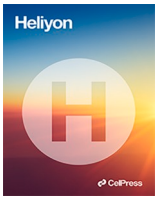
Heliyon 10 (2024) e40597

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Mechanism of intestinal flora affecting SLC2A9 transport function to promote the formation of hyperuricemia

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ARTICLE INFO

Keywords:
Obesity
Hyperuricemia
Intestinal flora
SLC2A9
XOD

ABSTRACT

Objective: To investigate the structural characteristics of the intestinal flora in obese-hyperuricemic (HUA-W) patients and the mechanisms by which they promote the formation of hyperuricemia.

Methods: 120 human fecal samples (60 cases in HC, 30 cases in HUA-N, and 30 cases in HUA-W) and 40 cases in the colonic tissues (20 cases in HC, 10 cases in HUA-N, and 10 cases in HUA-W) were collected. The intestinal flora of faeces was detected by 16s rRNA method; and the expression of SLC2A9 on human colon tissues was detected by RT-qPCR method and immunofluorescence method. 40 obese-hyperuricemia rat models were established (10 rats in Model, 10 rats in HC-FT, 10 rats in HUA-N-FT, and 10 rats in HUA-W-FT), and 10 rats were set up in Control; and the level of uric acid in rat serum, the levels of xanthine oxidase (XOD) activity and uric acid in intestinal fluid were examined. SLC2A9^{+} Caco-2 cells were produced to simulate the Transwell uric acid transport model, and the Caco-2 cells and SLC2A9^{+} Caco-2 cells were grown in five different culture media (Blank, Germ-free, HC-germ, HUA-N-germ and HUA-W-germ), and the uric acid levels in the upper and lower layers of the chambers were detected.

Results: The HUA-W intestinal flora showed significant specificity, with a decrease in Bacteroidota and Bacteroidia and an increase in Escherichia and Ruminococcus. There were no significant differences in the fluorescence intensity of the SLC2A9 protein and the SLC2A9 mRNA levels in the colon tissues of the HUA-N and HUA-W ($P = 0.447$, $P = 0.152$, $P = 0.4799$ and $P = 0.965$, respectively). In rat animal experiments, uric acid levels were significantly higher ($P < 0.05$) and XOD activity was significantly higher ($P < 0.05$) in intestinal fluid of HUA-W-FT. In Transwell experiments with SLC2A9^{+} Caco-2 cells, uric acid levels were increased in the upper compartment and decreased in the lower compartment of HUA-W-germ.

Conclusion: HUA-W intestinal flora may increase XOD activity in the intestinal tract and improve the ability of uric acid transporter protein SLC2A9 to reabsorb uric acid, providing a new theoretical basis for the pathogenesis of hyperuricemia.

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https://doi.org/10.1016/j.heliyon.2024.e40597
Received 1 September 2024; Received in revised form 19 November 2024; Accepted 20 November 2024
Available online 29 November 2024

1. Introduction

Hyperuricaemia is a syndrome of metabolic abnormalities characterised by increased uric acid levels in serum caused by disturbances in purine metabolism. A serum uric acid level of more than 420 μmol/L on two occasions not on the same day is called hyperuricemia [1]. In recent years, the prevalence of hyperuricaemia has increased significantly and is becoming younger. The overall prevalence of hyperuricaemia in China is 14 %, and it has become another common metabolic disease after diabetes [2].

Hyperuricaemia can be divided into excessive uric acid production and insufficient uric acid excretion, and about 90 % of hyperuricaemia is caused by insufficient uric acid excretion. Uric acid is mainly excreted through the kidneys and intestines, with the intestines becoming the main route of excretion in renal insufficiency [3]. The function of ABCG2 transporter protein in intestinal mucosal epithelial cells to secrete uric acid and SLC2A9 transporter protein to reabsorb uric acid regulates peripheral serum uric acid levels in humans [4,5].

Obesity is a health condition caused by excessive accumulation of body fat and is usually defined by calculating body mass index (BMI). According to the World Health Organisation (WHO) criteria, a BMI of 28 and above is considered obese [6,7]. Clinical studies have shown that there are significantly more obese than normal weight individuals with hyperuricaemia and that serum uric acid levels are positively correlated with BMI, triglyceride levels and total cholesterol levels [8].

Intestinal flora is an important metabolic "organ" in the human body as it contains different enzymes and can participate in the energy metabolism of the organism. It is an important metabolic "organ" of the human body. Intestine flora can influence the development of diseases such as gout, obesity, type II diabetes, and metabolic syndrome [9]. It has been found that there are structural changes in the intestinal flora in high-fat-fed rat models, and Berberine, a traditional Chinese medicine extract, can alter the functional structure of the intestinal flora and play a role in the regulation of lipid metabolism [10]. Iram Liaqat et al. [11] raised mice with obese people faeces along with high fat diet or normal diet for 3 months and found that five abundant germ were detected in the faecal intestinal flora of mice with obese people faeces along with high fat diet or normal diet as compared to mice with high fat diet alone or normal diet, namely *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, Morganella spp. and Klebsiella acidophilus. It has also been documented that XOD-producing germ, such as Streptomyces and Pseudomonas, can break down purine molecules such as ATP to produce uric acid [12,13].

Therefore, the present study intends to start from the interrelationship between intestinal flora and colonocytes in order to reveal the structural characteristics of intestinal flora and its mechanism of promoting the formation of hyperuricemia in patients with obesity-hyperuricemia, and seeks to provide new evidences and ideas for the study of the pathogenesis of hyperuricemia.

2. Materials and methods

2.1. Clinical case collection

According to the diagnostic criteria of hyperuricemia (two non-same-day serum uric acid values $\geq$ 480 μmol/L) and normal uric acid (two non-same-day 300 μmol/L $\leq$ serum uric acid values $\leq$ 420 μmol/L), as well as the diagnostic criteria of obesity (BMI $\geq$28) and normal body weight (18.5 $\leq$ BMI $<$ 25), Chinese Han males aged between 18 and 70 years old were collected, divided into three groups: normal uric acid group (HC), normal weight-hyperuricaemia group (HUA-N) and obese-hyperuricaemia group (HUA-W). The samples were collected from September 2023 to February 2024 in outpatient clinics, medical examination centres and inpatient volunteers of Ningbo No.2 Hospital. All study subjects were unrelated, and the trial was approved by the Ethics Committee of Ningbo No. 2 Hospital in accordance with the ethical guidelines of the Declaration of Helsinki, with all volunteers signing an informed consent form. Volunteers with other autoimmune diseases such as rheumatoid arthritis, cancer, and blood diseases were excluded. Recorded data included volunteers' age, height, weight, and peripheral serum uric acid and lipid (cholesterol, high-density lipoprotein, low-density lipoprotein, and triglyceride) levels.

2.2. Animal experiment

A total of 50 4-week-old male SD rats weighing about 200–220 g were purchased from Shanghai Slaughter Laboratory Animal Co. The animals were housed in the SPF barrier environment of the Animal Experimentation Centre of Zhejiang University of Traditional Chinese Medicine (room temperature 25 °C, relative humidity 40 %–60 %, continuous illumination for 12 h per day, ordinary feed, free water and food). All animal experiments were conducted in strict accordance with the guidelines set by the Animal Ethics Committee of Zhejiang University of Traditional Chinese Medicine.

2.3. Cellular experiment

The Caco-2 cell model is a human clonal colon adenocarcinoma cell that is structurally and functionally similar to differentiated small intestinal epithelial cells and contains enzyme lineages associated with the brush border epithelium of the small intestine. Under cell culture conditions, cells grown on porous, permeable polycarbonate membranes can fuse and differentiate into intestinal epithelial cells, forming a continuous monolayer suitable for experiments simulating intestinal transit in vivo. The Caco-2 cell line in this experiment was purchased from the cell bank of the Chinese Academy of Sciences (catalog number: SCSP-5027).

2.4. 16s rRNA sequencing of human faecal intestinal flora

After collection, faecal samples were processed using a special collector and snap-frozen in liquid nitrogen and transferred to −80 °C for storage. DNA was extracted using a faecal flora DNA extraction kit, and the integrity, concentration and purity of DNA were examined by agarose gel electrophoresis and spectrophotometry. Next, PCR amplification of bacterial V3 and V4 regions was performed using bacterial amplification universal primers 343F and 798R, and gene sequencing was performed using an Illumina MiSeq sequencer. Bioinformatics analyses included microbial community structure, α-diversity (by Chao 1, Shannon, Simpson and Observed indices) and β-diversity (by NMDS and PCoA analyses).

2.5. Immunofluorescence and mRNA detection of SLC2A9

Immunofluorescence detection of SLC2A9 included antigen repair, membrane disruption, and closure of colon tissue sections, incubation of SLC2A9 primary antibody and fluorescent secondary antibody, followed by DAPI staining, and finally visualisation with a digital pathology slide scanner. Expression detection of SLC2A9 was performed by RNA extraction through the kit and mRNA levels were detected by RT-qPCR, with β-actin as an internal reference, and the relative expression was calculated by ΔΔCt value.

2.6. Establishment of obesity-hyperuricaemia rat model and experimental grouping

Male SD rats, 4 weeks old, were acclimatised and fed with Slaughter's high-fat diet (36 % fat) for 7 weeks, of which potassium oxonate 250 mg/kg intraperitoneally and yeast paste 30 g/kg by gavage were started at the end of the 6th week of the high-fat diet, once a day for 7 days; body weights were measured at the end of the 8th week of the high-fat diet, and fasting blood was taken for uric acid measurement to validate the model, and a body weight of more than 15 % of the normal body weight was considered obese. The body weight exceeding 15 % of the normal group body weight was regarded as obesity standard. After the model was established, 4 groups were randomly assigned: 10 animals in the model group (Model), 10 animals in the HC faecal transplantation group (HC-FT), 10 animals in the HUA-N faecal transplantation group (HUA-N-FT), 10 animals in the HUA-W faecal transplantation group (HUA-W-FT); and 10 animals in the blank control group (Control) were set up at the same time.

2.7. Faecal transplantation and intestinal fluid experiment

Ampicillin solution (0.5 g/L) was added to the drinking water every 3 days for 1 week starting at the end of the 7th week of high-fat diet feeding. Starting at week 8, HC, HUA-N or HUA-W faeces collected and cryopreserved were homogenised in sterile PBS, followed by faecal gavage once a day for 3 days to complete faecal flora transplantation. On the 4th day after faecal transplantation, rats in each group were anaesthetized, and a small midline incision was made on the scraped abdomen to expose the intestines, locate the distal part of the ileum, and empty the ileal contents. The ileum is ligated with a 0-gauge wire to form an ileal loop approximately 3 cm long, ensuring that mesenteric vessels are avoided to maintain blood supply. After loop ligation, samples of the loop fluid were collected from each group of rats at the end of week 10, and uric acid levels were measured using a uric acid test kit. In addition, the loop samples at the end of week 10 were divided into sterile and bacterial samples: sterile loop samples were filtered through a 0.45 μm centrifuge filter, centrifuged at 14,000 g for 1 min to remove bacteria and stored at - 80 °C; bacterial loop samples were centrifuged at 3,000 g for 3 min to remove contaminants and stored at - 80 °C.

2.8. Lentiviral vector construction of $SLC2A9^{+}$ Caco-2 cells and cellular intervention

Recombinant lentivirus was constructed using pHBLV-CMV-MCS-3FLAG-EF1-ZsGreen-T2A-PURO vectors and a triple plasmid system (pSPAX2, pMD2G, and a shuttle plasmid carrying the SLC2A9 gene), and then packaged and infected into Caco-2 cells to obtain Caco-2 cells that stably and highly expressed SLC2A9. Caco-2 cells stably expressing SLC2A9 were identified by RT-qPCR and Western Blot, and preserved for future use. Then, in vitro cultured Caco-2 cells or SLC2A9+ Caco-2 cells were inoculated into the upper culture layer of Transwell chambers, and 3 replicates were set in each group. After 24 h of incubation, transfer medium containing 0.5 mM uric acid was added to the lower culture medium and incubation was continued for 30 min under five different medium conditions (Blank, Germ-free, HC-germ, HUA-N-germ and HUA-W-germ). Finally, the upper and lower cultures were collected and tested for uric acid levels after decontamination using a 0.45 μm centrifugal filter.

2.9. Statistical methods

Data were analyzed using SPSS 20.0 software. Continuous variables with normal distribution were expressed as mean ± standard deviation, and *t*-test or ANOVA was used to compare the differences between groups; continuous variables with skewed distribution were expressed as quartiles, and rank-sum test was used to compare the differences between groups. $P < 0.05$ was considered as statistically significant difference.

3. Results

3.1. The structure of HUA-W intestinal flora is significantly different

There were no statistically significant differences in age, BMI, and lipid levels in HUA patients compared with HC (all $P > 0.05$). In HUA-N and HUA-W, there were significant differences in BMI, age, and HDL levels ($P < 0.05$), and no statistically significant differences in uric acid and other lipid levels (all $P > 0.05$).

Diversity analyses of the intestinal flora were performed: HC and HUA did not differ significantly in Chao 1 index ($P = 0.420$), Shannon index ($P = 0.760$), Simpson index ($P = 0.840$) and Observed index ($P = 0.420$). The results showed significant differences in the structural composition of the flora by NMDS analysis (HC and HUA: Stress = 0.153; HUA-N and HUA-W: Stress = 0.134) and PCoA analysis (HC and HUA: $P = 0.001$; HUA-N and HUA-W: $P = 0.01$) (Fig. 1A).

Analysis of differential intestinal flora in HC and HUA showed that, at the phylum level, the abundance of Campilobacterota was significantly higher in HC than in HUA; and at the genus level, the abundance of Alphaproteobacteria and Campylobacteria was significantly higher in HC than in HUA ($P < 0.001$). Differential flora analysis of HUA-N and HUA-W showed that the abundance of Bacteroidota and Bacteroidia was significantly higher in HUA-N than in HUA-W ($P < 0.001$) (Fig. 1B).

LEfSe analyses showed that HC was mainly enriched in Rhodobacterales/Rhodobacteraceae and Alphaproteobacteria; HUA was mainly enriched in Bacilli, Lactobacillales, Muribaculacea; HUA-N was mainly enriched in Bacteroidota/Bacteroidia/Bacteroidaies/Bacteroidaceae/Bacteroides and Prevotella; HUA-W was mainly enriched in Escherichia and Ruminococcus (Fig. 1C).

3.2. HUA-W intestinal flora transplantation affects uric acid and XOD levels in rats

Serum uric acid levels were significantly higher in Model compared with Control; serum uric acid levels were significantly lower in HC-FT and higher in HUA-W-FT compared with Model, and all were statistically different ($P < 0.05$); whereas serum uric acid levels were not significantly altered in HUA-N-FT compared with Model ($P > 0.05$) (Fig. 2A).

At the end of the 10th week, samples of rat intestinal fluid were taken from each group to test the uric acid level, and we found that the uric acid level of Model intestinal fluid was significantly higher than that of Control; the uric acid level in the intestinal fluid of HC-FT was significantly lower compared with that of Model, and the uric acid level in the HUA-W-FT was significantly higher ($P < 0.05$),

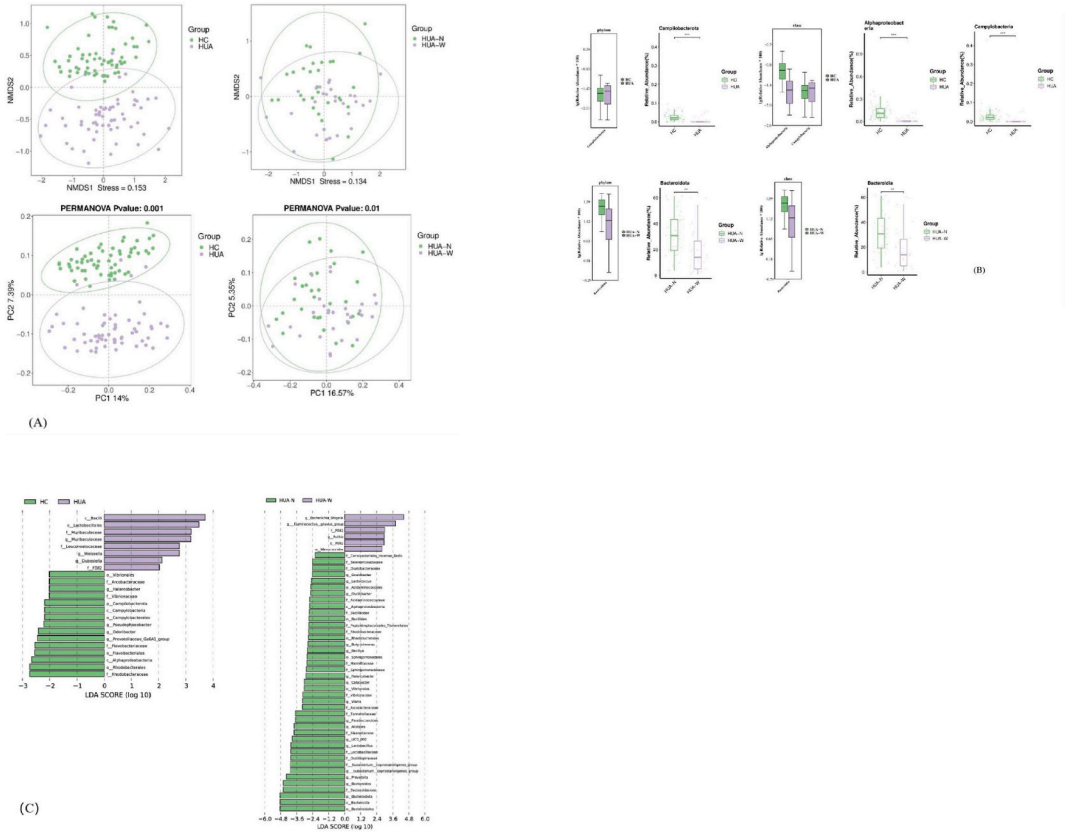

Fig. 1. Intestinal flora analysis. (A) The β-diversity analysis indicates species diversity in the intestinal flora between HC and HUA, and between HUA-N and HUA- W. (B) Analysis of species differences between groups. (C) LEfSe analysis bar chart.

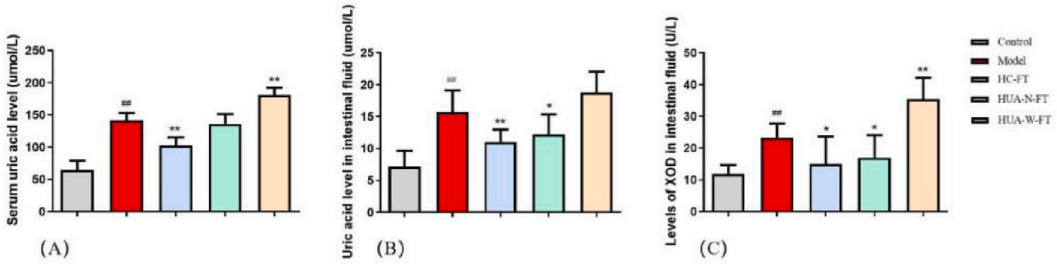

Fig. 2. The levels of uric acid and XOD in rats among groups. (A) The serum uric acid level in rats. (B) The uric acid level in intestinal fluid. (C) The levels of XOD in intestinal fluid. (Model vs Control, ##P < 0.01; FT vs Model, **P < 0.01).

whereas the uric acid level in the HUA-N-FT levels were not significantly altered ($P > 0.05$) (Fig. 2B).

At the end of the 10th week, samples of rat intestinal fluid from each group were taken to test the XOD activity, and we found that the XOD activity of Model intestinal fluid was significantly higher than that of Control; the XOD activity of rat intestinal fluid from HC-FT and HUA-N-FT was significantly lower, and that of HUA-W-FT was significantly higher compared with that of Model ($P < 0.05$) (Fig. 2C).

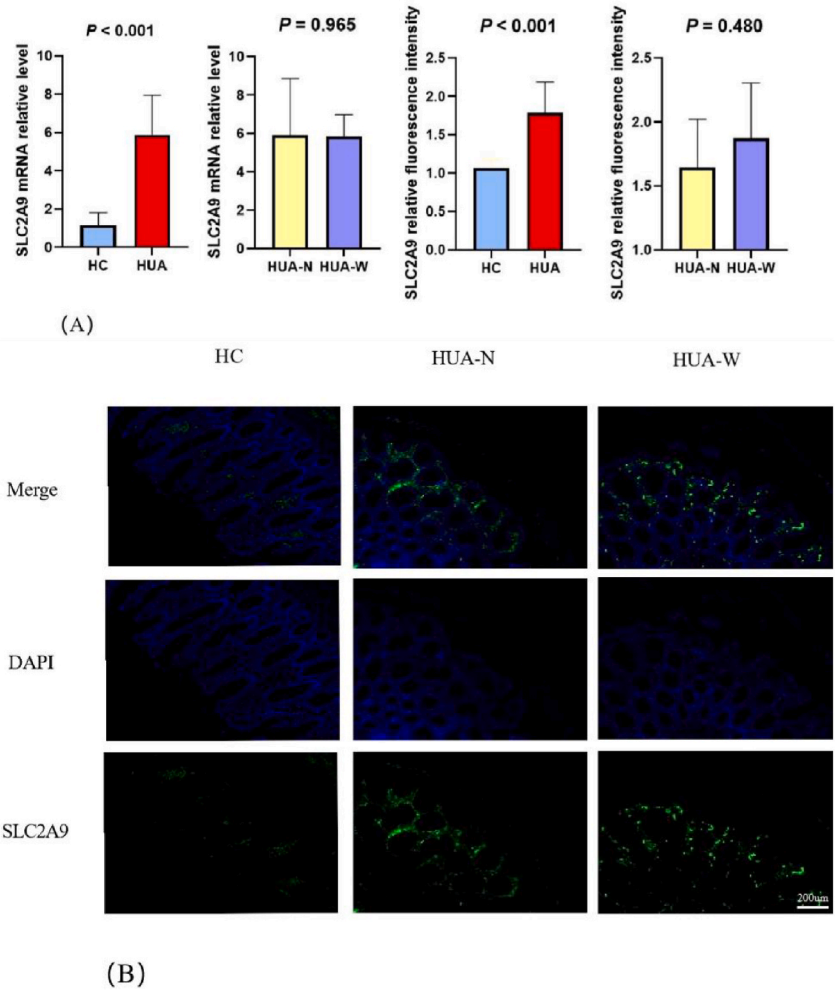

Fig. 3. SLC2A9 expression levels in colon tissues among groups. (A) SLC2A9 mRNA levels by Western Blot. (B) SLC2A9 protein levels by Immunofluorescence.

3.3. Comparison of SLC2A9 expression levels in colon tissues from different groups

SLC2A9 mRNA levels were significantly higher in HUA colon tissues than in HC ($P < 0.001$), but there was no significant difference in SLC2A9 mRNA levels between HUA-N and HUA-W colon tissues in HUA ($P = 0.965$) (Fig. 3A).

The fluorescence intensity of SLC2A9 protein was significantly higher in HUA colon tissues than in HC ($P < 0.001$), but there was also no significant difference in SLC2A9 protein fluorescence intensity between HUA-N and HUA-W colon tissues in HUA ($P = 0.480$) (Fig. 3B).

3.4. Effect of rat intestinal fluid on the reabsorption of uric acid by Caco-2 cells

In the Transwell models of Caco-2 cell and SLC2A9$^+$ Caco-2 cell, we found that, compared with Blank, Germ-free, HC-germ, and HUA-W-germ had significantly higher uric acid levels in the upper compartment and significantly lower uric acid levels in the lower compartment ($P < 0.05$), but HUA-N-germ's uric acid levels were not significantly altered in both upper and lower compartments ($P > 0.05$). In conclusion, Germ-free, HC-germ, and HUA-W-germ significantly increased uric acid levels in the upper ventricular layer and decreased uric acid levels in the lower ventricular layer in both the Caco-2 cell and the SLC2A9$^+$ Caco-2 cell models, and the effect was especially pronounced in the SLC2A9$^+$ Caco-2 cell model (Fig. 4).

4. Discussion

16S rRNA gene sequence analysis is a method commonly used to study the structure and diversity of microbial communities, especially in the study of intestinal flora, which is widely used to provide an important basis for the study of the relationship between intestinal microorganisms and host health and disease [14]. Intestine flora plays an important role in the pathogenesis of metabolic diseases such as diabetes, hypertension, heart disease and tumours [15,16]. In recent years, treatments targeting intestinal flora have also played an increasingly important role [17].

The intestinal tract contains a wide variety of microorganisms, which together with the intestinal mucosal barrier, intestinal metabolites, and the intestinal immune system form a complex ecosystem that plays an important role in the regulation of human metabolism, and is considered to be an important "organ". The enterohepatic cycle is an important physiological circulatory process that takes place in the body between the liver and the intestines. When we ingest food, the nutrients are absorbed into the intestine. Subsequently absorbed nutrients are transported to the liver through the portal venous system. Subsequently liver metabolites such as cholesterol and bile acids are secreted through bile to the intestine, where a portion of the bile acids in the bile are reabsorbed in the intestine and circulate back to the liver again [18].

Min Gong et al. [19] showed that obesity and hyperuricemia are interrelated, and Kailai Panlu et al. [20] found a causal relationship between obesity and hyperuricemia by Mendelian randomisation analysis based on the available whole genome, and Yuan Zhao et al. [21] showed that tea polyphenols regulate the intestinal flora, resulting in the significant enrichment of Bacteroidetes and Oscillospira family microorganisms and the almost disappearance of Peptostreptococcaceae family, with potential therapeutic efficacy for the treatment of obesity. Bacteroidetes and Oscillospira families of microorganisms significantly enriched and the Peptostreptococcaceae family almost disappeared, with potential therapeutic efficacy in treating obesity. Yu Lou [22] et al. used two-sample Mendelian randomisation to assess the causal relationship between intestinal microbial genera and hyperuricemia and gout and found that Escherichia was positively associated with uric acid levels. Meanwhile, Junqing Hu and Zhilu XU indicated that both Escherichia and Ruminococcus were associated with obesity, respectively [23,24]. Qiulan Lv et al. [25] also found that the levels of the key bacterium, Bacteroidota and Bacteroidia, decreased during the development of hyperuricaemia and were accompanied by an increase in Ruminococcus. Meanwhile, Crane et al. showed that XOD was released from intestinal tissues and body fluids and was active in intestinal fluids during infection with enteropathogenic Escherichia (EPEC) and Shiga toxin Escherichia (STEC) [26].

XOD is known to be involved in the process of purine metabolism in the body. It oxidises purines to hypoxanthines, which are then further oxidised to uric acid. The function of xanthine oxidase is essential for maintaining uric acid homeostasis. In addition to its role in purine metabolism, xanthine oxidase is also involved in the production of nitric oxide, which regulates vascular function and blood

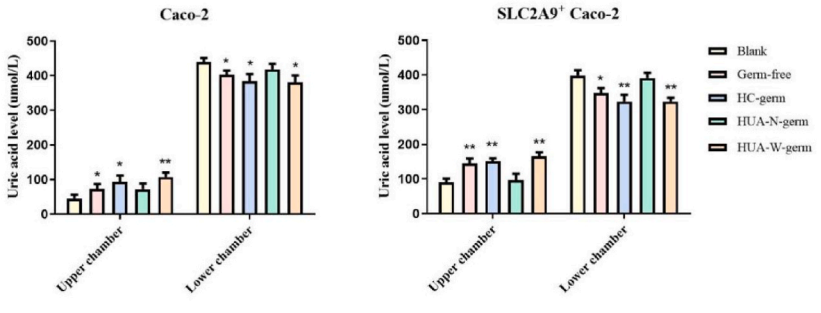

Fig. 4. The levels of uric acid in upper chamber or lower chamber among different groups (all germs vs Blank, *P < 0.05 , **P < 0.01).

pressure. XOD-producing germ, such as Escherichia, can secrete XOD, a key enzyme in uric acid metabolism; while Lactobacillus germ in the intestine can maintain XOD activity through a short-chain fatty acid-dependent mechanism [27,28].

Intestinal cells have a large number of uric acid transporter proteins that play an important role in the transport of uric acid. The uric acid transporter protein SLC2A9 is encoded by the *SLC2A9* gene, also known as GLUT9, and is voltage-dependent. It consists of a transmembrane domain with intracytoplasmic NH2 and COOH terminals and an extracellular N-glycosylation site. It is mainly found in the kidney and intestine and regulates uric acid reabsorption [29]. In the intestinal epithelium, SLC2A9 is abundantly expressed in the jejunum, ileum, and colon, and is mainly located in the apical and basolateral membranes of intestinal epithelial cells. Serum uric acid levels are elevated in intestinal cell-specific *SLC2A9* knockout mice, and mice lacking the *SLC2A9* gene are prone to metabolic syndrome (hyperuricemia, hypertension, hyperglycaemia, hyperlipidaemia), suggesting that SLC2A9 mediates the excretion of uric acid from the intestine [30].

In this experiment, the detection of human faecal intestinal flora by the 16s rRNA method revealed that in the HUA-W, Bacteroidota and Bacteroidia decreased, while Escherichia and Ruminococcus increased, which is in line with the findings of Qiulan Lv [25]. The Ruminococcus may be XOD-producing like Escherichia, resulting in increased levels of XOD in the intestine, which catabolises molecules such as ATP into uric acid, resulting in increased levels of uric acid in the intestine, while at the same time, some of the XOD can be absorbed into the bloodstream, resulting in an increase in XOD activity in the peripheral blood.

In addition, we examined the expression of SLC2A9 protein in human colon tissues; as well as fecal transplantation into rat ileum and rat intestinal loop fluid into Transwell model to explore whether fecal intestinal flora of the HUA-W increased the capacity of reabsorption of uric acid by the uric acid transporter protein SLC2A9 and whether it increased the expression of the uric acid transporter protein SLC2A9. Possibly due to insufficient molecules such as ATP being depleted in the intestine, we did not find that HUA-W intestinal flora significantly enhanced the ability of uric acid transporter protein SLC2A9 to reabsorb uric acid in Caco-2 cells in our cellular experiments (there was no statistically significant difference). However, the reabsorption was still enhanced compared to the other groups, which in turn led to higher blood levels of uric acid. However, we did not find that the intestinal flora of the HUA-W could increase the expression of SLC2A9 in the colon tissue (Fig. 5).

The present study has the following shortcomings. Firstly, no further exploratory studies on specific intestinal flora and their metabolites or uric acid metabolism in animals and cells were conducted. And secondly, XOD was not traced, so it is not directly clear which gut flora secretes and produces XOD. Even so, this study analyzed the structure of intestinal flora in different populations and rat models through a large number of samples, and used SLC2A9^{+}Caco-2 cells and the transwell model to simulate uric acid transport, which revealed the potential mechanism of the role of specific flora on uric acid metabolism. This multilevel and multimodeling study design is an important addition to existing HUA studies, especially providing a new theoretical basis for the pathological mechanisms of HUA.

5. Conclusions

In conclusion, the intestinal flora of HUA-W may increase the activity of XOD and the reabsorption of uric acid by the uric acid transporter protein SLC2A9, which may lead to an increase in the production of uric acid in the intestinal tract as well as an increase in the reabsorption of uric acid, which may contribute to the formation of hyperuricemia, and provide new theoretical bases for the pathogenesis of hyperuricemia.

CRediT authorship contribution statement

Ying Ying: Writing – original draft, Resources, Project administration, Methodology, Investigation. **Yi Zhang:** Writing – review & editing. **Jing Sun:** Software. **Yong Chen:** Supervision. **Huaxiang Wu:** Supervision.

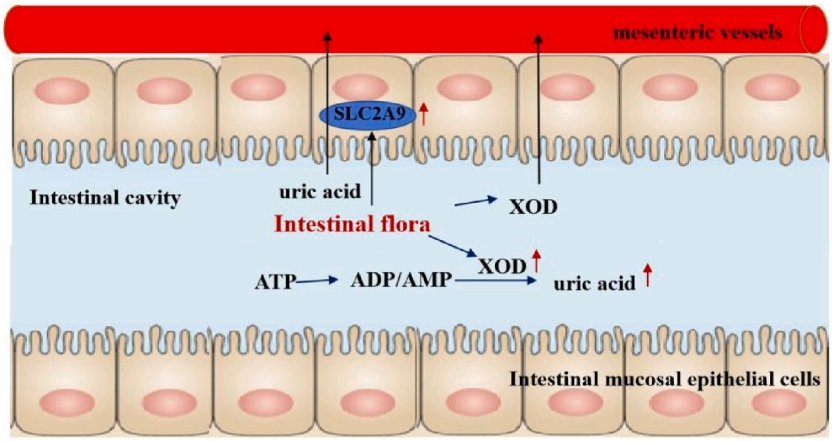

Fig. 5. Mechanism diagram of Intestinal flora affecting uric acid levels.

Ethics approval

This study was reviewed and approved by Human Ethics Committee of Ningbo No.2 Hospital with the approval number: YJ-NBEY-KY-2023-117-01, and by Animal Ethics Committee of Zhejiang University of Traditional Chinese Medicine with the approval number: 20230619-17.

Data availability statement

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

Funding

This study was funded by Medical Scientific Research Foundation of Zhejiang Province, China (Grant 2022KY328), HwaMei Research Foundation of Ningbo No.2 Hospital, China (Grant 2024HMKYA03), Key discipline Foundation of Ningbo No.2 Hospital, China (Grant No.2023-Y04) and National Natural Science Foundation of China (Grant No.82374393).

Declaration of competing interest

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.

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