Research Article

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Effect of probiotic administration of *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactiplantibacillus plantarum* ATCC 14917 on metabolic profiles in an IBS-D rat model: a metabolomic analysis approach

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Abstract

Background: Gut microbiota dysbiosis is associated with diarrhea-predominant irritable bowel syndrome (IBS-D). Visceral hypersensitivity (VH) is a hallmark symptom.

Methods: Twenty-five female rats were allocated to either a control group (sham stress, $n = 5$) or a stressed group exposed to a 10-day water avoidance (WA) stress protocol to induce VH ($n = 20$). After stress induction, stressed rats received a 4 week intervention with phosphate-buffered saline (PBS), *Bifidobacterium animalis* subsp. *lactis* BB-12 (P1), *Lactiplantibacillus plantarum* ATCC 14917 (P2), or their combination (P1P2). Control rats were administered PBS. Visceral hypersensitivity was assessed using abdominal withdrawal reflex (AWR) scores, and metabolic changes in plasma, liver, and distal colon were analyzed using proton nuclear magnetic resonance (^{1}H NMR)-based metabolomics.

Findings: Water avoidance stress induced VH, as evidenced by higher AWR scores ($P < 0.05$) and lower pain thresholds compared to controls (38.47 $\pm$ 3.78 versus 29.48 $\pm$ 2.52; $P < 0.001$). Probiotics significantly reduced AWR scores at 20 and 80 mmHg and increased pain thresholds in comparison with the WA + PBS group ($P < 0.05$). Metabolomic profiling revealed that WA + PBS rats showed significant dysregulation in energy, amino acid, one-carbon, and lipid metabolism. Notable changes included elevated sarcosine, acetate, taurine, glutamate, tryptophan, 2-hydroxybutyrate, urea, and allantoin, with reduced *O*-acetylcarnitine in plasma; increased succinate, *myo*-inositol, isoleucine, threonine, glutamine, betaine, pyroglutamate, and *O*-phosphocholine with decreased taurine in the liver; and various amino acids, ketone bodies, glucose, glycerol, and one-carbon metabolites in the colon ($P < 0.001$). Probiotic supplementation largely restored these metabolic changes, with clustering analyses supporting the normalization of metabolic signatures.

Conclusions: Probiotic supplementation ameliorated VH and reversed IBS-D-associated metabolic disruptions, illustrating its therapeutic potential and providing insights into the underlying mechanisms.

Supporting information may be found in the online version of this article.

Keywords: diarrhea-predominant irritable bowel syndrome; metabolomics; metabolites; probiotics

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common condition. It is defined by symptoms of abdominal discomfort, bloating, and altered bowel habits, and used to be classified as a functional gastrointestinal (GI) disorder.[1,2] According to the Rome IV diagnostic criteria, IBS can be divided into four subtypes: diarrhea-predominant (IBS-D), constipation-predominant (IBS-C), mixed (IBS-M), and unclassified (IBS-U), with IBS-D being the most common.[3]

Recent research has highlighted the role of gut microbiota imbalance, or dysbiosis, in the onset and progression of IBS.[4-6] The brain–gut–microbiota axis is central to IBS pathophysiology, mediating bidirectional communication between the gastrointestinal tract and the central nervous system and linking intestinal dysfunction to psychiatric comorbidities, particularly anxiety and depression.[2,5,7] Microbiota-derived metabolites, including neurotransmitters (histamine, serotonin, glutamate, γ-aminobutyric acid, noradrenaline, dopamine, and acetylcholine), tryptophan, short-chain fatty acids (SCFAs), bile acids, hypoxanthine, and vitamins D and B_6, are reported as biomarkers correlated to the pathogenesis of IBS.[6,8] The multifactorial nature of IBS complicates its pathogenesis; however, genetic predispositions, food sensitivity, abnormal GI motility, psychosocial stress, gut mucosal immune activation, low-grade inflammation, bile acid malabsorption, infection, and post-infectious changes are believed to contribute to its manifestation.[5,9]

Animal models are invaluable for deciphering the biological pathways involved in human diseases and in therapy.[7] The water avoidance (WA) stress model is commonly employed to study stress-induced gastrointestinal disease in rodents, effectively reproducing the psychiatric stress in IBS-D.[7,10,11] The model meets the main validity criteria for preclinical studies on IBS. It has construct validity, as chronic WA stress leads to visceral hypersensitivity (VH), colonic dysmotility, anxiety-like behavior, and immune activation (mast cell infiltration and cytokine release). It has face validity, through observable IBS-like symptoms like altered defecation patterns, increased sensitivity to colorectal distension (CRD), and stress-dependent behavioral changes. It also has predictive validity, as pharmaceutical interventions that are clinically demonstrated to benefit in IBS are effective in alleviating WA stress-induced symptoms.[11-15] By placing rodents on a small platform surrounded by water, the model subjects the rodent to both psychological and physical stress, activating the hypothalamic–pituitary–adrenal (HPA) axis and inducing anxiety-like behavior, thus simulating the comorbid psychiatric disorders commonly observed in IBS.[7,10] The pathophysiological consequences are duration dependent; a single 1 hour exposure elicits delayed VH; exposure for 3 days consecutively impairs intestinal permeability; and 4–10 days lead to diarrhea-like symptoms and gut microbiota alterations.[16,17]

Visceral hypersensitivity, a hallmark of IBS, is exaggerated perception of visceral stimuli due to gut–brain axis dysregulation, dysfunctional pain processing in the brain, and peripheral lesions like increased intestinal permeability, mucosal immune activation, and low-grade inflammation, collectively contributing to urgency, abdominal pain, as well as bloating.[12,14,18]

The imbalanced gut microbiota linked to IBS has led to an interest in probiotics as a potential therapeutic option.[19] Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[20] Numerous meta-analyses have evaluated the efficacy of mono- and multi-strain probiotics in IBS, often yielding inconsistent and contradictory results.[5,21,22] Several studies suggested that certain probiotics might help some patients with IBS but it remains unclear whether particular strains or species are consistently more effective, or whether specific symptoms or subtypes of IBS are more likely to respond positively to their use.[19]

Bifidobacterium spp. and *Lactobacillus* spp. are the most commonly used probiotic strains in the context of IBS due to their prevalence and the ratio of aerobes to anaerobes.[5] Mechanistically, probiotics exert beneficial effects by modulating gut microbiota composition, intestinal barrier function, regulating the immune system, and gut-brain communication.[18]

A clinical study by Hong *et al.*[23] demonstrated that the administration of probiotic fermented milk containing *Lactobacillus* sp. HY7801, *Bifidobacterium longum* HY8004, and *Lactobacillus brevis* HY7401 can improve intestinal barrier function and reduce serum glucose and tyrosine levels in IBS patients. Another study conducted by Noorbakhsh *et al.*[24] suggested that the synbiotic yogurt containing *L. plantarum* ATCC 14917 and *L. fermentum* ATCC 14931 leads to significant changes in IBS-D metabolism, notably affecting one-carbon metabolism. Recent research also indicated that *Lactiplantibacillus plantarum* D266 supplementation demonstrated efficacy in colonic physiology and enteric neurons through the action of microbial tryptophan metabolites.[25]

Based on this body of evidence, the present study selected *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactiplantibacillus plantarum* ATCC 14917 for investigation. *Bifidobacterium animalis* subsp. *lactis* BB-12 is one of the most extensively studied probiotics, with more than 130 human clinical trials supporting its safety and efficacy, and with documented resistance to gastric acid and bile, mucus adherence, and immunomodulatory activity.[22,26] Clinical research has shown its effectiveness in increasing bowel movement frequency, reducing abdominal discomfort, and improving stool consistency while colonizing the gut microbiota in IBS patients.[27-30] Likewise, *L. plantarum* strains have shown efficacy in IBS with randomized controlled trials reporting reductions in abdominal pain, bloating, and flatulence, with sustained effects during follow up and high patient-reported efficacy.[24,31-35] In addition, a previous *in vitro* evaluation further confirmed the probiotic potential of both strains, including their tolerance to gastrointestinal conditions, adhesion capacity, and antimicrobial activity.[36]

The study aimed to assess VH and associated metabolic changes using a 4 week probiotic intervention with *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactiplantibacillus plantarum* ATCC 14917, both individually and in combination, across three different biospecimens – plasma, liver, and distal colon – in a rat model of IBS-D. The research examined the therapeutic potential of probiotics in IBS-D and the metabolic pathways involved to inform future therapeutic strategies.

MATERIALS AND METHODS

Animals

Twenty-five female Wistar–Kyoto rats were purchased from the animal laboratory of Mashhad University of Medical Sciences (Mashhad, Iran). The rats were kept under controlled conditions (room temperature, 23 ± 2 °C; approximately 30% relative humidity; and a 12 h lighting cycle; lights on at 7:00 a.m.) with free access to standard chow and tap water. After 2 weeks of acclimation, 7-week-old rats weighing 135–165 g were randomly divided

into two main groups: a stressed group ($n = 20$), which underwent a 10-day WA stress protocol, and a control group ($n = 5$), with sham stress. The stressed rats were subdivided into four groups, each receiving a different treatment. The experiment consisted of the following five groups ($n = 5$/group): (1) the control + PBS group treated with 1 mL phosphate-buffered saline (PBS); (2) the WA + PBS group treated with 1 mL PBS; (3) the WA + P1 group treated with 1 mL *Bifidobacterium animalis* subsp. *lactis* BB-12; (4) the WA + P2 group treated with 1 mL *Lactiplantibacillus plantarum* ATCC 14917; and (5) the WA + P1P2 group treated with 1 mL of an equal mixture of P1 and P2. The intervention lasted 28 days after the induction of the IBS-D model in the rats. Each group had two cages that housed two or three rats together. The experimental design is presented in Fig. 1.

Ethics statement

All procedures were approved by the animal ethics committee of the Ferdowsi University of Mashhad, Mashhad, Iran (IR.UM.REC1400.051).

Sample collection

At the end of the intervention, the rats were anesthetized with a ketamine/xylazine solution (87.5/12.5 mg kg^{-1} body weight, Alfasan Co, Netherlands) by intraperitoneal injection. Blood samples were withdrawn from each rat's heart into a lithium-heparinized tube and centrifuged at 3000 × *g* for 10 min at 4 °C to separate the plasma. The plasma obtained was then transferred quickly into a sterile cryotube and stored at −80 °C. After cervical dislocation, the left lobe of the liver was removed, washed with cold normal saline on an ice pack, and immediately snap-frozen with liquid nitrogen. A segment of the distal colon was also dissected, gently flushed out twice with cold normal saline to remove feces and mucus, and then snap-frozen in liquid nitrogen.[37] All samples were stored at −80 °C until further metabolomics analysis.

Probiotics treatment

Frozen culture of *Lactiplantibacillus plantarum* ATCC 14917 (Molecular Biotechnology Laboratory, Ferdowsi University of Mashhad, Mashhad, Iran) and freeze-dried *Bifidobacterium animalis* subsp. *lactis* BB-12 (Chr. Hansen, Hoersholm, Denmark) were obtained and cultured in De Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) and MRS with 0.05% (w/v) filter-sterilized L-cysteine·HCl (Sigma-Aldrich, St.Louis, USA) broth for 18 h at 37 °C under aerobic and anaerobic conditions, respectively. Bacterial pellets were harvested by centrifugation at 5000 × *g* for 10 min, washed twice, and subsequently resuspended to a final dilution of 2.1×10^8 colony-forming units per milliliter (CFU mL^{-1}).[36] During the intervention, all rats received 1 mL of a freshly prepared suspension containing 2.1×10^8 CFU mL^{-1} of probiotics or a PBS solution as a placebo, administered once daily by oral gavage between 10:00 and 11:00 a.m.

Irritable bowel syndrome model establishment

Water avoidance stress rat model

The rats were exposed to WA stress to induce the rat IBS-D model, as described in earlier work.[38,39] Based on a body weight-to-surface ratio of approximately 7–9 g cm^{-2}, each rat was placed on a glass block (4.3 × 4.3 × 6.0 cm) positioned at the center of a white semi-transparent plastic container (41.5 × 34.5 × 18.5 cm) filled with room-temperature water to 1 cm below the block height, for 1 h daily over 10 consecutive days between 8:00 and 11:00 a.m. Control rats were placed in identical containers without water. Fecal pellets expelled during the 1 h WA stress were counted to evaluate colonic motility, following validated procedures.[10] Rats were weighed daily before WA stress to monitor changes from the baseline.

Assessment of VH to colorectal distension

Visceral hypersensitivity was assessed on the 11th and 39th day by measuring the abdominal withdrawal reflex (AWR) in response to colorectal distension (CRD) – an established and validated method.[40] After being fasted for 16 h, each animal was lightly anesthetized using isoflurane. Subsequently, a lubricated polyethylene balloon (5 cm in length and 1 cm in diameter) was tied to the 6 French nasogastric (NG) feeding tube with a surgical suture. Then, the balloon was gently inserted into the rectum and secured to the tail with zinc oxide tape. The handmade barostat was assembled by connecting one end of the tube to a three-way stopcock, a 60 mL syringe as an air pump, and a sphygmomanometer (Accumed, Morbio Inferiore, Switzerland). The rats were placed into a 20 × 8 × 8 cm glass box and allowed to recover fully prior to the measurement (15–30 min). The inserted balloon was inflated progressively, with an increment of 10 mmHg s^{-1}, until it reached 80 mmHg, to assess the pain threshold. Behavioral responses at constant CRD pressures (20, 40, 60, and 80 mmHg) were evaluated by the semi-quantitative AWR score system. The duration of the CRD measurements was 20 s, with 4 min intervals repeated three times for each rat. Abdominal reflex scores were

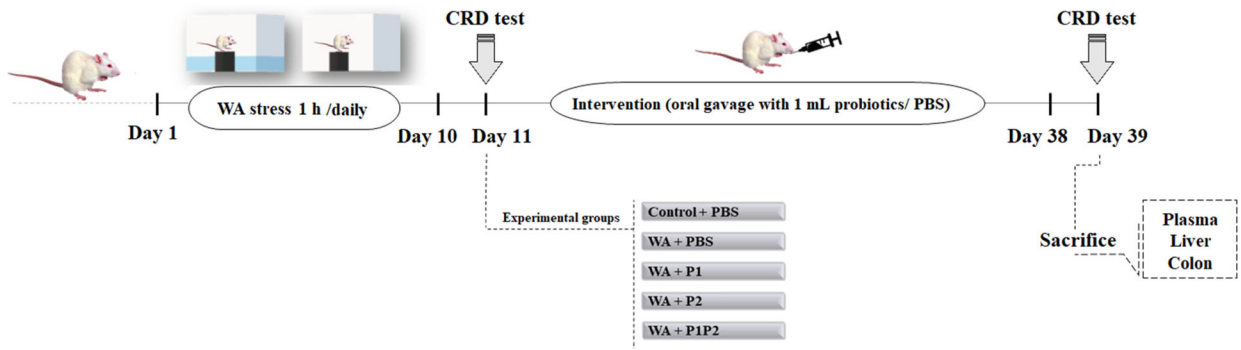

Figure 1. Experimental timeline showing water avoidance (WA) stress exposure, group assignments (control + PBS: sham-stressed rats given phosphate-buffered saline (PBS); WA + PBS: stressed rats given PBS; WA + P1: stressed rats given *Bifidobacterium animalis* subsp. *lactis* BB-12; WA + P2: stressed rats given *Lactiplantibacillus plantarum* ATCC 14917; WA + P1P2: stressed rats given the combination of both strains), and the schedule for colorectal distension (CRD) testing and sample collection.

collected by a blinded observer using a scale ranging from 0 to 4 as follows: (0) lack of reaction, (1) stock-still or minimal motion of the head, (2) mild contraction of abdominal muscles, (3) intense contraction of abdominal muscles with lifting of the abdomen, and (4) body arching or lifting pelvic structure. The pain threshold was AWR-3 during the CRD.[41]

Proton nuclear magnetic resonance-based metabolomics analysis

Plasma

Plasma samples were thawed on ice and applied to pre-washed Nanosep centrifugal filters (3 kDa cutoff) (Pall Life Science, Port Washington, NY, USA). Centrifugation was performed at 10 000 × *g*, 4 °C for 2 h, followed by additional centrifugation at 13 000 × g for 7 h. For metabolite quantification, a mixture containing filtrate (310 μL), sodium phosphate buffer (150 μL, 0.4 mol.L^{-1}, pH 7.0), D_2O (45 μL), Milli-Q water (65 μL), and sodium-3-(trimethylsilyl)-2,2,3,3-tetra-deuteriopropionate (TSP-d_4) solution as an internal standard (30 μL, 5.8 mmol.L^{-1}) (Cambridge Isotope Laboratories, Tewksbury, USA) was prepared for each sample, and 560 μL of the final mixture was added to a 5-mm nuclear magnetic resonance (NMR) tube.[24]

Liver and distal colon tissue

The rat liver and distal colon samples were extracted as described in previous studies with minor modifications.[42,43] In brief, the liver samples (100 mg) were cut into pieces and homogenized with an Ultra-Turrax (Janke and Kunkel, Staufen, IKA Werke, Germany) in ice-cold methanol-chloroform (2:1 v/v, 3 mL) for 1 min, followed by sonication for 30 min. Following the addition of 1 mL of ice-cold chloroform and 1 mL of ice-cold water, each sample was centrifuged at 1800 × *g* for 35 min at 4 °C after being vortexed for 1 min. The aqueous supernatant (2.200 mL out of 3 mL of the polar phase) was collected, dried for 4–5 h using a SpeedVac (Savant, SVC 100H, Savant Instruments Inc., New York, USA), and then redissolved in 520 μL of sodium phosphate buffer (0.135 mol.L^{-1}, pH 7.0). Reconstituted samples were centrifuged through pre-washed Nanosep centrifugal filters at 12 000 × *g* for at least 1 h at 4 °C. Each 5 mm NMR tube contained 350 μL of filtrate, 170 μL of sodium phosphate buffer (0.135 mol.L^{-1}, pH 7.0), 50 μL of D_2O, and 30 μL of TSP-d_4. Distal colon samples were prepared using the same procedure, except that homogenization was extended to 3–5 min to achieve a homogeneous solution.

Metabolomics analysis of samples

High-resolution one-dimensional proton nuclear magnetic resonance (^{1}H NMR) measurements of all biospecimens collected at the end of each intervention period were performed using a 600 MHz NMR spectrometer (Bruker Avance III 600, Rheinstetten, Germany) equipped with a cryoprobe and an autosampler. The *zgesgp* pulse sequence (Bruker Spectrospin) was performed on all spectra with 128 scans and 65.5 K data points over a spectral width of 17 942.58 Hz at 298 K with 1.82 s acquisition time per scan and 4.0 s relaxation delay. Free induction decays (FIDs) were multiplied by a 0.3 Hz line-broadening factor prior to Fourier transformation. Acquired NMR spectra were processed using Bruker TopSpin (version 4.1) software. TSP-d_4 served as an internal reference at δ 0.0 ppm, and baseline distortions were corrected. Spectra were further processed with ChenomX NMR Suite version 7.1 Profiler (ChenomX Inc., Edmonton, AB, Canada) for phase correction. ^{1}H NMR signals were identified using the Human Metabolome Database (HMDB; http://www.hmdb.ca/), Chenomx reference library, and previous literature[24,43] In total, 60 metabolites in plasma, 62 in the aqueous extract of the liver, and 52 in the aqueous extract of the distal colon were selected for quantification.

Statistical analysis

Univariate statistical analyses were conducted using SPSS Statistics 27.0 software. Comparisons of body weight, pain thresholds, and AWR scores across different groups (before and after the intervention) and fecal pellet output (FPO) (during the WA stress session) were analyzed using one-way analysis of variance (ANOVA) with Duncan's post hoc test applied where appropriate. The same statistical method was used to compare the concentrations of discriminatory metabolites identified by multivariate models between groups. Data are presented as means ± standard deviation (SD), and statistical significance was defined as a $P < 0.05$.

Multivariate data analysis was performed on the biospecimen metabolite concentrations datasets using SIMCA-P version 14.1 (Umetrics, Umeå, Sweden) at the end of the intervention (on day 39). A principal component analysis (PCA) model was applied as an unsupervised analysis on unit variance (UV)-scaled data to identify potential groupings or outliers using Hotelling's T^2 (95% confidence interval, CI). No strong outliers were detected in the biospecimens dataset. A partial least squares discriminant analysis (PLS-DA) model was also conducted as a supervised analysis to improve the separation of sample classes and identify the discriminative metabolites among the five groups. A cross-validated analysis of variance (CV-ANOVA) was carried out to verify the reliability and validity of the PLS-DA models. Models with CV-ANOVA *P* less than 0.05 were considered significant. R^2Y, representing the percentage of variation in the dataset explained by the model (the goodness of fit), and Q^2, representing the percentage of variation in the dataset predicted by the model (the goodness of prediction), were reported to describe the quality of models. Metabolites were deemed discriminative if they exhibited variable importance for the projection (VIP) value greater than 1, with a corresponding jackknife-based 95% CI not close to or including zero.[24,43,44]

RESULTS

Model evaluation

As Fig. 2(A) shows, exposure to WA stress for 1 h daily for 10 days increased FPO in the stressed rats ($n = 20$) in comparison with the control group ($n = 5$). The mean number of FPOs per hour in the stressed rats was 3.33 ± 0.71, whereas the control group had a mean of 0.88 ± 0.59 ($P < 0.01$). In response to CRD, the AWR scores in the stressed group were significantly higher at all constant pressures than the control group on day 11 ($P < 0.001$ at 20 and 40 mmHg, $P = 0.004$ at 60 mmHg, and $P = 0.03$ at 80 mmHg) (Fig. 2(B)). Accordingly, WA stress resulted in VH, as reflected by increased FPO and AWR scores compared with the control group. Remarkably, a 4 week probiotic supplementation, either alone or together, significantly reduced AWR scores at 20 and 80 mmHg in comparison with the WA + PBS group ($P < 0.018$ at 20 mmHg and $P = 0.001$ at 80 mmHg) (Fig. 2(C)). Evaluation of pain thresholds conducted before and after the intervention also revealed a substantial increase in mean pain thresholds within the probiotic-treated groups ($P = 0.029$ for the WA + P1 group and $P < 0.001$ for both the WA + P2 and WA + P1P2 groups). In contrast, no statistically significant changes

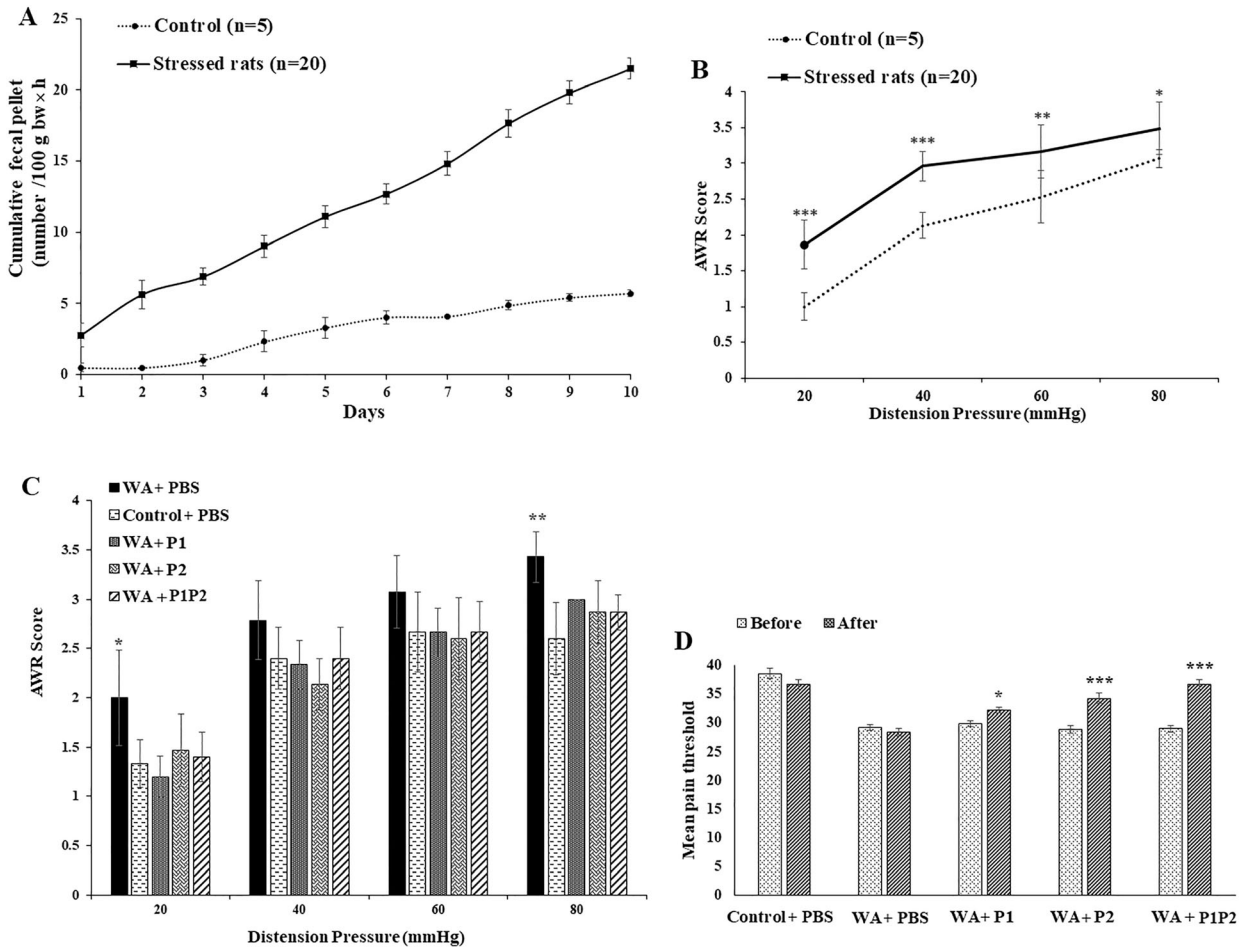

Figure 2. A comparison of the water avoidance (WA) stress effect on the experimental groups before (on day 11) and after 4 weeks of treatment intervention (on day 39). (A) cumulative fecal pellet output (FPO). (B) Abdominal withdrawal reflex (AWR) score to colorectal distension (CRD) test on day 11 and (C) after intervention. (D) Mean pain thresholds before and after intervention. Each point represents the mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

were observed in the pain thresholds of the control + PBS and WA + PBS groups before and after the intervention. Furthermore, the findings suggest that the combination of probiotics was more effective in enhancing pain thresholds than individual administration (mean pain threshold = 36.67 ± 3.46, $P < 0.001$) (Fig. 2(D)). There were no significant differences in the percentage of body weight gain during the WA stress or intervention periods among the groups (data are shown in Supporting Information, Fig. S1).

Proton nuclear magnetic resonance metabolomics analysis of samples at the end of the intervention period

Plasma metabolome

The PCA model of all the plasma data, using the first two principal components (PCs), showed a clear separation between the WA + PBS group and other groups along the first component, with the probiotic-treated groups closely clustering with the control + PBS group (Supporting Information, Fig. S2A). Further investigations using a PLS-DA model confirmed this separation (Fig. 3). The two-component fitted PLS-DA model (two PCs in total) parameters were: $R^2X = 30.6\%$, $R^2Y = 41\%$, $Q^2 = 23.1\%$, and CV-ANOVA $P = 0.021$, indicating that the model's separation of groups was statistically significant despite the relatively low Q^2 and R^2Y. Fourteen out of 60 metabolites were identified as discriminatory among the groups along the first component, with 13 found significant based on ANOVA (Supporting Information, Table S1). The WA + PBS group exhibited significantly higher plasma levels of sarcosine, acetate, taurine, tryptophan, glutamate, urea, allantoin, glucose, and 2-hydroxybutyrate than other groups. In

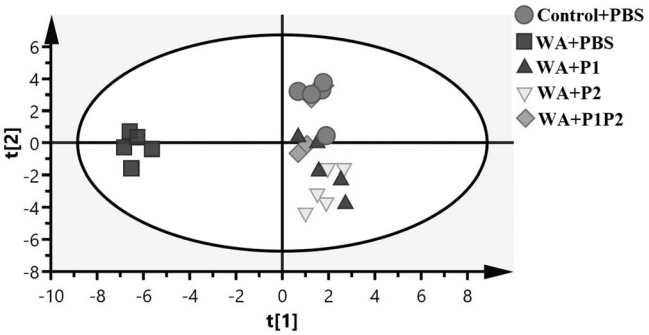

Figure 3. Score plot of partial least-squares discriminant analysis (PLS-DA) model to compare changes in plasma metabolome of all experimental groups ($n = 25$; five samples per group) at the end of the intervention. $R^2X = 30.6\%$, $R^2Y = 41\%$, $Q^2 = 23.1\%$, CV-ANOVA $P = 0.021$.

Table 1. Discriminative metabolites and their concentrations (μmol L^{-1}) in the plasma from all experimental groups on day 39 ($n = 25$ with five per group; total plasma metabolites = 60)

Metabolites	Concentration (μmol L^{-1})					*P*
	Control + PBS	WA + PBS	WA + P1	WA + P2	WAS + P1P2	
Sarcosine	2.73 ± 0.27^{a}	5.26 ± 0.27^{b}	2.44 ± 0.29^{a}	2.09 ± 0.46^{a}	2.36 ± 0.79^{a}	< 0.001
Acetate	112.30 ± 39.8^{a}	266.39 ± 42.9^{b}	122.14 ± 18.56^{a}	133.17 ± 33.69^{a}	158.72 ± 22.92^{a}	< 0.001
Taurine	313.32 ± 43.50^{a}	405.10 ± 28.03^{b}	320.86 ± 25.26^{a}	302.01 ± 27.31^{a}	312.74 ± 17.39^{a}	< 0.001
Creatine	332.54 ± 21.92^{bc}	233.3 ± 21.64^{a}	356.17 ± 16.41^{c}	303.6 ± 37.80^{b}	336.33 ± 30.84^{bc}	0.01
Tryptophan	7.59 ± 1.66^{a}	14.32 ± 5.26^{b}	7.43 ± 1.07^{a}	6.50 ± 1.22^{a}	6.99 ± 0.47^{a}	< 0.001
2-Hydroxybutyrate	16.06 ± 3.6^{a}	39.12 ± 5.97^{c}	20.59 ± 5.41^{ab}	25.70 ± 9.37^{b}	18.89 ± 3.07^{ab}	< 0.001
Glutamate	89.88 ± 14.81^{a}	130.65 ± 20.35^{b}	71.3 ± 20.45^{a}	78.74 ± 23.84^{a}	77.8 ± 6.39^{a}	< 0.001
O-Acetylcarnitine	10.72 ± 2.34^{b}	6.91 ± 0.23^{a}	11.88 ± 1.53^{b}	11.50 ± 1.41^{b}	12.14 ± 2.33^{b}	< 0.001
Urea	10 265.66 ± 364.35^{a}	11 863.9 ± 848.44^{b}	9576.35 ± 1213.04^{a}	9219.68 ± 659.21^{a}	9976.96 ± 802.55^{a}	< 0.001
Glucose	8872.07 ± 581.46^{a}	10 617.96 ± 619.04^{b}	8557.36 ± 801.51^{a}	8115.46 ± 819.45^{a}	9130.83 ± 1340.39^{a}	0.003
Allantoin	55.28 ± 8.94^{a}	79.63 ± 4.60^{c}	55.59 ± 6.96^{a}	69.99 ± 4.36^{b}	65.47 ± 3.78^{b}	< 0.001
Glutamine	531.06 ± 46.09^{b}	410.21 ± 23.89^{a}	518.44 ± 38.12^{b}	502.36 ± 59.45^{b}	496 ± 60.52^{b}	0.018
N-Acetylcysteine	9.45 ± 1.15^{b}	7.54 ± 0.55^{a}	8.71 ± 0.47^{b}	9.68 ± 0.67^{b}	9.45 ± 1.16^{b}	0.005

Note: Values are expressed as means ± SDs. Values within a row with different superscript letters are significantly different at $P < 0.05$, based on univariate statistics, including Duncan's post hoc test.

contrast, the levels of creatine, *O*-acetylcarnitine, *N*-acetylcysteine, and glutamine were significantly lower than those in the other groups ($P < 0.05$). Whether used alone or in combination, the probiotic intervention effectively restored the altered metabolites found in the WA + PBS group to levels similar to those observed in the control + PBS group. Table 1 presents the data.

Liver aqueous extract metabolome

The PCA score plot of all liver aqueous extract metabolite data demonstrated clear separation of the WA + PBS group from the others (Supporting Information, Fig. S3A). A better distinction emerged after plotting the PLS-DA score using the first two components (four PCs in total) with the following model parameters: $R^2X = 41.5\%$, $R^2Y = 39.4\%$, $Q^2 = 22.6\%$, and CV-ANOVA $P = 0.049$ (Fig. 4). Nineteen out of 62 metabolites were identified as discriminatory along the first component, with 15 found significant based on ANOVA (Supporting Information, Table S2). In the WA + PBS group, there was a considerable increase in the levels of succinate, *myo*-inositol, isoleucine, threonine, glutamine, methylamine (MA), pyroglutamate, propionate, betaine, glucose, *S*-adenosylhomocysteine (SAH), and glutamate in comparison with the other groups. Conversely, aspartate and taurine levels significantly declined relative to the other groups ($P < 0.05$). Propionate levels in the WA + P2 group and *O*-phosphocholine levels in the WA + P1P2 group exhibited no significant differences in comparison with the WA + PBS group ($P < 0.05$) (Table 2).

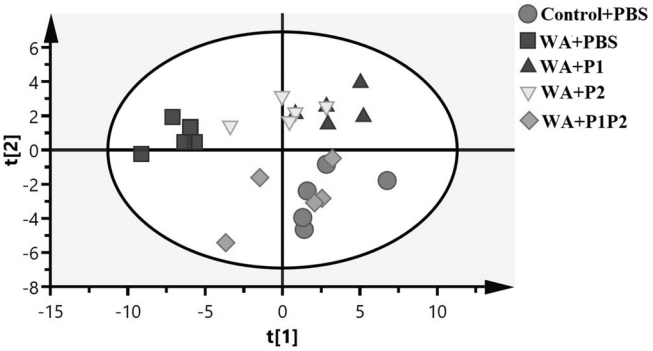

Figure 4. Score plot of partial least-squares discriminant analysis (PLS-DA) model to compare changes in the liver metabolome of all experimental groups ($n = 25$; five samples per group). The WA + PBS group is separated along the first predictive component with $R^2X = 41.5\%$, $R^2Y = 39.4\%$, $Q^2 = 22.6\%$, and cross-validated ANOVA $P = 0.049$.

Distal colon aqueous extract metabolome

Similar to the plasma and liver analyses, a fitted PCA model of the distal colon aqueous extract metabolites showed a clear separation of the WA + PBS group along the first component compared with the others (Supporting Information, Fig. S4A). Using the first two components (four PCs in total), a PLS-DA model was established. The fitted two-component PLS-DA model revealed that probiotic groups clustered with the control + PBS group and were distinct from the WA + PBS group (Fig. 5), with model parameters: $R^2X = 83.2\%$, $R^2Y = 38\%$, $Q^2 = 25.6\%$, CV-ANOVA $P = 0.038$. Out of 52 metabolites, 33 were discriminative along the first component of the PLS-DA model (Supporting Information, Table S3). Elevated levels of branched-chain amino acids (leucine, isoleucine, and valine), aromatic amino acids (tryptophan, phenylalanine, and tyrosine), proline, methionine, lysine, alanine, histamine, asparagine, glutamate, serine, threonine, sarcosine, *N*, *N*-dimethylglycine (DMG), *β*-alanine, *N*-methylhydantoin (NMH), MA, SAH, *sn*-glycero-3-phosphocholine (GPC), acetone, acetate, glycerol, cytidine, ethanolamine, xanthosine, trimethylamine (TMA), pyroglutamate, 3-hydroxybutyrate, niacinamide, and pyruvate were observed in the WA + PBS group. In probiotic-treated groups, the levels of these metabolites were downregulated ($P < 0.001$) (Table 3).

DISCUSSION

In the current study, IBS-D was induced in rats by subjecting them to WA stress for 1 h daily over ten consecutive days, thereby mimicking a state of chronic psychological stress. In line with prior studies,[10,38,39] WA stress resulted in a significant increase in FPO,

Table 2. Discriminative metabolites and their concentrations (μmol g^{-1}) in the liver aqueous extracts from all experimental groups on day 39 (n = 25 with five per group; total liver metabolites = 62)

	Concentration (μmol·g^{-1})					
Metabolites	Control + PBS	WA + PBS	WA + P1	WA + P2	WAS + P1P2	P
Succinate	0.36 ± 0.06^{a}	0.99 ± 0.13^{c}	0.33 ± 0.05^{a}	0.56 ± 0.07^{b}	0.39 ± 0.04^{a}	< 0.001
myo-Inositol	0.86 ± 0.11^{a}	1.75 ± 0.25^{b}	0.99 ± 0.06^{a}	1.02 ± 0.13^{a}	1.03 ± 0.20^{a}	< 0.001
Isoleucine	1.78 ± 0.22^{a}	2.63 ± 0.17^{c}	1.83 ± 0.28^{a}	2.22 ± 0.09^{b}	1.96 ± 0.11^{a}	< 0.001
Threonine	2.58 ± 0.32^{a}	3.27 ± 0.22^{c}	2.61 ± 0.08^{ab}	2.86 ± 0.16^{b}	2.76 ± 0.12^{ab}	< 0.001
Glutamine	3.36 ± 0.54^{a}	5.20 ± 0.66^{b}	3.64 ± 0.62^{a}	3.36 ± 0.37^{a}	3.31 ± 0.36^{a}	< 0.001
Methylamine	0.05 ± 0.01^{a}	0.09 ± 0.01^{b}	0.05 ± 0.02^{a}	0.06 ± 0.02^{a}	0.06 ± 0.02^{a}	0.002
Pyroglutamate	1.36 ± 0.18^{a}	2.23 ± 0.24^{c}	1.61 ± 0.34^{ab}	1.87 ± 0.16^{b}	1.70 ± 0.30^{ab}	< 0.001
Propionate	0.17 ± 0.05^{a}	0.33 ± 0.01^{c}	0.18 ± 0.04^{ab}	0.25 ± 0.07^{bc}	0.24 ± 0.08^{ab}	0.003
Betaine	4.13 ± 0.53^{a}	6.35 ± 0.52^{b}	4.19 ± 0.78^{a}	3.70 ± 0.41^{a}	5.29 ± 0.33^{a}	< 0.001
Glucose	69.24 ± 3.40^{a}	91.11 ± 10.51^{b}	59.09 ± 16.31^{a}	59.64 ± 13.70^{a}	69.15 ± 8.28^{a}	0.002
Taurine	11.93 ± 0.36^{b}	7.92 ± 1.06^{a}	11.40 ± 0.82^{b}	11.14 ± 0.84^{b}	13.17 ± 2.80^{b}	< 0.001
Aspartate	5.98 ± 0.73^{c}	3.91 ± 0.62^{a}	5.20 ± 0.96^{bc}	5.37 ± 0.47^{bc}	4.82 ± 0.49^{b}	0.002
SAH	0.19 ± 0.03^{a}	0.24 ± 0.00^{b}	0.20 ± 0.02^{a}	0.19 ± 0.02^{a}	0.21 ± 0.02^{a}	0.024
Glutamate	6.12 ± 0.61^{a}	8.83 ± 0.55^{c}	6.88 ± 0.94^{ab}	7.67 ± 0.44^{b}	7.25 ± 0.8^{b}	0.002
O-Phosphocholine	3.19 ± 0.44^{b}	3.80 ± 0.19^{c}	2.82 ± 0.18^{ab}	2.47 ± 0.35^{a}	3.87 ± 0.41^{c}	< 0.001

Note: Values are expressed as means ± SDs. Values within a row with different superscript letters are significantly different at $P < 0.05$, based on univariate statistics, including Duncan's post hoc test.
Abbreviation: SAH, *S*-adenosylhomocysteine.

reflecting enhanced colonic motility, and an elevated AWR score, confirming the successful induction of VH. The 4 week probiotic intervention effectively increased pain thresholds and decreased AWR scores at 20 and 80 mmHg, suggesting an alleviation of VH. The combined probiotic treatment yielded better improvements in pain thresholds than individual administration.

Research has demonstrated that probiotic supplementation can effectively attenuate stress-induced VH through various mechanisms. For example, a 10-day treatment with *Lactobacillus farciminis* markedly decreased visceromotor responses to CRD in female Wistar rats subjected to repeated WA stress; with more pronounced effects observed in females than males.[38] Probiotics also preserve intestinal barrier integrity under stress conditions and prevent bacterial translocation. In WA stress models, administration of *Lactobacillus helveticus* and *Lactobacillus rhamnosus* successfully inhibited bacterial adhesion and translocation to mesenteric lymph nodes.[45] Studies have also clarified how probiotics can affect the brain–gut axis. A study conducted in 2018 by Ait-Belgnaoui *et al.* discovered that co-administration of *Lactobacillus helveticus* and *Bifidobacterium longum* reduced chronic stress-induced VH by suppressing stress hormones and modifying glucocorticoid receptor expression in principal brain regions in the stress pathway.[46] A study by McVey Neufeld *et al.*[47] discovered that *Lactobacillus rhamnosus* GG's soluble mediators improved visceral hypersensitivity caused by early life stress, normalizing corticosterone levels as well as reversing differential spinal cord expression of genes. Likewise, Neufeld *et al.*[48] showed that these mediators alleviated maternal separation-induced visceral pain hypersensitivity, thereby affirming that particular bioactive compounds can ameliorate stress-induced pain. Taken together, the results of the current study are consistent with prior research,[49,50] further supporting the therapeutic potential of probiotics in alleviating stress-induced VH.

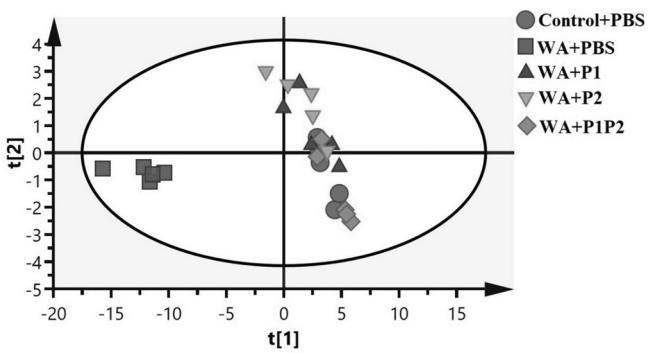

Figure 5. Score plot of partial least-squares discriminant analysis (PLS-DA) model to compare changes in the distal colon metabolome of all experimental groups (n = 25; five samples per group). The WA + PBS group is separated along the first predictive component with R^2X = 83.2%, R^2Y = 38%, Q^2 = 25.6%, cross-validated ANOVA P = 0.038.

In this study, a metabolomics approach was applied to assess metabolic alterations across the experimental groups in plasma, liver, and distal colon samples collected at the end of the intervention. The analysis revealed that 13 out of 60 plasma metabolites, 15 out of 62 liver metabolites, and 33 out of 52 distal colon metabolites exhibited significant changes between groups, highlighting the distal colon as a major site of metabolic dysregulation in response to stress.

Glycerol, the backbone of fatty acids, can be converted to pyruvate through glycolysis or glucose by gluconeogenesis.[51] Alanine serves as a substrate for gluconeogenesis or contributes to hepatic energy metabolism.[52] Succinate, a key intermediate of the tricarboxylic acid (TCA) cycle, is associated with reactive oxygen species (ROS) production when accumulated.[53] In the WA + PBS group, increased levels of these metabolites in the liver and colon, along with elevated plasma glucose levels, indicate a shift in energy metabolism. In patients with IBS-D, high glucose levels have been associated with low-grade inflammation, which

Table 3. Discriminative metabolites and their concentrations (μmol g^{-1}) in the distal colon aqueous extracts from all experimental groups on day 39 ($n = 25$ with five per group; total distal colon metabolites = 52)

	Concentration (μmol g^{-1})					
Metabolites	Control + PBS	WA + PBS	WA + P1	WA + P2	WA + P1P2	*P*
DMG	0.01 ± 0.00^{a}	0.04 ± 0.00^{b}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.01 ± 0.00^{a}	< 0.001
NMH	0.02 ± 0.01^{a}	0.09 ± 0.01^{b}	0.03 ± 0.01^{a}	0.03 ± 0.01^{a}	0.03 ± 0.01^{a}	< 0.001
Proline	2.01 ± 0.36^{a}	6.30 ± 0.80^{b}	2.42 ± 0.45^{a}	2.55 ± 0.54^{a}	1.94 ± 0.46^{a}	< 0.001
Tyrosine	1.36 ± 0.28^{a}	5.56 ± 0.93^{b}	1.82 ± 0.48^{a}	1.90 ± 0.50^{a}	1.23 ± 0.26^{a}	< 0.001
Leucine	2.26 ± 0.38^{a}	10.88 ± 2.14^{b}	3.02 ± 0.76^{a}	3.16 ± 0.74^{a}	2.30 ± 0.59^{a}	< 0.001
Lysine	2.01 ± 0.38^{a}	6.78 ± 1.15^{b}	2.33 ± 0.27^{a}	2.37 ± 0.69^{a}	1.75 ± 0.26^{a}	< 0.001
Phenylalanine	1.13 ± 0.15^{a}	4.85 ± 0.92^{b}	1.55 ± 0.43^{a}	1.61 ± 0.38^{a}	1.11 ± 0.17^{a}	< 0.001
Acetone	0.04 ± 0.01^{a}	0.13 ± 0.02^{b}	0.04 ± 0.01^{a}	0.05 ± 0.01^{a}	0.04 ± 0.01^{a}	< 0.001
Methionine	0.06 ± 0.02^{a}	0.71 ± 0.18^{b}	0.07 ± 0.04^{a}	0.10 ± 0.07^{a}	0.03 ± 0.01^{a}	< 0.001
Isoleucine	1.14 ± 0.20^{a}	5.03 ± 1.09^{b}	1.51 ± 0.34^{a}	1.60 ± 0.35^{a}	1.06 ± 0.16^{a}	< 0.001
Acetate	0.57 ± 0.07^{ab}	1.32 ± 0.12^{c}	0.58 ± 0.11^{ab}	0.69 ± 0.14^{b}	0.50 ± 0.10^{a}	< 0.001
Valine	2.09 ± 0.41^{a}	7.51 ± 1.53^{b}	2.76 ± 0.57^{a}	2.84 ± 0.61^{a}	1.96 ± 0.33^{a}	< 0.001
Glycerol	3.62 ± 0.71^{a}	9.75 ± 1.01^{b}	3.18 ± 1.12^{a}	3.08 ± 0.70^{a}	3.01 ± 0.50^{a}	< 0.001
Methylamine	0.03 ± 0.01^{ab}	0.11 ± .03^{c}	0.03 ± 0.01^{ab}	0.04 ± 0.01^{b}	0.02 ± 0.00^{a}	< 0.001
Cytidine	0.13 ± 0.07^{ab}	0.50 ± 0.05^{c}	0.13 ± 0.04^{ab}	0.18 ± 0.09^{b}	0.09 ± 0.01^{a}	< 0.001
Histamine	0.61 ± 0.15^{a}	1.99 ± 0.34^{b}	0.82 ± 0.13^{a}	0.79 ± 0.25^{a}	0.58 ± 0.09^{a}	< 0.001
Alanine	5.48 ± 0.86^{a}	13.38 ± 1.64^{b}	6.04 ± 0.91^{a}	6.33 ± 1.22^{a}	5.73 ± 1.22^{a}	< 0.001
Ethanolamine	1.36 ± 0.22^{a}	3.21 ± 0.21^{b}	1.45 ± 0.30^{a}	1.59 ± 0.42^{a}	1.39 ± 0.26^{a}	< 0.001
Tryptophan	0.24 ± 0.04^{a}	1.02 ± 0.26^{b}	0.31 ± 0.06^{a}	0.35 ± 0.10^{a}	0.22 ± 0.03^{a}	< 0.001
Asparagine	1.08 ± 0.17^{a}	3.38 ± 0.63^{b}	1.44 ± 0.34^{a}	1.44 ± 0.34^{a}	1.11 ± 0.23^{a}	< 0.001
Threonine	1.74 ± 0.24^{a}	4.37 ± 0.55^{b}	2.16 ± 0.50^{a}	2.12 ± 0.43^{a}	1.62 ± 0.45^{a}	< 0.001
Xanthosine	0.01 ± 0.00^{a}	0.05 ± 0.01^{b}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.00^{a}	< 0.001
Sarcosine	0.95 ± 0.13^{a}	2.7 ± 0.45^{b}	1.2 ± 0.31^{a}	1.22 ± 0.23^{a}	0.93 ± 0.29^{a}	< 0.001
GPC	1.43 ± 0.43^{b}	3.26 ± 0.44^{c}	1.41 ± 0.41^{b}	1.23 ± 0.39^{ab}	0.86 ± 0.25^{a}	< 0.001
SAH	0.04 ± 0.01^{a}	0.15 ± 0.03^{b}	0.06 ± 0.02^{a}	0.06 ± 0.03^{a}	0.04 ± 0.01^{a}	< 0.001
Trimethylamine	0.02 ± 0.00^{a}	0.04 ± 0.00^{b}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.01^{a}	< 0.001
Pyroglutamate	0.65 ± 0.19^{a}	1.25 ± 0.17^{b}	0.66 ± 0.08^{a}	0.73 ± 0.11^{a}	0.54 ± 0.10^{a}	< 0.001
3-Hydroxybutyrate	0.11 ± 0.05^{a}	0.44 ± 0.05^{c}	0.16 ± 0.05^{a}	0.25 ± 0.08^{b}	0.09 ± 0.02^{a}	< 0.001
β-Alanine	0.10 ± 0.01^{a}	0.21 ± 0.04^{b}	0.11 ± 0.03^{a}	0.12 ± 0.03^{a}	0.09 ± 0.01^{a}	< 0.001
Niacinamide	0.19 ± 0.02^{a}	0.38 ± 0.04^{b}	0.23 ± 0.08^{a}	0.24 ± 0.06^{a}	0.19 ± 0.03^{a}	< 0.001
Pyruvate	0.08 ± 0.02^{a}	0.19 ± 0.04^{b}	0.07 ± 0.02^{a}	0.08 ± 0.04^{a}	0.05 ± 0.01^{a}	< 0.001
Glutamate	5.36 ± 0.79^{a}	9.03 ± 0.62^{b}	5.90 ± 0.84^{a}	6.09 ± 1.37^{a}	4.93 ± 0.75^{a}	< 0.001
Serine	4.9 ± 0.78^{a}	9.5 ± 1.49^{b}	5.6 ± 1.24^{a}	5.9 ± 1.12^{a}	4.3 ± 1.19^{a}	< 0.001

Note: Values are expressed as means ± SDs. Values within a row with different superscript letters are significantly different at $P < 0.05$, based on univariate statistics, including Duncan's post hoc test.
Abbreviations: DMG, *N, N*-dimethylglycine; NMH, *N*-methylhydantoin; GPC, *sn*-glycero-3-phosphocholine; SAH, *S*-adenosylhomocysteine.

may contribute to alterations in glycolysis or impaired nutrient absorption.[8,23,50] Plasma creatine concentrations were decreased in the WA + PBS group, consistent with its role in adenosine triphosphate (ATP) recycling and epithelial integrity, both of which are relevant to IBS-D pathophysiology.[54] The current study also found increased levels of ketone bodies, including 3-hydroxybutyrate and acetone, in the colon of the WA + PBS group, suggesting metabolic shifts that may be linked to energy depletion caused by diarrhea.[55] In accordance with previous research,[24,56,57] probiotic treatment, either alone or in combination, restored the levels of energy-related metabolites, aligning with the normalization of glycolytic and energy homeostasis markers, suggesting a potential improvement in glycolysis and energy balance.[23]

Elevated acetate levels were observed in the plasma and colon of the WA + PBS group, along with increased hepatic propionate levels. The link between SCFAs and IBS remains controversial, as studies have reported both increased and decreased SCFA levels. However, a recent systematic review suggests that fecal propionate and acetate levels are correlated with IBS, with propionate being proposed as a potential biomarker for IBS-D diagnosis.[6,9] Acetate and propionate are produced through the microbial fermentation of carbohydrates and specific amino acids, and variations in their levels reflect gut microbiota composition shifts.[6,58] Following the intervention, plasma and colonic acetate levels returned to levels comparable with those in the control + PBS group, suggesting a restoration of gut microbiota balance.[59] Although hepatic propionate levels decreased across all treatment groups, this reduction did not reach statistical significance in the WA + P2 group. Gut microbiota alterations in the WA + PBS group were accompanied by changes in microbial metabolites, including increased colonic levels of TMA and MA, both derived from choline metabolism, as well as NMH, a creatinine-derived metabolite.[24,60-62]

In line with previous studies,[49,56,63-66] the findings of the current study suggest a metabolic shift in amino acid metabolism in the IBS-D rat model, particularly affecting branched-chain and aromatic amino acids. Given the extensive proteolytic activity in the intestine, largely mediated by the gut microbiota,[58] the increased amino acid levels observed in the distal colon may be attributed to enhanced peptide catabolism resulting from gut microbiota alterations, increased epithelial turnover associated with low-grade inflammation, or impaired amino acid absorption in IBS.[67] The conversion of glutamine to glutamate is crucial for maintaining neurotransmitter balance. Stress or infection can lower plasma glutamine levels, increasing demands on the immune system and organs such as the gut and liver.[68] Consistent with earlier studies,[50,57] the probiotic intervention effectively downregulated amino acid levels, restoring them to control + PBS levels, suggesting a potential regulatory effect of probiotics on microbial metabolism and host-microbiome interactions. The normalization of plasma urea levels following probiotic treatment also indicates the restoration of nitrogen balance, which may help regulate proteolytic bacterial activity and mitigate its metabolic consequences.[69]

N-Acetylcysteine and *O*-acetyl-carnitine are recognized for their neuroprotective roles in modulating inflammation and oxidative stress. The former acts as a potent antioxidant glutathione (GSH) precursor and the latter provides acetyl-CoA for energy production.[70] In this study, the WA + PBS group exhibited significantly lower plasma levels of these compounds. Elevated pyroglutamate levels, a marker of GSH deficiency, along with increased 2-hydroxybutyrate, suggest a shift in ATP production potentially linked to reduced cysteine availability or increased GSH utilization in response to oxidative stress.[71,72] Increased plasma allantoin levels also indicate heightened ROS activity, consistent with oxidative stress-associated metabolic alterations.[73]

Prior research has indicated a correlation between IBS-D and a shift in one-carbon metabolism, with elevated serum sarcosine (*N*-methylglycine) levels emerging as a potential biomarker in this context.[24] One-carbon metabolism encompasses a complex network of interrelated metabolic pathways, including the folate, choline, and methionine cycles, which serve as essential 'housekeeping' mechanisms that underpin cellular growth and function.[60,74] Consistent with prior research, the results of this study demonstrate increased betaine (trimethylglycine) levels in the liver, DMG in the colon, and sarcosine in both plasma and colon samples. Elevated colonic methionine levels and increased SAH were also observed in both the liver and colon in the WA + PBS group. These findings suggest that WA-induced stress may disrupt methylation homeostasis, potentially mediated by inflammatory pathways, mirroring the metabolic alterations previously reported in IBS-D patients.[24,75] A 4 week probiotic intervention restored these metabolite levels, supporting prior evidence that probiotics modulate one-carbon metabolism and methyl group flux.[24,76]

Taurine is a sulfur-containing β-amino acid present in various animal tissues and it has been proposed that it could play a protective role against oxidative stress and cellular injury.[77] In the current study, plasma taurine levels were elevated, while hepatic taurine levels were reduced in the WA + PBS group, suggesting a compensatory response to oxidative stress, as previously reported.[78] In patients with IBS-D, increased urinary taurine levels have been associated with a reduced population of *Lactobacillus* species and gut microbiota dysbiosis.[24] The observed decline in hepatic taurine levels may indicate its increased utilization in the synthesis of taurine-conjugated bile acids, thereby reducing its hepatic availability.[77] Probiotic supplementation normalized taurine levels, potentially through its modulatory effects on gut microbiota composition and overall gut health.[79]

Consistent with previous findings,[56,67,80] WA stress resulted in significant elevations in GPC and ethanolamine in the distal colon, along with increased *O*-phosphocholine levels in the liver. Given the role of these metabolites in lipid metabolism and membrane integrity maintenance, these changes likely reflect intestinal epithelial damage and gut barrier dysfunction associated with inflammation.[81] Liver *myo*-inositol levels were also higher in the WA + PBS group than other groups, potentially indicating a shift in glucose or lipid metabolism or serving as an adaptive response to osmotic or oxidative stress.[82,83] The probiotic-treated groups exhibited a trend toward downregulation of these metabolites, suggesting a modulatory effect on intestinal lipid metabolism. However, *O*-phosphocholine levels remained unchanged in the group receiving both probiotics, which may be attributed to complex interactions between probiotic strains and their dose-dependent effects on liver metabolism.

CONCLUSION

The NMR-based metabolomics approach used in this study revealed significant metabolic alterations in energy metabolism, amino acid pathways, one-carbon metabolism, and lipid metabolism in a rat model of IBS-D. Oral administration of *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactiplantibacillus plantarum* ATCC 14917 effectively alleviated IBS-associated VH and restored most perturbed metabolic pathways. These findings underscore the potential of metabolomic analysis of biological fluids and tissues in providing novel insights into the pathophysiology of IBS-D and its potential therapeutic strategies. Further studies are warranted to elucidate the contributions of host and microbiome factors to these metabolites and to define the strain-specific mechanisms of probiotics in IBS-D. Such studies could involve advanced multi-omics approaches to map the interactions between probiotic strains, gut microbiota, and host metabolism comprehensively. Clinical trials with larger cohorts and extended follow-up periods are also needed to validate these results.

AUTHOR CONTRIBUTIONS

Nazila Dardmeh: Writing – original draft (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead). Ali A. Moazzami: supervision (lead); resources (lead); software (lead); formal analysis (lead); writing – review and editing (equal); methodology (supporting); conceptualization (equal); funding acquisition (equal). Masoud Yavarmanesh: project administration (lead); supervision (lead); resources (lead); writing – review and editing (equal); conceptualization (supporting); funding acquisition (equal); methodology (supporting). Maryam M. Matin: resources (supporting). Hamid Noorbakhsh: writing – original draft (supporting); writing – review and editing (supporting); conceptualization (supporting); methodology (supporting).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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