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The Protective Effect of *Roseburia faecis* Against Repeated Water Avoidance Stress-induced Irritable Bowel Syndrome in a Wister Rat Model

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Roseburia faecis, a butyrate-producing, gram-positive anaerobic bacterium, was evaluated for its usefulness against repeated water avoidance stress (WAS)-induced irritable bowel syndrome (IBS) in a rat model, and the underlying mechanism was explored. We divided the subjects into three groups: one without stress exposure, another subjected to daily 1-hour WAS for 10 days, and a third exposed to the same WAS regimen while also receiving two different *R. faecis* strains (BBH024 or R22-12-24) via oral gavage for the same 10-day duration. Fecal pellet output (FPO), a toluidine blue assay for mast cell infiltration, and fecal microbiota analyses were conducted using 16S rRNA metagenomic sequencing. Predictive functional profiling of microbial communities in metabolism was also conducted. FPO and colonic mucosal mast cell counts were significantly higher in the WAS group than in the control group (male, $P = 0.004$; female, $P = 0.027$). The administration of both BBH024 (male, $P = 0.015$; female, $P = 0.022$) and R22-12-24 (male, $P = 0.003$; female, $P = 0.040$) significantly reduced FPO. Submucosal mast cell infiltration in the colon showed a similar pattern in males. In case of fecal microbiota, the WAS with *R. faecis* group showed increased abundance of the *Roseburia* genus compared to WAS alone. Moreover, the expression of a gene encoding a D-methionine transport system substrate-binding protein was significantly elevated in the WAS with *R. faecis* group compared to that in the WAS (male, $P = 0.028$; female, $P = 0.025$) group. These results indicate that *R. faecis* is a useful probiotic for treating IBS and colonic microinflammation.

Key Words *Roseburia faecis*, Irritable bowel syndrome, Rats, Probiotics

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder, and symptoms include chronic abdominal pain and change in bowel habits without structural abnormalities [1]. The etiology of IBS is multifaceted, encompassing stress, increased mucosal permeability, mucosal immune activation, food hypersensitivity, transient infection-altering intestinal microbiota, neuroplasticity, altered enteroendocrine metabolism, and genetic polymorphisms [2]. Stress is a key factor in the onset, maintenance, and aggravation of IBS symptoms. Stress induces changes in colonic functions, including the increased permeability, increased mucus secretion, alteration in motility, myenteric nerve activation, serotonin release, and the development of visceral hypersensitivity in rats [3]. Repeated water avoidance stress (WAS) is a well-known animal model of stress that increases psychological components and imitates the experiences of the ongoing environmental life in humans. WAS is known to elevate parameters such as fecal pellet output (FPO), colonic mast cell counts, mucosal cytokine levels, and induce alterations in gut microbiota composition [4,5]. Increased FPO, which reflects altered colonic transit time, is associated with IBS [6]. Moreover, as stress is a key factor for inducing IBS, corticotropin-releasing factor, which produced in response to stress, increases intestinal paracellular permeability via mast cells infiltration to mucosa and inducing proinflammatory cytokines, such as interleukin (IL)-1β [4,7].

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Probiotics can change the composition and balance of gut microbiota and inhibit intestinal microinflammation through anti-inflammatory properties [8,9]. A well-recognized protective function of gut microorganisms is the production of short-chain fatty acids (SCFAs), including butyric acid. Butyric acid serves as an energy source and immune system modulator, contributing to the maintenance of intestinal epithelial integrity [10-13].

Another beneficial effect of probiotic intake is the improved bile acid (BA) metabolism [14,15]. BAs are amphipathic molecules produced in the liver that solubilize lipids into micelles for digestion and absorption [16]. Patients with IBS-diarrhea have a high proportion of primary BAs and a relatively low proportion of secondary BAs in the serum and feces [17,18]. In enterohepatic circulation, which denotes the movement of BA molecules from the liver to the small intestine and back to the liver, the conjugation of primary BAs to secondary BAs by the gut microbiota plays a crucial role [19-21].

Given that probiotics are composed of microorganisms, it is important to assess their impact on the human gut microbiome when studying their potential benefits. The *Roseburia* genus exhibits high butyrate production by breaking down indigestible carbohydrates [22,23]. Notably, most *Roseburia* strains were initially isolated from human fecal samples, and in healthy adults, *Roseburia* spp. account for approximately 3%-15% of the total bacterial counts in feces [24,25]. Moreover, fecal samples collected from 40 pairs of twin cohorts showed the disappearance of *Roseburia* in patients with inflammatory bowel disease (IBD) [8,26]. *Roseburia intestinalis*, the most well-known species in *Roseburia* genus, shows various immunomodulatory functions by decreasing proinflammatory cytokine IFNr and IL-17 production and increasing anti-inflammatory cytokine IL-22 under IBD-associated dysbiosis [27]. Ruan et al. [28] demonstrated the possibility of *R. intestinalis* as a therapeutic agent in a dextran sulfate sodium (DSS)-induced mouse colitis model. *R. faecis*, another strain in the *Roseburia* genus, produces bacteriocin-like substance (BLIS) in vitro [29], which is a type of abtibiotic produced by certain microorganisms that inhibits the growth of the same or related species. Bacterial strains, which produce BLIS could also be considered as probiotics such as lactic acid bacteria which are generally regarded as probiotics [30].

In a previous study, we observed that WAS induced colonic microinflammation, as evidenced by increased FPO and mucosal mast cell counts. Notably, these effects were sex-specific, with female rats showing a stronger response. Interestingly, our research also found that a 10-day treatment regimen of *Lactobacillus farciminis* effectively alleviated symptoms in the female WAS-induced IBS model. However, it is important to note that this therapeutic intervention did not yield similar positive outcomes in male rats [5]. Based on this background, we hypothesized that *R. faecis* could exert a protective effect against WAS-induced colonic microinflammation and show sex differences in its anti-inflammatory effects. Thus, this study aimed to evaluate whether the effect of two different strains of *R. faecis* isolated from a healthy Korean adult stool donor on the WAS-induced IBS is sex-specific in Wistar rats and to investigate the underlying mechanism including fecal microbial change.

MATERIALS AND METHODS

Isolation of *R. faecis* strains and culture conditions

Fecal samples were collected from healthy Korean adult donors and transferred to an anaerobic chamber. Briefly, fecal samples were vortexed and filtered in 1× phosphate-buffered saline (PBS) using a 100 μm cell strainer (SPL Life Sciences Co. Ltd.) to remove undigested food and small particles. Serial dilutions were performed on plate dilutions of 10^{-6} to 10^{-8} on Fastidious Anaerobe Broth (Neogen) agar supplemented with 5% sheep blood. After culturing at 37°C for 3 days single colonies were selected for bacterial species identification. Finally, 16S rRNA gene sequencing (Macrogen) was performed after pure culture. The viable isolated bacterial culture was stocked at –80°C with 20% glycerol.

Based on the above-mentioned process, two different *R. faecis* strains, BBH024 and R22-12-24, were identified. Both BBH024 and R22-12-24 were cultured in DSM 104 medium (DSMZ; Leibniz Institute) in an anaerobic chamber (5% CO_2, 5% H_2, 90% N_2 condition) maintained at 37°C. After culturing on DSM 104 agar plates for 1 day, *R. faecis* cells were collected by scraping with a loop and resuspended in anaerobic PBS containing 20% glycerol (v/v). The number of cells was determined using a Quantom Tx Microbial Cell Counter (CronyTek). Wistar rats were administered *R. faecis* by gavage at a dose of 1.0×10^9 CFU/day.

Animals

Male and female Wistar rats (Orient Co., Ltd.) were housed in cages (2 animals/cage) maintained at 23°C under a 12/12-hour light/dark cycle. The rats were housed under specific pathogen-free conditions with ad libitum-only Purina rat chow and water without enrichment. After one week of adaptation, 7-week-old male and female Wistar rats weighing approximately 200 g were used in the experiments. A total of 36 rats of both sexes (18 male and 18 female) were divided into three groups—six rats in the control group, six rats in the WAS group, and six rats in the WAS group with probiotics for each experiment. This study was conducted according to the recommendations of the Guide for the Care and Use of Laboratory Animals in South Korea. All of the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital (IACUC No. BA-2112-333-002-04).

Repeated WAS

The WAS was performed as described previously [5]. To ac-

count for potential variations in stress responses due to different postures resulting from the weight disparity between male and female rats, distinct glass platforms were employed. In other words, depending on sex, each Wistar rat was placed on a different glass platform (5.8-cm length × 5.8-cm width × 6.0-cm height for male rats and 5.0-cm length × 5.0-cm width × 6.0-cm height for female rats), which had been fixed to the center of a standard plastic cage (26.7-cm length × 48.3-cm width × 20.3-cm height) filled with warm water (25°C) to 1 cm below the height of the platform, for 1 hour for 10 consecutive days between 8 and 10 am. The rats in the no-stress group were placed on the same platform, which was then attached to a container not filled with water for 1 hour. The rats were kept in pairs in their home cages and placed individually in their WAS cages. To assess stress levels resulting from each WAS session, the total number of fecal pellets expelled by each rat was tallied at the conclusion of the 1-hour session.

Measurement of colonic mucosal mast cell using toluidine blue staining

To evaluate the mast cell activation status, the number of mast cells in the colonic mucosa was counted. For tissue preparation, 1 cm tissues were removed from the cecum and anus, and 1 cm of the proximal part of the colon was collected. The samples were fixed in 10% buffered formalin. Tissue specimens embedded in paraffin blocks were cut perpendicular to the lumen into 4 mm-thick sections and stained with toluidine blue. The number of purple-stained mast cell granules in the colonic mucosa area was counted and divided by the total area of the colonic mucosa (the number of mast cells/colonic mucosal area [μm^2]).

Measurements of the concentration of BAs and SCFAs

To measure the concentrations of SCFAs and BAs, feces were collected immediately after defecation, 10 days after WAS sessions. All fecal samples were immediately frozen in liquid nitrogen and stored at –80°C. Frozen feces were homogenized in 2 mL of ice-cold PBS by vortexing and incubated at 4°C for 20 minutes. After incubation, the homogenates were centrifuged at 12,000 × *g* for 20 minutes and the supernatants were transferred to fresh tubes. The concentration of BAs in fecal samples was quantified in these supernatants utilizing enzyme-linked immunosorbent assay (ELISA) kits (Rat Cholic acid ELISA Kit [MBS266826]; Rat chenodeoxycholic acid ELISA Kit [MBS7273638]; general deoxycholate [DC] ELISA Kit [MBS2700679]; Rat Lithocholic acid ELISA Kit [MBS7273582], MyBioSource Inc.), following the manufacturer's recommendations. Furthermore, acetic acid and butyric acid in each sample were separated and measured using an Agilent 1100 series instrument (Agilent) equipped with a C18 column (ZORBAX Eclipse XDB-C18, analytical 4.6 × 150 mm, 5-Micron; Agilent) and a UV detector (210 nm). The mobile phase consisted of 90% 10 mM KH2PO4 and 10% acetonitrile, in the same manner as described in our previous studies [31,32].

Real-time quantitative PCR

mRNA was isolated from the colon tissue using TRIrizol reagent (Invitrogen) according to the manufacturer's instructions, and mRNA was quantified using a NanoDrop (ND-1000; Thermo Scientific). Complementary DNA (cDNA) was synthesized using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR (RT-qPCR) was performed using SYBR Green I Master Mix and an ABI Viia7 instrument. The transcript levels of β-actin were used for sample normalization.

Fecal sample collection and metagenome sequencing

Bacterial genomic DNA was extracted from frozen fecal samples using a QIAamp DNA Stool Mini Kit (Qiagen), according to the manufacturer's recommendations. In the case of BBH024, we could not perform NGS because of inappropriate stool preparation. DNA quantity and quality were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and electrophoresed using 2% agarose gel. For MiSeq library amplicon preparation, the target gene, the V3-V4 region of the 16S rRNA, was initially amplified using the 341-F (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′) and 805-R (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′) primers. Subsequently, the V3-V4 PCR amplicons were linked to the Illumina indices and adapters from Nextera® XT Index Kit (Illumina). Short DNA fragments were eliminated using the Prep™ DNA Purification Kit (Favorgen). To quantify the PCR amplicons, we used the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific). Sequencing was conducted at CJ Bioscience Inc. using a MiSeq system (Illumina). Non-specific, non-target, and chimeric amplicons were removed from the pre-filter during quality control to exclude reads with short lengths and low Q values. Using operational taxonomic unit (OTU) information (number of OTUs and sequences in each OTU), α-diversity, Shannon indices were analyzed using EzBioCloud (CJ Bioscience Inc.). Selected taxa were created using GraphPad Prism (ver. 8.01; GraphPad Software Inc.) to visualize sample differences.

Functional profiling of the fecal microbial community using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt)

Phylogenetic investigation of the communities was conducted using PICRUSt to predict the functional profiles of the microbial community. This analysis enabled us to infer alterations in the functional markers of the microbiota based on the KEGG database [33].

Statistical analyses

Data are expressed as mean ± SEM. Continuous and categorical variables were compared among groups (no-stress, WAS, and WAS with probiotics) using Kruskal–Wallis and Fisher's exact tests, respectively. *P*-values < 0.05 were considered statistically significant. All statistical analyses were performed using the SPSS version 20.0 software (IBM Corp.).

RESULTS

The relieving effect of *R. faecis* on IBS-related symptoms

Exposure to WAS for 10 days consistently increased FPO during the 1-hour session in both male and female groups when compared to that in the control group with no stress, across all experiments (Fig. 1A-1D). The mean FPO was also significantly higher in the WAS group than in the control group in all experiments for both sexes (Fig. 1E and 1F, $P < 0.001$). Moreover, the WAS with probiotics groups also showed a significantly higher FPO than the no-stress group in both BBH024 (males, $P < 0.001$; females, $P = 0.035$) and R22-12-24 (males and females, $P < 0.001$). However, comparing the WAS group to WAS with probiotics groups, there was a significant decrease in FPO in the WAS with probiotics group for both BBH024 (male, $P = 0.015$; female, $P = 0.022$) and R22-12-24 (male, $P = 0.003$; female, $P = 0.004$). When we compared sex differences, BBH024 showed a lower FPO in females than in males ($P = 0.005$). The number of mast cells in the colonic submucosa was counted using toluidine blue-stained slides (Fig. 1G and 1H). In all experiments, the WAS groups showed a significantly increased number of mast cells in the submucosa (BBH024 males, $P = 0.006$; BBH 024 females, $P = 0.006$; R22-12-24 males, $P = 0.041$; and R22-12-24 females, $P = 0.025$). The administration of probiotics significantly reduced the number of mast cells in males (BBH024, $P = 0.030$; R22-12-24, $P = 0.005$).

Molecular responses due to repeated WAS and *R. faecis* supplement

To assess the effects of WAS alone and WAS with probiotics on the colonic mucosa at the molecular level, we performed RT-qPCR (Fig. 2). The expression of mucosal serine protease gene (*PRSS*) was significantly increased in the R22-12-24 male WAS group compared to that in the control group (Fig. 2B: *PRSS1*, $P = 0.009$; Fig. 2D: *PRSS2*, $P = 0.009$). In the BBH024 and R22-12-24 experiments, females showed increased mRNA levels of *PRSS* in the WAS group compared to those in the control group, but the difference was not statistically significant. Moreover, the WAS with probiotic group showed lower levels of *PRSS* gene expression than the WAS group, but the difference was not statistically significant. Furthermore, the mRNA expression of the inflammatory cytokines *IL-1β* and *IL-6* was compared between the control and WAS groups. In R22-12-24 experiments, the male WAS group showed significantly higher expression of *IL-1β* than the control group (Fig. 2F, $P = 0.028$); contrastingly, the WAS with probiotics group showed lower expression of *IL-1β* than the WAS alone group, but the results were not statistically significant. In the BBH024 experiments, the male WAS with probiotics group showed significantly lower mRNA levels of *IL-6* (Fig. 2G) than the control group ($P = 0.025$ vs. $P = 0.028$). Female groups in the BBH 024 experiments showed a similar tendency as the male groups, but the difference was not statistically significant. However, in the R22-12-24 experiment (Fig. 2H), the male WAS group showed significantly higher expression of *IL-6* than the control group ($P = 0.047$), whereas the female WAS with probiotics group showed a significantly higher expression of *IL-6* than the WAS group ($P = 0.028$).

Roseburia faecis as candidate probiotic in terms of butyric acid production and BA conjugation

As *R. faecis* is a known butyric acid producer [22], we confirmed the butyric acid-producing ability of the two candidate *R. faecis* strains (Figure S1A). The concentrations of SCFAs in the spent media were measured 24 hours after 0.5% supplementation of the culture media. BBH024 and R22-12-24 successfully produced butyric acid. Accordingly, we measured the fecal butyric acid concentration in WAS-exposed rats to investigate whether the two *R. faecis* strains can produce butyric acid in the intestinal environment (Fig. 3A and 2B). Compared with the control and WAS groups, the WAS with probiotics group showed no statistically significant differences. Increased fecal primary BA and BA malabsorption are known features of patients with IBS-associated diarrhea [15,18]. Thus, we confirmed the BA-modifying abilities of the two candidate *R. faecis* strains (Figure S1B). Twenty-four hours after the addition of primary BA, cholic acid (CA), chenodeoxycholic acid (CDCA) each to culture media, secondary BA, deoxycholic acid (DCA), and lithocholic acid (LCA) levels were measured in the spent media. Both BBH024 and R22-12-24 successfully converted primary BAs to secondary BAs. Subsequently, we measured fecal BA concentration in the WAS-treated rats to investigate whether the two *R. faecis* strains can conjugate primary BAs to secondary BAs in the intestinal environment (Fig. 3C-3F). In the case of CA conjugation to DCA in the R22-12-24 experiment (Fig. 3D), the female WAS with R22-12-24 group showed a significantly lower CA/DCA ratio than the control ($P = 0.029$) and WAS groups ($P = 0.025$). No other significant differences were observed in BA concentrations.

Changes in fecal microbiota in response to exposure to repeated WAS and R22-12-24 supplement

To evaluate the influence of WAS and R22-12-24 on the

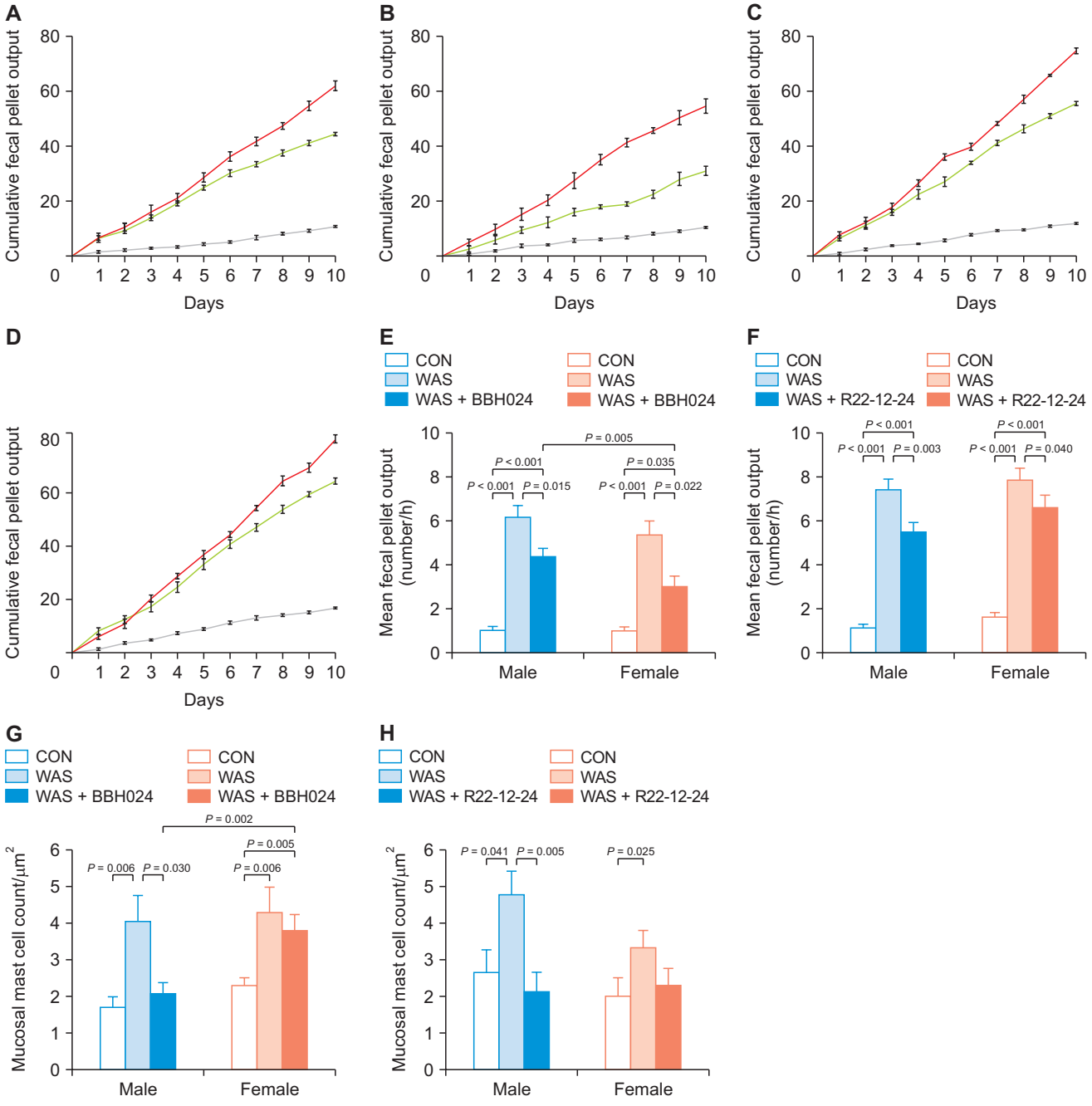

Figure 1. The fecal pellet output (FPO) and mast cell count from each repeated water avoidance stress (WAS) experiment with two different *Roseburia faecis* strains as probiotics candidates. FPO data represent the 10-day sessions with weight calibration for male and female rats in the BBH024 experiments. The difference between male and female body weight was normalized by multiplying female FPO with the ratio of male body weight to female body weight. The cumulative FPO differed significantly between WAS-exposed groups and no-stress group in the later stages of BBH024 experiments (A) male and (B) female, as well as the R22-12-24 experiments (C) male and (D) female. The mean FPO of (E) BBH024 experiment and (F) R22-12-24 experiment indicated a significant reduction in FPO in WAS with probiotics group with statistical significance. The mucosal mast cells were counted from toluidine blue stained colonic tissue slides about each gavaged *R. faecis* strain, (G) BBH024, and (H) R22-12-24. The mast cell count in the colonic mucosa was divided into the area of the colonic mucosa. CON, control.

gut microbiota, we performed 16s rRNA sequencing of fecal samples (Fig. 4). The α-diversity of the observed OTUs (Fig. 4A) and Shannon index (Fig. 4B) showed no statistical differences among the control, WAS and WAS with R22-12-24 groups regardless of sex. In the case of β-diversity derived from the generalized UniFrac distance, principal coordinate analysis (PCoA) was performed on males (Fig. 4C, $P = 0.037$) and females (Fig. 3D, $P = 0.011$). The relative

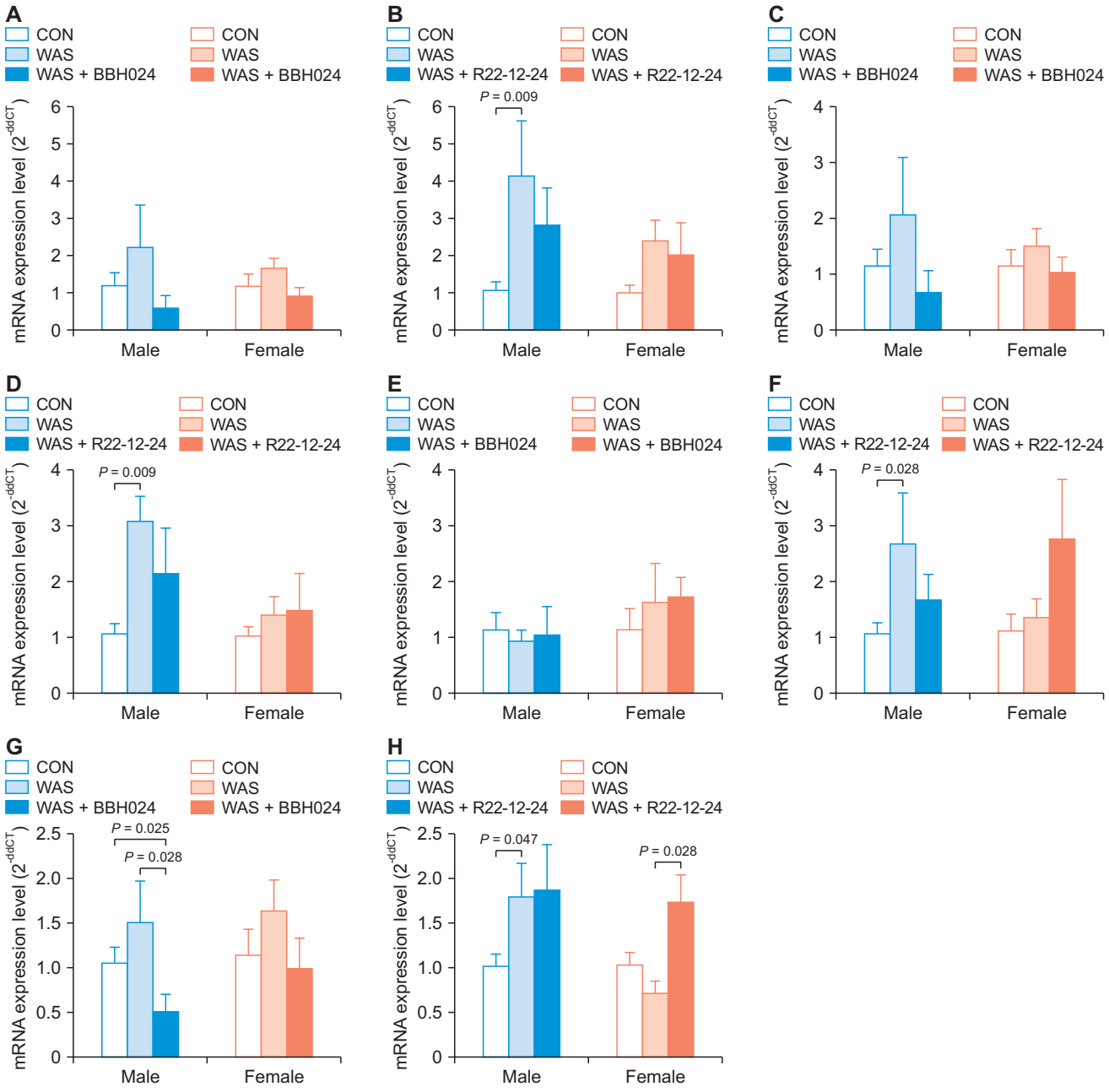

Figure 2. Colonic mucosal mRNA expression from each repeated water avoidance stress (WAS) experiment with two different *Roseburia faecis* strains as probiotics candidates. Mucosal qPCR analysis of serine protease (*PRSS*) gene 1 (*PRSS1*: A, B) and 2 (*PRSS2*: C, D) revealed increased expression in the stress group compared to that in the control (CON) group in both males and females, although statistical significance was not reached. The mRNA expression of the inflammatory cytokine *IL-1β* (E, F) was significantly increased in the male stress group compared to that in the control group in R22-12-24 gavaged experiments. Furthermore, the inflammatory cytokine *IL-6* (G, H) showed increased gene expression in the male stress group compared to that in the control group and showed a statistically significant decrease in expression in the BBH024 gavaged group.

abundance (%) of each group at the phylum level represents distinctions in gut microbiota between sexes, observed not only between control groups but also between the WAS and WAS with R22-12-24 groups (Fig. 4E). In the case of *Firmicutes*, the male WAS group showed a lower abundance than the control group (control, 80.7%; WAS, 61.4%; $P = 0.028$), whereas the female WAS group showed an increased abundance compared to the control group (control, 52.5%; WAS, 67.9%; $P = 0.028$). Moreover, females with WAS in the R22-12-24 group showed restored *Firmicutes* levels compared to those in the WAS group, and the results were significant (WAS + R22-12-24, 53.3%; $P = 0.010$). Additionally, in males, *Bacteroidetes* was more abundant in the WAS group than those in the control group (control, 15.8%; WAS, 33.3%; $P =$

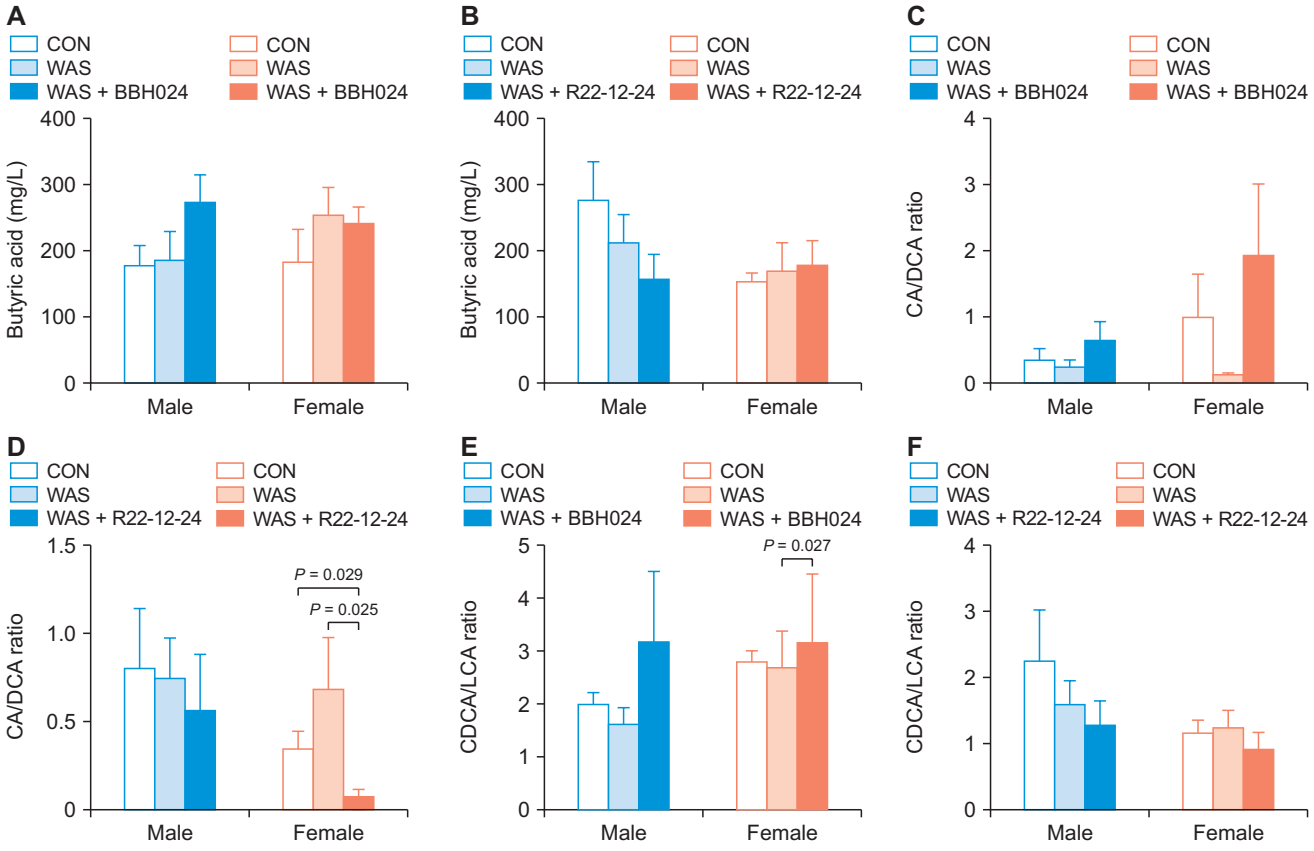

Figure 3. Butyric acid and bile acid (BA) concentrations in fecal samples from repeated water avoidance stress (WAS) experiments with two different *Roseburia faecis* strains as probiotic candidates. (A) The butyric acid concentration in feces after 10 days of WAS sessions. Unexpectedly, there was no statistical significance in both (A) BBH024 and (B) R22-12-24 strains and no correlation with previous cultured media results was noted. (C, D) The ratio of cholic acid (CA, primary BA) to deoxycholic acids (DCAs, secondary BA) in feces after 10 days of WAS sessions. In the case of R22-12-24, females showed significantly decreased levels of CA/DCA ratio (D, $P = 0.025$). (E, F) The ratio of chenodeoxycholic acid (CDCA, primary BA) to lithocholic acids (LCA, secondary BA) in feces after 10 days of WAS sessions. In the case of BBH024, females showed significantly decreased levels of CDCA/LCA ratio (E, $P = 0.027$). CON, control.

0.045), and in females, *Verrucomicrobia* was more abundant in the control than in the WAS group (control, 23.6%; WAS, 4.9%; $P = 0.011$). Among the numerous observed genera, the abundance of the *Roseburia* genus, which we administered as a probiotic, was increased in the WAS with R22-12-24 group compared to that in the WAS group (Fig. 4F: male, $P = 0.045$; female, $P = 0.054$).

Predictive functional profiling of microbial communities in various metabolism

We conducted predictive functional profiling of microbial communities regarding metabolism related to carbohydrates, amino acids, lipids, nucleotides, and energy based on the KEGG modules. The representative modules in each group are indicated as heat maps (Fig. 5A and 5B). Modules were selected based on a *P*-value lower than 0.05 in more than two pair comparisons from the Kruskal–Wallis H test between two groups. In particular, M00053, a nucleotide metabolism-related module involved in deoxyribonucleotide biosynthesis, showed WAS- and R22-12-24-related relative abundances (Fig. 5C). In males, the administration of R22-12-24 elevated the relative abundance of M00053 compared to that in the control and WAS groups (compared to control, $P = 0.047$; compared to WAS, $P = 0.018$). In contrast, in females, the WAS group exhibited a lower relative abundance of M00053 than that in the control group (WAS group, $P = 0.045$; WAS with R22-12-24 group, $P = 0.045$).

We also conducted detailed predictive functional profiling of microbial communities based on the KEGG orthology, and the representative modules in each group are indicated as heatmaps (Fig. 5D and 5E). Orthologs were selected based on a *P*-value lower than 0.05 in more than two pair comparisons from the Kruskal-Wallis H test between the two groups. Especially, the relative abundance of K02073 (Fig. 5F), which encodes a D-methionine transport system substrate-binding protein, was significantly decreased in the WAS group (male, $P = 0.045$; female, $P = 0.028$) and was restored in the WAS with R22-12-24 group (male, $P = 0.028$; female, $P = 0.025$).

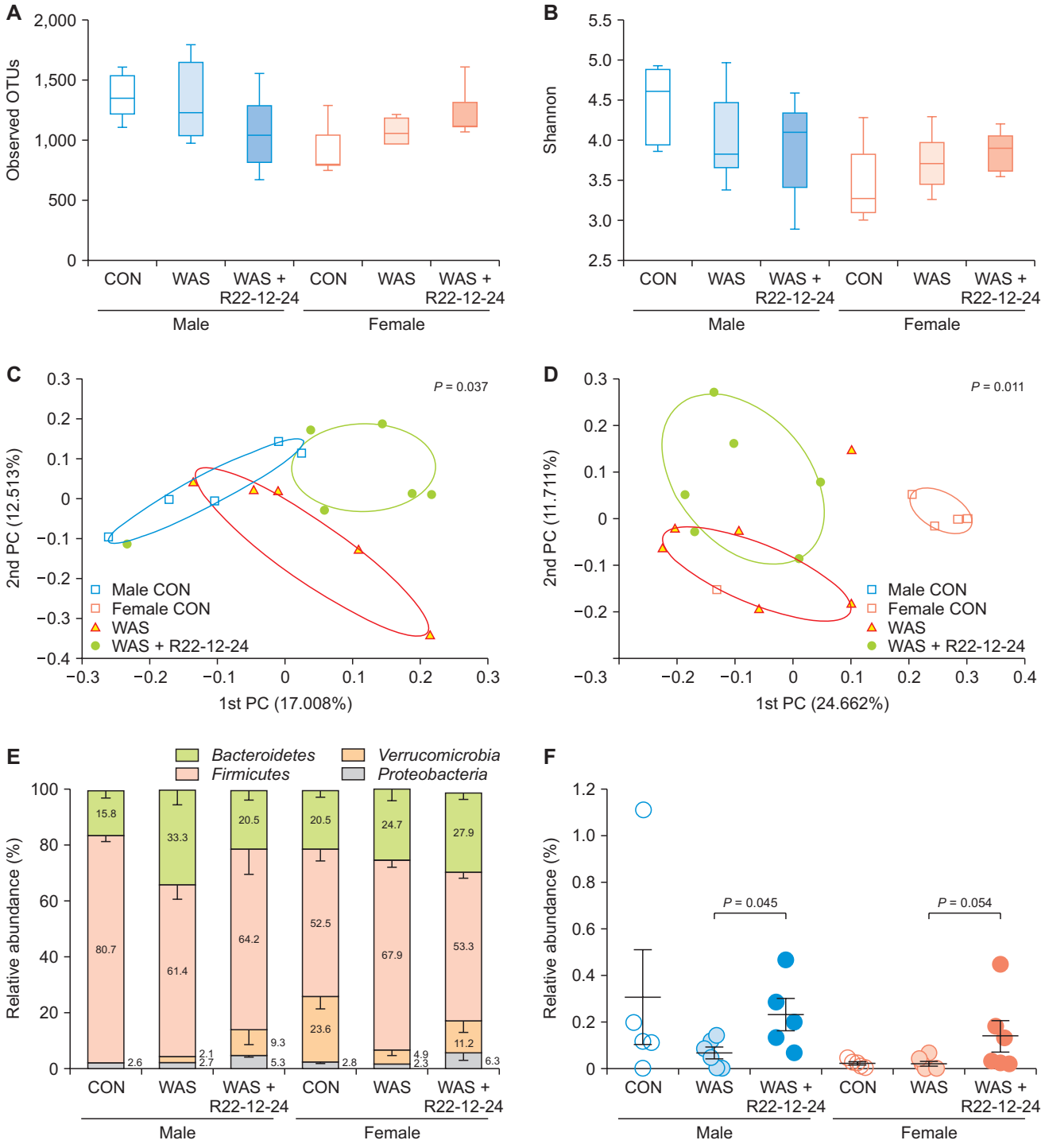

Figure 4. Fecal microbiome characteristics in rats exposed to repeated water avoidance stress (WAS) with *Roseburia faecis* R22-12-24 as a potential probiotic. The α-diversity observed operational taxonomic units (OTUs) (A) and Shannon (B) were decreased in male stress-exposed groups while the female stress-exposed group showed increased α-diversity, but there was no statistically significant. The β-diversity was derived from generalized UniFrac distance and represented through principal coordinates analysis (PCoA) male (C, $P = 0.037$) and female (D, $P = 0.011$) separately. Both male and female stress groups exhibited significant separation from the control (CON) group. The taxonomic composition of each group in the phylum level (E) highlighted sex-based differences between males and females subjected to WAS. At the genus level, the orally gavaged genus *Roseburia* (F) showed slightly increased levels in R22-12-24 gavaged group compared to those in the stress group both in males ($P = 0.045$) and females ($P = 0.054$).

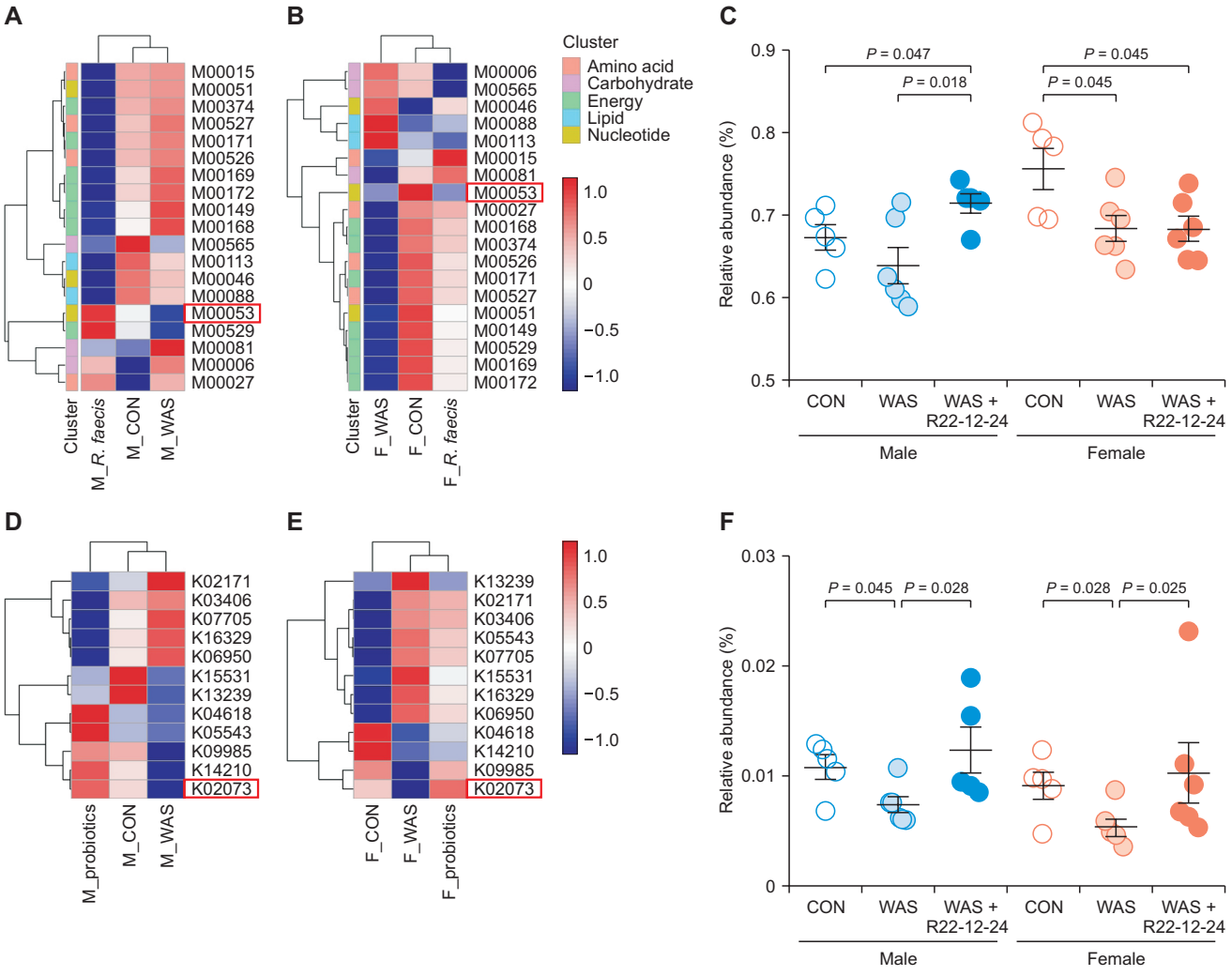

Figure 5. Predictive functional profiling of microbial communities, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, regarding metabolism related to carbohydrate, amino acid, lipid, nucleotide, and energy. Panels (A) and (B) represent male (M) and female (F) data, respectively. Panel (C) illustrates module M00053, which is the module about deoxyribonucleotide biosynthesis (ADP/GDP/CDP/UDP→dATP/dGTP/dCTP/dUTP), the significantly different modules for both sexes are shown in the scatter graph. Panels (D) and (E) depict predictive functional profiling of microbial communities through the KEGG orthology database for males and females, respectively. Panel (F) focuses on K02073, which is the gene encoding D-methionine transport system substrate-binding protein, are shown in the scatter graph with both sexes. WAS, water avoidance stress; CON, control.

DISCUSSION

In this study, we investigated the effects of WAS, a form of chronic psychological stress, on several parameters. Notably, WAS led to a substantial increase in FPO, indicative of accelerated bowel transit times akin to those seen in individuals with diarrhea-predominant IBS. Furthermore, we observed elevated counts of submucosal mast cells following a 10-day regimen of WAS exposure. *R. faecis*, a probiotic candidate based on butyric acid production and BA modification ability, significantly improved WAS-induced IBS-related symptoms, whereas there were no consistent protective effects, similar to those of FPO and mast cell counts. However, in terms of the gut microbiota, the *Roseburia* genus showed a significantly increased abundance in the R22-12-24-gavaged group compared to that in the WAS groups. Moreover, not only the relative abundance difference between males and females, but also the alpha- and β-diversity, and even the functional profile of the microbial community showed sex differences.

The beneficial effects of probiotics include: the reinforcement of the mucosal barrier, modulation of the inflammatory response, and maintenance of healthy gut microflora. In a previous study, we reported that WAS-induced upregulation of proinflammatory cytokine *IL-1β* and *IL-6* gene expression in rats was restored by Lactobacillus farciminis in females [5]. Similarly, another probiotic Lactobacillus paracasei MSMC39-1 showed reduction of TNF-α secretion in DSS-induced colitis rats [34]. Even though the same *R. faecis* species

was used under the same experimental conditions, BBH024 showed reduced *IL-6* gene expression in the WAS with probiotics group, whereas R22-12-24 demonstrated reduced *IL-1β* gene expression. Furthermore, a correlation analysis between mast cell counts and *PRSS* gene expression (Figure S2), both of which are known to be associated with the severity of IBS symptoms, and showed distinct results. Mast cells increase in patients with IBS [35], and their relationship with hypersensitivity [36] has been reported in several human and animal studies. Mast cells play various roles, including the control of intestinal permeability [37], neuronal stimulation through the brain-gut axis [38], and visceral hypersensitivity [39]. As inflammatory modulators, mast cell produce various proteases for protective purpose [40]. The trypsin-like activity of *PRSS*, a serine protease produced by mast cells, enhances intestinal permeability and causes visceral hypersensitivity [41]. Therefore, we expected a positive correlation between mast cell count and *PRSS* gene expression. We suggest that probiotic strains should be examined carefully because of the differences in *R. faecis* species from different sources.

As the gut microbiota is important in human diseases, various omics tools have been used to identify key effectors of the gut microbiota. Metataxonomic sequencing of highly conserved 16s ribosomal RNA is commonly used to identify the composition of bacterial communities in the gut microbiota [42]. With numerous studies on the taxonomic diversity of microbiota under healthy and diseased conditions, scholars have attempted to not only determine harmful bacterial strains for the host but also the microbial metabolism that influences the host, such as butyric acid-producing ability. PICRUSt can help predict the genes present in the microbiota community based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [43]. In the case of M00053 (Fig. 5C), the deoxyribonucleotide biosynthesis module initiates l-glutamine synthesis [44]. Moreover, some studies have reported that glutamine supplements enhance IBS management with low-FODMAP diets [45], and glutamine supplements in patients with post-infectious IBS improved gastrointestinal symptoms in a randomized controlled trial [46]. Together with the fact that the male WAS with R22-12-24 group showed reduced FPO and colonic submucosal mast cell counts (Fig. 1F and 1H), we assumed that the improved effect of glutamine on IBS symptoms may pass through the gut microbial module M00053, and this module could also be enhanced by *R. faecis* administration.

Moreover, in the orthology analysis, K02073 (Fig. 5F), which encodes the D-methionine transport system substrate-binding protein, showed significantly inhibited abundance in the WAS groups, which was restored by probiotic administration. As methionine is an essential amino acid [47], the inhibition of K02073 may imply inhibition of the growth of the whole gut microbiota. Thus, we assumed that chronic stress inhibits the growth of the gut microbiota, thereby causing IBS-related symptoms. In addition, probiotic administration may improve the gut environment, facilitating the growth of gut-resident beneficial bacteria. In addition, increased methionine transport from the host intestinal environment to the microbial community in the WAS with *R. faecis* group may prevent carcinogenesis via the Hoffman effect, which refers to the cancer cell-specific metabolic dependence on exogeneous methionine [48]. Notably, limiting methionine intake decreases inflammation by relieving metabolic imbalances [49], improving BA conjugation and lipid metabolism [50], and improving age- and obesity-associated inflammatory cytokine responses [51]. Therefore, we assume that the increased level of the K02073 ortholog not only represents vitalization of the gut microbiota, but also prevents the carcinogenic mucosal environment via microinflammatory responses.

Several limitations are associated with this study. First, our findings regarding the WAS IBS model reveal a different gender-related pattern compared to prior research. Previously, we demonstrated significant gender differences in the context of WAS-induced microinflammation, with females exhibiting increased vulnerability to stress than males [4,5]. However, in the present study, the WAS-induced inflammatory effects were not reversed (Fig. 1H, 2B, 2D, and 2F). As we tried hard to follow a strict protocol of exposure time and period to WAS, probiotic gavage conditions, and animal sacrifice conditions, we carefully assumed that sex bias from the researcher may have affected the animal model. It has been reported that the sex of the human researcher affects animal model results, which may even result from stress activation by the human-male scent [52]. In future studies, we have a plan to compare the effects of the sex of the human researcher on WAS model results. Second, unlike the in vitro test, the two *R. faecis* groups did not show a remarkable difference in butyric acid concentration and BA conjugation ratio in feces compared to the other groups. However, the metabolic activity of the gastrointestinal microflora can be modified by various factors such as changes in pH depending on the region of the intestine, intestinal motility and structure, host diet, and even bacterial competition [53]. It is difficult to explain the reason for the decline in *R. faecis* metabolism, but we can assume that the intestinal environment might not be favorable for producing butyric acid or modifying BA. Third, the RT-qPCR results in the WAS groups in both the BBH024 and R22-12-24 experiments did not appear to be constant. In the case of the *PRSS* gene (Fig. 2A-2D), despite statistical differences, the gene expression in the control, WAS, and WAS with probiotic groups in both males and females was similar. However, the inflammatory genes (Fig. 2E-2H), especially in the female WAS group, showed different expression patterns compared to those in the control group in the BBH024 and R22-12-24 experiments. It is difficult to recognize the reason for the difference between experiments in the WAS groups; thus, we performed RT-qPCR, at least in duplicate, to reduce irregular values. Moreover, no significant difference was observed in the levels of *IL-1β*, which were found to be increased in the

female WAS group compared to those in the male group in a previous study [4]. We assumed that the difference resulted from measuring the colonic tissue at the mRNA level in the present study, rather than measuring the colonic mucosa at the protein level in a previous study. Finally, 16s rRNA sequencing of fecal samples was performed only in the R22-12-24 experiment. Due to budget constraints, we selected the R22-12-24 experiment to perform the gut microbial analysis according to the BA modification results in the female R22-12-24 administrated group (Fig. 3D).

In conclusion, constant probiotic administration significantly relieved IBS-related symptoms and FPO, and reduced colonic submucosal mast cell counts. Moreover, although BBH024 and R22-12-24 are the same *R. faecis* species, their abilities for butyric acid production and BA modification differ depending on the environmental conditions and show sex differences. Taken together, our results suggest *R. faecis* is a probiotic that can be used for treating chronic stress-induced IBS. In addition, it prevented this effect, which could be related to its anticarcinogenic effects.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.2023.28.3.93.

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