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Galacto-oligosaccharides promote urate excretion via enhanced microbial production of *N*-isovaleroylglycine

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ABSTRACT: Dysbiosis of gut microbiota has been implicated in the development of hyperuricemia (HUA), which accelerates the progression of macrovascular and microvascular disorders. Galacto-oligosaccharides (GOS), a soluble fiber, is a potent regulator of the microbial community, however, its role in the management of HUA remains obscure. This study aimed to investigate the impact of GOS on HUA in murine models. Multi-omics analysis and germ-free mice were employed to identify the key microbial effectors of GOS. Subsequently, targeted knock-down with adeno-associated virus 9 in the kidney was employed to evaluate the relevance of ATP-binding cassette subfamily G member 2 (ABCG2) in the regulation of GOS-mediated uric acid clearance. GOS alleviated HUA by enhancing renal urate excretion. The phenotype of increased urate excretion was transmissible with fecal microbial transplantation from donor mice fed with GOS. Integrative 16s rRNA sequencing and metabolomics analysis identified *N*-Isovaleroylglycine (*N*-IVG) as the key microbial effector mediating the uric acid-lowering effect of GOS. Mechanistically, microbiota-derived *N*-IVG suppressed the nucleus translocation and activation of nuclear factor- κ B (NF- κ B), which in turn boosted the expression and co-localization of ABCG2 and PDZ-domain-containing 1 in proximal tubule brush border membrane. Inhibition of renal ABCG2 but activation of NF- κ B abolished the uric acid-lowering effect of *N*-IVG. Eight-week intervention with GOS effectively increased the production of *N*-IVG, and enhanced renal urate excretion. GOS may provide therapeutic benefits in combating HUA by a targeted promotion of the microbial production of *N*-IVG.

Keywords: Hyperuricemia, Galacto-oligosaccharides, *N*-Isovaleroylglycine, ABCG2

1. Introduction

Hyperuricemia (HUA), characterized by an elevation in serum uric acid (UA), has become the second most common metabolic disease worldwide¹. Disruption to the dynamic equilibrium between endogenous urate synthesis and urate excretion impacts serum UA². Though asymptomatic in most cases, prolonged exposure to high UA is linked to a spectrum of diseases, such as gout, chronic kidney disease, and

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cardiovascular and cerebrovascular disorders, posing a major threat to all societies³. Given the side effects of existing hypouricemic agents⁴, there is an urgently unmet need for the identification of safe and effective alternatives for the management of HUA. Recently, dysbiosis of gut microbiota has been implicated in the progression of HUA. For example, the abundances of *Prevotella*, *Ruminococcus*, and *Lactobacillus* were found to be significantly lower in a rat model of HUA⁴. The phenotype of elevated UA could be transmitted through fecal microbial transplantation (FMT)⁵, whereas, administration of *Bifidobacterium* and *Lactobacillus* was effective in lowering serum UA in murine models⁴. Our recent study also found that hippuric acid, a key microbial metabolite, lowered serum UA via enhanced intestinal urate excretion in an ATP-binding cassette subfamily G member 2 (ABCG2)-dependent manner⁶, further highlighting the potential of targeting gut microbiota for the management of HUA. Given the crucial role of gut microbiota in UA homeostasis, the use of prebiotics, a potent regulator of the complex microbial community⁷, which selectively stimulates the growth and/or activity of beneficial microorganisms, to modulate UA is of increasing interest.

Galacto-oligosaccharides (GOS) is a kind of oligosaccharides formed by β -galactosidase transgalactosylation. GOS arrives at the colon intact and consequently increase the abundance of specific primary and secondary degraders, resulting in an expanded beneficial microbial species⁸. As a pleiotropic prebiotic capable of stimulating the growth of *Bifidobacteria* and *Lactobacillus*⁹⁻¹¹ and attenuating metabolic disorders via reduced inflammation¹², has gained a lot of attention due to its wide applications in functional foods^{13, 14}.

In this study, we interrogated the role of GOS in murine models of HUA, and explored the key microbial metabolite and molecular mechanisms whereby GOS alleviated HUA, offering new insights into the prebiotic properties of GOS.

2. Materials and methods

2.1 Materials

Galacto-oligosaccharides (Product code: GOS-70S, total galactooligosaccharide content $\geq 70\%$, moisture content $\leq 5.0\%$, lactose content $\leq 22.0\%$, glucose content $\leq 10\%$) was purchased from Tate & Lyle Co., Ltd. (Guangzhou, China).

2.2. Animal model

All animal experiments were approved by the Animal Care and Utilization Committee of Sun Yat-sen University (No. 2023-026). Eight-week male mice on C57BL/6J background were obtained from Shenzhen Gnotobio Biotechnology Co., Ltd. and housed under germ-free conditions, or purchased from Zhuhai BesTest Biotechnology Co., Ltd. and housed in specific pathogen free (SPF) conditions at Sun Yat-sen University. The animals were housed in groups of four per cage, with free access to food and water under a strict 12-h light/dark cycle at a controlled temperature ($23 \pm 2^\circ\text{C}$). To establish an HUA model, mice were fed with hyperuricemia (HUA) diet, consisting of 2% uric acid (UA) and 4% oxonic acid, an inhibitor of uricase (LAD3001M-X2, Nantong Trofeo Feed Technology Co. Ltd., China) for 4 weeks, as previously described⁶. Control group was

fed with a control diet (LAD3001M, Nantong Trofeo Feed Technology Co. Ltd., China).

To evaluate the effects of galacto-oligosaccharides (GOS) or *N*-Isovaleroylglycine (*N*-IVG) on UA metabolism, HUA mice were gavaged with GOS dissolved in phosphate-buffered saline (PBS) at 500 mg/kg/day, a dose commonly used in metabolic disease research¹⁵ or *N*-IVG dissolved in PBS at 50 mg/kg/day to mimic the level detected in mice fed with GOS, and mice were gavaged with benzbromarone, a widely used drug for lowering UA, dissolved in PBS at 6.25mg/kg/day, on a daily basis, while the control group received an equivalent volume of PBS as a solvent control.

To confirm the role of microbial metabolism in the protective effect of GOS against HUA, fecal microbial transplantation (FMT) was performed as previously described¹⁶. Briefly, 100 mg of fresh stools obtained from mice fed with PBS or GOS for 4 weeks (donor mice) were homogenized in 1 mL of sterile PBS buffer containing 0.5g/L cysteine as reducing agent, followed by filtration through a 100 µm strainer in an anaerobic chamber. Stool samples from the same donor groups were pooled and fecal slurry was transferred into recipient mice fed with a HUA diet. Before FMT, recipient mice were treated with antibiotics cocktail (ampicillin 1g/L, metronidazole 1g/L, neomycin 1g/L and vancomycin 0.5g/L) for 1 week to eliminate their gut microbiota¹⁶. After a 3-day wash-out period to eliminate antibiotics, mice were gavaged once daily for three consecutive days in the first week of colonization, and during the remaining three weeks, fecal slurry was introduced every other day (200µl per mice) to reinforce colonization.

To determine the role of gut microbiota in the production of *N*-IVG, eight-week SPF mice, antibiotics-treated SPF mice and germ-free mice fed with normal chow diet were subjected to targeted measurement of *N*-IVG.

Mouse adeno-associated virus 9 (AAV9) delivery system was used to knock down *ABCG2* specifically in the kidney. HBAAV9-Ksp-cadherin-mir30-m-*ABCG2*-EGFP and HBAAV9-Ksp-cadherin-EGFP (negative control) were obtained from Hanbio Technology Co. Ltd. (Shanghai, China). Mice receiving AAV9 vector (150 µL, 1×10^{12} genome copies) through tail vein were fed with HUA diet and gavaged with *N*-IVG at 50mg/kg/day or PBS as solvent control for 4 weeks before further analysis.

All animal experimental procedures were performed with a sample size of eight ($n=8$ per group).

2.3. Cell lines and culture conditions

Human Kindey-2 (HK-2) cells were obtained from American Type Culture Collection. HK-2 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 mg/mL streptomycin, under 37°C with 5% CO₂ humidity. HK-2 cells were seeded on filter inserts to undergo a process of spontaneous differentiation for 3 days, acquiring functional characteristics akin to renal enterocytes, before subjected to subsequent experiments. All *in vitro* experiments were repeated 6 times unless otherwise stated.

2.4. Determination of UA excretion in mice

Inosine-stimulated test was employed to assess UA excretion capacity as previously described¹⁷, with minor modification. Briefly, following an overnight fast, inosine solution (187.5mg/kg) was orally gavaged,

and serum samples were collected from tail vein at 0, 30, 60 and 120 min after inosine gavage to determine the level of UA. After centrifugation at 3500 rpm at 4°C for 15min, concentrations of serum UA were determined by commercial kit from Nanjing Jiancheng Co., Ltd, according to the manufacturer's instruction.

2.5. Determination of hepatic UA synthesis

The synthesis of UA in the liver was evaluated by the activity of xanthine oxidase (Solarbio, BC1095) and the adenosine deaminase (YuanYe Biotechnology, R22208), following the manufacturer's instruction.

2.6. UA excretion from the intestine

To assess intestinal urate excretion, mice were anesthetized by intraperitoneal injection of urethane and cannulated with polyethylene tubing (Hibiki size 8) at the upper duodenum and middle jejunum to make an intestinal loop at the upper half of the intestine, as previously described¹⁸. The intestinal contents were slowly removed by alternating saline and air infusions. Subsequently, an efflux buffer (saline containing 0.3 mM potassium oxonate) was introduced into the intestinal loop, and both ends of the loop were closed with syringes. The efflux buffer in the loop was collected by syringes and urate concentrations at 20, 40, 60, 80, 100 and 120 min after infusion were quantified (Uric Acid Kit, Nanjing Jiancheng). UA excretion from the intestine was calculated using the following equation: intestinal urate excretion = (urate concentration in the intestinal loop) × (volume of efflux buffer in the intestinal loop) × (length of the whole small intestine/length of the intestinal loop).

2.7. UA excretion from the kidney

To determine urate excretion through kidney, urinary samples were collected using metabolic cage for 24h after 4 weeks of intervention. UA and creatinine levels in serum and urine were determined by UA kit and creatinine kit, respectively from Nanjing Jiancheng Co., Ltd. Kidney urate excretion was evaluated by FE_{UA} and 24h UUA. FE_{UA} was calculated as the ratio between the renal clearance of uric acid to the renal clearance of creatinine, while 24h UUA was calculated by 24h urinary volume × urinary urate, as previously described¹⁹.

2.8. 16S rRNA sequencing and analysis

Mouse fecal DNA was extracted from frozen stools using MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit (Thermo Fisher Scientific, MA, USA). The concentration and purity were monitored on 1% agarose gels. 16S rRNA sequencing of the V3-V4 regions was performed on Illumina MiSeq platform (Illumina, San Diego, California, USA) by Novogene Co., Ltd (Beijing, China). MiSeq (Illumina) fastq files were analyzed using Qiime2 (v.2018.4.0). Sequences were denoised and binned to amplicon sequencing variants (ASVs) using DADA2 (Divisive Amplicon Denoising Algorithm 2)²⁰. ASVs were taxonomically classified based on the feature-classifier plugin (Silva-138-nb-classifier).

Alpha diversity of the gut microbiota, including Shannon and Simpson indexes, were calculated for each sample using R package *vegan* (v.2.6-6.1). Beta diversity was assessed based on Bray-Cutis distance matrix, and permutational multivariate analysis of variance (PERMANOVA) was performed to evaluate the

association between microbial composition and GOS treatment, with 999 permutations.

For further analysis, only ASVs prevalent in at least 60% of the samples were included. Significantly altered ASVs after GOS supplementation were identified using empirical Bayes estimation and exact tests based on the negative binomial distribution with R package *edgeR* (v.4.2.1), with false-discovery rate (FDR)-adjusted *P* value < 0.05 considered as significant. Spearman correlation analysis was employed to determine the association of differential ASVs with UA-related phenotypes in mice. Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation for the 16s rDNA was performed by PICRUST2²¹, and assigned to metabolic modules using Gomixer²². *P* values were adjusted by the Benjamini-Hochberg method to control for multiple comparisons, and an adjusted *P* value < 0.05 was considered as significant.

2.9. Untargeted metabolomics profiling and analysis in mice

For serum metabolic profiling in mice, an integrated method for large-scale detection, identification, and quantification of metabolites was performed²³. Briefly, 50 μ L of serum samples were thawed on ice, vortexed for 10sec, followed by the addition of 300 μ L of methanol-acetonitrile with internal standards. The mixture of serum and internal standards was vortexed for 3min, and the supernatants were recovered by centrifugation at 12,000rpm for 10 min at 4°C. Then, 10 μ L of the supernatant taken from each sample was pooled together to create a “mixed sample”, followed by a full scan mode with QTRAP® 6500+ system to acquire a high-resolution data including RT, Q₁, and Q₃. Peak annotation of the mixed sample was performed with the in-house database of Metware Co., Ltd (Wuhan, China), followed by a transfer of the ion pair information to the triple quadrupole (QQQ) instrument. Finally, individual serum samples were analyzed by QQQ in multiple reaction monitoring (MRM) mode to get a more accurate quantification of metabolites. The sample extracts were analyzed using an LC-ESI-MS/MS system (UPLC, ExionLC AD, <https://sciex.com.cn/>; MS, QTRAP® System, <https://sciex.com/>). The analytical conditions were as follows, UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μ m, 2.1mm*100 mm); column temperature, 40°C; flow rate, 0.4 mL/min; injection volume, 2 μ L; solvent system, water (0.1% formic acid): acetonitrile (0.1% formic acid); gradient program, 95:5 V/V at 0 min, 10:90 V/V at 11.0min, 10:90 V/V at 12.0 min, 95:5 V/V at 12.1min, 95:5 V/V at 14.0min. Mass spectrometry (MS) detection was performed by triple quadrupole-linear ion trap mass spectrometry (QTRAP), equipped with an electrospray ionization (ESI) source. Full scans with a range of *m/z* 50-1000 were acquired in LIT and QQQ scans with positive and negative ion modes and controlled by Analyst 1.6.3 software (Sciex). The ESI source parameters were as follows: source temperature 500°C; ion spray voltage (IS) 5500 V (positive), -4500 V (negative); ion source gas I (GSI), gas II (GSII), curtain gas (CUR) was set at 55, 60, and 25 psi, respectively; the collision gas (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100mmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period. For the analysis of metabolomics data, log-transformation was performed before subjected to downstream analyses. Orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed using R package *ropls* (v.1.36.0). The contribution of each metabolite to the classification was determined by

VIP value. Differentially enriched metabolites were identified by empirical Bayes estimation and exact tests based on the negative binomial distribution with the R package *edgeR* (v.4.2.1). Significantly altered metabolites after GOS intervention were identified based on a threshold of both $VIP > 1$ and $FDR\ P\ value < 0.05$. Spearman correlation analysis was further conducted to interrogate the association of differentially enriched metabolites with key ASVs and UA-related phenotypes. P values were adjusted by the Benjamini-Hochberg method to control for multiple comparisons.

2.10 Targeted determination of *p*-Cresol glucuronide and *N*-Isovaleroylglycine

A targeted analysis was carried out by high-performance liquid chromatography–mass spectrometry (HPLC-MS/MS) to quantify *p*-Cresol glucuronide and *N*-IVG in serum samples. Serum samples were mixed with a solution containing acetonitrile/methanol (v/v=4:1), internal standards (*p*-Cresol glucuronide- D_7 at 500 ng/mL and glutamic acid- ^{13}C at 100 ng/mL), and then vortexed for 10 min. Proteins were precipitated by centrifugation at 18,000g at 4°C for 15 min. Then, the supernatants were carefully transferred to new microtubes and filtered into bottles for LC-MS analysis using 1290 Infinity II HPLC system (Agilent Technologies) interfaced to a 6495 Triple Quadrupole MS system (Agilent Technologies) equipped with an ESI. The chemical separation was achieved on a Poroshell 120 Aq-C18 column (4.6×100mm, 2.7 μm, Agilent). The autosampler temperature was set to 4°C and the injection volume was 1 μL. A binary eluent system was used at a flow rate of 0.3 mL/min. Mobile phase A was aqueous buffer containing 2 mmol/L ammonium acetate, and mobile phase B was acetonitrile with 0.1% formic acid. The gradient was programmed as follows: 0-1 min 80%-60% B, 1-2 min 60%-40% B, 2-3 min 40%-20% B. The MS determination was carried out using an electrospray interface operated in the positive (*N*-IVG) and negative (*p*-Cresol glucuronide) mode with MRM. The mass-to-charge ratios of the precursor-to-product ion reactions monitored (m/z) were: 283.0→106.9 for *p*-Cresol glucuronide; 160.1→56.9 for *N*-IVG, respectively. The analytical response was linear in the range of 1.5-1000 ng/mL for *p*-Cresol glucuronide, and 1.5-2000 ng/mL for *N*-IVG, respectively. The MS raw data were processed by Mass Hunter software (Agilent, version B.08.00).

2.11 Cell counting kit-8 (CCK-8) assay for HK-2 cells

HK-2 cells viability was detected using cell counting kit-8 (CCK-8). HK-2 cells were seeded in 96-well plates, followed by different concentration of *N*-IVG incubation for another 24h, as previously described²⁴. Cell growth was evaluated with the cell counting kit-8 (CCK-8) detection assay (10 μL/well, APExBIO, USA), according to the manufacturer's instructions.

2.12 Quantitative real-time PCR

Total mRNA was extracted from tissues or HK-2 cells using Trizol reagent (Thermo Scientific, USA), followed by the conversion into cDNA using GoScript Reverse Transcription Mix (Promega, USA) according to the manufacturer's instruction. cDNA was then subjected to real-time PCR analysis using a real-time PCR system (Applied Biosystems, QuantStudio 6 Pro, Sweden) and Universal SYBR Green Fast qPCR Mix (Vazyme Technology, China). The quantitative measurements were determined using the $2^{-\Delta\Delta CT}$ method, and

the results were normalized with β -actin as an internal control. All primer sequences used in this study were listed in **Table S1**.

2.13 Western blotting analysis

Total protein was extracted with RIPA Lysis Buffer (Beyotime Biotechnology, China). Cytoplasmic or nuclear protein were extracted with nuclear and cytoplasmic protein extraction kit (Beyotime Biotechnology, China). These proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), before being transferred onto nitrocellulose membranes (PVDF, Millipore, USA). The membranes were blocked with 5% skimmed milk for 2h at room temperature and then incubated with various primary antibodies overnight at 4°C, including β -actin (Santa Cruz; 1:10000), ABCG2 (ABclonal, 1:1000), PDZK1 (ABclonal, 1:1000), IL-1 β (Proteintech, 1:500), Histone-H3 (Proteintech, 1:5000), NF- κ B p65 (Cell Signaling Technology, 1:1000) and Phospho-NF- κ B p65 (Cell Signaling Technology, 1:1000). After washing to remove any unbound antibodies, the membranes were incubated with HRP-conjugated secondary antibodies (1:2000) at room temperature for another 2h. The specific bands were detected using an enhanced chemiluminescence (ECL, Pierce Chemical), and the quantification was performed using ImageJ software.

2.14 Immunofluorescence staining and quantification

Frozen tissue sections (5 μ m) or HK-2 cells were fixed with 4% paraformaldehyde, followed by permeabilization with Triton X-100 for 8 min. After blocking non-specific binding sites with goat serum for 10 min at room temperature, sections or cells were incubated with various primary antibodies, including ABCG2 (ABclonal, 1:100), PDZK1 (ABclonal, 1:250) and phospho-NF- κ B p65 (Cell Signaling Technology, 1:1000), overnight at 4°C to facilitate antibody-antigen interactions. Subsequently, the sections and cells were incubated with corresponding secondary antibodies for additional 2h at room temperature in dark. Membrane was stained with wheat germ agglutinin (WGA, Servicebio, 1:200) and nucleus were stained with DAPI (Beyotime Biotechnology, 1:500). Images were acquired using Zeiss LSM-900 confocal microscope, and the data were analyzed using ZEN microscope software (Carl Zeiss AG, Germany) and ImageJ software.

2.15 Immunohistochemistry staining and quantification

Kidney was harvested, fixed with 4% paraformaldehyde and embedded in paraffin, and 5 μ m sections were prepared. After endogenous peroxidase activity was inhibited by 3% hydrogen peroxide for 15 min, sections were incubated overnight with anti-IL-1 β (Proteintech, 1:50), followed by staining with horseradish peroxidase-conjugated secondary antibody (Servicebio, 1:100). The target proteins were visualized with 3,3'-diaminobenzidine in the presence of hydrogen peroxide, and sections were counterstained with Harris hematoxylin (Servicebio, China). The slides were scanned with TissueFAXS Plus ST (TissueGnostics, Austria), and the intensity of positive staining was quantified using ImageJ software.

2.16 In vitro experiments in HK-2 cells

To induce inflammation, HK-2 cells were challenged with UA 500 μ g/mL for 48h²⁵, before subjected to N-IVG incubation at indicated concentrations or PBS control for another 24h. To overexpress NF- κ B, HK-2

cells were treated with Phorbol 12-myristate 13-acetate (MedChemExpress, USA) at 10 ng/mL for 6h as described²⁶, before subjected to *N*-IVG incubation at indicated concentrations or PBS control for another 24h. To knockdown ABCG2 in HK-2 cells, siRNA was designed and synthesized by RiboBio (Guangzhou RiboBio Co., Ltd., China). HK-2 cells were transfected with siRNA targeting *ABCG2* using Lipofectamine 3000 for 48h, following the manufacturer's instructions. Cells were then treated with *N*-IVG at indicated doses or PBS control for an additional 24h before subjected to further analysis. The non-specific siRNA control (NC siRNA) was used as a negative control. The primer sequences of small interfering RNA were listed in **Table S2**. Subsequently, the cells were lysed and subjected to the determination of UA transport and Western blotting analysis, as described above.

2.17 Quantification and statistical analysis

Experimental data were presented as mean $\pm$ SEM, and all the analyses were performed by GraphPad Prism (V.9.0). Comparison between groups were performed by two-tailed Student's *t*-test or one-way ANOVA followed by Dunnett's multiple comparison tests, unless otherwise stated. Two-sided *P*-value < 0.05 was considered as significant.

3. Results and discussion

3.1 GOS attenuates HUA in mice through enhanced renal urate excretion

To explore the protective effect of GOS against HUA, GOS supplementation was performed in a mouse model of HUA and compared with benzbromarone, a widely used urate-lowering agent targeted in renal UA excretion (**Fig. 1A**)²⁷. Administration of GOS for 4 weeks reduced serum UA to a level comparable to normal controls. Of note, the UA-lowering effect of GOS was comparable to benzbromarone (**Fig. 1B**). Though no significant changes in hepatic enzymes responsible for purine biosynthesis were observed (**Fig. 1C and 1D**), UA clearance was enhanced by 40% and 30% in mice treated with GOS and benzbromarone (**Fig. 1E**), suggesting that GOS lowered UA via enhanced excretion. Given that kidney and gut are two major regulators of urate excretion², we further explored the potential mechanisms whereby GOS facilitated urate excretion. In line with the enhancement of renal rather than intestinal UA excretion observed with benzbromarone, administration of GOS led to around 50% and 70% increase in the fractional excretion of urate (FE_{UA}) and urinary excretion of urate during 24h (**Fig. 1F and 1G**), two major indicators of renal UA excretion^{28, 29}, whereas no obvious improvement in intestinal urate excretion was found (**Fig. 1H**). Conversely, the expression of ABCG2, a major transporter of urate secretion², was found to be 2-fold and 1.5-fold higher at both transcriptional and protein levels in the kidney, whereas no significant increase in other transporters responsible for urate reabsorption and excretion in the kidney was observed (**Fig. 1I-1K**). Given that the apical localization of ABCG2 is essential for its function³⁰, we further evaluated its localization in the proximal tubule brush border membrane. Of note, supplementation of GOS substantially enhanced the expression of PDZ domain-containing 1 (PDZK1), a scaffold protein that binds to several urate transporters and mediates their subcellular localization³¹, at both the mRNA and protein levels in the kidney (**Fig. 1L and 1M**), and

enhanced the co-localization of PDZK1 and ABCG2 complex in the renal brush border (Fig. 1N). Collectively, these findings demonstrated that GOS ameliorated HUA via enhanced kidney urate excretion.

Figure 1

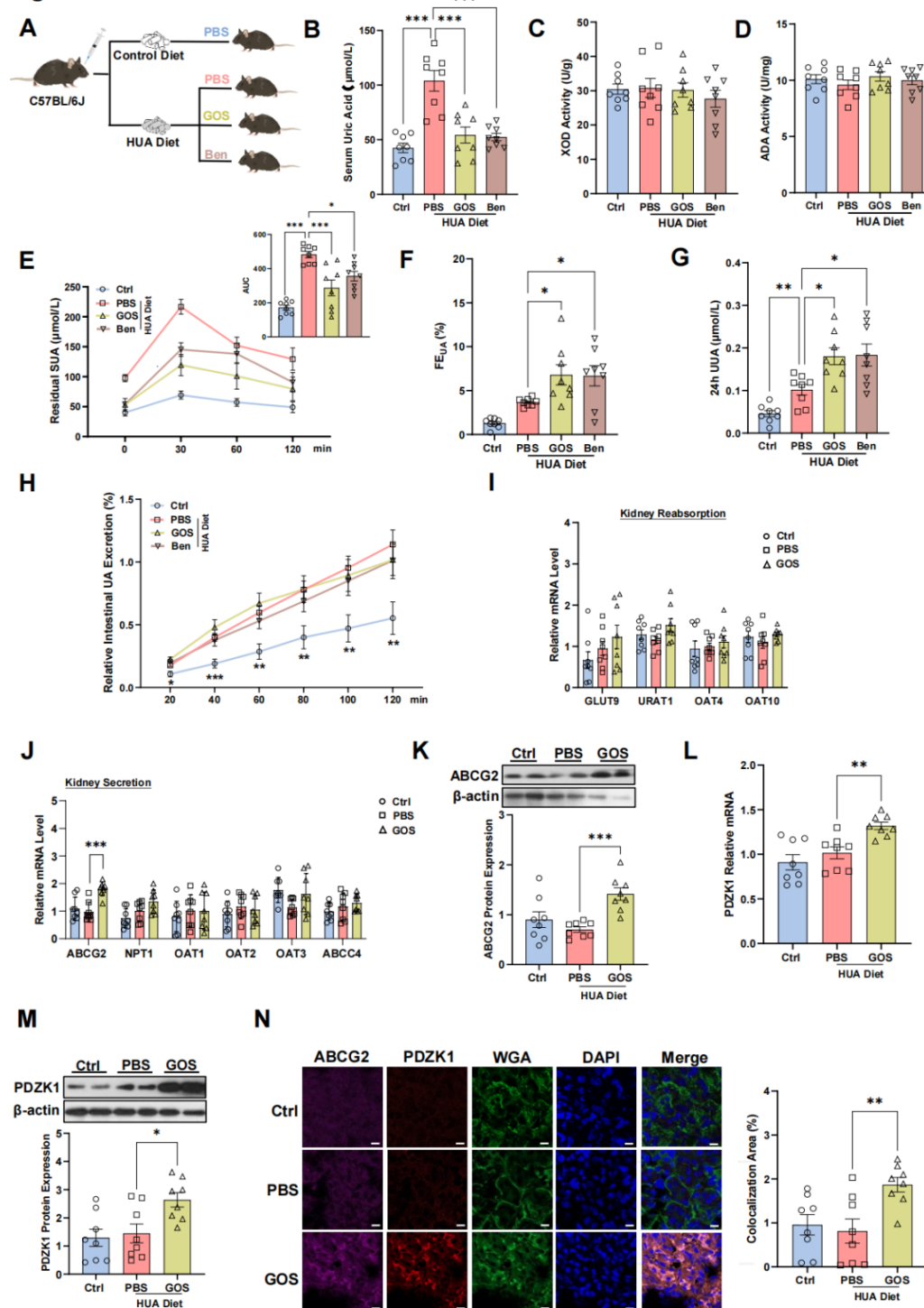


Fig. 1. GOS supplementation attenuates hyperuricemia in mice. (A) Experimental design. (B) Serum uric acid. Hepatic (C) xanthine oxidase and (D) adenosine deaminase activity. (E) Serum uric acid after oral inosine load over 2h. (F) Estimated glomerular filtration rate. (G) Urinary excretion of urate during 24h. (H) Intestinal uric acid excretion over 2h. Gene expression of (I) urate reabsorption and (J) urate secretion in the kidney. (K) Representative immunoblotting and quantification of ABCG2 in the kidney. (L) Relative mRNA expression of PDZK1 in the kidney. (M) Representative immunoblotting and quantification of PDZK1 in the kidney. (N) Co-localization of ABCG2 and PDZK1 in the proximal tubule brush border membrane (scale bar=10 μm). Data were shown as mean $\pm$ SEM ($n=8$ per group). P values were determined by one-way ANOVA followed by Dunnett's test (compared to the PBS group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. HUA, hyperuricemia; XOD, xanthine oxidase; ADA, adenosine deaminase activity; SUA, serum uric acid; ABCG2, ATP-binding cassette subfamily G member 2; PDZK1, PDZ domain-containing 1.

3.2 FMT from GOS-treated donor mice alleviates HUA in recipient mice

Given the prebiotic nature of GOS, conventional antibiotics-treated mice were transplanted with microbiota from donor mice fed with PBS control or GOS (**Fig. 2A**) to explore whether the UA-lowering effect of GOS was dependent on the gut microbiota. Mice colonized with microbiota from donors fed with GOS demonstrated a 45% reduction in serum UA, primarily through an enhanced excretion and clearance of UA with minimal effect on purine biosynthesis (**Fig. 2B-2E**). Consistent with the observation in mice supplemented with GOS (**Fig. 1F-1H**), remarkable increases in FE_{UA} and urinary excretion of urate over 24 hours were observed only in mice colonized with microbiota from donors fed with GOS, with no significant difference in intestinal urate excretion (**Fig. 2F-2H**). Moreover, transfer of the microbiota from donor mice treated with GOS led to a 2-fold and 2.3-fold increase in ABCG2 and PDZK1 at protein level, respectively in the kidney of recipient mice (**Fig. 2I-2L**). Similarly, the co-localization of PDZK1 and ABCG2 complex in renal brush border was found to be 110% higher in mice receiving microbiota from donors fed with GOS (**Fig. 2M**). Taken together, the above findings suggested that the ameliorative effect of GOS against HUA was at least partially attributable to the alterations of gut microbiota.

Figure 2

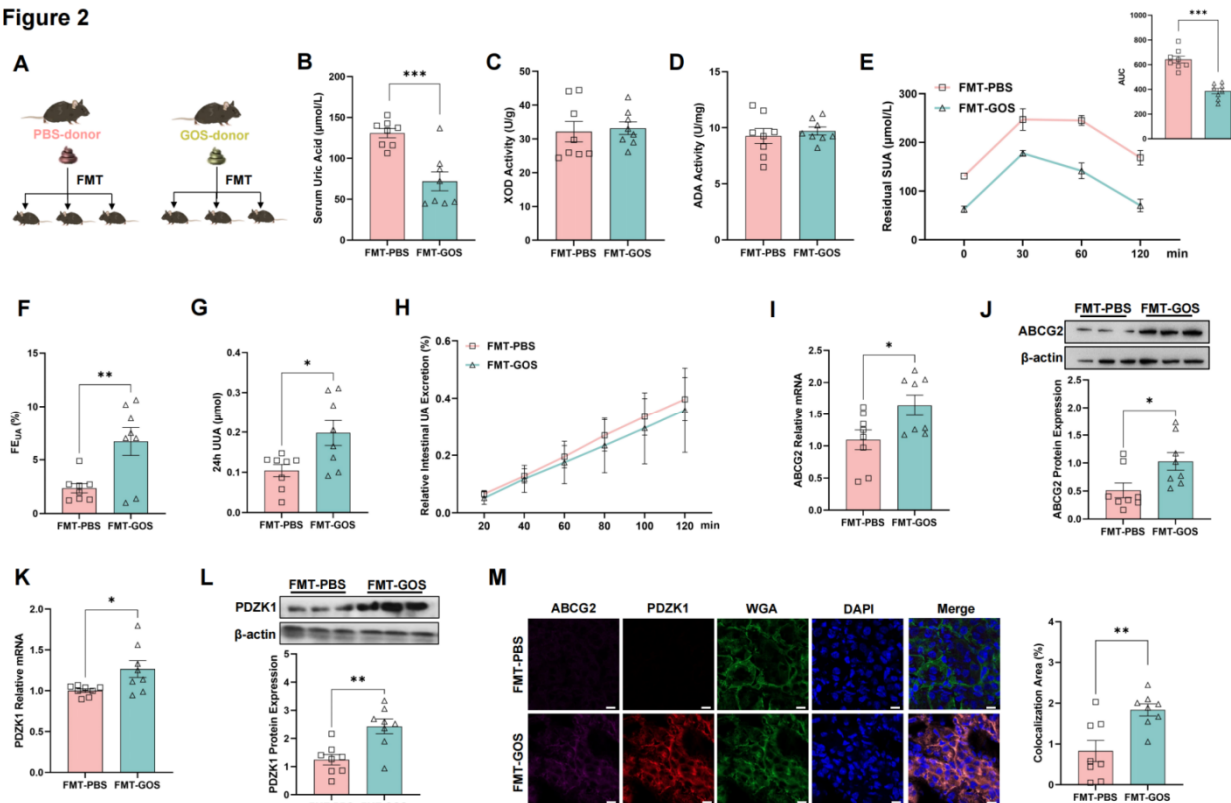


Fig. 2. Transfer of GOS-shaped microbiota alleviates hyperuricemia in recipient mice. (A) Experimental design. (B) Serum uric acid. Hepatic (C) xanthine oxidase and (D) adenosine deaminase activity. (E) Serum uric acid after oral inosine load over 2h. (F) Estimated glomerular filtration rate. (G) urinary excretion of urate during 24h. (H) Intestinal uric acid excretion over 2h. Expression of ABCG2 at (I) mRNA and (J) protein level in the kidney. Expression of PDZK1 at (K) mRNA and (L) protein level in the kidney. (M) Co-localization of PDZK1 and ABCG2 in the proximal tubule brush border membrane (scale bar=10 μm). Data were shown as mean ± SEM (n=8 per group). *P* values were determined by Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. FMT, fecal microbiota transplantation; HUA, hyperuricemia; XOD, xanthine oxidase; ADA, adenosine deaminase activity; SUA, serum uric acid; ABCG2, ATP-binding cassette subfamily G member 2; PDZK1, PDZ domain-containing 1.

3.3 GOS supplementation augments microbial production of *N*-Isovaleroylglycine

To further delineate how microbial metabolism mediates the UA-lowering effect of GOS, an integrative analysis of 16S rRNA sequencing and serum metabolomics was employed in HUA mouse model gavaged with PBS or GOS. Though there was no significant difference in α -diversity (**Fig. 3A and 3B**), principal coordinates analysis based on amplicon sequence variants (ASVs) showed a significant separation of microbial composition in mice fed with PBS or GOS ($P=0.001$, **Fig. 3C**). Specifically, several ASVs, mainly from the *Bacteroidia*, *Bacilli* and *Gammaproteobacteria* class, were found to be differentially enriched in mice fed with PBS or GOS (FDR $P < 0.05$, **Table S3, and Fig. 3D**). Notably, ASV32, ASV54, ASV12 and ASV84, all of which belonging to the *Bacteroidia* class, were found to be remarkably higher in mice fed with GOS compared to those treated with PBS (**Fig. 3D**), and were negatively associated with serum UA (**Fig. 3E**). More importantly, all these four ASVs demonstrated a strong positive correlation with indices associated with renal urate excretion, including FE_{UA} (%), and 24h UUA and the expression of ABCG2 in the kidney (**Fig. 3E**), further suggesting that increase in *Bacteroidia* class was related to the UA-lowering effect of GOS. Functional inference further demonstrated that metabolic modules responsible for amino acid metabolism, including aspartate degradation (MF0011), methionine degradation (MF0023), phenylalanine degradation (MF0007), glutamine degradation (MF0034) and glycine degradation (MF0016), were significantly enriched in the microbiota of mice fed with GOS (**Table S4 and Fig. 3F**). Collectively, the above findings indicated that increased abundance of *Bacteroidia* and altered amino acid metabolism might be involved in the beneficial effect of GOS on renal UA excretion.

Given that small molecules always function as transducers mediating the metabolic effect of gut microbiota³²⁻³⁴, we next investigated how serum metabolites linked GOS-shaped microbiota to attenuated HUA. A clear segregation of metabolic profiles was found in mice gavaged with PBS or GOS (**Fig. 3G**), with 11 metabolites substantially increased while 4 metabolites decreased in mice administered with GOS (variable importance in the projection [VIP] > 1 , FDR $P < 0.05$, **Fig. 3H**). Among them, only three metabolites, including 3-sulfocatechol, p-Cresol glucuronide and *N*-Isovaleroylglycine (*N*-IVG), demonstrated a positive association with upregulated ASVs belonging to the *Bacteroidia* class, kidney urate excretion, and renal ABCG2 expression (**Fig. 3I**), suggesting that they were potential effectors of GOS. Of note, the increases in p-Cresol glucuronide and *N*-IVG was further supported by the functional inference of altered gut microbiota shaped by GOS (**Fig. 3J**). Specifically, enhanced phenylalanine degradation in mice fed with GOS led to an increased production of L-tyrosine and p-Cresol (MF0007, **Fig. 3F**)³⁵, which in turn resulted in a marked increase in the biosynthesis of p-Cresol glucuronide with the help of UDP glucosyltransferase (**Fig. 3J**)³⁶. Moreover, higher capacity for aspartate and glutamine degradation (MF0011 and MF0034, **Fig. 3F**) in the microbiota of mice gavaged with GOS resulted in an enhanced production of L-Leucine and glycine. Consistent with previous report that *Bacteroidota* class promoted the production of isovaleric acid³⁷, isovaleric acid was further transformed into isovaleryl-CoA. Increased isovaleryl-CoA and glycine subsequently led to an enhanced biosynthesis of *N*-IVG with the help of glycine N-acyltransferase in the liver (**Fig. 3J**)³⁸. Given

the semi-quantitative nature and potential inaccuracy of untargeted metabolomics technique³⁹, targeted measurement was subsequently performed and demonstrated that only *N*-IVG, but not p-Cresol glucuronide, was found to be 1.4-fold higher in mice following GOS supplementation, compared to those treated with PBS control (**Fig. 3K**). Similarly, circulating *N*-IVG was elevated to a similar level in recipient mice compared to that in donors fed with GOS, whereas no obvious difference in p-Cresol glucuronide was found (**Fig. 3L**). Importantly, the remarkably lower levels of *N*-IVG in mice exposed to antibiotics cocktail and raised in germ-free facilities (**Fig. 3M**) further demonstrated that the production of *N*-IVG was dependent on the presence of gut microbiota. Taken together, these findings implied that increased *N*-IVG might function as the key microbial effector linking the ameliorative effect of GOS on renal urate excretion.

Figure 3

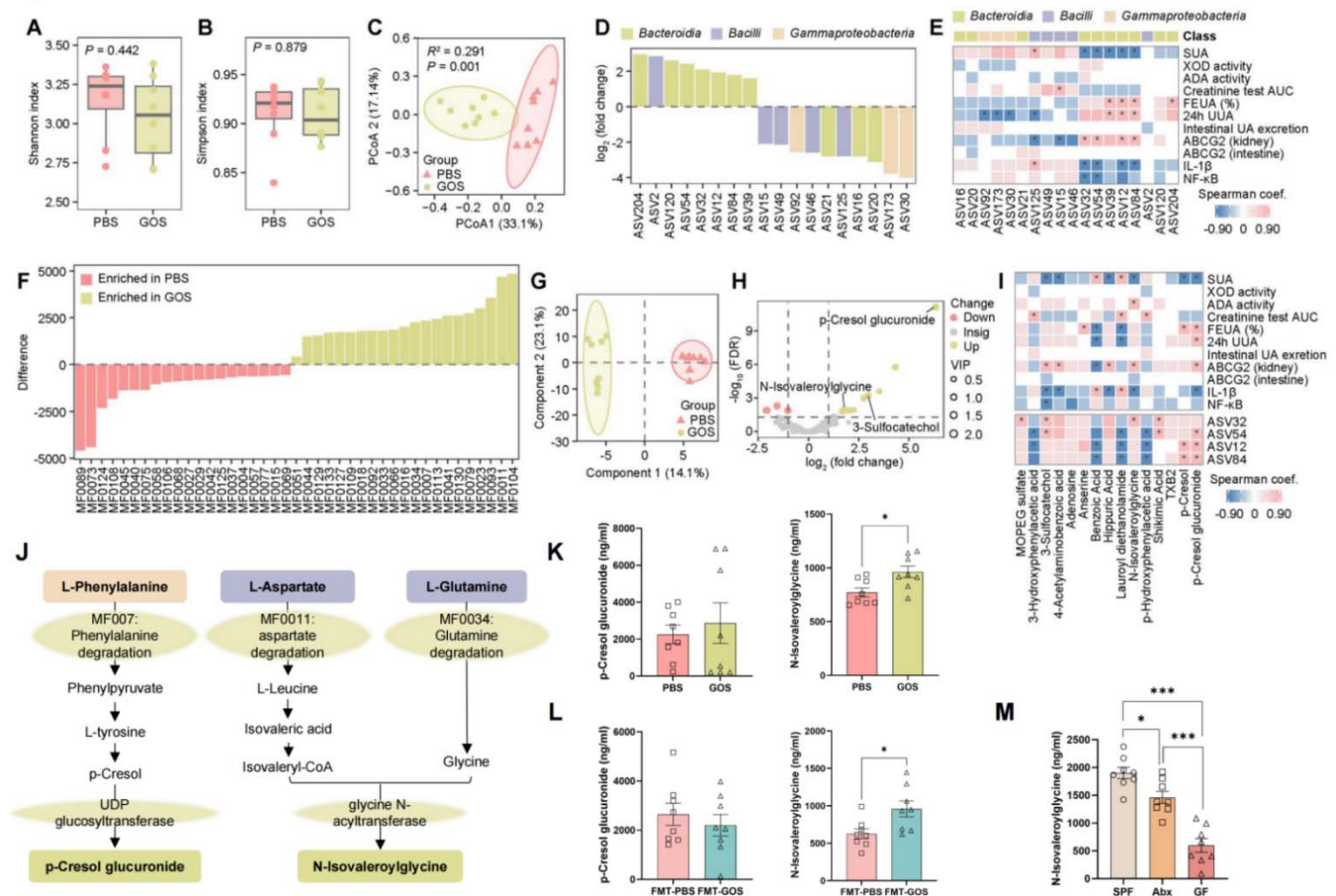


Fig. 3. GOS supplementation enhances microbial production of *N*-Isovalerylglycine. α -Diversity in terms of (A) Shannon index and (B) Simpson index, and (C) beta diversity of the gut microbiota in HUA mouse model treated with GOS or PBS. Each point represented one mouse. (D) Significantly altered ASVs. (E) Heatmap of the Spearman's correlation between significantly altered ASVs and biochemical indices. Red and blue indicated positive and negative correlations, respectively. (F) Differentially enriched metabolic modules. (G) Score scatter plot (OPLS-DA) of serum metabolites in mice gavaged with GOS or PBS. (H) Volcano plot analysis of metabolites significantly altered after GOS treatment. Blue and red indicated increased and decreased metabolites, respectively. (I) Heatmap of the Spearman's correlations between significantly altered metabolites and biochemical indices or ASVs. Red and blue indicated positive and negative correlations, respectively. All *P* values were adjusted for multiple comparisons with BH method. Data were shown as mean $\pm$ SEM (*n*=8 for each group). (J) Schematic summary showing the microbial production of p-Cresol glucuronide and *N*-IVG. Serum levels of p-Cresol glucuronide and *N*-IVG in mice treated with (K) PBS or GOS, or (L) mice receiving fecal microbial transplantation from donors fed with PBS or GOS. *P* values were determined by Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (M) Serum level of *N*-IVG in SPF mice, mice receiving antibiotics cocktail or germ-free mice. *P* value was determined by one-way ANOVA followed by Tukey's test. ASV, amplicon sequence variant; OPLS-DA, orthogonal partial least squares-discriminant analysis; FMT, fecal microbiota transplantation.

3.4 *N*-Isovaleroylglycine facilitates renal UA excretion via the activation of ABCG2

We then interrogated whether supplementation of *N*-IVG served as a UA-lowering strategy in a ABCG2-dependent manner. Exposure to *N*-IVG led to minimal effect on the variability of HK-2 cells (**Fig. S1A**), suggesting that *N*-IVG was safe. When HK-2 cells were further exposed to *N*-IVG mimicking the median level detected in mice fed with GOS, a remarkable increase in ABCG2 and PDZK1 expression and brush border co-localization of PDZK1 and ABCG2 complex (**Fig. S1B-S1E**). However, the increased expression and co-localization of PDZK1 and ABCG2 upon *N*-IVG stimulation was substantially attenuated in HK-2 cells exposed to small interfering RNA (siRNA) targeting ABCG2 (**Fig. S2**). To further determine the role of ABCG2 in *N*-IVG-induced urate excretion, targeted ABCG2 knockdown in the kidney was performed (**Fig. 4A**). Compared to PBS control, daily administration of *N*-IVG increased the level of *N*-IVG by 2-fold (**Fig. 4B**), reaching a level comparable to those observed in mice treated with GOS (**Fig. 3K**). Notably, with an approximately 50% reduction in the expression of ABCG2 (**Fig. 4C and 4D**), the UA-lowering effect of *N*-IVG was almost completely abolished (**Fig. 4E**). Similar to the observation in mice fed GOS, *N*-IVG supplementation exerted no obvious effect on purine biosynthesis (**Fig. 4F and 4G**), but led to nearly 40% decrease in cumulative UA following an inosine challenge (**Fig. 4H**). Further analysis demonstrated that such a reduction in serum UA was primarily driven by an enhanced renal urate excretion (**Fig. 4I and 4J**), while minimal effect on intestinal excretion was observed (**Fig. 4K**). Of note, the enhanced kidney urate excretion and UA-lowering effect induced by *N*-IVG were completely eliminated in mice receiving targeted knock down of ABCG2 in the kidney (**Fig. 4E-J**). Taken together, the above findings supported the biological relevance of renal ABCG2 in the regulation of *N*-IVG-mediated UA clearance.

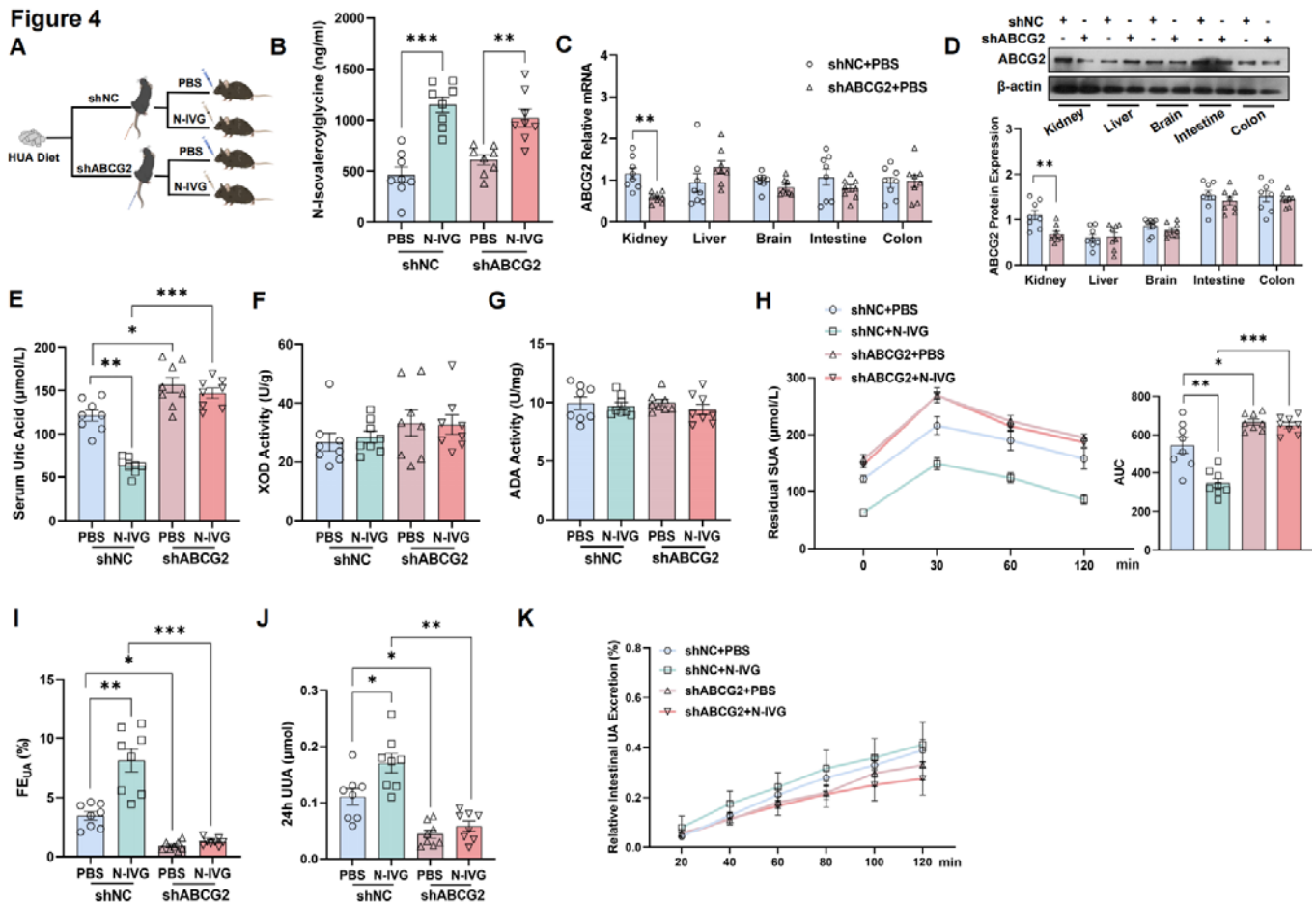
Figure 4

Fig. 4. Renal inhibition of ABCG2 abolishes the uric acid-lowering effect of N-Isovaleroylglycine. (A) Experimental design. (B) Serum N-Isovaleroylglycine. (C) Relative mRNA levels of ABCG2 in different tissues. (D) Representative immunoblotting and quantification of ABCG2 in the kidney. (E) Serum uric acid. Hepatic, (F) xanthine oxidase and (G) adenosine deaminase activity. (H) Serum uric acid after oral inosine load over 2h. (I) Estimated glomerular filtration rate. (J) urinary excretion of urate during 24h. (K) Intestinal uric acid excretion over 2h. Data were shown as mean $\pm$ SEM ($n=8$ for each group). P values were determined by two-way ANOVA followed by Tukey's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. HUA, hyperuricemia; N-IVG, N-Isovaleroylglycine; XOD, xanthine oxidase; ADA, adenosine deaminase activity; SUA, serum uric acid; ABCG2, ATP-binding cassette subfamily G member 2; PDZK1, PDZ domain-containing 1.

3.5 N-Isovaleroylglycine restores ABCG2 activity via the inhibition of NF- κ B

Given that the expression and function of ABCG2 is tightly regulated by inflammatory response⁴⁰⁻⁴², we then interrogated whether the beneficial effects of GOS and N-IVG on HUA were mediated by inflammasome activation. Remarkably, a 50% reduction in interleukin-1 β , a master regulator of proinflammation⁴³, was found in mice fed with GOS (**Fig. 5A-5C**). Consistently, a reduced accumulation of phospho-Nuclear Factor- κ B (NF- κ B) p65, an activated form of NF- κ B, in the nucleus was found in mice administered with GOS (**Fig. 5D-5E**). Similarly, interleukin-1 β expression was found to be 40% lower in mice gavaged with N-IVG compared to PBS control (**Fig. 5F-5H**), accompanied by a 45% reduction in the activation and nuclear translocation of NF- κ B in the kidney (**Fig. 5I-5J**). Consistent with previous report that chronic extracellular accumulation of UA boosted inflammation⁴⁴, UA exposure led to a remarkable increase in interleukin-1 β expression and NF- κ B activation (**Fig. S3**). Notably, such an increase in inflammasome activation was successfully reversed by N-IVG incubation (**Fig. S3**). More importantly, when the activity of NF- κ B was boosted by phorbol 12-myristate 13-acetate, a specific agonist of NF- κ B, the enhanced transcription and expression of ABCG2 in

HK-2 cells induced by *N*-IVG incubation was substantially attenuated (Fig. S4A-4D). Consistently, the co-localization of ABCG2 and PDZK1 in the brush border induced by *N*-IVG was largely abolished by a forced activation of NF- κ B (Fig. S4E). Collectively, these findings indicated that NF- κ B-ABCG2 axis was required for GOS and *N*-IVG-induced kidney UA excretion.

Figure 5

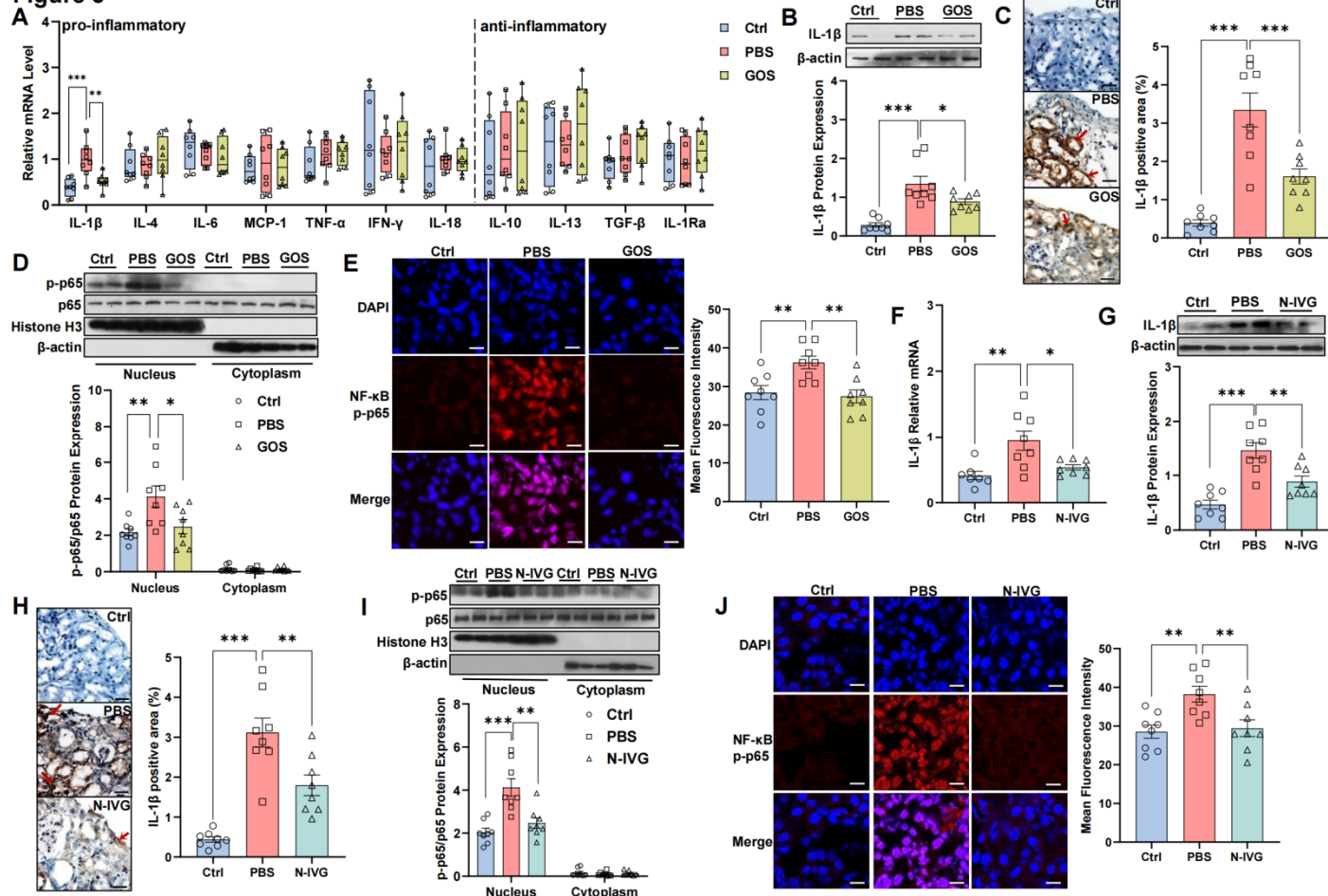


Fig. 5. *N*-Isovalerylglutamine promotes renal urate excretion via the inhibition of NF- κ B signaling pathway. Mice were gavaged with (A-E) GOS or (F-J) *N*-IVG for 4 weeks before analysis. (A) Relative mRNA expression of pro-inflammatory and anti-inflammatory markers in the kidney. (B, G) Representative immunoblotting and quantification of IL-1 β in the kidney. (C, H) Proximal convoluted tubule-localization of IL-1 β in the kidney (scale bar=20 μ m). (D, I) Representative immunoblotting and quantification of phosphorylated NF- κ B p65 in the nucleus. (E, J) Nucleus-localization of phosphorylated NF- κ B p65 in the kidney (scale bar=50 μ m). Data were shown as mean $\pm$ SEM ($n=8$ for each group). P values were determined by one-way ANOVA followed by Dunnett's test (compared to the PBS group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IL-1 β , interleukin-1 beta; *N*-IVG, *N*-Isovalerylglutamine; NF- κ B, nuclear factor kappa-B; IL-4, interleukin-4; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein-1; TNF- α , tumor necrosis factor- α ; IFN- γ , Interferon- γ ; IL-18, interleukin-18; IL-10, interleukin-10; IL-13, interleukin-13; TGF- β , transforming growth factor- β ; IL-1Ra, interleukin-1 receptor antagonist.

4. Discussion

Here, we provided evidence in animal models that supplementation of GOS enhanced renal urate excretion and attenuated the progression of HUA through an increased microbial production of *N*-IVG. Mechanistically, increased *N*-IVG, on the one hand, upregulated *ABCG2* transcription via the inhibition of NF- κ B nucleus translocation and activation, and on the other hand, facilitated its trafficking to the proximal tubule brush border membrane through binding to PDZK1. Collectively, GOS supplementation serves as an alternative dietary strategy for the management of patients with HUA.

HUA is a rising metabolic disorder in contemporary societies caused by abnormal UA metabolism, a complex physiological process involving multiple organ¹. Recently, the impact of gut microbiota, a major “metabolic organ”, on the regulation of UA hemostasis has gained increasing interests. When UA was secreted into the intestine, it could be rapidly metabolized by purine-degrading bacteria, including *Bacillota*, *Fusobacteriota* and *Pseudomonadota*⁴⁵. Inversely, an increase in UA would affect the intestinal environment, thus leading to an altered composition of gut microbiota in individuals with HUA, such as a rise in *Bacteroides caccae* and *Bacteroides xylanisolvens* and a depletion in *Faecalibacterium prausnitzii* and *Bifidobacterium pseudocatenulatum*⁴⁶. Moreover, when the fecal slurry of HUA mice were transplanted into normal mice, the latter showed a notable increase in serum UA⁴⁷, further suggesting that gut microbiota is causally involved in the pathogenesis of HUA. Therefore, targeting gut microbiota to alleviate HUA and its related comorbidities has attracted an increasing interest in recent years. In this regard, we reported for the first time that GOS, a well-known prebiotic, commonly found in dairy products, beans, and certain root vegetables^{48,49}, was effective in modulating the excretion and clearance of UA in murine models with HUA. Of note, an enrichment of ASVs belonging to the *Bacteroidia* class, previously found to be closely associated with favorable cardiometabolic traits⁵⁰, and a correction of dysregulated aromatic and branched-chain amino acids metabolism commonly seen in the microbiome of patients with HUA^{51,52} had been found in mice fed with GOS for only four weeks. Importantly, given its high solubility and stability, clean taste, and remarkable prebiotic functions, as well as excellent physicochemical characteristics which contribute to the improvement of appearance, flavor, texture and shelf life of food products, GOS had been permitted for use in several food categories in many countries, such as Japan, European Union and the United States⁵³⁻⁵⁵. As a widely used prebiotic, the effective dose of GOS was low while its tolerable dose was high. For instance, a clinical trial reported that a low dosage of 1.3-2.0 g/day for three weeks was sufficient to increase the relative abundance of *Bifidobacterium* in healthy women⁵⁶. Similarly, a dosage of 2.75 g/day had been found to be effective in the alleviation of functional constipation and in reducing the symptoms of bloating and abdominal pain within only one to two weeks¹¹. Of note, acute and subacute toxicity studies in rats established a no observed adverse effect level of GOS at least 1,000 mg/kg/day⁵⁷, suggesting a favorable safety profile. In 2021, the European Food Safety Authority demonstrated that for healthy adults, a daily intake of GOS not exceeding 16.2 grams, when used as a dietary supplement, was considered safe⁵⁸. While current urate-lowering therapies remain the cornerstone of hyperuricemia management, their efficacy can be limited by factors including treatment resistance, side effects, and a predominantly single-target mechanism^{59,60}. In this context, dietary supplements like GOS, which modulate gut microbiota and exhibit multi-faceted effects on uric acid homeostasis, present a promising candidate for alternative or synergetic management strategies. By concurrently addressing uric acid production, excretion, and associated inflammation via the gut-kidney axis, such a synergistic approach holds the promise of improved efficacy and minimized side effects. More studies in both animal models and humans are warranted to evaluate the synergistic effects and optimal combinations of GOS with existing drugs for the management of hyperuricemia. Collectively, our findings shed new light into the pleiotropic metabolic

benefits of GOS, and supported its use as an effective and safe strategy to alleviate HUA by targeting gut microbiota.

Epidemiological studies demonstrated that over 90% of the individuals with HUA suffered from impaired UA excretion to varying degrees⁶¹, with kidney accounting for approximately two-thirds of total UA excretion¹⁸. Therefore, renal UA excretion has long been a pivotal therapeutic target in the development of drugs for HUA. Several pharmacological agents widely used in clinical practice reduce UA through an enhancement of renal urate excretion, such as Benzbromarone, a urate transporter 1 (*URAT1*) inhibitor, and Probenecid, a dual inhibitor of *URAT1* and organic anion transporter 1 (*OAT1*). However, both drugs need to be used with caution, due to their potential hepatotoxicity, disturbances in the gastro-intestine, and an increased risk of kidney stones⁶²⁻⁶⁴. Similarly, lesinurad, a selective inhibitor of *URAT1* recently approved by the US Food and Drug Administration⁶⁵, is also undesirable owing to a relatively limited efficacy and potential nephrotoxicity⁶⁶. Given the rising prevalence of HUA globally⁶⁷, alternative therapeutic target with less side effects are in urgent need. In this context, we provided evidence that GOS supplementation enhanced renal urate excretion in a ABCG2-dependent manner, with minimal adverse effects on either liver or kidney function. As a high-capacity urate exporter primarily located on the apical membrane of proximal tubule in the kidneys⁶⁸, dysfunction of ABCG2 raised the risk of HUA and gout by nearly 60%^{18, 69}. Given the pivotal and complementary role of ABCG2 to classical drug target for the promotion of renal urate excretion⁷⁰, such as *URAT1* and *OAT1*, activation of ABCG2 by GOS served as a safe and effective synergetic approach to enhance the efficiency of urate excretion. Of note, in addition to GOS, several phytochemicals have also been reported to reduce UA via the activation of renal ABCG2. For instance, supplementation of punicalagin, a polyphenol found in the peel of pomegranate⁴², and α -viniferin, an oligostilbene of trimeric resveratrol⁷¹, promoted UA excretion through a dual activation of ABCG2 and *OAT1* in the kidney, accompanied by a reduction in inflammation^{42, 71}. Similarly, fisetin, a natural flavonoid found in many fruits and vegetables⁷², alleviated HUA by upregulating the expression of renal urate transporters, including *URAT1*, *OAT1* and ABCG2, and suppressing renal inflammation⁷². Moreover, *Lactobacillus plantarum*, a widely used probiotics in functional foods, was also found to enhance UA clearance from the kidney in a ABCG2-dependent manner, without any undesirable side effects in liver or kidney⁷³. Collectively, these findings suggest that the enhancement of renal ABCG2 activity represents an alternative therapeutic target with negligible side effects for the development UA-lowering agents. Moreover, given a complex interplay between inflammation and UA homeostasis, elevated UA and inflammatory responses reinforced each other, resulting in a vicious cycle⁷⁴. Consistently, a remarkable increase in IL-1 β expression and NF- κ B activity was found in HK-2 cells exposed to high levels of UA, which was readily reversed by GOS incubation in our study. Moreover, in line with previous report that chronic inflammation exacerbated HUA and gout primarily through the suppression of renal UA excretion⁷⁵, the enhanced expression and renal brush border co-localization of ABCG2 and its mediated UA transport induced by GOS was substantially attenuated when NF- κ B was forcedly activated in our study. Such an observation was further corroborated by previous report that NF- κ B downregulated the

transcription of *ABCG2* by directly binding to its promoter region at -30/-8 site⁷⁶. Moreover, punicalagin, an ellagitannin, upregulated the expression of renal *ABCG2* through the suppression of MAPK-NF- κ B axis in HUA mice⁴². Similarly, *Dendrobium officinale* six nostrum, a traditional Chinese medicine, exerted its hypouricemic effect via the augmentation of *ABCG2* in the kidney, through an LPS/TLR4/NF- κ B signaling pathway⁷⁷, lending further support to the relevance of renal NF- κ B-*ABCG2* axis in the management of HUA. Taken together, NF- κ B-*ABCG2* axis in the kidney functioned as a common and safe pathway whereby dietary interventions, prebiotics, and pharmacological treatments alleviate HUA.

As a derivative synthesized by the conjugation of glycine and isovaleric acid with glycine N-acyltransferase⁷⁸, *N*-IVG was previously known as a biomarker of kidney function with controversial conclusions⁷⁹⁻⁸¹. For example, reduced renal clearance of *N*-IVG was reported to be associated all-cause mortality in a cohort conducted in patients with chronic kidney disease⁸⁰, whereas, no clinically or statistically significant associations were identified between the clearance of *N*-IVG and incident cardiovascular events⁷⁹. Importantly, all these findings were based on purely mathematical associations and the accuracy of metabolite identification and absolute quantification of *N*-IVG determined by untargeted metabolomics warranted further investigation. In this connection, through an integration of multi-omics analysis and targeted measurements in germ-free mice, we expanded existing knowledge by demonstrating that *N*-IVG was a gut microbiota-dependent metabolite. GOS intervention in murine models led to approximately 1.5-fold increase in *N*-IVG, reaching around 1000 ng/mL in the circulation, whereas, its level was decreased by 300% in germ-free mice. Of note, the biosynthesis of *N*-IVG relied on a co-metabolism of gut microbiota and the host, whereby gut microbiota provided the substrate while the host enzyme executed the final conjugation. As was proposed by our integrative analysis, supplementation of GOS substantially enhanced the degradation of aspartate and glutamine in the gut microbiota, resulting in an increased production of isovaleryl-CoA and glycine. After entering the liver through portal vein, microbiota-derived isovaleryl-CoA and glycine were further transformed into *N*-IVG with the help of mitochondrial enzyme glycine N-acyltransferase (**Fig. 3J**). Furthermore, both *in vivo* and *in vitro* analyses demonstrated that increased *N*-IVG enhanced the transcription of *ABCG2* and facilitated its brush border co-localization primarily through an inhibition of the nucleus translocation and activation of NF- κ B, whereas, the beneficial effect on renal urate excretion was largely abolished when *ABCG2* was silenced. Although increasing number of studies are reporting the modulation of human physiology by the gut microbiota via small molecules, only a fraction of them have been experimentally demonstrated to date⁸², which substantially hinders the development of therapies targeting gut microbiota. Therefore, the identification of *N*-IVG as a microbial product and NF- κ B-*ABCG2* as its downstream target advances our knowledge of bacterial physiology and the underlying molecular mechanisms whereby gut microbiota maintain metabolic health.

5. Conclusions

In conclusion, our findings uncover increased microbial production of *N*-IVG as the main effector of GOS to lower serum UA via rescuing the abundance and function of *ABCG2*, which in turn facilitates renal

urate excretion. From a clinical perspective, our study proposes an innovative and safe strategy for the management of HUA by targeted modulation of microbial metabolism with GOS.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Sequencing data for all samples was deposited at the NCBI SRA with accession PRJNA1214586. Other data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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