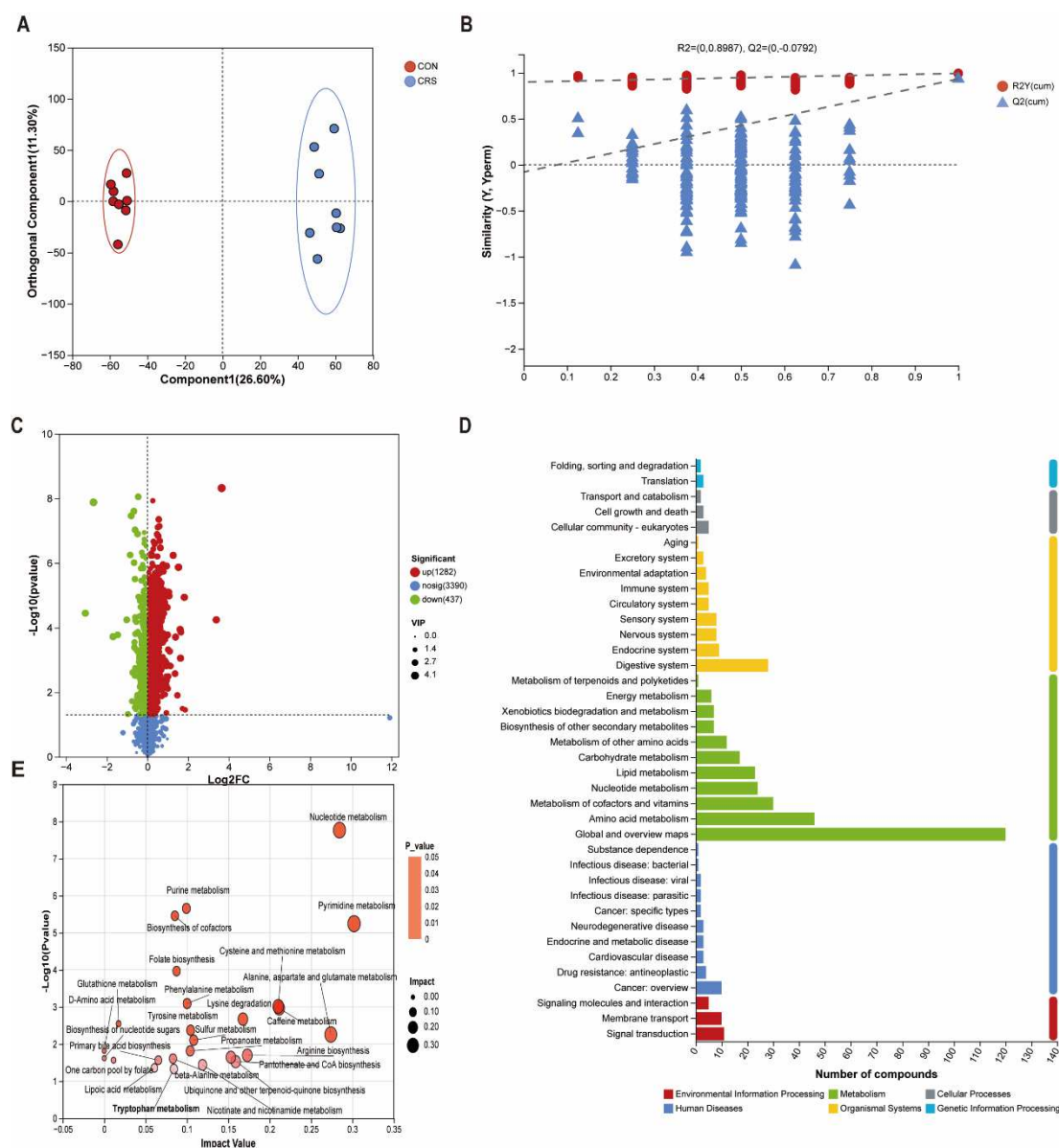


Figure 4. Cross-tissue metabolomic alterations in CRS mice. (A-C) Volcano plots of differential metabolites in the spleen (A), serum (B), and hippocampus (C). (D) Venn diagram showing shared and tissue-specific differential metabolites. (E) KEGG pathway enrichment in the spleen, serum, and hippocampus; bubble size indicates the number of differential metabolites and color indicates the P value. Differential metabolites met VIP > 1.0 and P < 0.05. Fold change was calculated as the mean mass-response ratio for CRS relative to CON. CON, control; CRS, chronic restraint stress; KEGG, Kyoto Encyclopedia of Genes and Genomes; VIP, variable importance in projection.

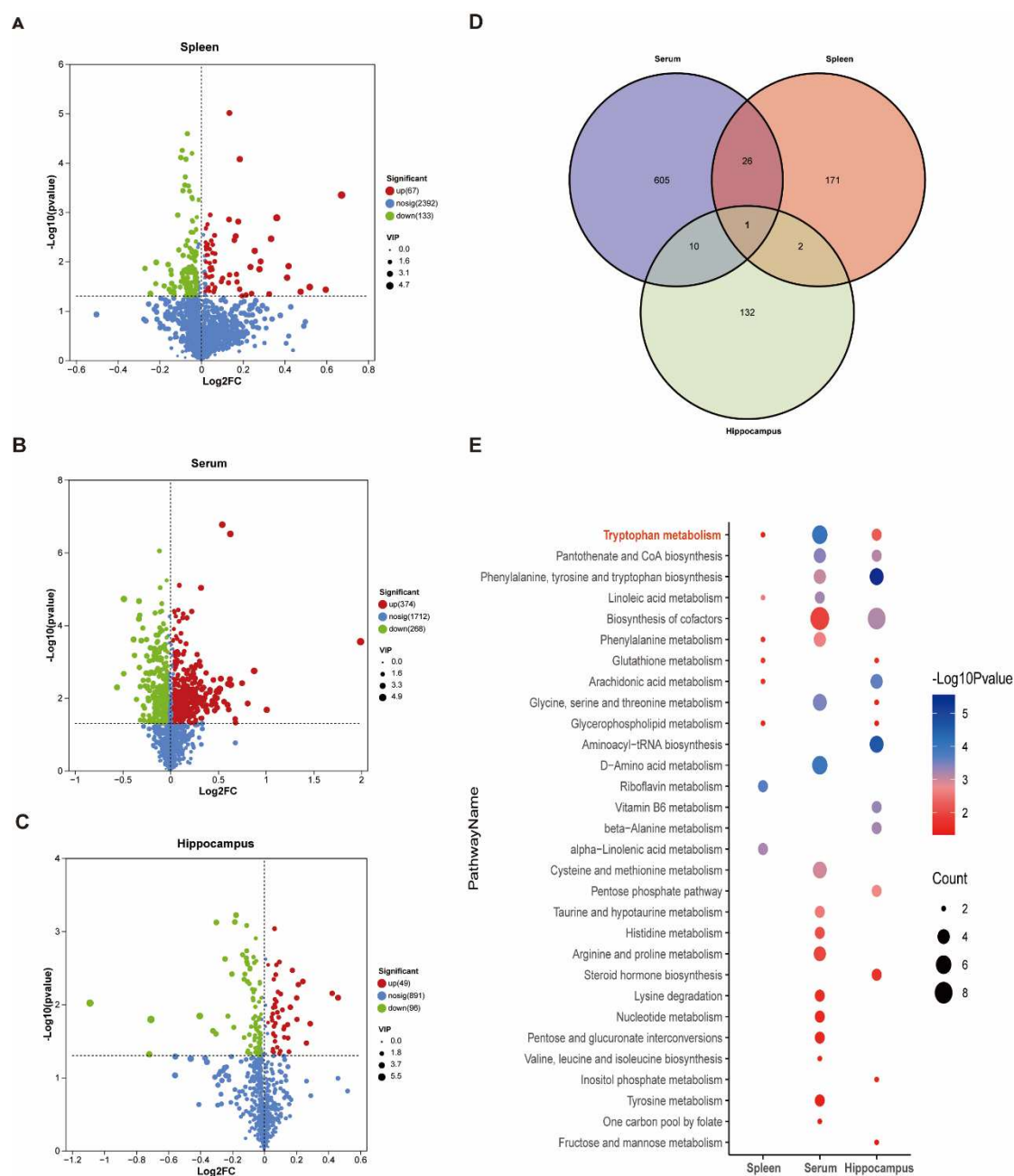


Figure 5. WGCNA identifies metabolite modules associated with depressive-like behaviors. (A) Hierarchical clustering dendrogram of metabolites based on topological overlap, with assigned module colors. (B) Module-trait correlation heatmap showing correlation coefficients and P values for module eigengenes and behavioral measures. (C) Distribution of metabolite classes across seven non-grey modules. (D) KEGG pathway analysis of the blue module, turquoise module, and their union. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IT, immobility time; FST, forced swim test; SP, sucrose preference; CT, center time; CoT, corner time; TST, tail suspension test; WGCNA, weighted gene co-expression network analysis.

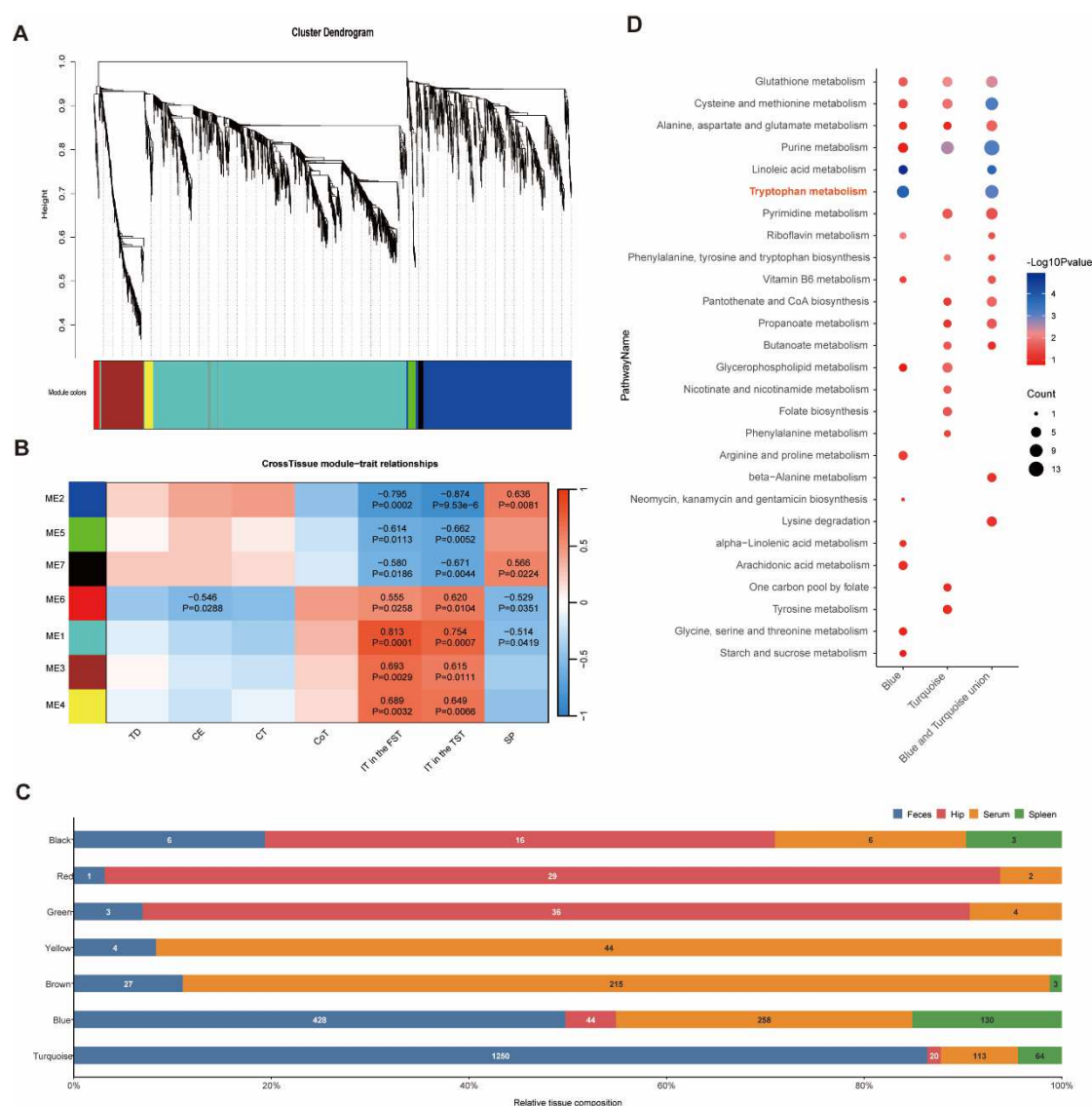
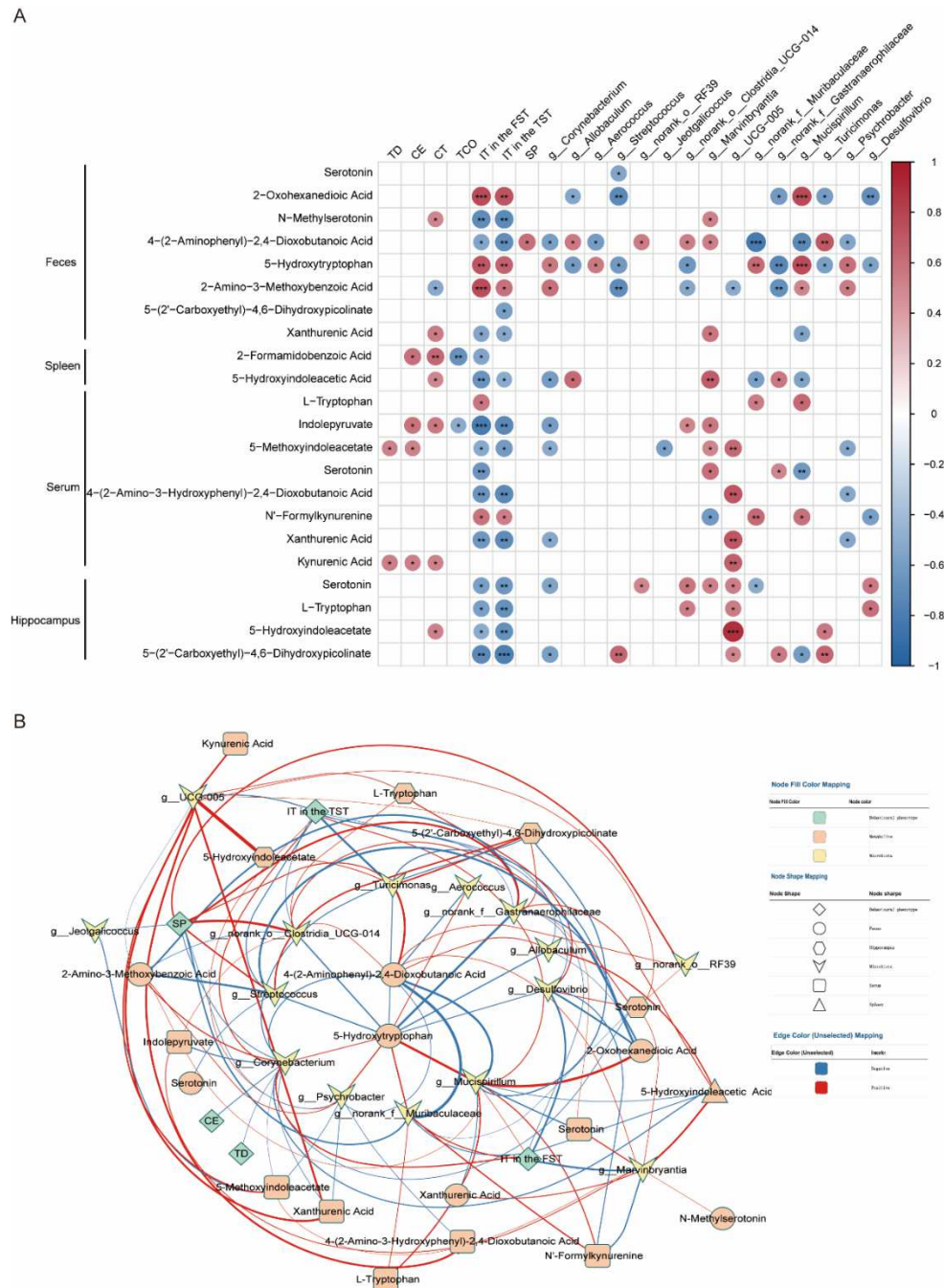


Figure. 6. Cross-tissue tryptophan-related associations link gut taxa, metabolites, and behaviors. (A) Spearman correlation heatmap relating tryptophan-associated metabolites from feces, serum, spleen, and hippocampus to bacterial genera and behavioral measures. (B) Integrated network retaining associations with nominal $P < 0.05$ and $|r| > 0.5$. Red edges indicate positive correlations, blue edges indicate negative correlations, and edge thickness is scaled by $|r|$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. DLB, depressive-like behavior; IT, immobility time; FST, forced swim test; SP, sucrose preference; CT, center time; CoT, corner time; TST, tail suspension test.



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