Pro-Glu-Trp (PEW) and Leu-Leu-Trp (LLW) are peptides derived from whey protein digestive products; both peptides exhibit xanthine oxidase inhibitory activity in vitro. However, it remains unclear whether these peptides can alleviate hyperuricemia (HUA) in vivo. In this study, we investigated the roles of PEW and LLW, both individually and in combination, in alleviating HUA induced by potassium oxonate and hypoxanthine. Together, PEW and LLW exhibited synergistic effects in reducing the serum levels of uric acid (UA), creatinine, and blood urea nitrogen, as well as increasing the fractional excretion of UA. The combined treatment with PEW and LLW inhibited UA synthesis, promoted UA excretion, and restored renal oxidative stress and mitochondrial damage. Moreover, the combined treatment alleviated dysbiosis of the gut microbiota, characterized by increased helpful microbial abundance, decreased harmful bacterial abundance, and increased production of short-chain fatty acids. Taken together, these results indicate that the combination of PEW and LLW mitigate HUA and kidney injury by rebalancing UA synthesis and excretion, modulating gut microbiota composition, and improving oxidative stress.

Key words: peptide, hyperuricemia, uric acid synthesis, uric acid transporters, gut microbiota

INTRODUCTION

Hyperuricemia (HUA) is characterized by an abnormally elevated serum uric acid (SUA) concentration caused by a disorder in purine metabolism (Liu et al., 2014). In recent years, the number of individuals with HUA has increased worldwide owing to changes in dietary patterns and lifestyles (Benn et al., 2018). The pooled prevalence of HUA in the general population of China was as high as 17.4% (ranging from 15.5 to 24.6% by region) until 2019 (Huang et al., 2020). In contrast, the prevalence of HUA is even higher in some Western countries, threatening global public health (Liu et al., 2020). The impact of HUA is widely felt, as monosodium urate crystals form when SUA concentrations exceed their solubility (6.8 mg/dL) and these crystals precipitate in the joint tissue, causing gout (Eleftheriadis et al., 2017). Moreover, HUA is a primary risk factor in the pathogenesis of chronic diseases, such as cardiovascular disease, diabetes, and chronic nephrosis (Grassi et al., 2014; Abeles, 2015; Hu and Wu, 2019). Therefore, HUA has become an important public health issue that must be addressed.

Excessive uric acid (UA) synthesis and insufficient UA excretion can lead to the disruption of UA homeostasis in the body, thereby inducing HUA (Ichida et al., 2012; Strilchuk et al., 2019). Uric acid is the ultimate product of purine metabolism in humans, in which a variety of enzymes are involved, such as phosphoribosyl pyrophosphate synthetase (PRPS), purine nucleoside phosphorylase (PNP), adenosine deaminase (ADA), and xanthine oxidase (XOD). The inhibition of these enzymes can effectively reduce SUA levels (Liu et al., 2019). The kidneys and intestines, which excrete 65% to 75% and 25% to 35% of the uric acid in the body, respectively, are 2 vital sites for UA elimination (Benn et al., 2018). Typically, UA excretion by the kidney is regulated by UA transporters, of which urate transporter 1 (URAT1) and glucose transporter 9 (GLUT9) are key players in the renal tubular reabsorption of UA, whereas ATP-binding cassette superfamily G member 2 (ABCG2) and organic anion transporters 1/3 (OAT1/3) are typically involved in UA excretion (Bobulescu and Moe, 2012). The impairment of these transporters can affect UA excretion process, leading to HUA (Xu et al., 2017). Additionally, increasing evidence suggests that the gut microbiota participates in purine and UA metabolism (Xu et al., 2021). A high-purine diet can result in the dysregulation of gut microbiota. Recent studies have revealed that the gut microbiota is significantly altered during the pathogenesis and palliation
of HUA (Guo et al., 2016; Bian et al., 2020; Xu et al., 2021). Thus, the modulation of the gut microbiota is a promising new therapeutic target for HUA.

Currently, several drugs (e.g., allopurinol, benzbumaron, and febuxostat) are in clinical use; however, they are frequently associated with undesirable side effects (Mehmood et al., 2019). In recent years, bioactive peptides have attracted great interest for prophylaxis and therapy of HUA because of fewer adverse effects, easy absorption, and high specificity (Sarmadi and Ismail, 2010). Some protein hydrolysates from sea cucumbers, tuna, and walnuts have been shown to reduce SUA levels and protect against kidney injury (Li et al., 2018; Han et al., 2020; Wan et al., 2020). Wan et al. (2020) showed that sea cucumber hydrolysates attenuated HUA by inhibiting UA production, promoting UA excretion, and mitigating disorders of the gut microbiota. Accordingly, some potential anti-hyperuricemic peptides (WPPKN, ADIYTE, AAAAGAKAR, PGACSN, WML, and FH) with XOD inhibitory activity have also been purified and identified (Li et al., 2018; He et al., 2019; Liu et al., 2019). These peptides have been demonstrated in vitro to inhibit XOD activity by interacting with key residues around XOD active pockets. However, investigation of the anti-hyperuricemia mechanisms of these peptides in vivo is still in its infancy. Crossing the barrier of gastrointestinal digestion and intestinal absorption to reach the target site is a prerequisite for bioactive peptides to exert physiological effects in vivo (Sontakke et al., 2016). Pro-Glu-Trp (PEW) and Leu-Leu-Trp (LLW) were identified in our previous study (Qi et al., 2022) as two XOD-inhibitory peptides that were screened from peptides that survived whey protein gastrointestinal digestion and passed through a Caco-2 cell monolayer. They exhibit high XOD-inhibitory activity and are partially absorbed by the intestine in their intact form (Qi et al., 2022). However, whether PEW and LLW attenuate HUA and gut microbiota dysbiosis remains unclear.

The purpose of the present study was to examine the anti-hyperuricemic effects of PEW and LLW in HUA rats. The underlying mechanisms were explored from the perspectives of UA synthesis and excretion and the gut microbiota. Furthermore, the protective effects of PEW and LLW against kidney injury and oxidative stress were evaluated, providing insights into new strategies for the treatment of HUA.

**MATERIALS AND METHODS**

**Experimental Animals and Treatments**

Thirty-six male Sprague Dawley rats (weighing 200 ± 20 g) were obtained from Liaoning Changsheng Technology Co. Ltd. (Benxi, China) and kept in a controlled environment of 22 ± 2°C, 50 ± 5% humidity, and normal 12 h light/12 h dark cycle with a standard diet and water. Animal experiments were approved by the Institutional Animal Care and Use Committee of Harbin Institute of Technology (permit number: IA-CUC-2022049).

After acclimatization for one week, the rats were randomly divided into 6 groups (n = 6 per group): normal control (NC), model control (MC), positive control (PC), PEW only, LLW only, and PEW+LLW (P+L; Figure 1A). All rats except those in the NC group were administered potassium oxonate (PO; 500 mg/kg) and hypoxanthine (500 mg/kg) to establish the HUA rat model. After modeling for 7 consecutive days, the PC group received a daily oral gavage of 15 mg/kg allopurinol; the PEW and LLW groups received 60 mg/kg PEW and LLW solution, respectively; the P+L group received 30 mg/kg each of PEW and LLW solution; and the NC and MC groups were orally administered 0.5% sodium carboxymethyl cellulose aqueous solution daily. The peptides PEW and LLW (purity ≥98%) were synthesized using a solid-phase procedure, purified, and desalted by Sangon Biochem (Shanghai, China). These treatments were administered for 21 d. Urine and feces were collected from rats the day before euthanasia. Serum samples were collected 1 h after final administration. After the rats were euthanized, their visceral organs were excised and weighed. One kidney was fixed in 4% paraformaldehyde, and other tissues were snap frozen and stored at −80°C.

**Biochemical Analyses**

Levels of SUA, urine UA (UUA), serum creatinine (SCr), urine creatinine (UCr), and BUN levels were analyzed using commercial kits (JianCheng, Nanjing, China). The fractional excretion of UA (FEUA) was calculated according to the following formula: FEUA (%) = (UUA × SCr)/(SUAn × UCrn) × 100. Activities of XOD and ADA in the serum and liver, and malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activity in the kidney were determined using commercial kits (JianCheng, Nanjing, China).

**Histopathological Examination**

Kidney tissue was fixed in 4% paraformaldehyde solution, dehydrated, and embedded in paraffin. Sections were cut (5 μm thick) and stained with hematoxylin and eosin, and the prepared slides were examined under a light microscope.
Figure 1. Effects of PEW, LLW, and their combined intervention on biochemical indexes and renal pathology of hyperuricemic rats. Experimental scheme of animals (A). SUA = serum uric acid (B). UUA = urine uric acid. (C). SCr = serum creatinine (D). BUN (E). UCr = urine creatinine (F). FEUA = fractional excretion of uric acid (G). ## P < 0.01, ### P < 0.001 vs. NC group; * P < 0.05, ** P < 0.01, *** P < 0.001 vs. MC group. Values are presented as means ± SD (n = 6). Renal pathological changes (H); the obvious damage is marked by arrows. NC = normal control; MC = model control; PC = positive control; PEW = Pro-Glu-Trp; LLW = Leu-Leu-Trp; PEW+LLW = combined treatment of PEW and LLW.
Quantitative Real-Time PCR Analysis

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR) were performed as previously described (Chen et al., 2022). Briefly, total RNA was extracted from liver and renal tissues using a TRIzol reagent kit (Vazyme, Nanjing, China). RNA was reverse transcribed into cDNA using a cDNA Synthesis Kit purchased from Vazyme. Finally, cDNA was quantified by qRT-PCR using an ABI QuantStudio 3 System. Details of the primer sequences are listed in Supplemental Table S1 (https://doi.org/10.17632/sfcv6b9bnz.1; Qi et al., 2023). β-Actin was used as an internal control to normalize the data, and the $2^{-\Delta\Delta C_T}$ method was used to calculate mRNA expression levels.

Western Blot Analysis

Kidney tissues were lysed with radio immunoprecipitation assay lysis buffer (Solarbio, Beijing, China) for the extraction of protein samples, 40 µg of which were denatured, separated by 10% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes. Subsequently, the membranes were blocked with 5% BSA and incubated with primary antibody at 4°C overnight, followed by secondary antibody at room temperature for 1 h. Primary antibody against OAT1 was purchased from Abcam (Cambridge, UK). The primary antibodies against ABCG2 and OAT3 were purchased from Affinity Biosciences (San Jose, CA). Primary antibodies against URAT1, GLUT9, and β-actin, as well as secondary antibodies, were purchased from Proteintech Group Inc. (Wuhan, China). Eventually, the immunoreactive bands were visualized with enhanced chemiluminescence reagent, and the grayscale value of the band was quantified using Image J software (National Institutes of Health, Bethesda, MD). All results were normalized to β-actin.

Immunohistochemical Staining

The tissue sections were deparaffinized, rehydrated, and then pretreated in a microwave oven with citrate buffer for antigen retrieval. The sections were then incubated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity, and subsequently blocked with 5% goat serum for 30 min. Next, the sections were incubated with URAT1, GLUT9, or ABCG2 antibodies overnight at 4°C. The sections were washed 3 times with PBS and incubated with secondary antibodies conjugated to horseradish peroxidase at room temperature. The sections were then washed, visualized with a 3,3-diaminobenzidine solution, and counterstained with hematoxylin. Finally, the sections were dehydrated, cleared, and examined using a CX31 microscope (Olympus, Tokyo, Japan).

Transmission Electron Microscopy

The sections were imaged at 80 kV using a Zeiss 900 electron microscope (Zeiss, Jena, Germany). Pretreatment of the tissue samples was performed as previously described (Chen et al., 2022).

Gut Microbiota Analysis

Fresh fecal samples from 4 groups of rats (NC, MC, PC, and P+L) were collected for 16S rRNA gene sequencing. As previously reported (Chen et al., 2022), total genomic DNA in the feces was extracted and quantified for amplification using specific primers with barcodes (16S rRNA gene V4 region): forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Next, paired-end sequencing was performed on a NovaSeq6000 platform (paired-end 2× 150-bp reads) provided by GUHE Info Technology Co. Ltd. (Hangzhou, China). The 16S rRNA sequencing data of the gut microbiota were analyzed using the microbial ecology platform (QIIME2, v1.8.0) and R packages (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria).

Determination of Short-Chain Fatty Acids

Fecal samples from rats were prepared and analyzed by gas chromatography, as previously reported (Xu et al., 2021). Briefly, fecal samples were extracted with 5× distilled water for 20 min on a horizontal shaker at 4°C. After centrifugation (18,000 × g, 4°C, 15 min), the supernatant was mixed with formic acid and filtered through a 0.22-µm aqueous membrane. Finally, the samples were analyzed using an Agilent 8890 gas chromatography system (Agilent Technologies, Santa Clara, CA).

Statistical Analysis

All data are presented as means ± standard deviation. One-way ANOVA followed by Duncan’s test was used to determine the differences between groups using SPSS (version 26.0; SPSS Inc., Chicago, IL). $P$-values <0.05 were considered significant.
RESULTS

PEW and LLW Attenuated HUA and Kidney Damage in Rats

To explore the anti-hyperuricemia effects of PEW and LLW, HUA rats were orally administered PEW and LLW individually or in combination for 21 d (Figure 1A). During the entire experimental period, the rats were in good condition, and no significant difference in body weight was observed between the groups ($P > 0.05$, Supplemental Figure S1; https://doi.org/10.17632/sfcv6b9bnz.1; Qi et al., 2023). We found no significant changes in the liver and spleen indices between the groups ($P > 0.05$), whereas the renal index of the rats significantly increased after PO and hypoxanthine administration ($P < 0.001$, vs. the NC group), which significantly ($P < 0.05$) recovered after peptide intervention. Additionally, as anticipated, the HUA rat model was successfully established, with a significant increase in SUA, SCr, and BUN and a significant decrease in UUA, UCr, and FEUA in the MC group compared with those in the NC group ($P < 0.01$; Figure 1B–G). The FEUA is the percentage of urate filtered by the glomeruli and excreted in the urine. Both PEW and LLW and their combination reversed these trends to varying degrees, and the effect of co-administration of PEW and LLW was superior to those of the 2 peptides alone, indicating that PEW and LLW exerted synergistic anti-hyperuricemia effects. Compared with the MC group, SUA, SCr, and BUN levels in the P+L group decreased by 48.51, 45.80, and 34.67%, respectively, and UUA, UCr, and FEUA levels increased by 108.59, 54.41, and 57.84%, respectively. As expected, the PC group showed better anti-hyperuricemia effects compared with the peptide-treated groups.

PEW and LLW Promoted UA Excretion in HUA Rats

We further explored the uricosuric effects of PEW and LLW and examined the mRNA and protein expression levels of renal UA transporters (GLUT9, URAT1, ABCG2, OAT1, and OAT3). In the MC group, the protein levels of the transporters responsible for UA reabsorption (GLUT9 and URAT1) were remarkably upregulated, whereas those of the transporters responsible for UA secretion (ABCG2, OAT1, and OAT3) were significantly downregulated ($P < 0.001$, vs. the NC group; Figure 3A and B). After PEW, LLW, and P+L treatment, the expression levels of these transporters were significantly restored ($P < 0.05$), especially in the PEW and P+L groups compared with the MC group, and their role in regulating UA transporters was stronger than that in the PC group. Similarly, the mRNA levels of these transporters (Supplemental Figure S2; https://doi.org/10.17632/sfcv6b9bnz.1; Qi et al., 2023) agreed

PEW and LLW Ameliorated Renal Pathological Injury in HUA Rats

Excessive UA levels in the body are often associated with kidney injury, which is a clinicopathological feature of HUA (Prasad Sah and Qing, 2015). Hematoxylin and eosin staining was used to detect renal pathological changes in HUA rats (Figure 1H). Rats in the NC group showed normal renal morphology, whereas serious histopathological injuries, such as renal tubule dilation, tubular epithelial cell abscission, glomerular atrophy, and inflammatory cell infiltration, were observed in the MC group. Compared with the MC group, allopurinol treatment markedly decreased the extent of renal lesions. Moreover, the administration of PEW, LLW, and P+L alleviated renal injury to varying degrees by weakening renal tubular lesions, glomerular atrophy, and inflammatory cell infiltration. Although some pathological changes such as renal tubular dilation and epithelial cell abscission persisted, especially in the LLW group, their severity was significantly lower compared with the MC group.

PEW and LLW Suppressed UA Synthesis in HUA Rats

As shown in Figure 2A, PRPS, PNP, ADA, and XOD are key enzymes involved in UA synthesis. To determine whether PEW and LLW affected UA synthesis in rats, the mRNA expression levels of these enzymes in the liver and the activities of XOD and ADA in the serum and liver were measured. The mRNA levels of PRPS, PNP, XOD, and ADA were significantly upregulated in the MC group ($P < 0.001$, vs. the NC group, Figure 2B–E). The LLW and P+L treatments significantly reversed these trends, with no significant differences between the LLW and P+L groups ($P > 0.05$). However, PEW significantly downregulated the expression of XOD and ADA ($P < 0.001$, vs. the MC group). Correspondingly, significantly elevated XOD and ADA activities in the serum and liver were also observed in the MC group compared with the NC group ($P < 0.01$; Figure 2F–I). We found that PEW, LLW, and their combination significantly inhibited XOD activity ($P < 0.001$, vs. the MC group). Notably, LLW and P+L, rather than PEW, played a significant role in inhibiting ADA activity compared with the MC group. Allopurinol significantly suppressed the activities of XOD and ADA but had no obvious effect on the mRNA expression levels of PRPS, PNP, XOD, and ADA ($P > 0.05$, vs. the MC group).
With the protein expression results, suggesting that the co-administration of PEW and LLW could affect the expression of UA transporters, thereby promoting UA excretion.

**Immunohistochemical Detection of URAT1, GLUT9, and ABCG2 Protein Expressions in Kidneys**

As presented in Figure 3C, the immunoreactivities of URAT1, GLUT9, and ABCG2 were observed in the renal tissue of rats. URAT1 and ABCG2 are localized in the brush border membranes of renal proximal tubule cells, whereas GLUT9 is expressed in the basolateral membranes. Furthermore, quantitative analysis of the positive area revealed increased expression of URAT1 and GLUT9 and decreased expression of ABCG2 in the MC group compared with the NC group (Figure 3D–F). In contrast, URAT1 and GLUT9 protein expression levels were significantly decreased, and those of ABCG2 were increased after allopurinol and peptide treatment. This result validated that the anti-hyperuricemic effects of PEW and LLW were partially mediated by the modulation of UA transporters.

**PEW and LLW Restored Renal Oxidative Stress and Mitochondrial Damage in HUA Rats**

Changes in the biochemical markers of oxidative stress in the kidneys were examined to confirm whether the activity of enzymes was affected by PEW or LLW (Figure 4A–D). We found that SOD, CAT, and GSH-Px activities decreased remarkably, and MDA content was significantly elevated in the MC group (P < 0.001, vs. the NC group). However, treatment with allopurinol reversed these changes. Administration of PEW, LLW, and their combination significantly enhanced the...
Figure 3. Effects of PEW, LLW, and their combined intervention on uric acid excretion pathway. The relative protein expression levels of GLUT9, URAT1, ABCG2, OAT1, and OAT3 normalized to β-actin in the kidneys (A). Representative figure of western blot analysis (B). Images of immunohistochemistry staining for URAT1, GLUT9, and ABCG2 in kidney tissues (C). Quantitative immunohistochemistry analysis of the expression of URAT1 (D), GLUT9 (E), and ABCG2 (F). URAT1, GLUT9, and ABCG2 immunoreactivity were evident in the proximal tubules of the kidney (gray arrows). #P < 0.05, ###P < 0.01, ####P < 0.001 vs. NC group; *P < 0.05, **P < 0.01, ***P < 0.001 vs. MC group. Values are presented as means ± SD (n = 6). NC = normal control; MC = model control; PC = positive control; PEW = Pro-Glu-Trp; LLW = Leu-Leu-Trp; PEW+LLW = combined treatment of PEW and LLW.
activities of SOD, CAT, and GSH-Px ($P < 0.01$), and lowered MDA levels in the MC group, where LLW exerted better results than P+L and PEW. These results suggest that PEW and LLW may exert a protective effect against UA-induced oxidative stress. Substantial mitochondrial damage occurs when the body is subjected to oxidative stress, which is the main cause of apoptosis in renal tubular epithelial cells (Yang et al., 2019). In the current study, we observed the mitochondrial structure in rat kidneys using transmission
The results revealed mitochondrial swelling accompanied by the disruption and disorganization of cristae in the kidneys of HUA rats, whereas PEW, LLW, and P+L treatments significantly alleviated mitochondrial lesions and reduced mitochondrial swelling.

**Combination of PEW and LLW Modulated Gut Microbiota and Short-Chain Fatty Acid Generation in HUA Rats**

Mounting evidence indicates that gut dysbiosis plays a critical role in HUA (Wang et al., 2022). Thus, 16S
Correlation Analysis Among Microbiota, SCFA Levels, and Other Parameters in HUA Rats

Spearman’s correlation analysis was conducted to study the correlation between the gut microbiota and biochemical parameters (Figure 6). The results revealed that intestinal flora and SCFA were notably correlated with biochemical indicators in HUA rats. More specifically, Alloprevotella, Bacteroides, Defluviitaleaceae_UGC-011, and Monoglobus were positively correlated with SUA, XOD, ADA, and BUN, and negatively correlated with ABCG2, OAT1, SOD, CAT, and GSH-Px. In contrast, Muribaculaceae, Lactobacillus, Phascolarctobacterium, Bifidobacterium, Ruminococcus, and Enterorhabdus showed the opposite correlation with these indicators. Moreover, SCFA concentration positively correlated with antioxidant capacity and negatively correlated with hyperuricemia and renal function impairment. Taken together, these results suggest that the beneficial effects of PEW and LLW in alleviating HUA may be related to the regulation of gut microbiota composition and SCFA concentration.

DISCUSSION

Hyperuricemia is a metabolic disease that threatens human health. In recent years, due to the side effects of first-line drugs, an increasing number of studies have focused on the use of natural ingredients as an efficacious way to manage HUA (Mehmood et al., 2019). Bioactive peptides as food-derived components exhibit effective and limited side effects in the treatment of obesity, type 2 diabetes, hypertension, and hypercholesterolemia (Udenigwe and Aluko, 2012; Redha et al., 2022). Both PEW and LLW were identified as potential UA-lowering peptides through our previous in vitro study (Qi et al., 2022). Here, we demonstrated that PEW and LLW synergistically attenuated HUA by re-balancing UA production and excretion and regulating the gut microbiota.

Diet has an important effect on the onset of HUA; a high-purine diet causes purine metabolism disorders and increases the risk of HUA (Han et al., 2021). In accordance with previous findings, we showed that purine metabolism in HUA rats was disturbed, manifested by abnormal expression or increased activity of related enzymes (PRPS, PNP, XOD, and ADA) during UA synthesis, which is closely related to increased level of SUA (Wan et al., 2020). PRPS, PNP, and ADA are primarily involved in the conversion of ribose-5-phosphate to hypoxanthine, whereas XOD is responsible for converting hypoxanthine to UA (Figure 2A). Several studies have reported that certain peptides (AAAAGAKAR,
GPAGPR, GPSGRP, and anserine) effectively suppress XOD activity in vivo and in vitro (Liu et al., 2019; Han et al., 2021; Fan et al., 2022). The underlying mechanism by which these peptides inhibit XOD may involve binding to XOD and preventing its interaction with hypoxanthine or xanthine (Zhong et al., 2021; Qi et al., 2022). Although PEW and LLW were previously identified as XOD inhibitory peptides, in the present study, these 2 peptides not only inhibited XOD activity in the serum and liver but also downregulated the mRNA levels of PRPS, PNP, XOD, and ADA, as well as the activity of ADA, indicating that PEW and LLW inhibit UA synthesis by affecting multiple targets. Allopurinol, a potent XOD inhibitor, had an extremely prominent inhibitory effect on XOD activity in the serum and liver, even lower than that in the NC group, but had no significant regulatory effect on the mRNA levels of PRPS; however, the combination of allopurinol and pallidifloside D significantly reduced PRPS mRNA expression. These results suggest that the peptides and allopurinol may have different mechanisms of action in alleviating HUA.

Relative insufficiency of UA excretion is another primary cause of HUA. Clinical data have shown that most patients with gout and primary HUA are associated with renal UA secretion disorder (Rieselbach et al., 1970). Meanwhile, decreased UA secretion has also been observed in animals with HUA (Zhang et al., 2018). In agreement with this, we found that FEUA levels were decreased in HUA rats, which was reversed by treatment with PEW and LLW (Figure 1G). Renal UA transporters play critical roles in HUA and kidney excretion and are considered promising therapeutic targets for HUA (Zhang et al., 2018). URAT1 and GLUT9 specifically mediate UA reabsorption by the proximal renal tubules. A previous study revealed that mutations in human GLUT9 and URAT1 can lead to the dys-
function of UA reabsorption, leading to hypouricemia (Claverie-Martin et al., 2018). In addition, ABCG2, OAT1, and OAT3 are involved in UA secretion, where OAT1 and OAT3 are responsible for the uptake of UA from the blood to intracellular tubular cells, whereas ABCG2 is responsible for the excretion of UA from tubular cells to the lumen (Xu et al., 2017). Studies have shown that the upregulation of these UA secretion transporters is associated with improved UA excretion (Han et al., 2020; Mehmood et al., 2020). In the current study, we examined the expression of these transporters using qRT-PCR, western blotting, and immunohistochemical analyses (Figure 3 and Supplemental Figure S2). Consistent with previous studies, abnormal expression of renal UA transporters was observed in PO- and hypoxanthine-induced HUA rats (Li et al., 2021; Lin et al., 2021). Compared with the MC group, the expression of GLUT9 and URAT1 was downregulated, and the expression of ABCG2, OAT1, and OAT3 was upregulated in all treatment groups. However, differences were evident in the regulatory abilities of these transporters among the groups. The combination of PEW and LLW exerted the strongest regulatory effects on these transporters. The effect is relatively weak when LLW was administered alone. Interestingly, we found that allopurinol also regulated the expression of these transporters, which has been reported in previous studies (Mehmood et al., 2020; Xu et al., 2021). These results suggest that PEW and LLW not only suppress the synthesis of UA but also promote the excretion of UA by regulating UA transporters. Similarly, Fan et al. (2022) reported that the expression of URAT1 and GLUT9 decreased in HUA mice treated with GPAGPR and GPSGRP.

Numerous studies have demonstrated that high UA levels can damage the body’s redox balance and release a large number of reactive oxygen species, thereby triggering oxidative stress (Sánchez-Lozada et al., 2008). XOD produces reactive oxygen species during UA production (Liu et al., 2021). Oxidative stress plays a key role in UA-induced renal injury; it not only activates NF-κB signaling pathway to release inflammatory cytokines but also promotes mitochondrial dysfunction, which is the main reason for apoptosis in renal tubule epithelial cells (Yang et al., 2019). When the kidney is damaged, renal UA excretion is disorganized, resulting in reduced UA excretion and increased serum UA levels. Thus, suppression of oxidative stress may be a feasible method for preventing HUA. In the present study, the abnormalities in antioxidant biomarkers (SOD, GSH-Px, CAT, and MDA) were significantly reversed by allopurinol and peptide treatments, with no significant differences between the PEW, LLW, and P+L groups (Figure 4). These results indicate that PEW and LLW exerted their antioxidant capacity and reduced oxidative stress in HUA, which was also confirmed by improvement to mitochondrial damage. Therefore, we inferred that the improvement in HUA-induced kidney injury and HUA by PEW and LLW may be related to the reduction in oxidative stress.

The gut microbiota is a group of microbes found in the colon that are thought to be associated with the development of various diseases (Du et al., 2022). The important role of the gut microbiota in HUA, which is involved in purine and UA metabolism, intestinal UA excretion, and intestinal inflammation, has been confirmed by an increasing amount of evidence (Han et al., 2021). A previous study demonstrated that the dysregulation of the gut microbiota in patients with HUA was associated with elevated serum UA levels (Wei et al., 2022). Intestinal flora disorders in animals has also been observed, including alterations in its diversity, composition, and metabolites (Xu et al., 2021). Based on our previous results, we found that PEW and LLW exert synergistic effects in alleviating HUA. Therefore, we studied the effects of the combination of PEW and LLW on the gut microbiota of HUA rats. Bioactive peptides have been previously shown to modulate the homeostasis of the gut microbiota (Wu et al., 2021). Our results indicate that the co-administration of PEW and LLW modifies gut microbial richness and diversity. In addition, it was noted that the combined treatment of PEW and LLW resulted in significant modifications in the intestinal bacterial structure of rats. At the phylum level, the increased abundance of Firmicutes and decreased abundance of Bacteroidetes were restored in the P+L group, which is in accordance with previous studies (Bian et al., 2020; Xu et al., 2021). Moreover, Alloprevotella and Bacteroides are considered potential pathogenic in the human gut, which decreased after P+L treatment. Alloprevotella is enriched in fecal samples of patients with chronic kidney disease, and Bacteroides are producers of LPS (Li et al., 2019; Chen et al., 2022). Additionally, the P+L treatment increased the abundance of Lactobacillus, Ruminococcus, and Bifidobacterium. Lactobacillus has the ability to synthesize uricase, which can further decompose UA into allantoin and reduce purine absorption in the gut (Wang et al., 2022). Ruminococcus and Bifidobacterium are related to the production of SCFA (Xu et al., 2021). In the present, we found that the SCFA concentration in the P+L group was significantly higher compared with the MC group. Short-chain fatty acids, especially propionic and butyric acids, have been reported to provide energy for UA excretion from cells in the intestinal wall (Nieuwdorp et al., 2014). Based on our findings,
modulating the composition of the gut microbiota and its metabolites may be a possible mechanism by which PEW and LLW ameliorate HUA. However, whether the beneficial effects of PEW and LLW are mediated by the gut microbiota requires further investigation using fecal microbiota transplantation.

In summary, our findings demonstrated that the combined treatment with PEW and LLW exhibited superior anti-hyperuricemia effects compared with either peptide alone in PO- and hypoxanthine-induced HUA rats. The efficacy of the combination of PEW and LLW was mainly mediated by inhibiting the activity of key enzymes in UA synthesis, regulating the expression of UA transporters associated with UA elimination, and modulating the composition of the gut microbiota (Figure 7). Moreover, PEW and LLW together exerted nephroprotective effects. These findings suggest that combining PEW and LLW may be a promising dietary strategy for the prevention and treatment of HUA.

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