

Dietary volatile molecules orchestrate microbiome-mitochondrial crosstalk via tryptophan metabolism to resolve intestinal stress

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Abstract

Dietary volatile organic compounds (VOCs) possess unique physicochemical properties that facilitate extensive tissue distribution, yet their potential as signaling molecules in host-microbe crosstalk and intestinal homeostasis remains largely unexplored. Here, we reveal that a black tea volatile fraction (BTVs) acts as a potent modulator of the intestinal microenvironment, rescuing alcohol-induced enteric stress in a rat model by orchestrating microbiome-mitochondrial crosstalk. BTVs supplementation effectively reverses alcohol-induced mucosal damage and barrier dysfunction by potently suppressing NLRP1B/GSDME-mediated pyroptosis. Integrated multi-omics analyses demonstrate that BTVs remodel the gut microbiota, specifically restricting the alcohol-induced expansion of dysbiotic taxa including *Clostridia_UCG-014* and *Escherichia-Shigella*, and fundamentally reprogram host-microbe metabolic interactions. Mechanistically, BTVs drive a metabolic shift in intestinal tryptophan utilization, upregulating indoleamine 2,3-dioxygenase 1 (IDO1)-dependent kynurenine pathway activity. This metabolic rewiring enhances the availability of precursors for NAD⁺ biosynthesis, which is critical for cellular resilience. Consequently, this NAD⁺ replenishment restores mitochondrial bioenergetics, re-establishes fatty acid oxidation, and sustains ATP production, thereby shielding the intestinal epithelium from metabolic exhaustion and pyroptotic cell death. Collectively, our findings conceptualize dietary plant volatiles not merely as sensory attributes, but as active inter-kingdom signaling molecules that safeguard intestinal integrity. By delineating the BTV-driven tryptophan-NAD⁺-mitochondria axis, this study provides a theoretical framework for utilizing volatile bioactive matrices to mitigate lifestyle-induced metabolic stress.

Introduction

The efficient absorption and utilization of dietary nutrients in the gastrointestinal tract are essential for maintaining systemic metabolic homeostasis and immune function [1]. The intestinal epithelium, in concert with a symbiotic gut microbiota, forms a highly dynamic barrier that orchestrates this nutrient flux while defending against luminal toxins. However, this delicate equilibrium is highly susceptible to environmental stressors. Excessive alcohol consumption profoundly disrupts nutrient absorption by damaging epithelial villus architecture, shortening intestinal transit time, and inhibiting key digestive enzymes, while simultaneously inducing gut dysbiosis and mitochondrial energetic deficits that further compromise the active transport of amino acids, vitamins, and other essential metabolites [2, 3]. Collectively, these derangements culminate in intestinal inflammation, increased mucosal permeability, and systemic metabolic disturbances [4].

Given the multifactorial nature of alcohol induced malabsorption, effective intervention must address structural integrity and the metabolic capacity of the gut. Conventional bioactive compounds, particularly polyphenols like curcumin, have shown promise in preclinical models, yet their clinical translation is hampered by poor bioavailability, a consequence of high molecular weight, hydrophobicity, and extensive first pass metabolism, which limits their access to intestinal target sites and thus their ability to restore absorptive function [5]. In contrast, plant-derived volatile compounds represent a relatively

underexplored class with distinct advantages in bioavailability [6]. Their low molecular weight and lipophilic nature enable rapid passive diffusion across biological membranes, allowing efficient accumulation in the intestinal mucosa and direct modulation of intracellular metabolic pathways, potentially circumventing the barriers that constrain larger phytochemicals [7, 8].

Black tea, a globally popular beverage, contains a complex mixture of volatile compounds, including monoterpenes such as linalool and geraniol [9, 10]. While these compounds have been reported to possess anti-inflammatory and antimicrobial properties [11], the potential of black tea volatile substances (BTVs) to mitigate alcohol-induced intestinal injury and reverse alcohol-induced nutrient malabsorption remains to be elucidated, as do the underlying mechanisms.

Based on these considerations, we hypothesized that BTVs alleviate alcohol induced intestinal stress by restoring the capacity for nutrient metabolism through coordinated actions on the epithelial barrier, the gut microbiome, and mitochondrial energy supply. Using a rat model of alcohol exposure, we first demonstrated that BTVs, predominantly enriched in linalool and geraniol, potently jejunal and ileal villus architecture, thereby maximizing the absorptive surface area, with efficacy markedly superior to that of curcumin. To elucidate the underlying mechanisms, we employed an integrated multi-omics approach. Our results reveal that BTVs selectively suppress the expansion of pathogenic *Escherichia Shigella* and *Clostridia_UCG 014*, alleviating local inflammation and preserving epithelial integrity, a prerequisite for efficient nutrient uptake. More critically, BTVs relieve the alcohol induced blockade of indoleamine 2,3 dioxygenase 1 (IDO1), restoring tryptophan pathway flux. This reactivation fuels the synthesis of NAD⁺ precursors and mitochondrial ATP synthesis, providing the energy necessary for active transport processes that drive the absorption and utilization of nutrients.

Collectively, our findings establish BTVs not merely as flavor components, but as a highly bioavailable class of agents that specifically target the recovery of alcohol impaired nutrient assimilation. By simultaneously repairing the physical absorptive surface, rebalancing the microbiome, and reenergizing key metabolic enzymes, BTVs offer a multipronged strategy to counteract lifestyle induced intestinal malabsorption. This work provides a new paradigm for the use of dietary volatiles in preserving gut metabolic resilience through the host microbiome mitochondria axis.

Results

Preparation and chemical components of BTVs

The preparation process of BTVs is illustrated in **Fig. 1a**. GC-MS analysis identified 13 primary volatile compounds (**Table 1, Fig. 1b**, the criteria for screening key volatiles is that area% > 1%). The mixture was dominated by alcohols, with linalool (41.19%) and geraniol (26.81%) being the most abundant components, followed by methyl salicylate (5.46%). These components constitute the characteristic floral and fruity aroma profile of Dian Hong black tea.

[Figure 1]

BTVs alleviates alcohol-induced intestinal morphological injury in rats

The experimental design involving male SD rats is illustrated in **Fig 2a**. To evaluate the protective effect of BTVS on the intestinal barrier, we performed H&E staining on the jejunum and ileum tissues of rats.

As shown in **Fig 2b**, alcohol exposure compromised jejunal integrity, characterized by epithelial shedding, apical villi autolysis (black arrows) and inflammatory infiltration (blue arrow). This damage was quantified by a significant decrease in villus height and the V/C ratio, despite unchanged crypt depth (**Fig 2c-e**). Treatment with curcumin and BTVs-H significantly restored the villus length ($p = 0.0233$ and $p = 0.0164$, respectively) and improved the V/C ratio ($p = 0.0407$ and $p = 0.0307$, respectively). No significant differences in crypt depth were observed among the groups in the jejunum (**Fig. 2d**).

Similar trends were observed in the ileum (**Fig. 2f-i**). Alcohol administration caused a drastic reduction in villus length (**Fig. 2g**, $p < 0.0001$) and V/C ratio (**Fig. 2i**, $p < 0.0001$), along with a significant increase in crypt depth (**Fig. 2h**, $p = 0.0431$), indicating hyperplasia due to injury. BTVs-H treatment effectively reversed these changes, significantly increasing villus length ($p < 0.0001$) and V/C ratio ($p = 0.0037$) compared to the alcohol group. Interestingly, while BTVs-L showed a trend towards improvement, it did not reach statistical significance in restoring villus length, suggesting that the protective effect of BTVs is dose-dependent. Moreover, the effect of BTVs-H is superior to that of curcumin. Collectively, these results suggest that BTVs, particularly at a high dose, effectively protects against alcohol-induced structural damage in both the jejunum and ileum.

[Figure 2]

BTVs alleviates alcohol-induced intestinal inflammation

To elucidate the molecular mechanisms underlying the protective effects of BTVs, we performed transcriptomic. As shown in **Fig 3a**, alcohol exposure significantly altered gene expression (260 upregulated and 107 downregulated differentially expressed genes vs. NS). Notably, BTVs-H treatment resulted in the downregulation of 655 genes and upregulation of 251 genes compared to the AISM group. GO enrichment analyses were conducted and the top 10 most significantly enriched pathways are shown in **Fig. 3b**, which indicate that alcohol-induced gene responses were concentrated in immune regulation, inflammatory response (GO:0006954; $p = 8.42 \times 10^{-6}$), and cell death-associated pathways. Notably, the inflammatory response pathway was significantly modulated by BTVs-H ($p = 5.6 \times 10^{-7}$) (**Fig 3c**), suggesting that BTVs exerts its protective effect primarily by modulating inflammation. Specifically, alcohol upregulated pro-inflammatory genes like *Cxcl3* (C-X-C motif chemokine 3) and *S100a8* (**Fig. S1a**), which are critical for leukocyte recruitment and cytokine secretion [14,15]. BTVs-H effectively suppressed most inflammatory factors (**Fig. S1b**), notably reversing *S100a8* overexpression (**Fig. 3d**).

We further validated these findings at the protein level using DIA-based quantitative proteomics. A total of 137 proteins were upregulated and 128 were downregulated in the AISM group compared to NS, while BTVs-H treatment reversed the expression of numerous proteins (**Fig. 3e**). Alcohol-induced proteins

were enriched in pyroptosis-related pathways (**Fig. S1c**). Consistent with the transcriptomic data, the protein level of S100A8 was significantly elevated in the AISM group ($p = 0.0255$) and restored by BTVs-H ($p = 0.0064$) (**Fig. 3f**). Furthermore, we observed similar expression patterns for GSDME and NLRP1B, key markers of pyroptosis.

To pinpoint the core targets, we performed an integrated analysis of the transcriptomic and proteomic datasets. Venn diagrams identified 162 genes and 111 proteins that overlapped between the comparison groups (**Fig. 3g, left**). The intersection of these datasets highlighted S100A8 as a consistent target, showing concordant expression trends at both the gene and protein levels, as visualized in the heatmap (**Fig. 3g, middle**). Moreover, Pearson correlation analysis revealed a strong positive correlation between *S100a8* transcript levels and the protein abundance of GSDME ($r = 0.8602$, $p < 0.001$) and NLRP1B ($r = 0.8255$, $p < 0.001$) (**Fig. 3g, right**). These results suggest that BTVs alleviates alcohol-induced intestinal inflammation likely by targeting S100A8 and its downstream pyroptosis-related signaling.

[Figure 3]

BTVs Restores Alcohol-Induced Gut Microbiota Dysbiosis

Given that S100A8 upregulation is often driven by microbial products, we examined the gut microbiota to investigate whether the protective effects of BTVs are mediated through gut microbiota modulation [16]. The coverage for all samples exceeded 0.995, ensuring adequate sequencing depth to represent the microbial community (**Fig. 4a**). Alpha diversity analysis (Chao1, Shannon and Simpson) revealed no significant differences ($p > 0.05$), suggesting that alcohol did not drastically alter the overall species richness or evenness (**Fig. 4b-d**). To visualize the β -diversity of microbial communities across distinct environmental samples, we performed PCoA. The results revealed that the first two principal coordinate axes (PCoA1 and PCoA2) cumulatively accounted for approximately 33% of the total variation, suggesting the presence of notable structural variations among the samples. Alcohol treatment significantly altered the microbial community structure, and this alteration was partially reversed by BTVs treatment in a dose-dependent manner (**Fig. 4e**). A Venn diagram identified 321 ASVs shared across all four groups (**Fig. 4f**).

At the phylum level, Firmicutes, Bacteroidota, Proteobacteria, and Actinobacteriota were the dominant phyla, collectively accounting for over 97% (**Fig. 4g**). Alcohol administration led to a significantly expansion of Proteobacteria (+169.31%) and slightly increased Actinobacteriota (+74.02%). The significant expansion of the Proteobacteria is a potential feature of the disruption of the intestinal microecology and inflammation [17]. BTVs treatment reversed these trends (**Fig. S2a**).

At the genus level, *Lactobacillus* is the top abundant genera (**Fig. 3h**). Alcohol exposure induced dysbiosis characterized by a depletion of beneficial genera, including an 81.34% decrease in *Bacillus* and a 45.48% decrease in *Lactobacillus*, alongside a 3.54-fold increase in the opportunistic pathogen *Escherichia-Shigella* and 1.86-fold in *Clostridia_UCG-014*. BTVs intervention reversed these alterations,

slightly increasing *Bacillus* abundance (1.07-fold vs AISM group) and significantly suppressing *Escherichia-Shigella* to restore microbial homeostasis. (**Fig. S2b**).

To further explore the microbe-inflammation crosstalk, Pearson correlation analysis was conducted between differentially abundant genera and inflammatory gene expression (**Fig. 4i**). *Clostridia_UCG-014* exhibited significant positive correlations with multiple pro-inflammatory markers, including *S100a8* and *Cxcl3*. *Bacillus* showed positive correlations with *Cxcl9* and *Cxcl10*, suggesting a potential association with Th1 immune responses. Collectively, these results indicate that alcohol exposure induces intestinal inflammation by disrupting the microbial community structure, and BTVs treatment effectively attenuates this dysbiosis, potentially by modulating key taxa such as *Escherichia-Shigella*. **Fig 4j** summarizes the regulatory pathways.

[Figure 4]

BTVs reprograms intestinal metabolism and restores mitochondrial function-related metabolites in alcohol-exposed rats

Because mitochondrial dysfunction can result from sustained inflammation, we next performed untargeted metabolomics to map the metabolic landscape. Principal component analysis (PCA) revealed a distinct separation between the Saline and Alcohol groups, indicating a profound disturbance in the intestinal metabolome caused by alcohol (**Fig. 5a**). Notably, BTVs treatment, particularly at the high dose, shifted the metabolic profile away from the Alcohol group. Volcano plots further visualized the differentially expressed metabolites (DEMs). Compared to the Alcohol group, the BTVs-L and BTVs-H groups showed significant metabolic alterations, with 150 upregulated / 212 downregulated DEMs in the low-dose group and 45 upregulated / 191 downregulated DEMs in the high-dose group (**Fig. 5b**).

KEGG pathway enrichment analysis demonstrated that BTVs induced a comprehensive metabolic reprogramming, primarily clustering into three categories: amino acid metabolism, lipid metabolism, and vitamin metabolism (**Fig. 5c**), suggesting that BTVs restores the metabolic homeostasis disrupted by alcohol.

To identify key targets, we focused on 89 metabolites reversed by BTVs-H (**Fig. S3a**). BTVs-H normalized the accumulation of adenosine and dihydroorotic acid (**Fig. 5d-e**), a marker of mitochondrial dihydroorotate dehydrogenase (DHODH) dysfunction, indicating the restoration of ATP homeostasis and mitochondrial enzymatic activity.

Furthermore, BTVs-H alleviated membrane damage (reduced PC (16:0/0:0)) and resolved the blockade of mitochondrial fatty acid β -oxidation (reduced 2-Hydroxyadipic acid and hydroxy-fatty acids) (**Fig. 5f-i**).

In parallel, BTVs-H restored the depleted levels of Lumichrome and 3-Hydroxyanthranilic acid (precursors for FAD and NAD⁺, respectively), suggesting reactivation of the mitochondrial electron transport chain (**Fig. 5j-k**).

Consistent with these metabolic shifts, proteomic analysis confirmed that BTVs-H significantly enriched pathways related to mitochondrial inner membrane organization and transport (**Fig. S3b**). Collectively, these results demonstrate that BTVs mitigates alcohol-induced injury by reprogramming metabolic fluxes, specifically through the restoration of mitochondrial structural integrity and cofactor supply.

[Figure 5]

BTVs Ameliorate Alcoholic Intestinal Stress by Rescuing the IDO1-Tryptophan-NAD⁺ Axis to Restore Mitochondrial Energy Homeostasis

To pinpoint the master molecular switch connecting BTV-induced protein changes to these metabolic recoveries, we performed a convergent analysis of the proteomic and metabolomic datasets. Venn diagram analysis of the differentially expressed proteins and metabolites revealed 111 proteins and 89 metabolites that were significantly altered in AISM group and subsequently reversed by BTVs-H treatment (**Figure 6a**). Among these, IDO1 and its downstream catabolite, 3-Hydroxyanthranilic acid, emerged as the convergent regulatory axis. As 3-Hydroxyanthranilic acid is a critical precursor for de novo NAD⁺ biosynthesis, this finding provided a mechanistic link between tryptophan utilization and the restoration of mitochondrial energy pools (ATP) (**Figure 6b**). Heatmap analysis demonstrated that alcohol exposure significantly increased the levels of Tryptophan, suppressed the rate-limiting enzyme IDO1, and downstream metabolites including 3-Hydroxyanthranilic acid, Kynurenic acid, and Xanthurenic acid. Notably, BTVs-H supplementation successfully reversed these suppressions, restoring the metabolic flux of this pathway.

We further validated its spatial expression in intestinal tissues using IHC and WB. Consistent with the proteomics findings, the IHC results confirmed that the robust expression of IDO1 in the normal intestinal mucosa (NS group) was markedly downregulated following alcohol stress (AISM group). However, intervention with BTVs-H effectively restored the mucosal IDO1 expression to near-normal levels (**Figure 6c-e**).

The de novo biosynthesis of NAD⁺ via the kynurenine pathway is a crucial physiological outcome of tryptophan metabolism, directly participating in cellular ATP production (**Figure 6b, bottom**). Based on the omics data, we hypothesized that the AISM-induced blockage of IDO1-mediated tryptophan metabolism disrupts intestinal energy homeostasis. To verify this, we quantified the intestinal ATP levels. As expected, AISM significantly depleted intestinal ATP content compared to the NS group ($p = 0.0262$). In contrast, BTVs-H treatment effectively replenished the ATP pool ($p = 0.0391$), indicating a recovery of cellular energy states (**Figure 6f**).

Finally, to establish a direct statistical link between the key enzyme IDO1 and the energy metabolic state, we performed a Pearson correlation analysis. The results revealed a significant correlation between IDO1 abundance and energy metabolism indicators ($r = -0.6342$, $p = 0.0111$) (**Figure 6f**). Collectively, these findings strongly suggest that BTVs ameliorates alcohol-induced intestinal stress by rescuing IDO1

expression, thereby reactivating tryptophan metabolism to maintain NAD⁺/ATP synthesis and intestinal energy homeostasis.

[Figure 6]

Discussion

Alcohol intake is known to profoundly disrupt intestinal nutrient absorption through damage to epithelial structures, dysbiosis, and mitochondrial dysfunction, yet effective interventions with high bioavailability remain scarce. In recent years, the volatile components of black tea and its fermented products have garnered increasing attention as promising bioactive raw materials [18]. Herein, we demonstrate for the first time that black tea volatile substances (BTVs) effectively restore alcohol-suppressed intestinal nutrient absorption and ameliorate alcoholic enteric stress through a concerted action: dietary volatile compounds from black tea act as potent inter-kingdom signaling molecules that remodel alcohol-induced gut dysbiosis, redirect intestinal tryptophan metabolism toward the IDO1-mediated kynurenine pathway and rescues mitochondrial bioenergetics. Notably, BTVs exhibit favorable gastrointestinal absorption and high intrinsic bioavailability, and their efficacy in alleviating alcohol-induced intestinal barrier damage is superior to that of curcumin. Collectively, these findings establish BTVs as a class of naturally derived, bioavailable agents that effectively mitigate alcohol-induced intestinal injury, offering a promising strategy to counteract lifestyle-related digestive dysfunction.

Compositional analysis identified linalool and geraniol as the primary active constituents in BTVs. Beyond defining the characteristic floral aroma of black tea [19], these volatile small molecules possess unique physicochemical properties that confer distinct pharmacokinetic advantages over traditional dietary polyphenols [19, 20]. Their low molecular weight and high lipophilicity facilitate efficient passive diffusion across lipid bilayers and the mucosal barrier, enabling rapid accumulation in target tissues, thereby overcoming the bottleneck of low bioavailability seen with non-volatile phytochemicals such as curcumin, which suffers from high hydrophobicity and extensive first-pass metabolism [21–23]. These properties facilitate rapid membrane permeation and intracellular access, potentially explaining why small molecules are more effective in sobering up than flavonoids.

Alcohol ingestion interferes with intestinal nutrient absorption and utilization through multifaceted mechanisms involving structural and functional damage to the gut. At the physical barrier level, alcohol directly damages the villi and microvilli of the intestinal epithelium, reducing the effective absorptive surface area while increasing intestinal permeability and compromising barrier integrity [24]. This facilitates the translocation of harmful substances into the bloodstream and triggers inflammatory responses, further disrupting nutrient uptake [25]. In addition, alcohol impairs normal intestinal motility, shortening the transit time of chyme and leading to incomplete digestion and reduced absorption [24]. Our histological findings demonstrate that BTVs effectively repair alcohol-induced damage to

intestinal epithelial villi and microvilli, increase villus length, and thereby maximize the recovery of nutrient absorption sites.

At the microbiome level, alcohol disrupts gut microbial homeostasis, altering community composition and function. This dysbiosis directly induces gastrointestinal symptoms and subsequently aggravates intestinal inflammation and barrier dysfunction [26–28]. Our study revealed that alcohol exposure significantly expanded pathogenic genera, e.g., *Clostridia_UCG-014* and *Escherichia-Shigella* while reducing beneficial bacteria, and BTVs intervention effectively reversed this trend. We postulate that this protective effect is partly attributable to the direct antimicrobial activity of volatile terpenes. For instance, linalool can disrupt Gram-negative bacterial membranes, thereby inhibiting LPS synthesis and bacterial proliferation [29]. By attenuating these upstream microbial triggers, BTVs subsequently suppress the pyroptosis pathway, thus preserving epithelial integrity.

At the enzymatic and energetic level, alcohol inhibits the activity of multiple enzymes in the intestinal brush border, particularly those critical for active nutrient transport [30]. Furthermore, mitochondria, as the core organelles sustaining epithelial barrier function, provide the ATP essential for material transport, and alcohol exposure severely compromises mitochondrial quality control [30–33]. Our results reveal that alcohol exposure induces profound remodeling of the gut metabolome, whereas BTVs intervention elicits a comprehensive metabolic reprogramming centered on amino acid, lipid, and vitamin metabolism. Among these, Tryptophan metabolism regulation serves as a critical pathway for maintaining mitochondrial homeostasis, preserving intestinal barrier function and alleviating intestinal inflammation [34, 35]. In this study, alcohol exposure significantly suppressed the expression of IDO1, a key enzyme in tryptophan metabolism, resulting in tryptophan accumulation and blockade of the downstream kynurenine pathway [36]. BTVs intervention successfully restored IDO1 expression and reactivated the metabolic flux. The biological endpoint of the kynurenine pathway is the de novo synthesis of NAD⁺, which directly participates in mitochondrial ATP production [37, 38]. We observed that alcohol exposure led to a marked depletion of intestinal ATP content, while BTVs intervention effectively replenished the ATP pool and restored mitochondrial fatty acid β -oxidation and structural integrity. Collectively, these results strongly confirm that BTVs reactivate tryptophan metabolic axis by restoring IDO1 expression, providing sufficient NAD⁺ and ATP to the intestinal epithelium, thus sustaining energy homeostasis and barrier integrity.

However, this study has certain limitations. For example, we did not compare the absorption and bioavailability of macromolecules versus small molecules within BTH. Future studies could employ fluorescence labeling or isotope tracing to track the in vivo absorption and subcellular localization of specific components following intragastric administration. As our findings rely heavily on omics data, further experimental validation is recommended to strengthen the credibility of the proposed mechanisms.

Conclusion

In summary, our study establishes black tea VOCs, predominantly linalool and geraniol, as highly bioavailable modulators of intestinal homeostasis. Rather than acting through isolated pathways, BTVs confer profound resilience against alcohol-induced enteric stress by orchestrating a coordinated crosstalk between the gut microbiome and host mitochondrial quality control. Central to this protection is the mechanistic rewiring of host-microbe metabolic networks: BTVs drive intestinal tryptophan utilization toward IDO1-dependent NAD⁺ biosynthesis, thereby replenishing the cellular bioenergetic pool, averting mitochondrial collapse, and ultimately silencing NLRP1B/GSDME-mediated pyroptosis. Crucially, the superior in vivo efficacy of BTVs compared to curcumin underscores a fundamental pharmacokinetic advantage. By circumventing the bioavailability bottlenecks that typically limit conventional large-molecular-weight polyphenols, these small-molecule volatiles achieve robust tissue distribution and target engagement. Ultimately, these findings expand our conceptual understanding of dietary volatiles from mere sensory attributes to active, inter-kingdom signaling molecules. By delineating the BTV-driven tryptophan-NAD⁺-mitochondria axis, this work provides a compelling theoretical foundation for developing next-generation, volatile-based therapeutic and nutritional strategies to combat lifestyle-induced metabolic enteropathies and preserve mucosal integrity.

Materials and Methods

Reagents

Curcumin (positive control, $\geq 95\%$ purity) was obtained from Shanghai Yien Chemical Technology (Shanghai, China). All other chemicals were analytical grade unless stated otherwise.

Preparation of black tea volatile substances

BTVs were prepared from Dian Hong black tea (provided by the Hangzhou Tea Research Institute, Hangzhou, China) using steam distillation [12]. The black tea leaves were appropriately ground and processed using a Spinning Cone Column (SCC). The operational parameters were optimized as follows: a solid-liquid ratio of 1:10 (w/v), a feed flow rate of 500 L/h, and a steam flow rate of 35 kg/h. The extraction was conducted at 90°C with a rotational speed of 350 r/min under a vacuum of -50 kPa. The primary and secondary condensation temperatures were maintained at 30°C and 5°C, respectively. The secondary condensate was collected until the mass ratio of the distillate to the raw tea material reached 1:1, yielding the BTVs.

Gas chromatography-mass spectrometry (GC-MS) analysis of BTVs

Volatile composition was analysed by headspace solid-phase microextraction coupled to GC-MS (SPME-GC-MS; Shimadzu, Kyoto, Japan). Samples were incubated with NaCl in headspace vials, extracted using an SPME fiber (Supelco, USA) at 60°C, and desorbed at 250°C for 3 min. Separation used an Agilent J&W DB-5MS column with helium carrier gas (2.5 mL/min constant flow). Oven programme: 40°C (2 min hold), ramp 6°C/min to 195°C, then 15°C/min to 280°C (2 min hold). Mass spectrometer operated in

electron impact mode (ion source 220°C, interface 280°C, m/z 33–500 scan). Compounds were identified by NIST library matching; key volatiles selected by area% > 1%.

Animal model and experimental design

Male Sprague-Dawley rats (180 ± 10 g, 6 weeks old; n = 50) were obtained from Chengdu Dashuo Experimental Animal Co., Ltd (License No: SCXK (chuan) 2020-030). All procedures were approved by the Ethics Committee of Chengdu University of Traditional Chinese Medicine (Approval No. 2022DL-017) and followed institutional animal welfare guidelines. Rats were housed under standard conditions (12-h light/dark cycle, 22 ± 2°C, ad libitum access to food and water) and acclimatized for 1 week before randomization into five groups (n = 10/group): control (normal saline, NS), alcohol-induced intestinal stress model (AISM), positive control (75 mg/kg curcumin), BTVs-L (0.45 mL/kg BTVs), and BTVs-H (9 mL/kg BTVs) [13]. Treatments were administered daily by gavage for 13 days. From day 5 to 13, model and treatment groups received 56% ethanol (10 mL/kg) 4 h before treatment to induce intestinal injury. On day 14, rats were first anesthetized using CO₂ inhalation in a designated chamber until unconsciousness was achieved, followed by cervical dislocation for euthanasia, and jejunum, ileum, and cecal contents collected for analysis.

Histological analysis

Jejunum and ileum tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned (4–5 µm), and stained with hematoxylin and eosin (H&E). Slides were (Panoramic Midi, 3DHISTECH Ltd., Budapest, Hungary), and evaluated using Slide Viewer software. Villus length (V), crypt depth (C), and the V/C ratio were quantified in ≥ 10 well-oriented fields per section using ImageJ.

RNA sequencing and transcriptomic analysis

Total RNA was extracted from intestinal tissues using TRIzol reagent. Concentration and purity were assessed by NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, USA). Libraries were prepared with NEBNext Ultra™ RNA Library Prep Kit (NEB, USA) and sequenced on an Illumina NovaSeq 6000. Differential expression analysis used edgeR (p < 0.05, |log₂FC| > 0.5). Gene Ontology (GO) enrichment was performed with Goseq R package.

Proteomic analysis

Proteins were extracted using SDT lysis buffer (4% SDS, 100 mM Trypsin-digested peptides (filter-aided sample preparation) were analyzed by data-independent acquisition (DIA) mass spectrometry on an Astral high-resolution mass spectrometer (Thermo Scientific). Bioinformatics used OmicStudio tools.

16S rRNA gene sequencing and microbiota analysis

Genomic DNA from cecal contents was extracted and amplified with barcoded primers targeting the V3-V4 region. Amplicons were sequenced on an Illumina platform. Amplicon sequence variants (ASVs) were processed with QIIME2 and DADA2. Alpha diversity (Chao1, Shannon, Simpson) and beta diversity

(principal coordinate analysis, PCoA) were calculated; differential abundance analysed at phylum and genus levels.

Untargeted metabolomics

Intestine samples were extracted with methanol:water (4:1) containing L-2-chlorophenylalanine (0.02 mg/mL). LC-MS/MS analysis used ACQUITY UPLC HSS T3 column (Waters) coupled to AB SCIEX Q-TOF mass spectrometer in positive and negative modes. Data processed for peak alignment and KEGG pathway annotation.

Immunohistochemistry (IHC) staining

The formalin-fixed, paraffin-embedded (FFPE) intestinal tissue blocks were sectioned at a thickness of 4 μ m. The sections were deparaffinized in xylene and rehydrated through a graded series of ethanol (100%, 95%, 85%, and 75%). For antigen retrieval, the slides were immersed in citrate buffer (pH 6.0) and heated in a microwave oven for 15 minutes. After cooling to room temperature, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 15 minutes in the dark. Subsequently, the sections were washed with PBS and blocked with 5% BSA at room temperature for 30 minutes. The slides were then incubated overnight at 4 °C with a primary antibody against IDO1 (Proteintech, 84063-3-RR). Following three washes with PBS, the sections were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 50 minutes at room temperature. The immunoreactive signals were visualized using a 3,3'-diaminobenzidine (DAB) substrate kit, resulting in a brown precipitate at the antigen site. The sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted with neutral resin. Finally, the slides were observed, and representative images were captured using a bright-field microscope (Panoramic Midi, 3DHISTECH Ltd., Budapest, Hungary).

Measurement of intestinal ATP levels

To evaluate the energy metabolism status of the gut, the ATP concentration in intestinal tissues was quantified using a commercial ATP Assay Kit (Cat. No. G4309; Servicebio, Wuhan, China). Briefly, intestinal tissue samples were accurately weighed and homogenized on ice with the lysis buffer provided in the kit at a predefined weight-to-volume ratio. The tissue homogenates were subsequently centrifuged at 10,000 g for 10 min at 4°C. The resulting supernatant was carefully collected for the subsequent assay. The ATP determination was performed strictly according to the manufacturer's instructions. The luminescence of the reaction mixture was measured using a microplate reader (SpectraMax D5, Molecular Devices, USA). The exact ATP concentration was calculated based on the standard curve generated simultaneously.

Western blot

Intestinal tissue was lysed in ice-cold radio immunoprecipitation assay lysis (RIPA) buffer (Solarbio, R0010) containing protease inhibitors. The protein concentration of lysates was quantified using a bicinchoninic acid (BCA) protein assay kit from Solarbio (XYW-3). Equal amounts of protein were loaded on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto a

nitrocellulose membrane (Millipore, Billerica, MA) after separation. The membrane was blocked with 5% non-fat milk at room temperature for 1 h, followed by incubation with the primary antibody at 4°C overnight. After incubation with the corresponding secondary antibody (MBL), the target protein was visualized by chemiluminescence. The primary antibodies and concentrations used for western blotting were IDO1 (Proteintech, 84063-3-RR) and β -actin (ABclonal, AC004).

Statistical analysis

All data are presented as mean $\pm$ standard deviation, with significant differences at $p < 0.05$. One-way analysis of variance (ANOVA) was performed using (GraphPad Prism 10.6), and post hoc analyses were performed using LSD, Tukey, and Tamhane T2 tests. Raw sequencing data were processed and analyzed using the Majorbio Cloud Platform (<https://cloud.majorbio.com>).

Abbreviations

alcohol-induced intestinal stress model	AIMS
amplicon sequence variants	ASV
black tea volatile substances	BTVs
crypt depth	C
C-X-C motif chemokine 3	CXCL3
data-independent acquisition	DIA
differentially expressed genes	DEGs
differentially expressed metabolites	DEMs
differentially expressed proteins	DEPs
dihydroorotate dehydrogenase	DHODH
gas chromatography-mass spectrometry	GC-MS
gene ontology	GO
hematoxylin and eosin	H&E
indoleamine 2,3-dioxygenase	IDO1
normal saline	NS
principal component analysis	PCA
principal coordinate analysis	PCoA
villus length	V
volatile substances	VS

Declarations

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Ethics declarations

The animal study was approved by Ethics Committee of Chengdu University of Traditional Chinese Medicine (Approval No. 2022DL-017) and followed institutional animal welfare guidelines.

Competing interests

The authors declare that they have no competing interests.

Authorship

Y.L. (first author): Data Curation, Formal Analysis, Visualization; Y.F. (first author): Methodology, Data Curation, Formal Analysis, Visualization, Writing- Original Draft; X.Y.: Methodology, Visualization; S.Z.: Writing- Review & Editing; P.W.: Resources, Supervision; Y.W. (Corresponding Author): Conceptualization, Funding Acquisition, Resources, Writing- Review & Editing; X.Y. (Corresponding Author): Conceptualization, Resources, Supervision, Writing- Review & Editing

Data Availability

All data generated or analyzed during this study are included in this article, and can be provided by the corresponding author upon reasonable request. All raw sequencing data of microbiome generated in this study is available in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1506890 and the raw sequencing data of transcriptome is available in the NCBI SRA under BioProject accession PRJNA1506863. The raw sequencing data of proteomics is deposited in Integrated Proteome Resources (iProX) under project ID IPX0018819000.

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Table

Table 1 is available in the supplementary files section

Figures

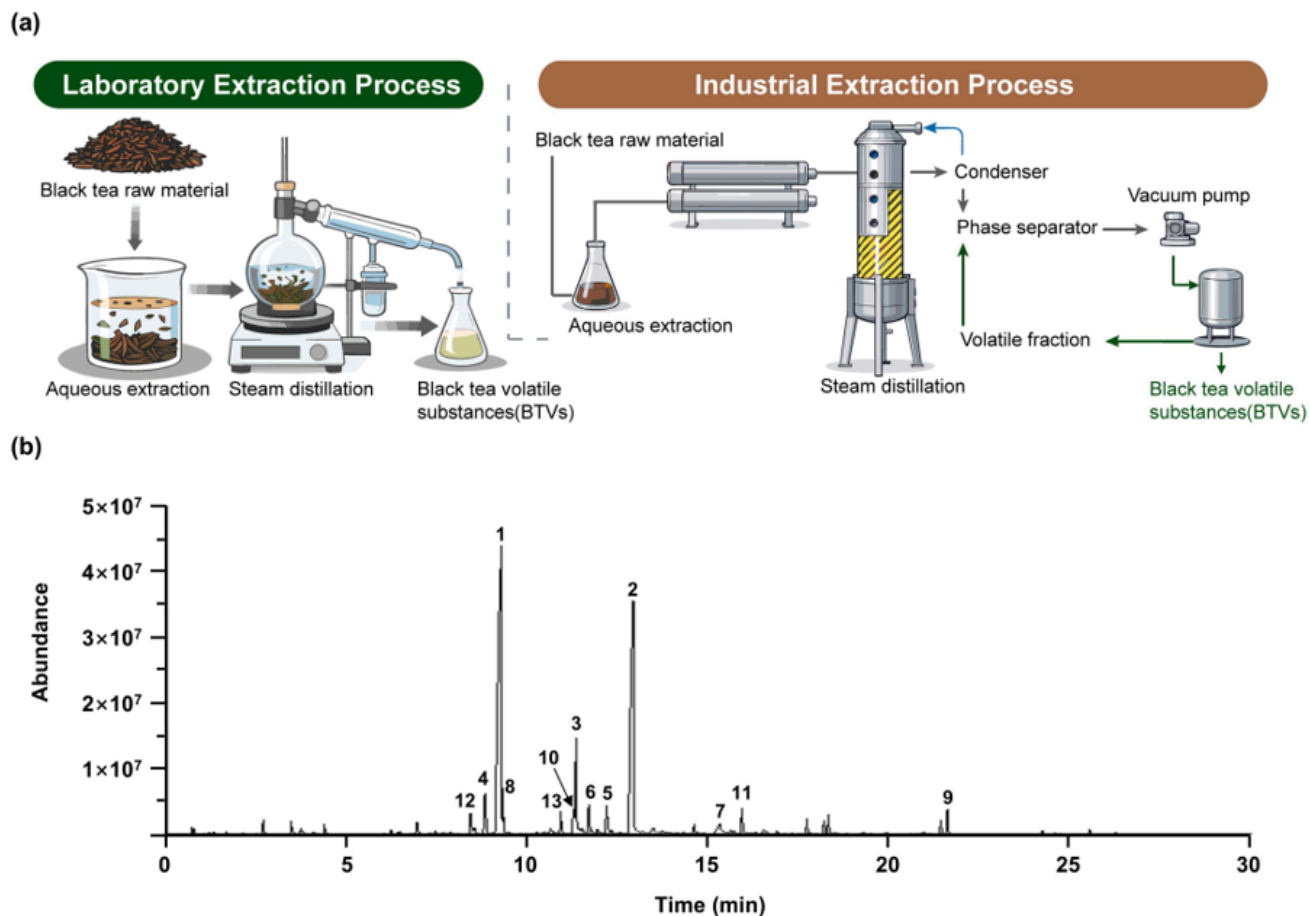


Figure 1

Preparation and major volatile composition of black tea volatile substances (BTVs).

(a) Flowchart of the BTVs production process from Dian Hong black tea via steam distillation and enrichment.

(b) Total ion chromatogram from GC-MS analysis, with relative abundance of key volatiles (area% >1%).

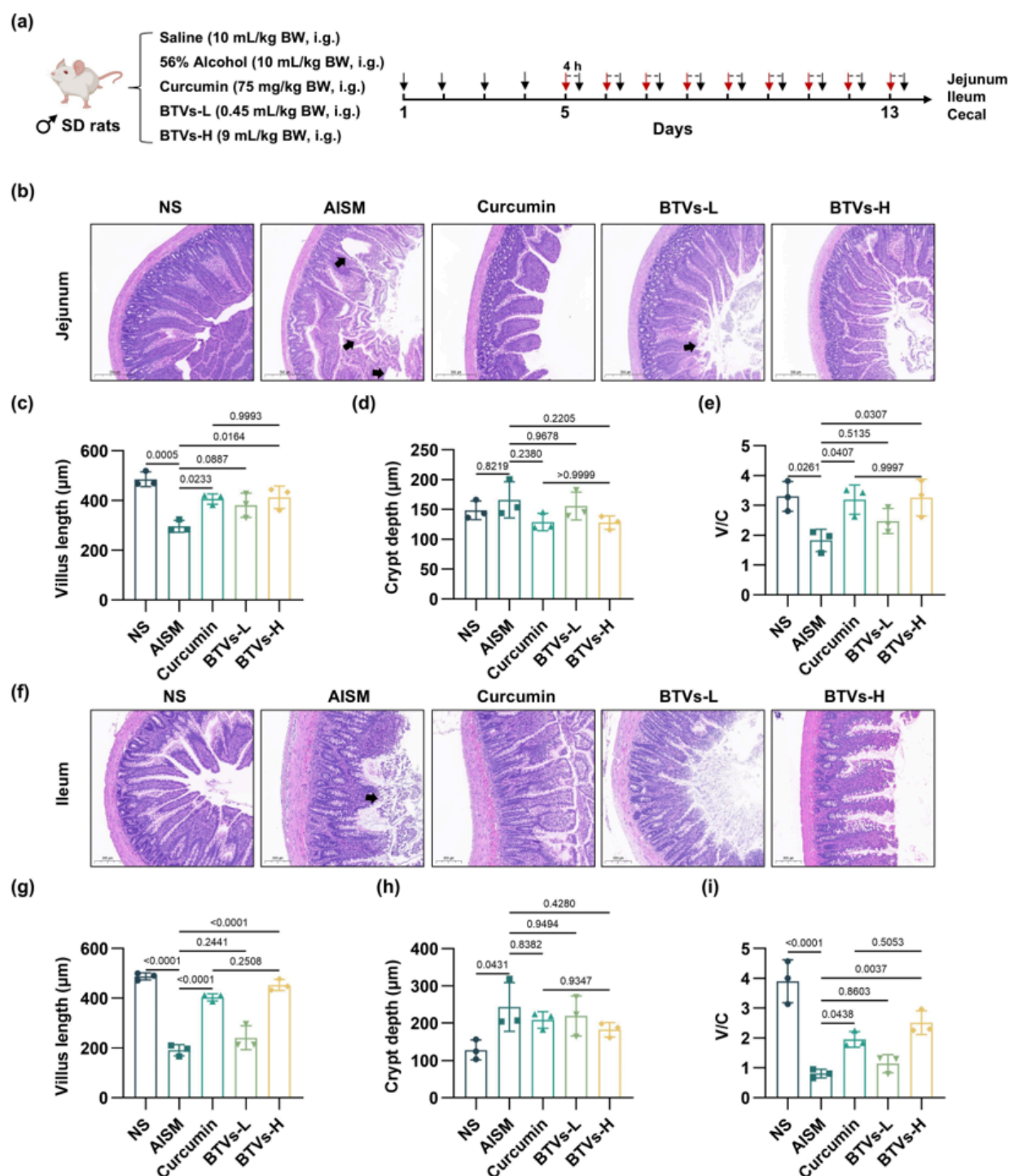


Figure 2

BTVs alleviates alcohol-induced morphological damage in rat jejunum and ileum.

(a) Schematic of the animal experimental design, including treatment groups and ethanol challenge timeline. Treatments were administered daily by gavage for 13 days (black arrows). From day 5 to 13, model and treatment groups received 56% ethanol 4 h before treatment (red arrows).

(b-e) Representative H&E - stained images of jejunum (b) and quantitative analysis of villus height (c), crypt depth (d), and villus height/crypt depth (V/C) ratio (e). Scale bars, 500 μ m

(f-i) Representative H&E - stained images of ileum (f) and corresponding quantitative analysis of villus height (g), crypt depth (h), and V/C ratio (i). Scale bars, 200 μ m. The black arrow indicates intestinal injury. Data are mean $\pm$ SD.

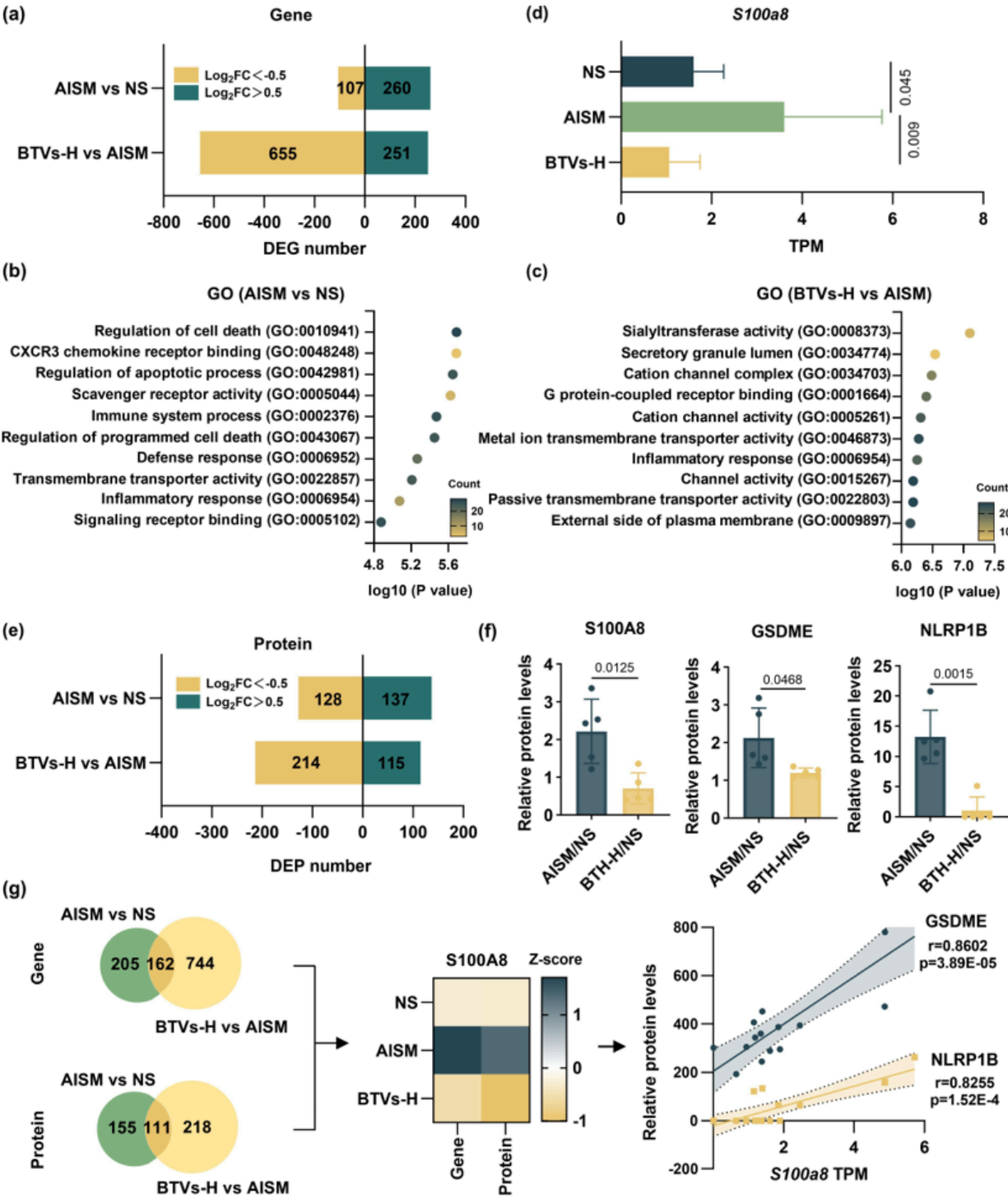


Figure 3

BTVs suppresses alcohol-induced intestinal inflammation and pyroptosis markers.

(a) Number of differentially expressed genes (DEGs; $p < 0.05$, $|\log_2FC| > 0.5$) across group comparisons.

(b-c) GO enrichment analysis of DEGs in the AISM vs. NS (b) and BTVs-H vs. AISM (c).

(d) Expression level of *S100a8* gene across groups.

(e) Number of differentially expressed proteins (DEPs, $p < 0.05$, $|\log_2FC| > 0.5$) across group comparisons.

(f) Relative protein levels of inflammation- and pyroptosis-associated markers.

(g) Integrated transcriptomic and proteomic analysis reveals differential expression of S100A8, and correlation analysis between S100a8 transcript levels and GSDME/NLRP1B protein levels.

Data are mean $\pm$ SD.

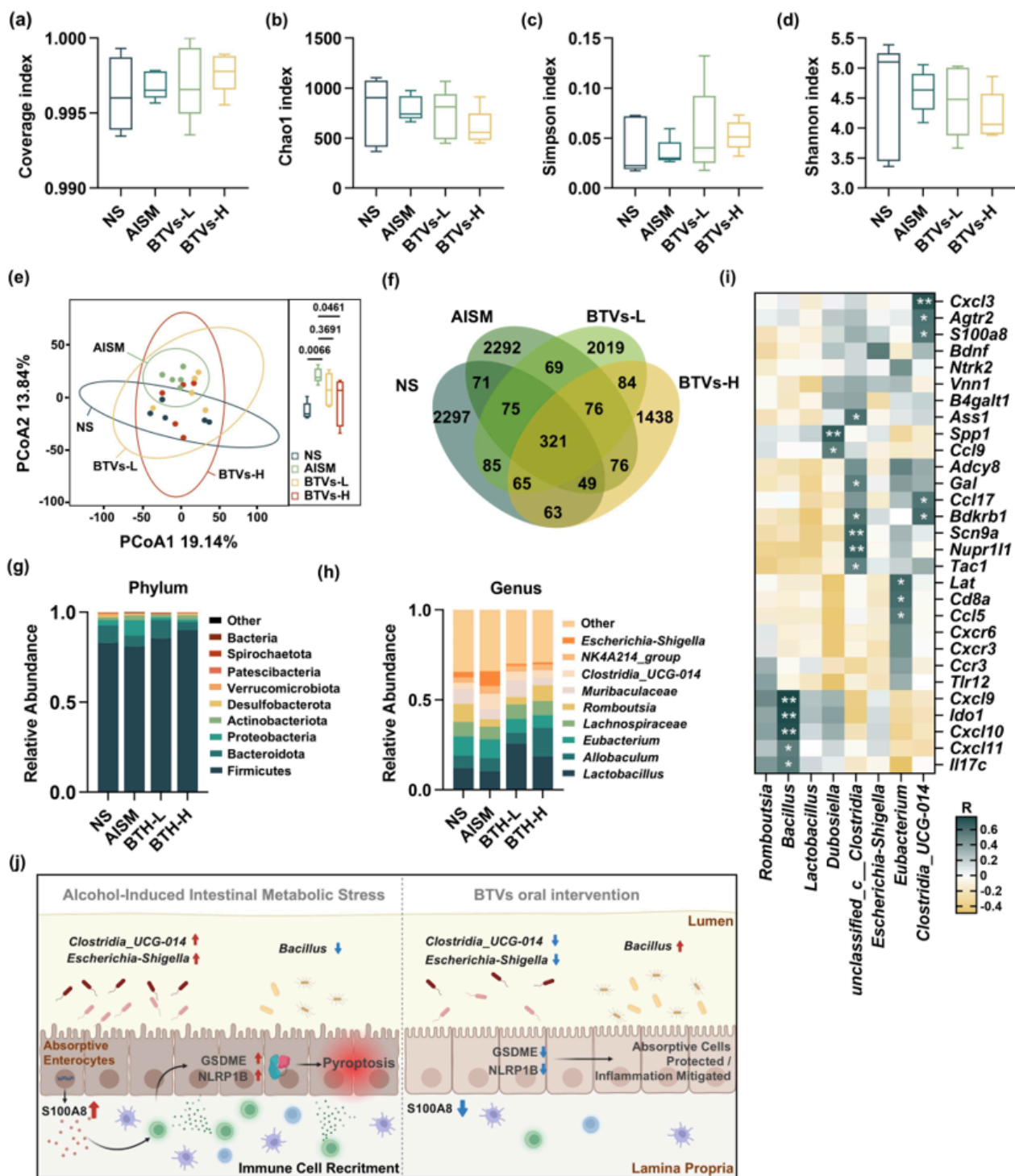


Figure 4

BTVs restores alcohol-induced gut microbiota dysbiosis in rats.

(a) Sequencing coverage across groups.

(b-d) Alpha diversity metrics (Chao1, Shannon, Simpson).

- (e) PCoA of beta diversity based on Bray-Curtis distance.
- (f) Venn diagram of shared and unique ASVs across groups.
- (g) Relative abundance of gut microbiota at the phylum level.
- (h) Relative abundance of gut microbiota at the genus level.
- (i) Pearson correlation heatmap between differentially abundant genera and inflammation-related gene expression.
- (j) BTVs dietary intervention orchestrates gut microbiome homeostasis and optimizes the intestinal microenvironment. The blue arrow indicates downregulation, and the red arrow indicates upregulation.

Data are mean $\pm$ SD where applicable.

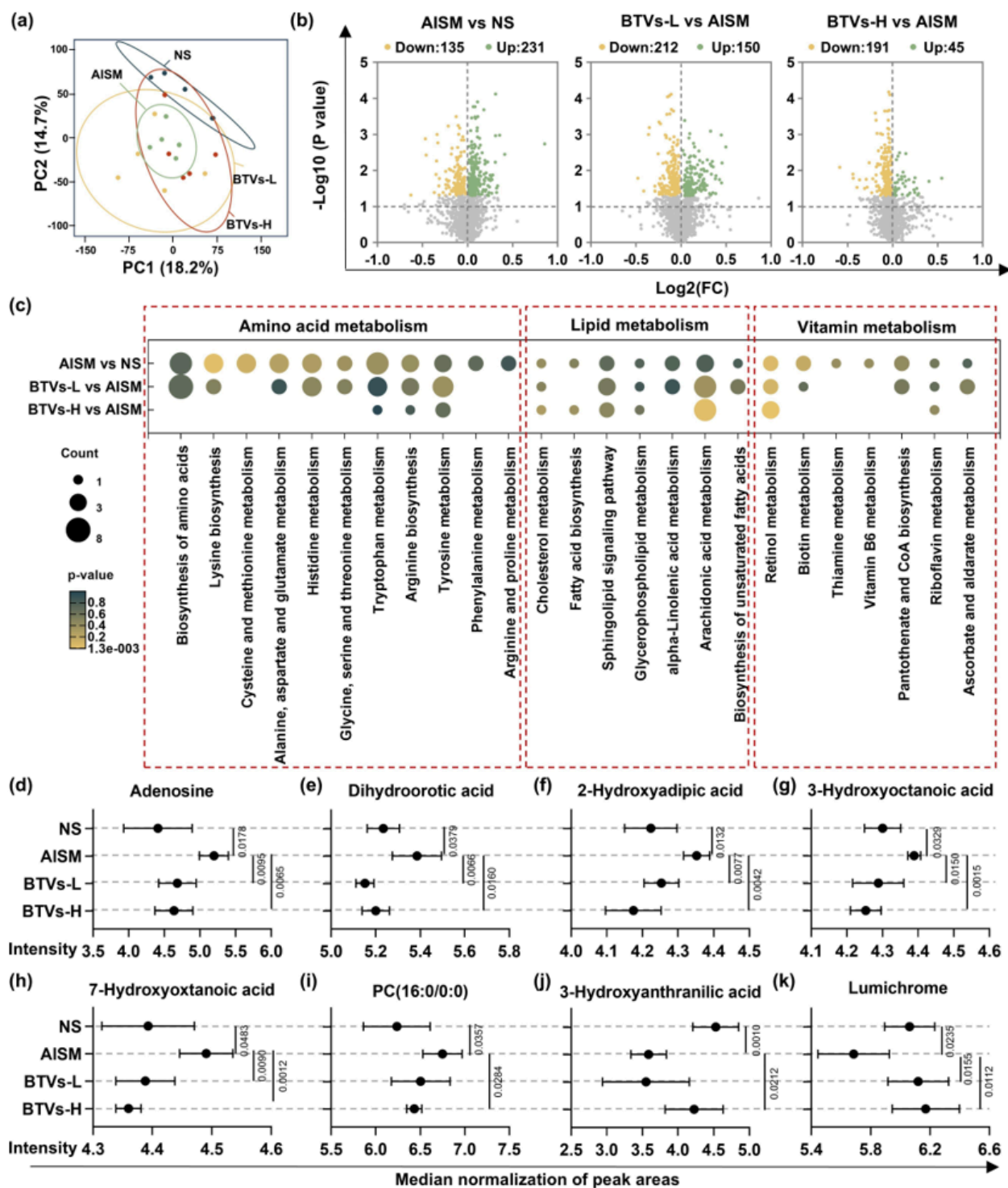


Figure 5

BTVs reprograms intestinal metabolism and restores mitochondrial function markers after alcohol exposure.

(a) PCA of untargeted metabolomics data across groups.

(b) Volcano plot of differential metabolites (VIP >1, $p < 0.05$).

(c) Bubble plot of enriched KEGG pathways among differential metabolites.

(d-k) Relative levels of representative metabolites affected by alcohol and reversed by BTVs-H (e.g., adenosine, dihydroorotic acid, phosphatidylcholine species, hydroxy-fatty acids, lumichrome, 3-hydroxyanthranilic acid).

Data are mean $\pm$ SD.

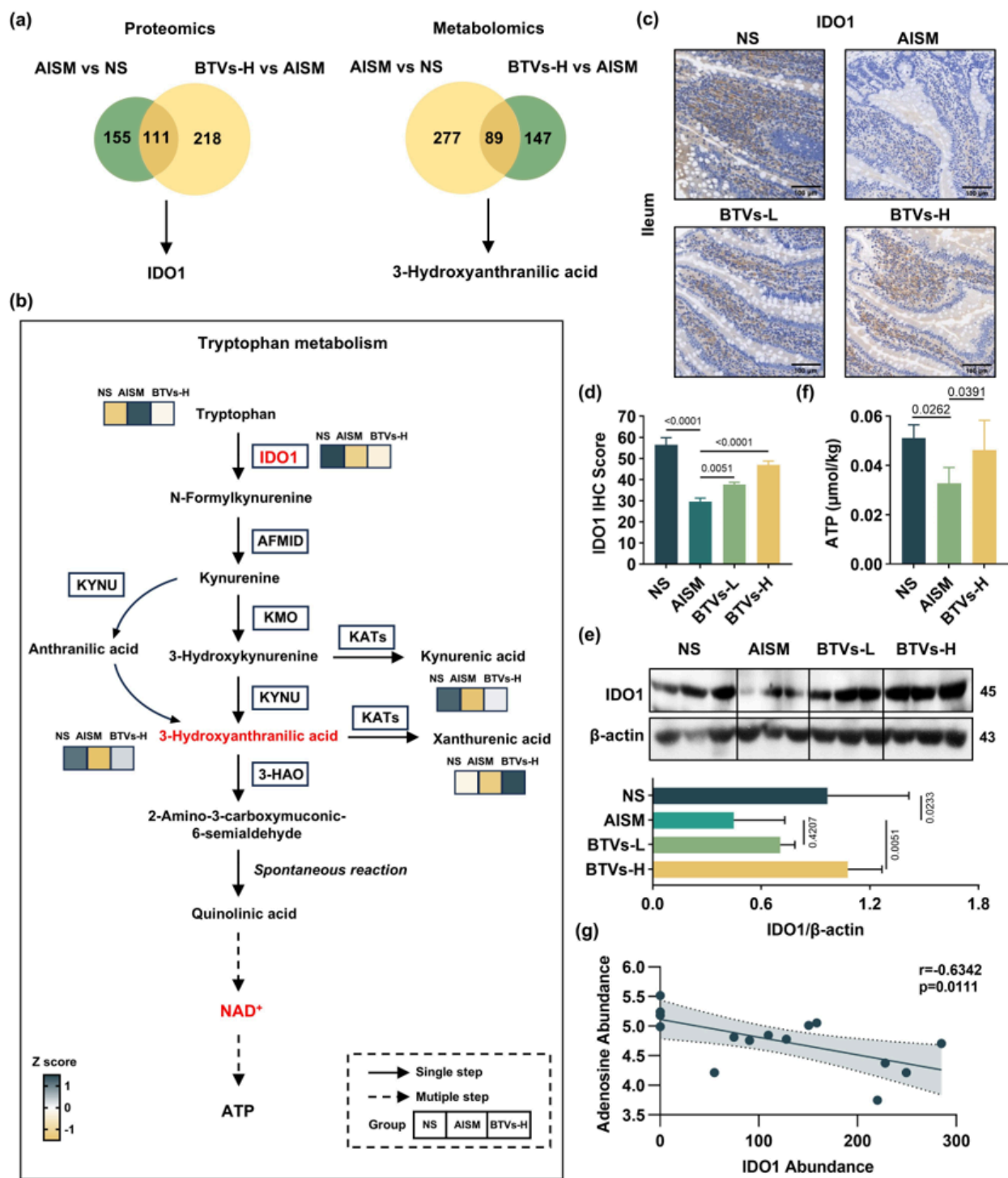


Figure 6

BTVs restore of mitochondrial energy homeostasis in AISM rats by reprogramming of IDO1-mediated tryptophan metabolism

(a) Venn diagrams illustrating the intersection of differentially expressed proteins (left) and metabolites (right) between the AISM vs. NS and BTVs-H vs. AISM groups. IDO1 and 3-hydroxyanthranilic acid were

identified as key overlapping targets.

(b) Schematic representation of the targeted tryptophan metabolism pathway. The colored boxes indicate the normalized relative abundance (Z-score) of specific enzymes and metabolites across the NS, AISM, and BTVs-H groups (from left to right).

(c-d) Representative IHC images and quantification showing the spatial expression and localization of IDO1 in the intestinal mucosa across different groups.

(e) Expression levels of IDO1 and quantification.

(f) Quantification of ATP levels in intestinal tissues.

(g) Pearson correlation analysis between intestinal IDO1 protein abundance and adenosine abundance.

Data are mean $\pm$ SD where applicable.

Supplementary Files

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