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Baseline gut microbiome as a predictive biomarker of response to probiotic adjuvant treatment in gout management☆

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ABSTRACT

Gout is characterized by dysregulation of uric acid (UA) metabolism, and the gut microbiota may serve as a regulatory target. This two-month randomized, double-blind, placebo-controlled trial aimed to investigate the additional benefits of coadministering Probio-X alongside febuxostat. A total of 160 patients with gout were randomly assigned to either the probiotic group (n = 120; Probio-X [1 $\times 10^{11}$ CFU/day] with febuxostat) or the placebo group (n = 40; placebo material with febuxostat). Coadministration of Probio-X significantly decreased serum UA levels and the rate of acute gout attacks ($P < 0.05$). Based on achieving a target sUA level (360 μmol/L) after the intervention, the probiotic group was further subdivided into probiotic-responsive (ProA; n = 54) and probiotic-unresponsive (ProB; n = 66) subgroups. Post-intervention clinical indicators, metagenomic, and metabolomic changes in the ProB and placebo groups were similar, but differed from those in the ProA group, which exhibited significantly lower levels of acute gout attack, gout impact score, serum indicators (UA, XOD, hypoxanthine, and IL-1β), and fecal gene abundances of UA-producing pathways (KEGG orthologs of K13479 and K01487; gut metabolic modules for formate conversion and lactose and galactose degradation). Additionally, the ProA group showed significantly higher levels ($P < 0.05$) of gut SCFAs-producing bacteria and UA-related metabolites (xanthine, hypoxanthine, bile acids) after the intervention. Finally, we established a gout metagenomic classifier to predict probiotic responsiveness based on subjects' baseline gut microbiota composition. Our results indicate that probiotic-driven therapeutic responses are highly individual, with the probiotic-responsive cohort benefitting significantly from probiotic coadministration.

1. Introduction

Gout is a chronic disease characterized by the deposition of monosodium urate crystals, presenting as painful and destructive arthritis in the context of hyperuricemia (HUA)[1]. Currently, lowering uric acid (UA) levels is considered a key point in the management of

☆ This trial was registered in the Chinese Clinical Trial Registry with the identifier number ChiCTR1900028232.

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gout. There are mainly two types of drugs used to manage gout: those that reduce UA synthesis (namely xanthine oxidase (XOD) inhibitors such as febuxostat) and those that promote UA excretion (such as benzbromarone). Although these drugs are effective in lowering UA levels, they can cause significant side effects, particularly allergies, renal insufficiency, diminished liver function, and dyslipidemia[2]. Therefore, the search for safe and highly effective treatments for gout holds considerable clinical value.

In the excretion of UA, about 70 % of urate is eliminated through the renal system, with the remaining 30 % being excreted via the intestinal pathway[3]. Therefore, gut microbiota is considered to be associated with gout development, and probiotics may represent a new treatment strategic option for gout[4,5]. A large body of research has observed alterations in the gut microbiota and metabolome in patients with gout. For example, the proportion of opportunistic pathogens, such as *Bacteroides*, *Prevotella*, and *Fusobacterium*, was found to be elevated in patients with gout compared to control subjects, while the polymicrobial cluster of *Lactobacillus*, *Bifidobacterium*, *Butyrivibrio*, and other beneficial bacteria that produce short-chain fatty acids (SCFAs) showed an opposite trend[6–13].

Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits on the host[14]. The potential of probiotics in preventing and managing gout has previously been explored in several rodent studies. For instance, *Lacticaseibacillus paracasei* (*L. paracasei*) X11 substantially reduced serum UA (sUA) levels by affecting UA metabolism-related enzymes (including adenosine deaminase and XOD) and transporters (GLUT9, NPT1, and URAT1) in mice [15]. Additionally, the application of *Akkermansia muciniphila* (*A. muciniphila*) reduced UA level, inhibited XOD activity, and increased fecal and urinary secretion of UA[16]. *Levilactobacillus brevis* MJM60390 led to an increase in intestinal *Rikenellaceae*, which may have contributed to a subsequent reduction in body UA levels by reabsorbing UA precursors like inosine and guanosine, thereby reducing XOD activity and alleviating kidney damage in HUA model mice[17]. Moreover, probiotic administration has demonstrated anti-inflammatory effects in HUA and gout models[15,16]. While probiotic administration seems to be effective in improving gout in rodents, it is important to note that UA metabolism in humans and rodents differs significantly. Rodents have a fully expressed liver uricase gene, whereas humans possess genetic mutations in this gene, leading to relatively high sUA levels[18].

There have been very few human intervention studies investigating the UA-lowing and gout-alleviating effects of probiotics. In a previous small-scale randomized controlled trial (RCT) study involving 25 male patients with gout and/or HUA, it was found that an 8-week intervention with a yogurt containing *Lactobacillus gasseri* PA-3 could effectively reduce sUA levels[19]. In clinical gout management, probiotics have been used in combination with conventional drugs, such as allopurinol and febuxostat, which lower body UA levels and inflammation, inhibited XOD activity, restore a healthier gut microbiota by enhancing beneficial and commensal microbes like bifidobacteria, lactobacilli, and enterococci[20–23]. The results reported in these studies suggest that the combined use of probiotics and conventional drugs is promising for managing gout with minimal side effects.

The probiotic product Probio-X comprises five different probiotic strains: *L. paracasei* Zhang, *Lactiplantibacillus plantarum* (*L. plantarum*) P-8, *Lacticaseibacillus rhamnosus* (*L. rhamnosus*) Probio-M9, *Bifidobacterium animalis* subsp *lactis* (*B. lactis*) Probio-M8, and *B. lactis* V9. These strains have been shown to confer beneficial effects on the host: *L. paracasei* Zhang alleviated kidney injury[24]; *L. plantarum* P-8 reduced inflammation[25]; *B. lactis* V9 affected the secretion of sex hormones in the pituitary-hypothalamus through the intestinal-brain axis[26]; and *L. rhamnosus* Probio-M9 and *B. lactis* Probio-M8 regulated the gut microbiota and increased intestinal SCFA-producing bacteria[27,28]. This study aimed to assess the add-on beneficial effects and mechanisms of Probio-X in alleviating gout when administered to patients alongside a conventional regimen (febuxostat). Clinical outcomes were evaluated by measuring changes in sUA levels, the acute flair rate, and participants' gut microbiome and metabolome after the intervention.

2. Materials and methods

2.1. Trial design

A two-month double-blind multicenter RCT was conducted across seven clinical centers of six hospitals: the Department of Rheumatology and Immunology of the Affiliated Hospital of Inner Mongolia Medical University, the Physical Examination Center of the Affiliated Hospital of Inner Mongolia Medical University, Inner Mongolia Autonomous Region People's Hospital, Hulunbeir people's Hospital, Bayannur Hospital, the Second Affiliated Hospital of Baotou Medical College, and the Third Affiliated Hospital of Inner Mongolia Medical University). This study was approved by the Ethical Committee of the Affiliated Hospital of Inner Mongolia Medical University (project number: 2018027) and was registered in the Chinese Clinical Trial Registry with the identifier number ChiCTR1900028232. Informed consent was obtained from all subjects prior to their participation.

The inclusion criteria were: (1) patients aged 18–70 years; (2) fulfillment of the European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) 2015 classification criteria for gout[29]; (3) a history of previous gout attack; (4) sUA levels higher than 480 μmol/L. The exclusion criteria were: (1) secondary HUA; (2) a severe acute gout attack within the past two weeks or requiring colchicine and/or glucocorticoid treatment; (3) abnormal renal and liver function (defined as alanine aminotransferase [ALT], aspartate aminotransferase [AST], and creatinine [Cr] levels 1.5 times above normal); (4) active peptic ulcer disease or gastrointestinal malignant tumor; (5) abnormal blood routine indexes (e.g., white blood cell count $< 4.0 \times 10^9$/L, platelet count $< 100 \times 10^9$/L , hemoglobin < 90 g/L) or other hematological diseases; (6) type I diabetes or poorly controlled type II diabetes (fasting blood glucose $\geq$ 8.5 mmol/L); (7) use of drugs that affect the metabolism or excretion of UA, such as azathioprine, thiazide diuretics and prednisone; (8) infectious diseases requiring antibiotic treatment; and (9) a history of allergy to lactic acid bacteria and their products.

2.2. Randomization and blinding

According to previous studies[30,31] and guidance from our collaborating doctors, it was anticipated that the 2-month treatment response rates for the probiotic and placebo groups would be 50 % and 25 %, respectively. Thus, calculating based on a significance level (α) of 0.05 and a power level (β) of 0.80, the minimized sample sizes would be 103 for the probiotic group and 34 for the placebo group. Considering the chances of subject withdrawal, the planned sample sizes for the probiotic and placebo groups were adjusted to 129 and 43, respectively.

Computer-generated random numbers were used to establish the randomized grouping sequences. Eligible participants were identified by clinicians, and then basic information was transmitted by telephone or email to a statistician, who determined treatment allocation based on the pre-established criteria. During the treatment, both researchers and participants were blinded to the treatment assignments until the study was completed. Eligible participants were randomly assigned in a 3:1 ratio to the probiotic and placebo groups. The probiotic and placebo materials were prepared as powders of identical appearance and taste, provided in individually sealed plastic sachets.

2.3. Subject recruitment and treatment

Firstly, 208 patients with gout were enrolled; however, 21 patients were excluded, resulting in 187 patients who were randomly grouped into probiotic and placebo groups in a ratio of 3:1. During the trial, 20 patients in the probiotic group and seven in the placebo group withdrew

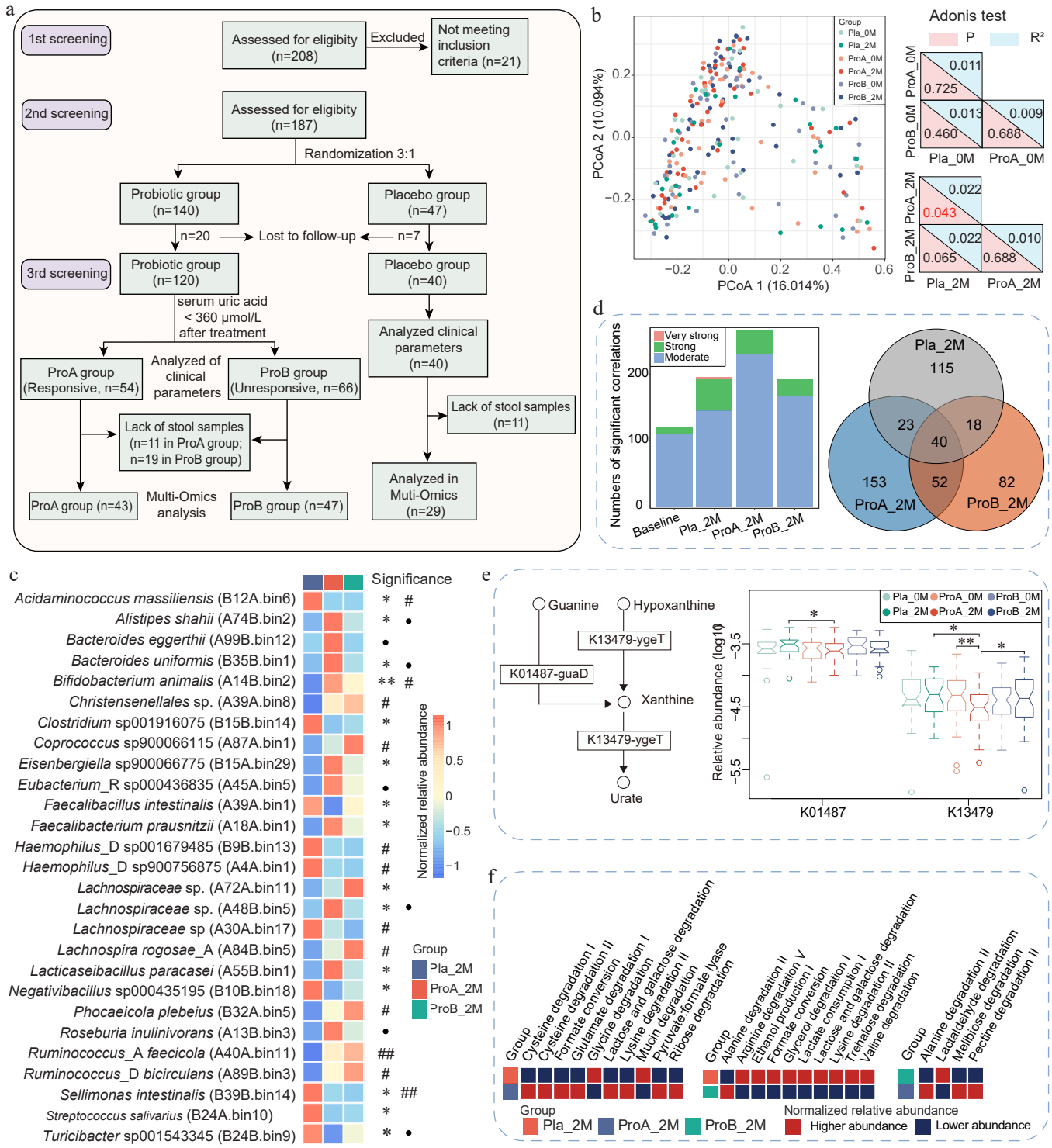

Fig. 1. Trial design and microbial changes of gout. (a) Flowchart of patient recruitment and study process. (b) Principal coordinate analysis (Bray-Curtis distance) score plot of the bacterial gut microbiota across three groups at different time points. Samples from each subgroup are represented by a different color. *P* and R^2 values are generated by the Adonis test. (c) Comparisons of the relative abundance of significantly differentially abundant SGBs identified after 2-month intervention. The color scale represents relative abundance (ranging from 1 to −1, indicating high to low abundance). Significant differences between the ProA and placebo groups: *, $P < 0.05$ and **, $P < 0.01$; between the ProB and placebo groups: #, $P < 0.05$ and ##, $P < 0.01$; between the ProA and ProB groups: •, $P < 0.05$. (d) Significant intragroup microbiota correlations at 0 M and 2 M intervention. Venn diagram shows common and specific correlations identified between groups. Moderate, strong, and very strong correlations are defined by Spearman's rho of 0.4, 0.6, and 0.8, respectively ($P < 0.05$). (e) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map showing the metabolic pathways of urate formation and corresponding changes in the relative abundance in urate-producing KEGG Orthologies (KOs). (f) Relative abundance of significantly differential gout- and inflammation-related gut metabolic modules after the 2-month intervention. Blue and red represent lower and higher relative abundance, respectively. Significant differences: *, $P < 0.05$; **, $P < 0.01$; ProA, probiotic-responsive group; ProB, probiotic-unresponsive group; Pla, placebo group; "0 M" and "2 M" represent the baseline before intervention and 2 months after intervention, respectively; n = 43 in the ProA group, n = 47 in the ProB group, n = 29 in the Pla group for fecal metagenomics analysis.

due to unwillingness to participate. Ultimately, 120 patients in the probiotic group and 40 in the placebo group completed the trial (Fig. 1a). All recruited patients received a prescribed pharmacological regimen of febuxostat based on the severity of their gout and physical condition throughout the trial.

Subjects in the probiotic group ingested a daily dose of two grams of Probio-X powder (3×10^{10} CFU/day), while subjects in the placebo group received two grams of placebo powder. Probio-X powder contained five probiotic strains (*L. paracasei* Zhang, *L. plantarum* P-8, *L. rhamnosus* Probio-M9, *B. lactis* Probio-M8, and *B. lactis* V9) along with excipients, whereas the placebo powder contained only excipients. Both Probio-X and placebo products were provided by JinHua YinHe Biological Technology Co., Ltd., Zhejiang, China, and were prepared according to ISO9001 and HALAL standards.

2.4. Outcome assessments

Blood and fecal samples, along with questionnaires, were collected at the start (month 0; 0 M) and the end (month 2; 2 M) of the trial. The primary outcome was the sUA level following probiotic intake, with a target sUA level of 360 μmol/L, as recommended in the 2016 updated EULAR evidence-based recommendations for the management of gout [32]. The secondary outcomes included acute gout attack rate during the treatment, lipids (triglycerides [TG], cholesterol [CHOL], high-density lipoprotein-cholesterol [HDL-c], and low-density lipoprotein-cholesterol [LDL-c]), clinical questionnaire scores (gout activity score [GAS], gout impact scale [GIS], and visual analogue scale [VAS]), as well as fecal microbiota and metabolites.

Safety outcomes included the recording of adverse events (e.g., bloating, nausea, diarrhea) that occurred throughout the course of the intervention. These were assessed by classifying the adverse events using the Common Terminology Criteria for Adverse Events version 5.0 during the study.

2.5. Determination of the levels of UA, blood lipids, ALT, AST, Cr, xanthine, hypoxanthine and XOD

Venous blood was drawn and centrifuged at 3000 rpm for 10 min to obtain serum samples. The levels of sUA, CHOL, HDL-c, LDL-c, Cr, and TG were estimated using the Cobas 8000 modular analyzer series (Roche Diagnostics Corporation, Indianapolis, IN, USA). Levels of interleukin-1-beta (IL-1β), xanthine, hypoxanthine, and XOD were determined using sandwich enzyme-linked immunosorbent assay kits (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China), according to the protocols provided by the manufacturer.

2.6. Shotgun metagenomic sequencing, contig binning, genome dereplication

All fecal samples were collected with sterile stool samplers. Due to the reluctance of some patients or their inability to submit faecal samples on time, a total of 119 patients (29 in the placebo group and 90 in the probiotic group, totally 238 samples) obtained stool samples at two time points, then 238 stool samples were subjected to shotgun sequencing on an Illumina NovaSeq platform (Tianjin Novogene Technology Co., Ltd., Tianjin, China). Raw metagenomic reads were analyzed according to our previous protocol[28]. Firstly, data were quality controlled sequentially by filtering of low-quality reads (length of reads < 60 nt) using the KneadData quality control pipeline (http://huttenhower.sph.harvard.edu/kneaddata; v0.7.5) and removing human contaminating reads with Bowtie2 (v2.3.5.1). A total of 1.58 Tb of clean data (6.63 ± 0.93 Gb per sample) remained in the dataset for downstream analysis. Secondly, clean data were assembled into contigs using MEGAHIT. Contigs greater than 2,000 bp were chosen for binning via MetaBAT2 and VAMB; and DAS Tool was used to dereplicate, aggregation, and scoring strategy with default options. Then, all bins were combined to obtain metagenome-assembled genomes (MAGs) using in-house scripts. CheckM was used to evaluate the completeness and contamination of MAGs, with MAGs having completeness $\geq$ 80 % and contamination $\leq$ 5 % considered high-quality[28]. Finally, all high-quality genomes were clustered, and the most representative genomes from each replicate set were selected by dRep to obtain species-level genome bins (SGBs) using the options "-pa 0.95".

2.7. Taxonomic annotation of SGBs and prediction of gut metabolic modules (GMMs)

Kraken2 and coverM were used to annotate SGBs via the NCBI non-redundant Nucleotide Sequence Database and to calculate the relative abundance of each SGB with the option "–min-read-percent-identity 0.95 –min-covered-fraction 0.4" (https://github.com/wwood/CoverM), respectively. The average content of SGBs in each contig was expressed as reads per kilobase per million. To determine the function of each SGB, the predicted open reading frames were compared against the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologies (KOs) database to identify metabolic abilities. Detailed metabolic modules of SGBs were identified by Omixer-RPM using the parameter "-c 0.66"[28], and the MetaCyc metabolic database was used to predict relevant GMMs encoded in the SGBs.

2.8. Fecal metabolomics by liquid chromatography-mass spectrometry

As the mass of some samples were insufficient to do metabolomics, there were only 19 patients (38 samples) met the norm, so we randomly chose 19 patients both in ProA and ProB groups, who were obtained twice samples and the mass of samples met the norm of metabolomics. After thawing, 20 mg of 114 samples (ProA group, n = 19; ProB group, n = 19; placebo group, n = 19; two time points for each subject) were vortexed for 3 min with 400 μL of a 70 % methanol-water internal standard extractant, then sonicated for 10 min in an ice water bath and left at −20 °C for 30 min. The mixture was centrifuged (12,000 rpm, 4 °C) for 10 min, and 300 μL of the supernatant was centrifuged again (12000 rpm, 4 °C) for 3 min. The supernatant was collected for analysis on an Agilent 6545 A Q/TOF (Agilent Technologies Inc., Santa Clara, CA, USA) in both positive and negative ion modes. A quality control sample was produced by mixing an equal volume of all samples. During instrumental analysis, one quality control sample was inserted into every 15 tested samples to monitor the repeatability of the analysis process. ProteoWizard was used to convert the original mass spectrometry data into mzML format, and the XCMS program was employed to extract, compare, and correct the retention time of peaks. The "sVR" method was used to correct the peak area and missing rate in each group of samples, with50 % of the peak value filtered out. After correction and screening, metabolite identification information was obtained by searching the laboratory database, comprehensive public databases, and MetDNA [33].

3. Statistical analyses

All statistical analyses were performed using R software (v.4.2.1), PASS (v.11.0.5), and IBM SPSS Statistics 25. Significant changes in sUA level after treatment (relative to < 360 μmol/L) between the two groups were calculated using IBM SPSS Statistics 25. Wilcoxon tests and t-tests were used to assess inter-group (probiotic group versus control group at the same time point) and intra-group (between data of the same group at 0 M and 2 M) differences (cut-off level: $P < 0.05$). Microbial species diversity analysis, Adonis test, and principal coordinate analysis (Bray-Curtis distance) were performed using various R packages, including vegan (v.2.5.6), optparse (v.1.7.1), and ggpubr (v.0.4.0). Spearman's correlation networks of SGBs were constructed by Cytoscape (v3.5.1), with cut-off levels of $|r| > 0.4$ (very strong, $|r| \geq 0.8$; strong, $0.6 \leq |r| < 0.8$; moderate, $0.4 \leq |r| < 0.6$) and $P < 0.05$. The quantitative fecal

Table 1
Baseline demographics and disease characteristics of enrolled patients with gout in probiotic and placebo group.

	Probiotic group (n=120)	Placebo group (n=40)	*P* value
Age	40.85±11.99	40.88±9.65	0.844
Sex, male (%)*	92.50	100.00	0.562
BMI (kg/cm2)	27.08±3.05	27.12±2.43	0.544
sUA (μmol/L)	551.74±58.29	569.12±58.09	0.084
ALT (U/L)	34.90±21.22	34.39±22.52	0.870
AST (U/L)	24.72±12.18	25.00±9.84	0.466
Cr (μmol/L)	80.01±12.53	80.26±16.23	0.950
TG (mmol/L)	2.17±1.38	1.81±1.21	0.061
CHOL (mmol/L)	4.78±0.80	4.59±0.97	0.253
LDL-c (mmol/L)	2.93±0.84	3.17±0.84	0.071
HDL-c (mmol/L)	1.02±0.24	1.00±0.26	0.766
Febuxostat intake (mg/d)	33.25±9.93	33.33±10.21	0.909
Gout related questionnaire scores			
GIS scores	87.98±5.72	89.12±6.23	0.288
GAS scores	6.45±0.47	6.45±0.49	0.970
VAS scores	7.74±0.87	8.06±0.96	0.063

Note: Data are expressed as means ± SD for continuous variables or numbers. sUA, serum urate acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cr, creatinine; TG, triglyceride; CHOL, cholesterol; LDL-c, low-density lipoprotein-cholesterol; HDL-c, high-density lipoprotein-cholesterol; GAS, gout activity score; GIS, gout impact scale; VAS, visual analogue scale; inter-group difference was calculated with Wilcoxon tests; * compared by chi-square test.

metabolomics data were imported into MetaboAnalyst (https://www.metaboanalyst.ca/MetaboAnalyst) for multivariate statistical analysis, with differential metabolites defined as fold change of > 1.5 or < 0.67, and $P < 0.01$. Continuous variables were described as mean and standard deviation (SD), while counting variables were described as frequency and percentage. Data were visualized using R and Adobe Illustrator.

4. Results

4.1. Coadministering probiotics lowered the sUA level and acute gout attack rate

At baseline, no significant differences were found in age, body mass index, the dose of febuxostat administration, or any of the monitored parameters between groups (Table 1). After 2-month treatment, the level of sUA in probiotic group was significantly lower than in the placebo group. A significantly higher proportion of subjects in the probiotic group achieved the target sUA level of < 360 μmol/L (45 % [54/120] in the probiotic group versus 17.5 % [7/40] in the placebo group, $P = 0.002$; Table 2). Additionally, the incidence of the acute gout attacks was significantly lower in the probiotic group (10 % [12/120] in the probiotic group versus 25 % [10/40] in the placebo group, $P = 0.017$; Table 2). No significant changes occurred in the serum levels of TG, ALT, AST, Cr, CHOL, LDL-c, HDL-c, and the scores of GAS, GIS, and VAS after the intervention between the two groups ($P > 0.05$, Table 2). In the longitudinal comparison, the sUA level decreased significantly in both groups, but the extent of sUA reduction was significantly greater in the probiotic group after the 2-month intervention (Table S1). Meanwhile, the scores of GAS, GIS, and VAS were also significantly lower in both the probiotic and placebo groups ($P < 0.001$, Table S1).

We then took a closer look at the data from the probiotic group. Based on therapeutic efficacy after the 2-month intervention, the patients could notably be subdivided into a probiotic-responsive group (ProA; n = 54) and a probiotic-unresponsive group (ProB; n = 66) based on the target sUA level of 360 μmol/L after the intervention. Conceivably, the magnitude of sUA reduction in the ProA group after the intervention was significantly greater than in both the ProB and placebo groups ($P < 0.0001$ in both cases), while the magnitude of sUA reduction was not significantly different between ProB and placebo groups (Table 2). Meanwhile, the incidence of acute gout attacks in the ProA group was significantly lower than in the ProB and placebo groups ($P < 0.05$ in both cases, Table 2). However, compared to baseline, the serum levels of both Cr and TG were significantly lower in the ProA group after the intervention ($P < 0.05$), and such differences were not evident in the ProB and placebo groups (Fig. 1c). Additionally, although the GIS score in all three groups (ProA, ProB, and placebo) decreased significantly after the 2-month intervention, the ProA group had a significantly lower level compared to the ProB group after the intervention ($P < 0.05$; Table 2). No adverse effects were reported during the intervention. These observations together suggest that coadministering Probio-X with febuxostat was more effective in protecting against acute gout attacks, lowering sUA, and improving other gout-related clinical indicators than taking febuxostat alone in approximately half of the participants.

Table 2
Comparison of table clinical manifestations and laboratory tests by probiotic-driven therapeutic responses after the intervention.

	Placebo group (n=40)	Probiotic group (n=120)	ProA group (n=54)	ProB group (n=66)	*P* value			
					Pro v.s. Pla	ProA v.s. Pla	ProB v.s. Pla	ProA v.s. ProB
Uric acid ≤ 360 μmol/L (%)*	7 (17.5 %)	54 (45 %)	54 (100 %)	0 (0 %)	0.002	-	-	-
Acute gout attack (%)*	10 (25 %)	12 (10 %)	2 (3.7 %)	10 (15.2 %)	0.017	0.002	0.209	0.038
△sUA (μmol/L)	−119.79±90.59	−178.89±97.14	−243.55±74.45	−125.99±80.01	0.020	3.77E−10	0.860	5.95E−13
△ALT (U/L)	−2.16±16.42	−3.36±22.24	−4.91±27.47	−2.10±16.70	0.858	0.982	0.794	0.630
△AST (U/L)	2.35±10.16	1.16±11.33	−0.07±11.29	2.17±11.27	0.351	0.194	0.686	0.180
△Cr (μmol/L)	−5.46±15.60	−9.544±48.15	−7.76±19.66	−10.99±62.40	0.982	0.422	0.804	0.193
△TG (mmol/L)	0.03±2.70	−0.20±1.50	−0.49±1.51	0.038±1.44	0.458	0.884	0.295	0.393
△CHOL (mmol/L)	−0.16±1.24	0.00±1.11	−0.13±1.09	0.11±1.11	0.271	0.369	0.199	0.502
△LDL-c (mmol/L)	0.24±1.03	0.11±0.96	0.26±1.01	−0.01±0.91	0.412	0.804	0.266	0.202
△HDL-c (mmol/L)	0.01±0.32	0.047±0.35	0.04±0.34	0.052±0.36	0.579	0.734	0.475	0.676
Gout related questionnaire scores								
△GIS scores	−16.50±6.75	−17.61±8.053	−19.54±8.33	−16.03±7.46	0.444	0.117	0.832	0.044
△GAS scores	−2.27±0.54	−2.30±0.64	−2.34±0.61	−2.28±0.66	0.998	0.725	0.990	0.523
△VAS scores	−5.94±1.20	−6.16±1.22	−6.18±1.26	−6.14±1.18	0.364	0.460	0.506	0.852

Note: Data are expressed as means ± SD for continuous variables or numbers. sUA, serum urate acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cr, creatinine; TG, triglyceride; CHOL, cholesterol; LDL-c, low-density lipoprotein-cholesterol; HDL-c, high-density lipoprotein-cholesterol; GAS, gout activity score; GIS, gout impact scale; VAS, visual analogue scale; △, Changes from baseline after 2 months of treatment; inter-group difference was calculated with Wilcoxon tests; * compared by chi- square test.

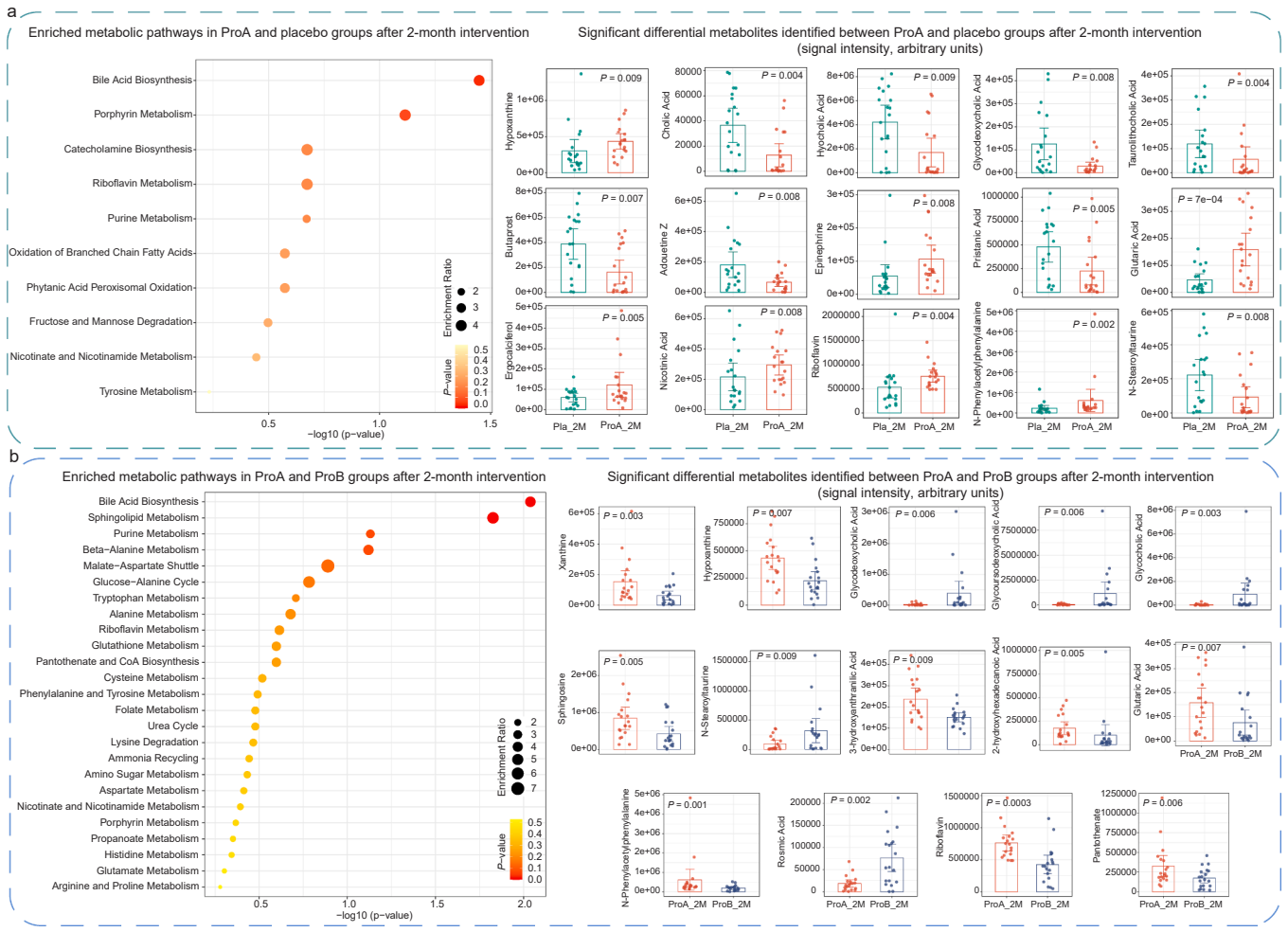

Fig. 2. Changes in the fecal metabolome of subjects after probiotic consumption. Significantly enriched metabolites and their related metabolic pathways in (a) the ProA and placebo groups; (b) the ProA and ProB groups after a 2-month (2 M) intervention. The identified differential metabolites and metabolic pathways exhibited significant differences between groups only after the 2-month intervention, but not at baseline. The amount of detected metabolite was measured by liquid chromatography-mass spectrometry. ProA, probiotic-responsive group; ProB, probiotic unresponsive group; Pla, placebo group; n = 19 in each group.

4.2. Coadministering probiotics modulated the gut microbiota structure and composition

A total of 422 SGBs were extracted from 238 samples, spanning 11 phyla, 14 classes, 29 orders, 52 families, 180 genera, and 409 species. No significant intra- or inter-group differences were detected in α-diversity among the placebo, ProA, and ProB groups ($P > 0.05$, Figure S1). However, a significant difference in gut microbial structure was observed between the responsive and placebo groups after the 2-month intervention ($P = 0.043$, $R^2 = 0.022$; Fig. 1b), while the unresponsive and placebo groups showed no obvious difference, suggesting a possible role of Probio-X in decreasing sUA levels by modulating gut microbes.

We then identified the differentially abundant microbiota among the three groups by pairwise comparison of their gut microbiota in response to clinical indicator improvements after the intervention (Table S2). A total of 27 SGBs showed significant differences in at least two groups only after the intervention, but not at baseline (Fig. 1c). These included significantly higher levels of *L. paracasei*, *Bifidobacterium animalis* (*B. animalis*), *Faecalibacterium prausnitzii* (*F. prausnitzii*), *Alistipes shahii*, *Lachnospiraceae* sp., and *Lachnospira rogosae*_A ($P < 0.05$) in the ProA and ProB groups, while significantly higher levels of *Acidaminococcus massiliensis*, *Sellimonas intestinalis*, and *Streptococcus salivarius* were found the in placebo group. Additionally, time-based variation in gut microbiota within each group identified significant changes in some SCFA-producing bacteria. For example, significantly fewer *Blautia_A* sp900066335 and *Ruminococcus_A faecicola* ($P < 0.05$) were detected in the placebo group compared to baseline, but not in the ProA and ProB groups after the intervention (Fig. 1d). Taken together, our results suggest that coadministering Probio-X favorably modulated the patients' gut microbial structure by increasing certain beneficial microbes, especially SCFAs-producing bacteria[34,35].

4.3. Coadministering probiotics modulated the gut microbiota interactions

Subsequently, we constructed overall and group-based SGB-level interactive gut microbial networks at baseline and after the 2-month intervention (Fig. 1d and S2). At baseline, 119 correlations were detected. While the microbial interconnectedness increased across all three groups at 2 M compared to baseline, the correlation network was notably stronger in the ProA group than in the placebo and ProB groups (Fig. 1d). The correlation networks for the ProA group after 2-month intervention were characterized by a higher number of significant and unique correlations, encompassing a much broader taxonomic spectrum. This included several well-recognized health- promoting microbes, such as *Bifidobacterium adolescentis* and *F. prausnitzii*_D, *Lachnospira eligens*, and *Bariatricus comes* (Fig. 1d). These findings suggest a stronger gut microbial interconnectedness in the ProA group compared to both the placebo and ProB groups. Our results indicate that probiotic-driven enhancements in the interconnectedness of patients' gut microbiota may be a key factor contributing to symptom

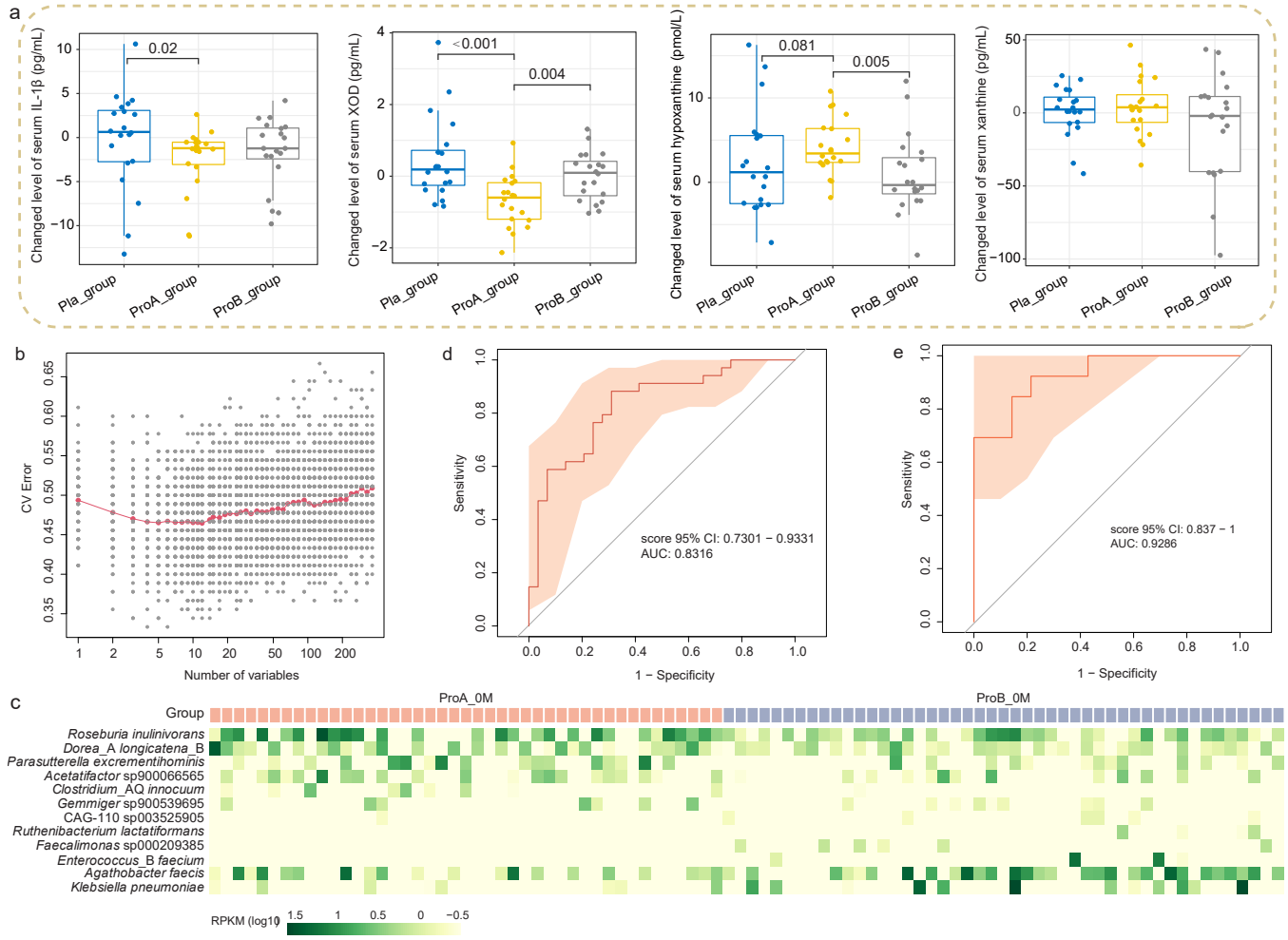

Fig. 3. Changes in serum indexes after a 2-month intervention and top species-level genome bins (SGBs) contributing to gout alleviation. (a) Differences in serum levels of interleukin (IL)-1β, xanthine oxidase (XOD), hypoxanthine, and xanthine after the 2-month intervention in the probiotic-responsive (ProA), probiotic-nonresponsive (ProB), and placebo (Pla) groups; n = 20 in each group. Random forest models were used to predict the top SGBs contributing to the symptom alleviation effect. (b) The cumulative variation (CV) error curve. (c) Heatmap showing the gut metagenomic classifier (the top 12 SGBs contributing most to the symptom alleviation effect, expressed in reads per kilobase per million, RPKM, shown in the color scale) between the ProA and ProB groups at baseline (0 M); n = 43 in the ProA group, n = 47 in the ProB group. (d, e) Receiver operating characteristic curves for the discovery and validation samples, respectively.

improvement in gout patients.

4.4. Coadministering probiotics modulated UA metabolism-related pathways

As xanthine is the direct precursor of UA, we calculated the gene abundance involved in the pathways of xanthine production and conversion to UA (K01487 and K13479, https://www.genome.jp/pathway; Fig. 1e). Our results showed that the ProA group had an overall lower level of these genes compared to the ProB and placebo groups (Fig. 1e). Coadministering Probio-X was also found to regulate multiple pathways, including those related to amino acid metabolism (lysine, cysteine, valine and glycine), mucin degradation, and formate conversion. The pathways of glycine and mucin degradation were enriched in the probiotic group, while the pathway for formate conversion was enriched in both the placebo and ProB groups (Fig. 1f).

4.5. Coadministering probiotics modulated the fecal metabolome

We analyzed the inter-group differences in the fecal metabolome after the 2-month treatment. In the PCA score plots, symbols representing the QC samples clustered together, reflecting good instrumental and chromatographic stability (Figure S3a). The partial least squares-discriminant analysis indicated that the gut metabolomes of the ProA and placebo groups, as well as ProA and ProB groups, were distinct from each other after the 2-month intervention (Figures S3b, S3c).

A total of 180, 105, and seven differential metabolites were detected between the ProA and placebo groups, the ProA and ProB groups, and the ProB and placebo groups after the 2-month intervention but not at baseline (cut-off level: fold changes > 1.5 or < 0.67, $P < 0.01$), respectively. The differential metabolites between the ProA and placebo groups mainly belonged to the pathways of bile acid biosynthesis, porphyrin metabolism, purine metabolism, riboflavin metabolism, and nicotinate and nicotinamide metabolism (Fig. 2a and Table S3), Significantly more hypoxanthine, ergocalciferol, nicotinic acid, riboflavin, and N-phenylacetylphenylalanine were detected in the ProA group than in the placebo group ($P < 0.01$), while cholic acid, hyocholic acid, glycodeoxycholic acid, taurolithocholic acid, and butaprost showed an opposite trend ($P < 0.01$). The differential metabolites between the ProA and ProB groups primarily belonged to the pathways of bile acid biosynthesis, purine metabolism, sphingolipid metabolism, riboflavin metabolism, and amino acid metabolism (tryptophan, alanine, and cysteine; Fig. 2b and Table S3). More xanthine, hypoxanthine, riboflavin, pantothenate, glutaric acid, and sphingosine were detected in the ProA group than in the ProB group ($P < 0.01$), while glycodeoxycholic acid, glycoursodeoxycholic acid, and glycocholic acid

showed an opposite trend ($P < 0.01$). These results suggest that Probio-X administration was accompanied by specific fecal metabolomics changes.

4.6. Coadministering probiotics affected serum IL-1β, xanthine, hypoxanthine, and XOD activity

To reconfirm our results, serum samples from 20 volunteers in the placebo, ProA, and ProB groups were randomly selected to measure serum levels of IL-1β, xanthine, hypoxanthine, and XOD before and after the intervention using sandwich enzyme-linked immunosorbent assays. The levels of IL-1β and XOD were significantly lower in the probiotic group compared to the placebo group ($P = 0.036$ in IL-1β, $P = 0.01$ for XOD; Figure S4), while hypoxanthine and xanthine levels were not significantly different between the two groups (Figure S4). In the responsive and non- responsive sub-groups, the levels of IL-1β and XOD were significantly lower in the ProA group compared to the placebo group ($P = 0.02$ for IL-1β, $P < 0.001$ in XOD; Fig. 3a). Additionally, XOD levels were significantly lower in the ProA group compared to the ProB group ($P = 0.004$; Fig. 3a). The level of hypoxanthine was significantly higher in the ProA group than in the ProB group ($P = 0.005$) and was non-significantly higher than in the placebo group after the intervention ($P = 0.081$). No significant difference was observed in the magnitude of change in xanthine levels across groups ($P > 0.05$). These results provide further evidence of the inhibitory effect of probiotic intake on inflammation and UA-production, particularly in the probiotic-responsive group. It is reasonable to infer that the anti-inflammatory and UA-lowering effects of probiotic intake may be mediated by modulating gut bacterial abundance, pathways, and metabolites, thereby alleviating the gout symptoms in patients.

4.7. Prediction of gout-specific microbiota features by random forest modeling

As our metagenomics and metabolomics results pointed to a specific relationship between the gut microbiota and treatment responsiveness, we therefore applied the random forest algorithm to generate a disease-response classifier for predicting subjects' treatment responses. The model was constructed based on the baseline gut bacterial SGB profiles of subjects in the ProA and ProB groups. After feature selection using 10-fold cross-validation, 12 SGBs were retained for optimal performance (Figs. 3b, 3c). The area under the curves for the discovery (70 %) and validation (30 %) cohorts reached 0.83 and 0.93, respectively (Figs. 3d, 3e). These results suggest that specific microbiota have the potential to predict the effects of probiotic administration.

5. Discussion

Gout is a metabolic disease characterized by dysregulation in UA metabolism. The number of patients with gout increases year on year, impacting their quality of life and contributing to social pressure and economic burdens. This study evaluated the efficacy of coadministering Probio-X with febuxostat in relieving gout in an RCT and found that around half of the subjects were responsive to the probiotic coadministration, showing improvement in clinical symptoms and gout-related indexes. We comparatively analyzed the changes in fecal metagenomes and metabolomes of responsive, unresponsive, and placebo subjects, identifying differential gut microbiota markers for the probiotic response. Our data provide interesting insights into the therapeutic responses in gout patients.

Urate-lowering therapy is the cornerstone of gout management; however, current uric acid-lowering drugs are frequently accompanied by long-term safety risks and low medication adherence[36], and the need for sustained sUA target maintenance has not been fully met. Our results showed that coadministering probiotics with febuxostat significantly reduced sUA levels, which is consistent with previous studies[20,22,23,37]. Additionally, we quantified the effect of probiotics by comparing it with the target level and found that Probio-X enhanced the efficacy of febuxostat in achieving a target sUA level. The acute gout attack rate also decreased with probiotic intervention, a finding that has rarely been reported before. Meanwhile, the serum Cr level decreased only in the probiotic group; an increase in Cr level is associated with kidney injury and is a key factor affecting UA excretion[38]. A decreased serum Cr level may imply an increase in UA excretion through the kidneys.

Another observation made in this study was that only around half of the study cohort was responsive to probiotic intake for symptom improvement. Almost all indicators did not show significant differences between the unresponsive and placebo groups after the 2-month intervention. In contrary, the larger magnitude of decrease in sUA level in responsive group was accompanied by a lower acute gout attack GIS score. The lower sUA level and acute gout attack meant better therapeutic effect of coadministration Probio-X; GIS is a disease-specific health-related quality of life measurement for patients with gout[39], and a lower GIS score indicates a higher quality of life of patients. The magnitude of reduction in TG level was also more pronounced in the responsive group than the placebo group. All these results showed that Probio-X improved gout disease and accompanied an improvement in the quality of life in around half of the patients.

Although the relationship between the gut microbiome and gout has not been fully elucidated, some studies have reported alterations in gut microbiota composition in gout compared to healthy controls[6,7]. In the present study, we found that the fecal microbiota of the responsive group had higher abundances of *B. animalis* and *L. paracasei* than the placebo group after the 2-month intervention, which was likely due to these species being part of the composition of Probio-X. The gut bifidobacterial abundance has been reported to be associated with gout development[40], and *L. paracasei* has been shown to suppress gout attack-related IL-1β secretion by inhibiting the activation of the NLRP3 inflammasome and inflammatory stress-induced caspase-1 activation [41], which was also observed in the present study. Some other probiotic-responsive microbes (*Alistipes shahii*, *Bacteroides uniformis*, *F. prausnitzii*, and *Lachnospiraceae* sp.) might have been involved in the observed UA-lowering effect. *Alistipes* was found to be associated with purine metabolism, and they are known as SCFA (acetate and propionate)-producers[42]; *Lachnospiraceae* and *F. prausnitzii* are well-known SCFA-productors, which are considered beneficial bacteria in various health conditions[43,44]. Some significantly depleted SGBs in the responsive group after the 2-month intervention (e.g., *Acidaminococcus massiliensis*, *Streptococcus salivarius*, and *Sellimonas intestinalis*) have been reported to be enriched in the gut of unhealthy individuals[45–47]. Besides, the fecal microbiota of the placebo group showed a decreased level of SCFA-producers after the 2-month intervention, while an opposite trend was observed in the probiotic group (both responsive and unresponsive groups), suggesting that the gut SCFA-producers might play a role in the observed gout treatment effect. Therefore, the clinical remissive effect of Probio-X was likely related to the regulation of specific gut microbiota in patients with gout.

The alterations in the gut microbiota were accompanied by considerable changes in the gut functional pathways and metabolome. In UA metabolism, probiotic intake down-regulated the gene expression of gut UA synthesis (K01478 and K13479), and increased the levels of hypoxanthine and xanthine in both fecal and serum samples, while decreasing serum XOD activity. Probiotic intake was also accompanied by increased pathways in glutamate degradation and decreased pathways in glycine degradation. Glutamine, produced by glutamate, enhances UA formation in gout[48], while glycine has shown an inverse association with gout[49]. These results provide more comprehensive evidence for probiotics inhibiting UA production than previous studies. Acute gout attack is mainly caused by an increased level of IL-1β, and both acute gout attack and IL-1β levels decreased with Probio-X in the present study. Gut bile acids may play an important role in this process.

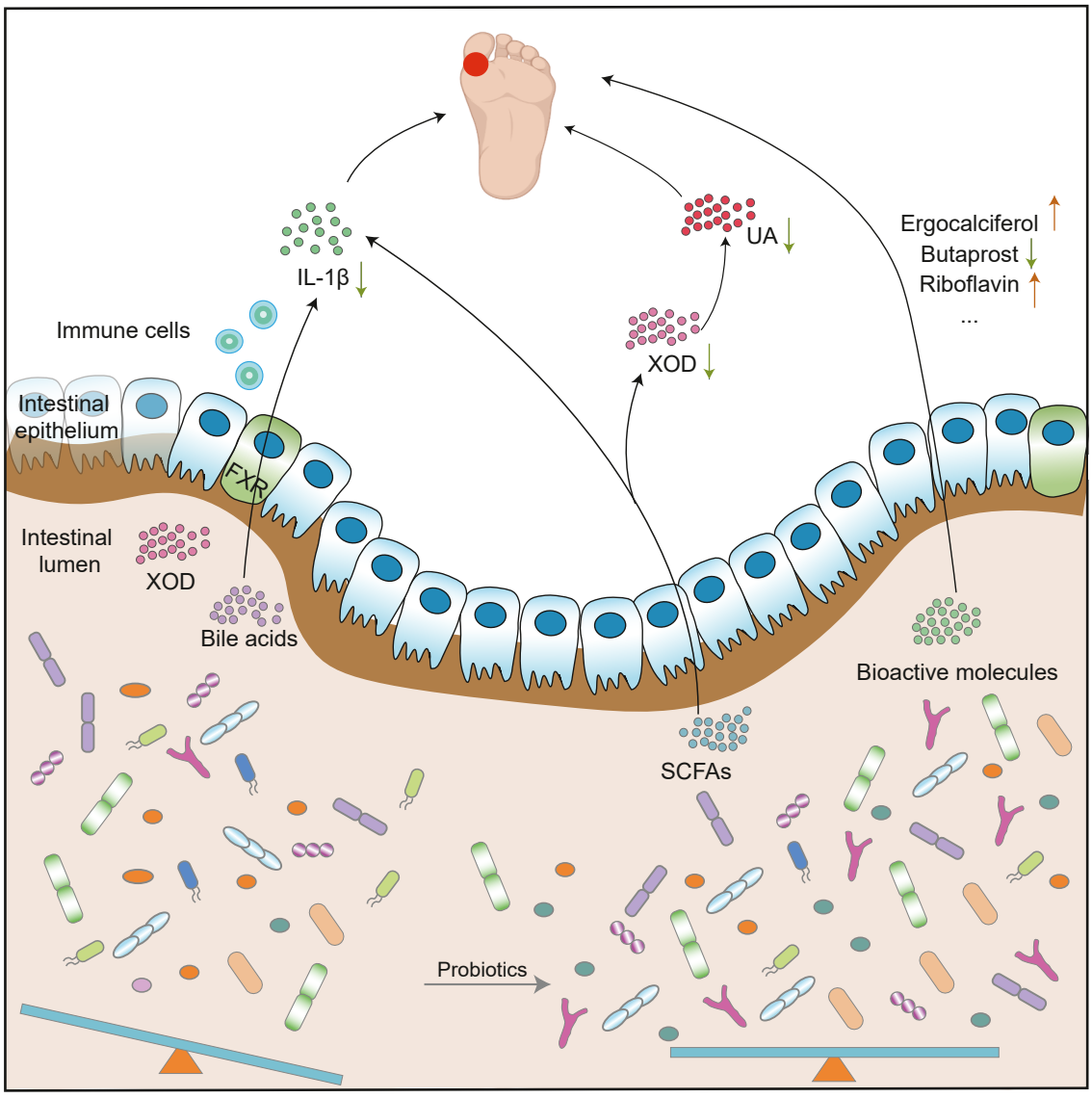

Fig. 4. Schematic diagram showing Probio-X-driven changes in host gut microbiome and metabolome, which may have contributed to the alleviation of gout. XOD = xanthine oxidase; SCFAs = short-chain fatty acids; IL-1β = interleukin-1β; FXR = Farnesoid X receptor.

Bile acids decreased in the probiotic group after the 2-month intervention and exert a variety of physiological functions, such as facilitating NLRP3 inflammasome activation[50], which can be activated by monosodium urate crystals and release IL-1β, thereby initiating gout flares. Bile acids also showed the ability to regulate insulin resistance, which is common in patients with gout, and lowering insulin resistance has promoted UA reduction[51]. Meanwhile, butaprost, a selective prostaglandin E receptor agonist, decreasd after probiotic intake and has been shown to lower the expression of IL-1β and NLRP3 in a mouse model through downregulating prostaglandin E2[52]. Therefore, the anti-inflammatory effect of probiotic coadministration was possibly achieved through the action of bile acids in suppressing NLRP3 inflammasome activation and butaprost in suppressing prostaglandin E2.

Additionally, probiotic intake has brought some indirect benefits. Firstly, an up-regulation of mucin degradation pathway was found in the responsive group. Mucin degradation accompanies the release of monosaccharides or amino acids, providing nutrients to other resident bacteria and beneficial bacteria like SCFA-producers[53], which is consistent with the changes observed in the bacterial correlation networks. Probiotic intake also suppressed the pathways of lactose and galactose degradation as well as formate conversion. Gout is known to cause abnormal galactose metabolism by affecting the activity of galactase, leading to excessive conversion of galactose to galactose-1-phosphate and its accumulation[54]. Formate contributes to purine and thymidylate synthesis[55], and the decrease in formate formation-related pathway is consistent with the therapeutic effect of probiotic co-consumption. Notably, lower vitamin D levels have been found to be associated with gout and the quality of life of patients with gout[56]. The prevalence of osteoporosis is higher among patients with gout than healthy subjects[57], and the increase in ergocalciferol (vitamin D_2) could restore serum vitamin D levels and enhance bone metabolism[58]. All these changes may contribute to the improvement in the quality of life of patients.

The probiotic-driven health effects and therapeutic responses are dependent on the endogenous gut microbiota of the subjects. We found that the fecal metagenome of the responsive subjects had more SCFA-producers and potential beneficial bacteria intrinsically present at baseline compared to the unresponsive ones. Human gut SCFA-producers promote intramicrobiota growth via cross-feeding among members of the microbial community[59]. Other beneficial microbes, such as lactobacilli, restrict gut colonization of pathogens (including

multidrug-resistant *Enterobacteriaceae*) through mechanisms like pH reduction, spatial and nutrient competition, and avoiding the development of a hostile environment[60]. Combined with the changes observed in the correlation networks and the mucin degradation pathway in the responsive and unresponsive groups after probiotic intervention, it is reasonable to speculate that Probio-X acts faster by interacting with the beneficial bacteria in the responsive group, while the probiotics inhibit the original pathogenic bacteria first in the unresponsive group. Therefore, these results highlight the variation in probiotic-driven responses between individuals and the importance of personalized probiotic design.

This study had some limitations. Firstly, the study population consisted solely of Chinese patients, limiting the generalizability of the findings to patients with gout from other countries, ethnicities, and clinical backgrounds. Secondly, due to patient preference and cost considerations, we did not evaluate the computed tomography results of the gout site. Thirdly, we did not conduct follow-up assessments after the intervention to evaluate the long-term effects of Probio-X on lowering sUA levels. Despite these limitations, the successful implementation of this multicenter RCT and the higher clinical response rate in the probiotic group compared to the placebo group suggest that the efficacy of Probio-X in the treatment of gout warrants further investigation.

Taken together, this multicenter RCT investigated the added beneficial effects of probiotic coadministration with the conventional regimen (febuxostat). Interestingly, we noted highly individualized probiotic-driven beneficial responses. Only around half of the patients experienced added beneficial effects from the perspectives of reduction in clinical indicator levels (including sUA, acute gout attack rate, gout impact score, IL-1β, and XOD activity), fecal metagenomics and metabolomics changes (increases in sequences of gut beneficial microbes like *F. prausnitzii*, *B. animalis*, and *L. paracasei*, and metabolites like xanthine, hypoxanthine and bile acids; Fig. 4), while the unresponsive subjects showed no obvious differences in the monitored parameters compared to the placebo group. The individualized responses towards probiotic intervention could be related to the subjects' endogenous gut microbiota composition. This work provides valuable information for future studies aiming to explore probiotic application in managing gout.

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CRediT authorship contribution statement

Hongbin Li: Methodology, Investigation, Conceptualization. **Heping Zhang:** Resources, Project administration, Funding acquisition, Conceptualization. **Teng Ma:** Visualization. **Jing Wang:** Data curation. **Dafu Man:** Conceptualization. **Xiangzheng Yuan:** Data curation. **Huiyun Li:** Data curation. **Lixia Pang:** Data curation. **Hui Shi:** Data curation. **Shuiming Ren:** Data curation. **Zhongjie Yu:** Software, Formal analysis. **Feiyan Zhao:** Writing – original draft, Visualization, Software. **Xin Shen:** Visualization, Software. **Ning Tie:** Project administration, Methodology, Data curation. **Lai-Yu Kwok:** Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

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Authors' contribution

Hongbin Li and Heping Zhang conceived and designed the trial. Feiyan Zhao, Zhongjie Yu, and Ning Tie supervised all experiments. Ning Tie, Jing Wang, Dafu Man, Xiangzheng Yuan, Huiyun Li, Lixia Pang, Hui Shi, and Shuiming Ren recruited patients and collected the data of all volunteers. Ning Tie, Feiyan Zhao, and Zhongjie Yu summarized data from all the centers. Feiyan Zhao, Teng Ma, and Xin Shen analyzed the metagenomic sequencing data. Feiyan Zhao wrote the main manuscript. Lai-Yu Kwok critically reviewed the manuscript and provided advice. All authors discussed the results and comments on the manuscript. All authors read and approved the final manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2024.107445.

Data Availability

Data will be made available on request.

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