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Chronic Low-Dose Phlorizin Intake Is Associated with Gut Microbiota Remodeling and Arachidonic Acid–Related Serum Metabolomic Changes in Healthy Mice

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ABSTRACT: Phlorizin, a major apple-derived dihydrochalcone glycoside, has been reported to contribute to metabolic and inflammatory regulation and has been linked to alterations in the gut microbiota across various disease models, but its relationship with the gut microbiota under normal physiological conditions remains unclear. This study aimed to characterize gut microbial ecology and serum metabolomic changes associated with chronic, low-dose phlorizin intake in healthy mice. Healthy male C57BL/6J mice received phlorizin orally at 30 mg/kg/day for 28 days. We assessed organ safety and histology, SGLT1/2 protein expression, glucose handling, gut microbiota, and the serum metabolome, and performed integrative correlation analysis.

Results showed that phlorizin was generally well tolerated. Serum ALT, AST, and BUN were significantly elevated, although histopathology showed no overt abnormalities; values remained within reference ranges, but safety still requires further evaluation. Neither SGLT1/2 protein expression nor indirect functional assessment (OGTT glucose tolerance and urinary glucose concentration) differed between groups, but total 24-h urinary glucose excretion was modestly higher in the phlorizin group, possibly reflecting a mild residual effect of phlorizin on SGLT-related glucose handling, whose specific basis requires further study. Phlorizin intake was associated with altered gut microbial diversity and composition, including enrichment of *Akkermansia*, *Alistipes*, and *Ligilactobacillus*, and with a distinct serum metabolic signature enriched in arachidonic acid (AA) and other PUFA-derived lipid mediator pathways, with several mediators showing lower relative abundance and correlating with specific microbial taxa.

Taken together, chronic low-dose phlorizin intake was associated with changes in gut microbiota and the serum metabolome, particularly within AA- and other PUFA-derived lipid pathways; whether these changes are independent

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of phlorizin's inhibition of SGLT function requires further exploration. As this was a correlational study, these findings provide a preliminary basis for future mechanistic studies of dietary polyphenol–host–microbiota interactions.

Keywords: Phlorizin; Gut Microbiota; Serum Metabolomics; Arachidonic Acid Metabolism; Multi-omics Integration

1. Introduction

Apple (*Malus domestica*) is a major temperate fruit crop and remains one of the fruits most frequently consumed worldwide. Its nutritional and health-related value has commonly been linked to a distinctive profile of apple polyphenols^[1, 2]. Within this group, phlorizin deserves particular attention. This dihydrochalcone glycoside is the predominant dihydrochalcone reported across *Malus* germplasm^[3, 4], and it is generally regarded as a marker bioactive compound of apples and apple-derived foods. For this reason, apples and their products constitute the main dietary source of phlorizin in humans^[1, 4].

Phlorizin is the first identified natural inhibitor of sodium–glucose cotransporters (SGLTs), blocking SGLT1-mediated intestinal glucose absorption and SGLT2-mediated renal glucose reabsorption^[4, 5]. However, its oral bioavailability is markedly limited by rapid intestinal β -glucosidase-mediated hydrolysis to its aglycone phloretin^[4]. This metabolic feature suggests that the physiological effects of phlorizin may not be fully explained by direct SGLT inhibition alone, but may also involve other intestinally mediated processes^[4, 6].

Phlorizin and phloretin have been reported to exert diverse health-promoting activities. In vivo studies have shown that phlorizin ameliorates cognitive impairment and depression-like behaviors^[7–9], protects against obesity-associated endotoxemia and insulin resistance^[10], and attenuates viral infection-related injury^[11]. Additional reported activities include anti-tumor^[12], glucose- and lipid-regulatory^[13], and renal-protective effects^[14]. Notably, many of these effects are increasingly associated with intestinal regulation, suggesting that the gut may represent a major interface through which phlorizin-derived signals influence host physiology. Despite this breadth of evidence, the effects of long-term, low-dose phlorizin intake under non-disease conditions remain insufficiently characterized.

The gut microbiota is now understood to participate actively in host metabolic and immune regulation, rather than simply occupying the intestinal tract. It contributes through the production of microbial metabolites, maintenance of epithelial barrier function, and multiple host–microbe signaling routes^[15–20]. Because phlorizin is extensively transformed in the intestine, the intestinal ecosystem is likely to be an important site at which its biological effects are shaped. At the same time, the circulating metabolome captures the net systemic response to dietary bioactive compounds and can therefore be used to assess whole-body metabolic changes after sustained phlorizin intake.

However, much of the current in vivo evidence comes from disease or injury models, including obesity, metabolic dysfunction, viral infection, neurobehavioral impairment, and intestinal inflammation^[7–14, 17–19]. Such settings are useful for testing therapeutic potential, but they also introduce a major interpretive problem: microbial changes may reflect the underlying disease state as much as the direct action of phlorizin. In addition, the systems-level connections among phlorizin-driven microbiota remodeling, circulating metabolic

signatures, and host immune–metabolic regulation remain incompletely defined. More generally, how phytochemicals reshape the microbiota to support systemic homeostasis under healthy physiological conditions is still not well resolved^[4, 16, 21, 22].

Based on our premise, we hypothesized that the administration of chronic low doses of phlorizin would change the composition and functional potential of the gut microbiota in healthy mice, and that these microbial changes would be linked to alterations in systemic metabolism. To test this hypothesis, healthy mice were given phlorizin orally for 28 days. Initially, we assessed the tolerability of the intervention and evaluated intestinal integrity, tissue SGLT protein expression, and the handling of glucose related to SGLT. Next, we combined shotgun metagenomic sequencing with untargeted serum metabolomics to analyze changes in gut microbial diversity, community composition, predicted metabolic potential, and circulating metabolite profiles. Subsequently, we performed integrative multi-omics correlation analyses to investigate the relationships between different microbial taxa and serum metabolic features, with a particular focus on arachidonic acid metabolism and other pathways derived from polyunsaturated fatty acids (PUFAs).

Overall, this study aimed to define the changes in gut microbiota and systemic metabolism associated with chronic low-dose phlorizin exposure under healthy physiological conditions. It also seeks to lay the groundwork for future investigations into the interactions between dietary polyphenols, the gut microbiota, and the host.

2. Materials and Methods

2.1 Animal model

All animal experiments were approved by the Laboratory Animal Ethics Committee of Peking University First Hospital (approval No. J2025035) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the institutional guidelines of Peking University Health Science Center. A total of 16 male C57BL/6 mice, aged 8 weeks, were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. The wild-type (WT) mice were housed under specific pathogen-free (SPF) conditions at a temperature of 20–24 °C and a relative humidity of 30–70%, with a 12 h light/12 h dark cycle and free access to standard rodent chow and water.

After 7 days of acclimatization in the SPF animal facility, the mice were randomly assigned to two groups: the vehicle group and the phlorizin-treated group, with 8 mice in each group. Mice in the phlorizin-treated group received phlorizin by intragastric administration at a dose of 30 mg/kg/day for 28 consecutive days, whereas mice in the vehicle group received an equal volume of the same solvent system without phlorizin for the same duration. Phlorizin was administered orally at 30 mg/kg/day. The dose was chosen before the experiment based on previous mouse studies that used approximately 20–100 mg/kg/day to examine effects on the gut microbiota and host metabolism^[10, 23–25]. Accordingly, 30 mg/kg/day was selected as a dose near the lower end of the published range. The solvent system consisted of 2% dimethyl sulfoxide (DMSO), 40% polyethylene glycol 300 (PEG300), 5% Tween 80, and 53% double-distilled water (ddH₂O).

2.2 Experimental Design of Phlorizin Intervention in Healthy Mice

To examine the effects of phlorizin on the intestinal microecosystem and host metabolic homeostasis under physiological conditions, we established a chronic phlorizin intervention model in healthy SPF male C57BL/6J mice. Phlorizin is a naturally occurring dihydrochalcone glycoside and was the first natural sodium-glucose cotransporter inhibitor to be identified; its chemical structure is presented in Figure 1a. As shown in Figure 1b, mice were acclimatized and then randomly assigned to either the vehicle group or the phlorizin group. Mice in the phlorizin group received 30 mg/kg/day phlorizin by oral gavage for 28 consecutive days, whereas mice in the vehicle group received the same volume of vehicle solution. On day 28, the mice were sacrificed, and heart, liver, kidney, intestine, blood, and fecal samples were collected for subsequent analyses.

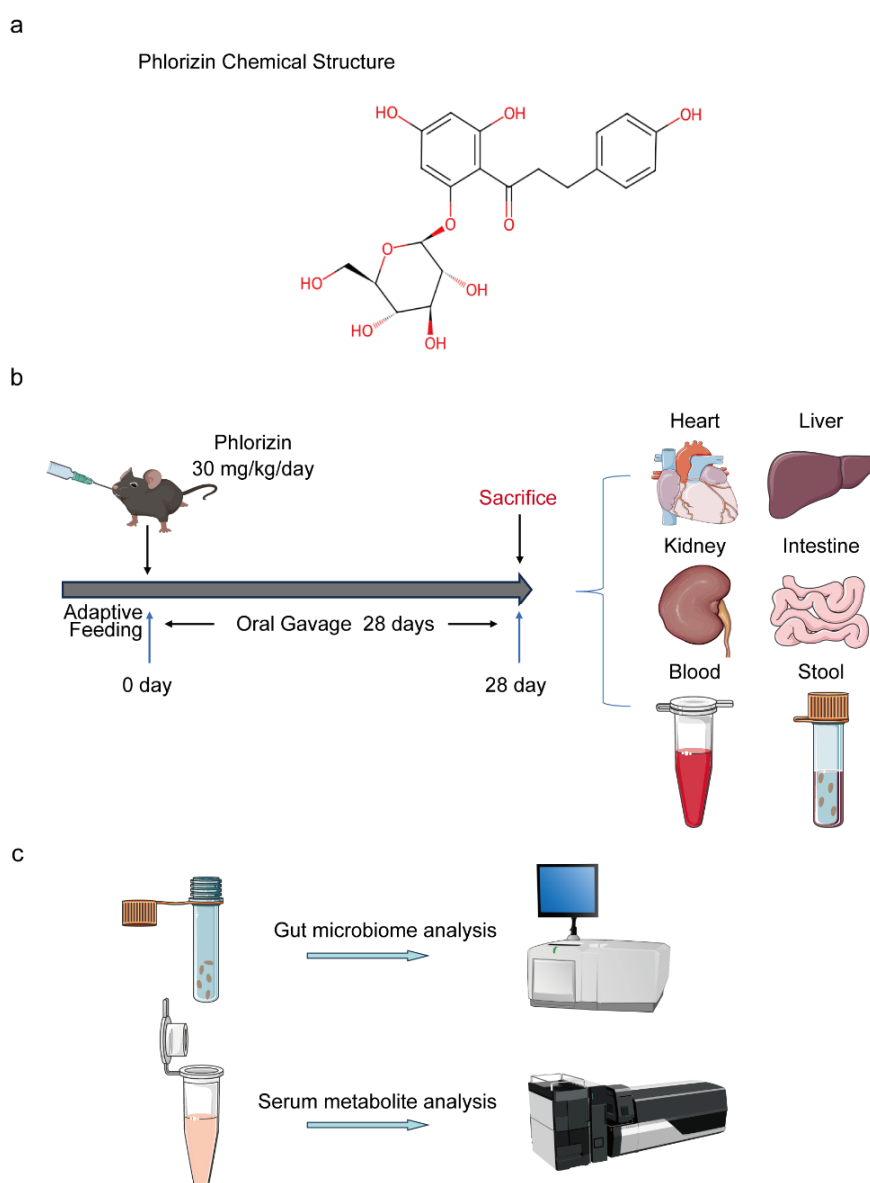


Figure 1. Schematic overview of the phlorizin intervention and multi-omics workflow. (a) Chemical structure of phlorizin. (b) Experimental design. After acclimatization, healthy C57BL/6J mice were orally administered phlorizin at 30 mg/kg/day or vehicle for 28 consecutive days. At the end of the intervention, mice were sacrificed, and heart, liver, kidney, intestine, blood, and fecal samples were collected for subsequent analyses. (c) Multi-omics analytical workflow. Fecal samples were subjected to shotgun metagenomic sequencing to characterize gut microbiota composition and functional potential, whereas serum samples were analyzed by untargeted metabolomics to profile host metabolic alterations.

The overall analytical workflow is shown in Figure 1c. Fecal samples were used for gut microbiome analysis to characterize microbial community alterations, whereas serum samples were subjected to metabolomic analysis to evaluate systemic metabolic changes. Together, this experimental design enabled integrated assessment of the gut microbiota–host metabolic responses to phlorizin intervention.

2.3 Serum biochemical analysis

Blood samples were collected from the retro-orbital venous plexus of mice and centrifuged at 4000 rpm for 10 min at 4 °C to obtain serum. Serum biochemical parameters were measured to evaluate liver, renal, and cardiac function. Liver function was assessed by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Renal function was evaluated by measuring blood urea nitrogen (BUN) and creatinine (CREA) levels. Cardiac injury-related parameters, including creatine kinase (CK) and lactate dehydrogenase (LDH), were also determined. All serum biochemical parameters were measured using an automatic chemistry analyzer (Chemray 800; Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China) with commercial assay kits supplied by Rayto, including ALT (Cat. No. S03030), AST (Cat. No. S03040), urea (Cat. No. S03036), creatinine (Cat. No. S03076), creatine kinase (Cat. No. S03024), and lactate dehydrogenase (Cat. No. S03034) assay kits. All measurements were performed according to the manufacturer's instructions. The reference intervals presented in Figure 2h were obtained from the package inserts provided with the corresponding assay kits and were used only as assay-specific contextual ranges.

2.4 Histological and immunohistochemical analysis

Heart, kidney, liver, and intestinal tissues were collected 4 weeks after phlorizin administration. The harvested tissues were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and cut into 4–6 µm sections. The sections were mounted on glass slides, deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) for histopathological evaluation. Periodic acid–Schiff (PAS) staining was also performed, with magenta staining indicating PAS-positive signals. PAS staining was used to evaluate the distribution and abundance of carbohydrate-containing substances, including glycoproteins and glycogen, thereby assisting in the assessment of intestinal mucosal barrier integrity and pathological injury. For immunohistochemistry (IHC), tissue sections were sequentially subjected to deparaffinization, rehydration, antigen retrieval, endogenous peroxidase blocking, and nonspecific binding blockade. The sections were then incubated overnight at 4 °C with primary antibodies against sodium-glucose cotransporter 1 (SGLT1; Proteintech, #30861-1-AP) or sodium-glucose cotransporter 2 (SGLT2; Proteintech, #24654-1-AP) at a dilution of 1:400. After incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature, immunoreactive signals were visualized using 3,3'-diaminobenzidine (DAB) substrate. The sections were counterstained with hematoxylin, dehydrated, mounted, and examined under a light microscope.

IHC staining was quantified by calculating the ratio of the positively stained area to the total tissue area using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

2.5 Untargeted metabolomics analysis

Untargeted metabolomics analysis was performed using an ultra-performance liquid chromatography–high-resolution mass spectrometry system consisting of a Thermo Vanquish Flex UPLC system coupled to a Thermo Q Exactive Plus mass spectrometer. Serum samples were thawed at 4 °C. For metabolite extraction, 100 µL of serum was mixed with 400 µL of ice-cold methanol. The mixture was incubated at −20 °C for 30 min to precipitate proteins and then centrifuged at $20,000 \times g$ for 10 min at 4 °C. The supernatant was transferred to a fresh vial for UPLC-HRMS analysis. Quality control samples were prepared by pooling equal aliquots of supernatant from all samples.

Chromatographic separation was performed using an ACQUITY UPLC HSS T3 column (100 mm $\times$ 2.1 mm, 1.8 µm; Waters). The mobile phase consisted of solvent A, containing 5 mmol/L ammonium acetate and 5 mmol/L acetic acid in water, and solvent B, acetonitrile. The flow rate was set at 0.3 mL/min, the injection volume was 4 µL, and the column temperature was maintained at 40 °C. The gradient elution program was as follows: 0–0.8 min, 2% B; 0.8–2.8 min, 2–70% B; 2.8–5.3 min, 70–90% B; 5.3–5.9 min, 90–98% B; 5.9–7.5 min, 98% B; 7.5–7.6 min, 98–2% B; and 7.6–10.0 min, 2% B.

Mass spectrometric detection was performed using a Q Exactive Plus high-resolution tandem mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples were analyzed in both positive and negative electrospray ionization modes. The electrospray ionization (ESI) source temperature was set at 350 °C. The spray voltage was +3500 V in positive ion mode and −3000 V in negative ion mode. The sweep gas pressure was set to 0 Arb, the auxiliary gas pressure to 10 Arb, and the sheath gas pressure to 35 Arb. Mass spectrometric data were acquired in full-scan and data-dependent acquisition (DDA) modes. For each acquisition cycle, the full-scan mass range was set to 70–1050 Da, with a resolution of 70,000 at m/z 200, an automatic gain control (AGC) target of 3×10^6 , and a maximum injection time of 100 ms. The top five precursor ions with signal intensities greater than 100,000 were selected for DDA-MS/MS analysis. The DDA resolution was set to 17,500 at m/z 200, with a maximum injection time of 50 ms and a dynamic exclusion time of 6 s. During data acquisition, one QC sample was injected after every 10 experimental samples to monitor system stability and correct batch-related systematic variation. For untargeted metabolomics data analysis, orthogonal partial least squares discriminant analysis (OPLS-DA) was performed to identify group-specific metabolic patterns.

Differential metabolites were identified using the following criteria: ratio ≥ 1.2 or ratio $\leq 1/1.2$, P value < 0.05 based on the Mann–Whitney U test, and variable importance in projection (VIP) > 1 . Pathway enrichment analysis was performed using MetaboAnalyst 5.0 based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, with significance assessed using the hypergeometric test.

2.6 Microbial DNA extraction and metagenomic sequencing

Total DNA was extracted from fecal samples using the Fecal Genome DNA Extraction Kit (AU46111-96, BioTeke, China) according to the manufacturer's instructions. DNA libraries were constructed using the TruSeq Nano DNA Library Preparation Kit Set (#FC-121–4001, Illumina, USA). Metagenomic libraries were sequenced on an Illumina NovaSeq 6000 platform with paired-end 150 bp reads at LC-Bio

Technology Co., Ltd. (Hangzhou, China). Raw sequencing reads were processed using fastp software (v0.23.4) to remove adaptor-contaminated reads, low-quality bases, and reads containing undetermined bases. The quality of the filtered reads was further assessed using fastp. Quality-filtered reads were aligned to the host genome using Bowtie2 (v2.2) to remove host-derived contamination. The remaining reads were then subjected to de novo assembly for each sample using MEGAHIT (v1.2.9). Coding sequences (CDSs) in the assembled contigs were predicted using MetaGeneMark (v3.26). CDSs from all samples were clustered using MMseqs2 (v15-6f452) to generate a nonredundant unigene catalog. Taxonomic annotation of the microbiota was performed using DIAMOND (v0.9.14) against the NR database. Differentially abundant taxa at different taxonomic levels were identified using the Wilcoxon rank-sum test, with significance defined as $P < 0.05$ and $|\log_2(\text{fold change})| > 1$. Microbial functional annotation was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

2.7 Integrated metagenomic and metabolomic analysis

Spearman correlation analysis was performed to assess pairwise associations among differentially abundant taxa, among differential metabolites, and between differentially abundant taxa and differential metabolites. Correlation pairs with Spearman's $\rho \geq 0.8$ and $P < 0.05$ were considered significant and selected for visualization. If significant pairs exceeded 20 in within-omics analyses or 50 in cross-omics analyses, only the top pairs with the smallest P values were displayed. Correlation heatmaps were used to visualize significant associations. Red indicated positive correlations, blue indicated negative correlations, and darker colors represented stronger correlations. Statistical significance was marked as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ for within-omics analyses, and as $*P < 0.05$ and $**P < 0.01$ for cross-omics analyses.

Further Mantel tests were performed to evaluate associations between differentially abundant taxa and differential metabolites. In the Mantel test heatmap, tile colors indicated the direction and strength of correlations, with red representing positive correlations approaching 1 and blue representing negative correlations approaching -1 . A correlation network was constructed using 10 differentially abundant taxa and 10 differential metabolites, in which edge color represented the P value and edge width represented the correlation coefficient, with thicker edges indicating stronger correlations.

2.8 Western Blotting

Kidney and intestinal samples were collected in a lysis buffer (10 mmol·L⁻¹ of Tris-HCl, pH 7.4, 100 mmol·L⁻¹ of NaCl, 1 mmol·L⁻¹ of EDTA, 1 mmol·L⁻¹ of EGTA, 1 mmol·L⁻¹ of NaF, 20 mmol·L⁻¹ of Na₄P₂O₇, 2 mmol·L⁻¹ of Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholic acid, 1 mmol·L⁻¹ of PMSF and 1 g·mL⁻¹ of aprotinin). The levels of SGLT1, SGLT2 and GAPDH were examined using Western blotting. Kidney and intestinal lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose membranes. Protein samples (30 µg) were separated via electrophoresis on 10% SDS polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were then incubated with primary antibodies for at least 8 h at 4 °C (SGLT1, 1:1000 dilution, Proteintech, #30861-1-AP; SGLT2, 1:1000

dilution, Proteintech, #24654-1-AP; GAPDH, 1:10,000 dilution, Cell Signaling Technology, #2118). After the membranes had been incubated with the corresponding HRP-conjugated secondary antibodies (ZSGB-BIO, Beijing, China), the protein bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, St. Burlington, MA, USA). Blotting images were obtained using a Syngene Gene Gnome-XRQ-NPC imager (Syngene Company, Cambridge, UK), and the proteins were quantified by calculating the grayscale value of each band using ImageJ 1.52 (Media Cybernetics, MD, USA).

2.9 Oral glucose tolerance test (OGTT) and 24 hours urinary glucose excretion (24-h UGE)

After 28 consecutive days of treatment, phlorizin administration was discontinued for 24 h to minimize potential acute effects on intestinal glucose absorption and renal glucose handling. Mice in the vehicle- and phlorizin-treated groups were then individually housed in metabolic cages for an additional 24 h to collect urine. During urine collection, mice had free access to food and water. Total urine volume was recorded, and urine samples were centrifuged to remove debris and stored at -80°C until analysis. Urinary glucose concentrations were measured using a commercial glucose assay kit (Cat. No. JL-T1044-96, Jianglai Biotechnology, China) according to the manufacturer's instructions. Twenty-four-hour urinary glucose excretion (24-h UGE) was calculated by multiplying the urinary glucose concentration by the total urine volume collected over 24 h.

After urine collection, mice were removed from the metabolic cages and fasted for 6 h with free access to water before undergoing an oral glucose tolerance test (OGTT). Baseline blood glucose was measured from tail-vein blood and recorded as the 0-min value. Mice were then orally administered glucose at 2 g/kg body weight. Blood glucose levels were measured at 15, 30, 60, 90, and 120 min after glucose administration using an Accu-Chek® Performa glucose meter (Roche Diagnostics GmbH, Mannheim, Germany). The area under the glucose curve (AUC) was calculated to assess glucose tolerance.

3. Result

3.1 Effects of Phlorizin on Serum Biochemistry and Organ Histology in Mice

Before exploring the mechanistic effects of phlorizin, we evaluated whether this intervention was systemically tolerated under physiological conditions. To this end, we measured serum biochemical indicators of hepatic, renal, and cardiac function and examined the histological integrity of major organs.

Serum biochemical analysis showed that 28 days of oral phlorizin administration at 30 mg/kg/day produced statistically significant increases in alanine aminotransferase (ALT) (Figure 2a, $P < 0.01$), aspartate aminotransferase (AST) (Figure 2b, $P < 0.05$), and blood urea nitrogen (BUN) (Figure 2c, $P < 0.01$) compared with vehicle group. In contrast, no significant between-group differences were observed in serum creatinine (CREA) (Figure 2d, $P > 0.05$), creatine kinase (CK) (Figure 2e, $P > 0.05$), lactate dehydrogenase (LDH) (Figure 2f, $P > 0.05$), or fasting blood glucose (Figure 2g, $P > 0.05$). The ALT, AST, and BUN values

remained within the assay-specific reference intervals provided in the corresponding manufacturer's package inserts (Figure 2h).

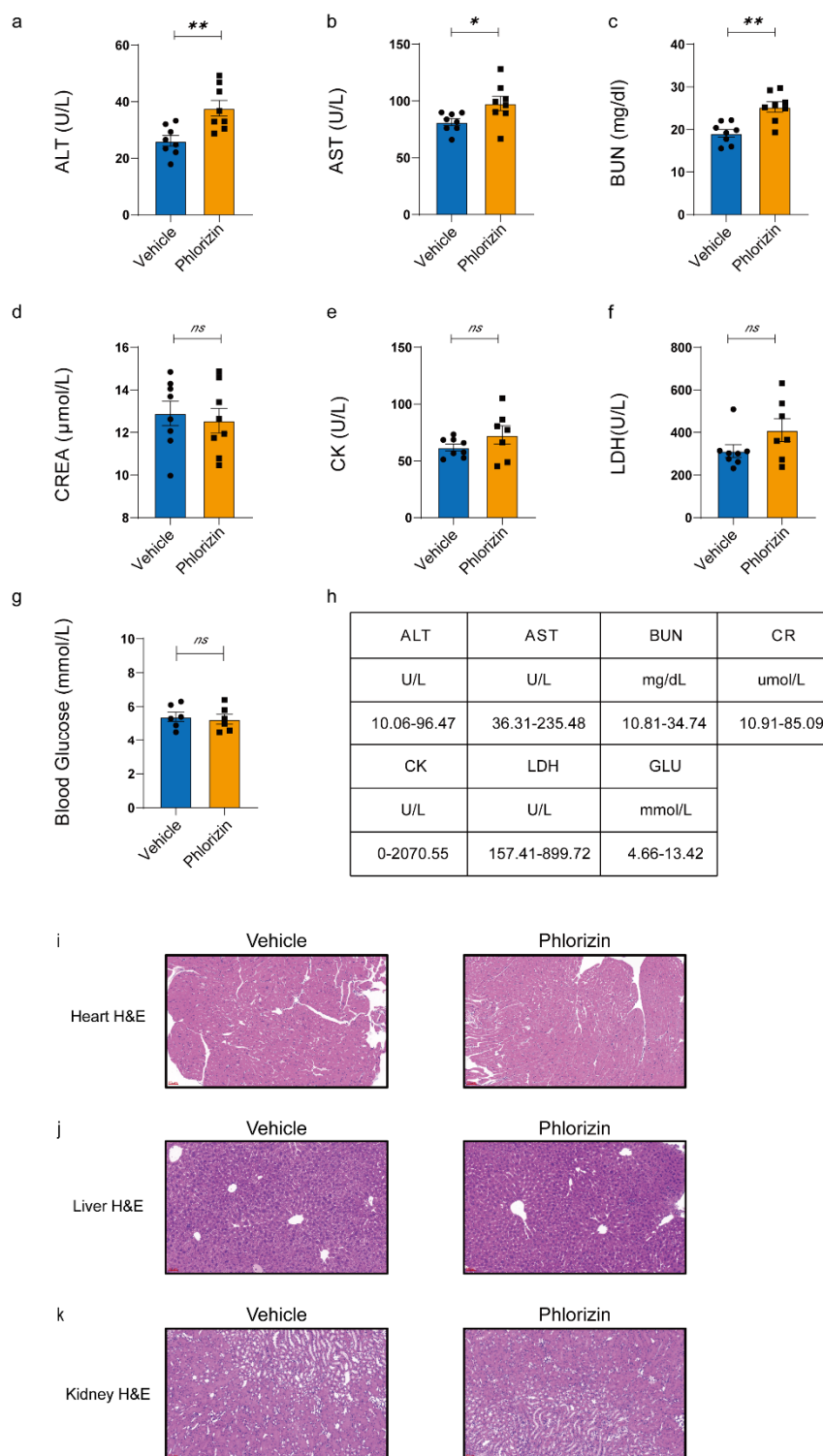


Figure 2. Systemic safety assessment of phlorizin in healthy C57BL/6J mice. (a–g) Serum biochemical markers of organ function: (a) alanine aminotransferase (ALT, n=8 per group); (b) aspartate aminotransferase (AST, n=8 per group); (c) blood urea nitrogen (BUN, n=8 per group); (d) creatinine (CREA, n=8 per group); (e) creatine kinase (CK, n=8 per group); (f) lactate dehydrogenase (LDH, n=8 per group); (g) fasting blood glucose (n=6 per group). (h) Normal reference ranges of serum biochemical parameters established for adult male C57BL/6J mice. (i–k) Representative hematoxylin and eosin (H&E)-stained histological sections of heart, liver, and kidney tissues (50 μ m, n=6 per group). Data are presented as mean $\pm$ SEM. Comparisons between two independent groups were performed using an unpaired two-tailed Student's t-test for normally distributed data or the Mann–Whitney U test for non-normally distributed data. * P < 0.05; ** P < 0.01; ns: not significant.

H&E staining showed no obvious histological abnormalities in the heart, liver, or kidney. Cardiac muscle fibers were orderly arranged, with no signs of degeneration, necrosis, or inflammatory cell infiltration (Figure 2i). Liver architecture was preserved, and no hepatocellular swelling, necrosis, steatosis, or portal inflammation was observed (Figure 2j). The kidneys also showed intact glomerular and tubular structures, with no evidence of tubular necrosis, glomerulosclerosis, or interstitial edema (Figure 2k).

Overall, 28 days of phlorizin treatment at 30 mg/kg/day caused no obvious histological damage to the heart, liver, or kidney. However, the increases in ALT and AST may suggest mild liver stress. The rise in BUN alone does not confirm kidney injury because serum creatinine and kidney histology were unchanged. Further studies with different doses, longer treatment periods, and more sensitive organ-specific markers are needed to better assess safety.

3.2 Phlorizin Preserves Intestinal Barrier Integrity Without Altering Tissue SGLT Protein Expression.

Intestinal barrier disruption is a well-established confounding factor in gut microbiota remodeling, while phlorizin, as a canonical SGLT inhibitor, exerts inhibitory activity on intestinal SGLT transporters. Given these considerations, we sought to determine whether phlorizin-treated mice exhibited signs of intestinal injury, and whether chronic administration at 30 mg/kg/day directly altered the tissue expression of SGLT1/2, thereby potentially contributing to the observed shifts in gut microbiota composition. To address these questions, we performed a systematic histological and immunohistochemical evaluation of the intestine and related target organs.

H&E staining of intestinal sections showed that phlorizin-treated mice maintained normal intestinal architecture, comparable to that of vehicle-treated controls. Villus height, crypt depth, and brush border morphology appeared normal, and no epithelial erosion, submucosal edema, or lamina propria inflammatory infiltration was observed in the small intestine or colon (Figure 3a). Periodic acid–Schiff (PAS) staining, which labels mucin glycoproteins secreted by goblet cells, showed no significant difference in PAS-positive mucin area between groups (Figure 3b–c, $P > 0.05$). This result indicates that phlorizin did not compromise mucus barrier production or secretory capacity. Immunohistochemical staining further showed that phlorizin did not alter the expression or tissue localization of major SGLT protein isoforms in their principal target organs. Quantitative analysis revealed no significant differences in the relative positive staining areas of intestinal SGLT1 (Figure 3d–e, $P > 0.05$), renal SGLT2 (Figure 3f–g, $P > 0.05$), or cardiac SGLT1 (Figure 3h–i, $P > 0.05$) between the two groups.

Furthermore, we performed Western blot analysis of kidney and intestinal tissue homogenates. Renal SGLT1 and SGLT2 levels were comparable between vehicle and phlorizin groups (Figure 3j). Intestinal SGLT1 levels were also comparable between groups (Figure 3k). Intestinal SGLT2 signal was minimal, consistent with its predominantly renal expression. Densitometric quantification confirmed no significant group differences in renal SGLT1 (Figure 3l, $P > 0.05$), renal SGLT2 (Figure 3m, $P > 0.05$), or intestinal SGLT1 (Figure 3n, $P > 0.05$) protein levels.

The results indicate that, at the dose employed in our study, phlorizin was not associated with any significant histopathological damage to the intestine, nor were any detectable changes in SGLT protein expression observed in the tissues examined.

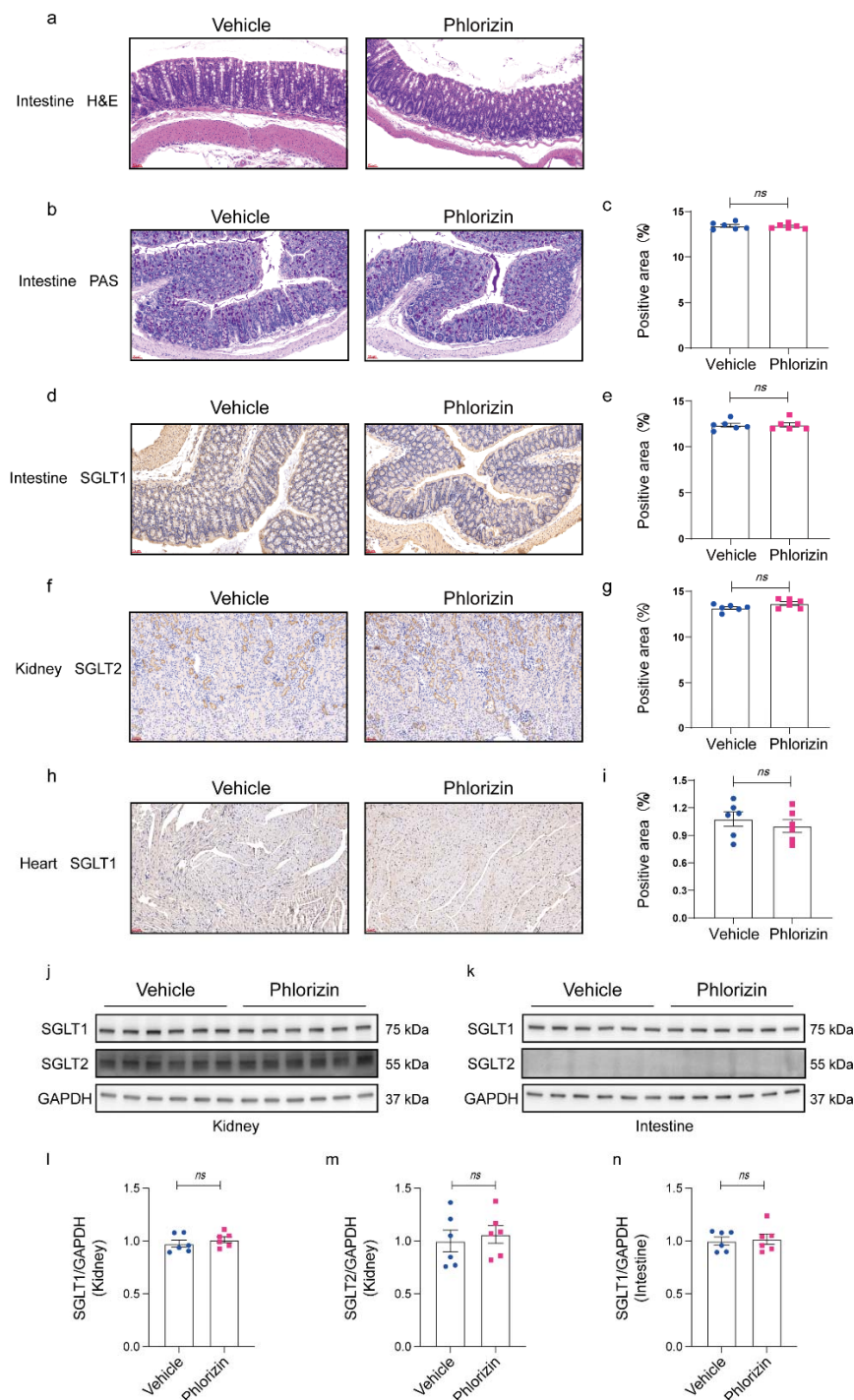


Figure 3. Intestinal barrier integrity and SGLT protein expression in phlorizin-treated mice. (a) Representative H&E-stained sections of small intestinal and colonic tissues demonstrating villus–crypt architecture (50 μ m). (b–c) Periodic acid–Schiff (PAS) staining of intestinal goblet cells and quantitative analysis of PAS-positive mucin-secreting area per unit field. (d–i) Immunohistochemical detection and semi-quantitative analysis of SGLT1 protein expression in the intestinal epithelium (d–e), SGLT2 protein expression in the renal proximal tubules (f–g), and SGLT1 protein expression in cardiac tissue (h–i). (j, k) Representative Western blots of SGLT1, SGLT2, and GAPDH in kidney (j) and intestine (k) tissues from vehicle and phlorizin groups. (l–n) Quantitative analysis of renal SGLT1 (l), renal SGLT2 (m), and intestinal SGLT1 (n) protein levels. Data are presented as mean $\pm$ SEM (n=6 per group). Comparisons between two independent groups were performed using an unpaired two-tailed Student's t-test for normally distributed data or the Mann–Whitney U test for non-normally distributed data. ns: not significant.

3.3 Indirect Assessment of SGLT-Related Glucose Handling Following Chronic Low-Dose Oral Phlorizin Administration

Protein expression and functional activity are distinct parameters, and unchanged protein expression does not necessarily indicate unchanged transporter activity. We therefore performed indirect functional assessments of SGLT-related glucose handling in vehicle- and phlorizin-treated mice, including an oral glucose tolerance test (OGTT) and measurement of urinary glucose excretion.

For the OGTT, mice were fasted for 6 h and then orally administered glucose at 2 g/kg body weight. Blood glucose concentrations peaked at 15 min in both groups and subsequently declined toward baseline over the 120-min observation period (Figure 4a). Glucose excursion profiles were comparable between groups, and the area under the curve (AUC) did not differ significantly (Figure 4b, $P > 0.05$). For urine collection, phlorizin treatment was discontinued for 24 h, after which mice were placed individually in metabolic cages, and urine was collected for an additional 24 h. Urinary glucose concentration did not differ significantly between the vehicle- and phlorizin-treated groups (Figure 4c, $P > 0.05$). However, total 24-h urinary glucose excretion was modestly but significantly higher in the phlorizin-treated group than in the vehicle group (Figure 4d, $P < 0.05$).

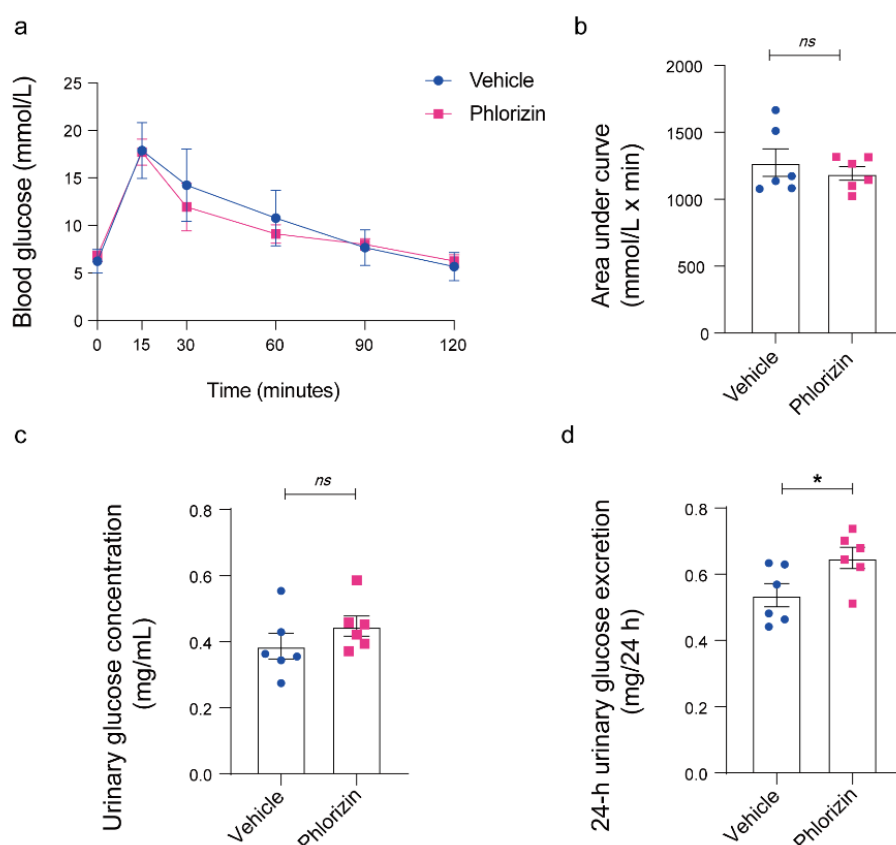


Figure 4. Glucose homeostasis and 24-hour urinary glucose excretion are preserved following chronic low-dose oral phlorizin administration. (a) Blood glucose concentrations at the indicated time points following an oral glucose challenge (2 g/kg) in vehicle- and phlorizin-treated mice. (b) Area under the glucose curve (AUC). (c) Urinary glucose concentration in 24-h urine samples collected from vehicle- and phlorizin-treated mice. (d) Total 24-h urinary glucose excretion, calculated by multiplying urinary glucose concentration by the corresponding 24-h urine volume. Data are presented as mean $\pm$ SEM ($n=6$ per group). Comparisons between two independent groups were performed using an unpaired two-tailed Student's *t*-test for normally distributed data or the Mann–Whitney *U* test for non-normally distributed data. ns: not significant.

Overall, chronic low-dose oral phlorizin treatment was not associated with detectable changes in glucose tolerance or urinary glucose concentration during the post-treatment assessment period. However, total 24-h urinary glucose excretion was modestly but significantly higher in the phlorizin-treated group. These indirect findings do not provide clear evidence of sustained renal SGLT inhibition during the post-treatment period.

3.4 Phlorizin Is Associated With Alterations in Gut Microbiota Diversity, Community Architecture, and Functional Metabolic Potential

To investigate gut microbial changes associated with chronic low-dose oral phlorizin administration, we performed shotgun metagenomic sequencing to systematically characterize alterations in microbial composition, community structure, and predicted functional potential.

Alpha diversity analysis showed that microbial richness did not differ between the two groups. The Chao1 richness estimator was comparable between the phlorizin and vehicle groups (Figure 5a). In contrast, microbial diversity was higher in the phlorizin group. Both the Shannon index (Figure 5b, $P < 0.01$) and the Simpson index (Figure 5c, $P < 0.01$) were significantly higher in the phlorizin group. Beta diversity was assessed using Bray–Curtis dissimilarity. Gut microbial community structure differed significantly between the two groups (ANOSIM: $R = 0.4722$, $p = 0.015$; Figure 5d). PCoA1 and PCoA2 explained 45.93% and 25.12% of the total variance, respectively (Figure 5e). These results indicate that phlorizin administration was associated with a shift in overall microbial community composition. *Bacteroidota* and *Bacillota* were the predominant phyla in both groups. Their relative abundance profiles were broadly similar at the phylum level (Figure 5f). More pronounced differences were observed at the genus and species levels (Figure 5g–h). LEfSe analysis showed enrichment of taxa belonging to *Akkermansia*, *Alistipes*, and *Ligilactobacillus* in the phlorizin group. In contrast, several *Bacteroides*, *Duncaniella*, and *Muribaculum* taxa were enriched in the vehicle group (LDA score > 2 ; Figure 5i).

Functional metagenomic annotation was performed using the Reporter score algorithm. Several metabolic pathways differed in their predicted representation between the two groups. Lysine degradation showed the strongest positive Reporter score. Glycerolipid metabolism, aminoacyl-tRNA biosynthesis, carbon metabolism, and several other pathways also showed positive Reporter scores (Figure 5j). These findings suggest differences in the encoded metabolic potential of the microbial community. Spearman correlation analysis showed that several phlorizin-enriched taxa were positively correlated with one another. These taxa included *Akkermansia muciniphila* and several *Alistipes* species. They were also negatively correlated with several taxa enriched in the vehicle group (Figure 5k). These associations indicate coordinated variation among microbial taxa but do not establish direct ecological or functional interactions.

Collectively, these data indicate that chronic low-dose phlorizin administration was associated with higher gut microbial diversity, shifts in community composition and taxonomic profiles, and differences in predicted metabolic potential. The functional and physiological significance of these microbial changes remains to be experimentally determined.

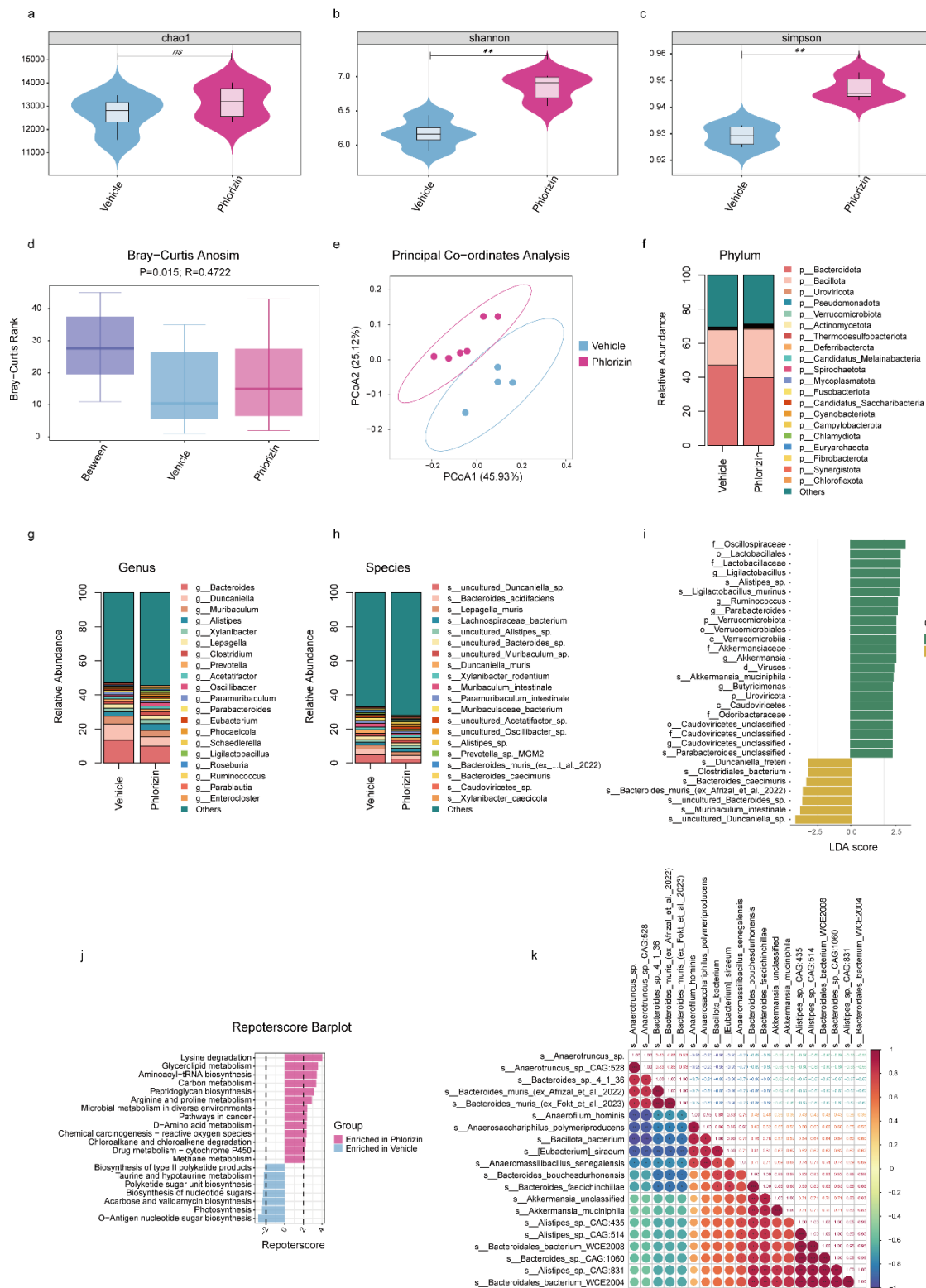


Figure 5. Effects of phlorizin on gut microbiota diversity, community structure, taxonomic composition, and functional potential. (a–c) Alpha diversity indices: (a) Chao1 richness estimator, (b) Shannon entropy index, and (c) Simpson diversity index.

(d–e) Beta diversity analysis: (d) Bray–Curtis dissimilarity boxplot with ANOSIM test and (e) principal coordinates analysis (PCoA) plot. (f–h) Relative taxonomic abundance at the (f) phylum, (g) genus, and (h) species levels. (i) Linear discriminant analysis effect size (LEfSe) cladogram showing differentially abundant taxa (LDA score > 2). (j) Reporter score analysis of differentially enriched microbial functional pathways. (k) Spearman correlation heatmap showing pairwise associations among differentially abundant taxa. Correlation coefficients are shown in the upper triangle, and colored circles in the lower triangle indicate correlation direction and strength. Red indicates positive correlations, blue indicates negative correlations, and darker colors indicate stronger correlations. Alpha diversity indices were compared between groups using the Wilcoxon rank-sum test. Spearman correlations were evaluated using two-tailed Spearman correlation analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: not significant. (Vehicle: 6 vs Phlorizin: 4)

3.5 Phlorizin Administration Is Associated with Alterations in the Serum Metabolome and Arachidonic Acid-Related Metabolites

Given the differences in gut microbial composition and predicted functional potential associated with phlorizin administration, we next characterized serum metabolic profiles using untargeted metabolomics.

The PLS-DA score plot showed separation between the vehicle and phlorizin groups. Components 1 and 2 accounted for 32.02% and 12.57% of the represented variance, respectively (Figure 6a). Using thresholds of $|\log_2(\text{fold change})| > 1$ and $P < 0.05$, 186 differential metabolites were identified. Of these, 104 showed higher relative abundance and 82 showed lower relative abundance in the phlorizin group (Figure 6b). KEGG enrichment analysis identified arachidonic acid metabolism as the most significantly enriched pathway among the differential metabolites (Figure 6c).

Hierarchical clustering showed group-associated differences in several metabolites. Several eicosanoid- and PUFA-related metabolites showed lower normalized abundances in the phlorizin group, including 12-HEPE, 17-HDoHE, and 6-trans-12-epi-leukotriene B4 (Figure 6d). Spearman correlation analysis further showed positive correlations among several differential metabolites, including 12-HEPE and 12-HETE (Figure 6e).

Collectively, chronic low-dose phlorizin administration was associated with distinct serum metabolomic profiles and significant enrichment of arachidonic acid metabolism among the differential metabolites. Although the observed metabolite correlations suggest coordinated variation, the physiological and inflammatory relevance of these changes remains to be established experimentally.

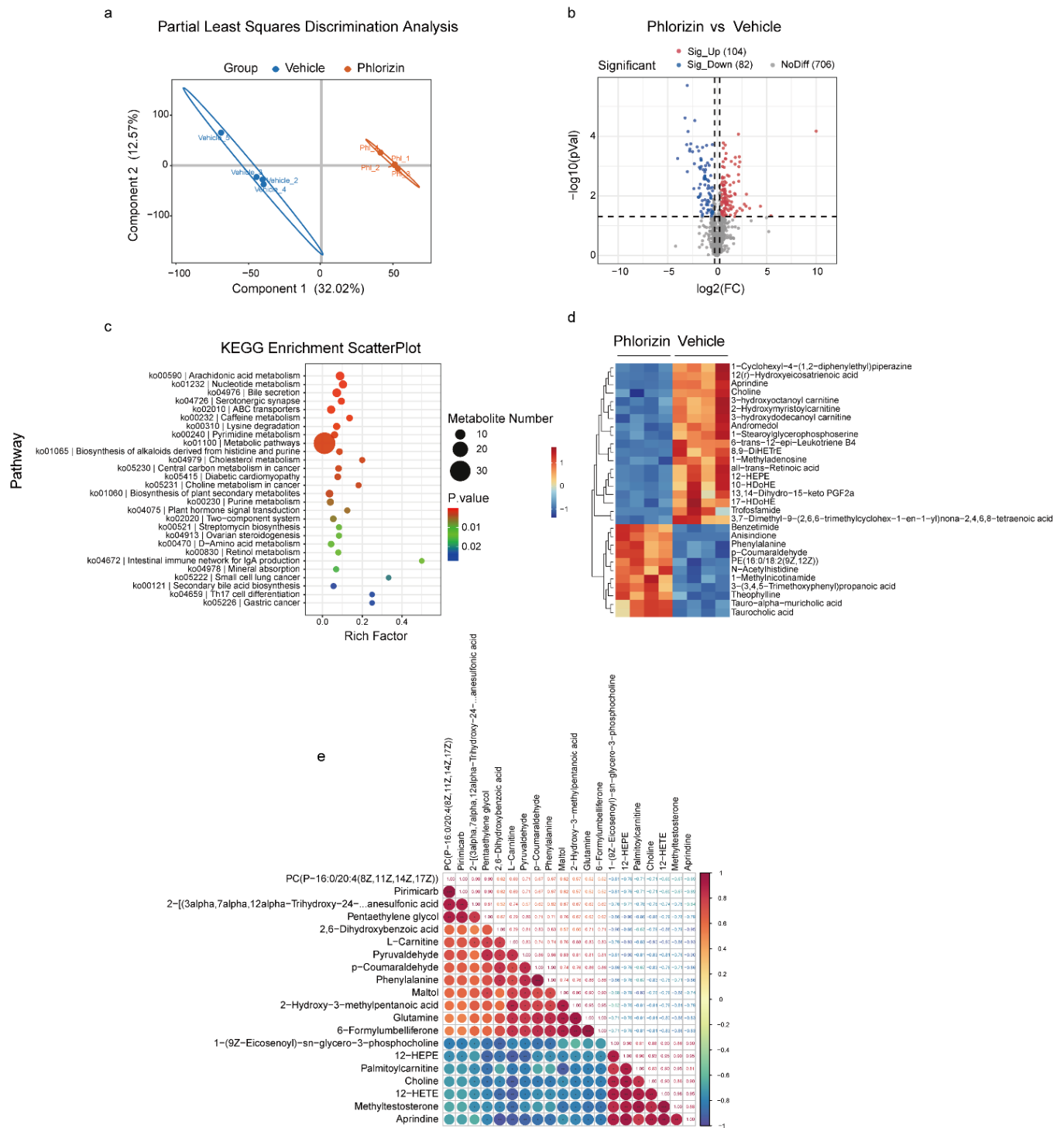


Figure 6. Effects of phlorizin on the host serum metabolome. (a) Partial least squares discriminant analysis (PLS-DA) score plot illustrating the global separation of serum metabolic profiles between vehicle and phlorizin groups ($n=4$ per group). **(b)** Volcano plot of differential serum metabolites (threshold: $|\log_2\text{FC}| > 1$ and $P < 0.05$); red and blue dots represent upregulated and downregulated metabolites in the phlorizin group, respectively. **(c)** KEGG pathway enrichment scatter plot of differentially abundant metabolites; bubble size represents the number of enriched metabolites per pathway; color intensity reflects statistical significance. **(d)** Hierarchical clustering heatmap of the top 50 differential metabolites; color scale represents row-normalized z-scores. **(e)** Spearman correlation heatmap of key differential metabolites. Correlation coefficients are shown in the upper triangle, and colored circles in the lower triangle indicate the direction and strength of correlations. Red represents positive correlations, blue represents negative correlations, and darker colors indicate stronger correlations. Statistical significance was determined using two-tailed Spearman correlation analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: not significant.

3.6 Integrative Correlation Analysis Reveals Covariation Between Gut Microbial Taxa and Serum Metabolites

To investigate the potential linkage between phlorizin-associated gut microbiota alterations and the observed systemic metabolic changes, we performed integrative multi-omics correlation analysis coupling differential microbial taxa with differential serum metabolites.

Mantel analysis identified multiple positive associations between feature-specific distance matrices across samples (Figure 7a). Notable associations included *Alistipes* sp. with 17-HDoHE (Mantel's $r = 0.762$, $p = 0.001$) and 12-HEPE ($r = 0.661$, $p = 0.003$), as well as an unclassified bacterium within the order *Clostridiales* with 12-HETE ($r = 0.810$, $p = 0.007$). In the metabolite–metabolite Spearman correlation matrix, 12-HEPE, 12-HETE, and 17-HDoHE showed positive pairwise correlations.

The Spearman correlation heatmap further revealed structured relationships between individual microbial taxa and serum metabolite features (Figure 7b). Several *Bacteroides*-related taxa and one *Muribaculaceae*-related taxon showed positive correlations with a metabolite cluster containing 12-HEPE, 12-HETE, 17-HDoHE, and other lipid-related metabolites. In contrast, *Akkermansia muciniphila*, *Alistipes* sp., and several *Clostridium*- and *Lactobacillus*-related taxa generally showed negative correlations with this cluster.

Overall, these exploratory analyses suggested coordinated variation between selected gut microbial taxa and serum metabolite features. Mantel analysis only shows similarity in sample-level distance patterns. It does not indicate whether taxon abundance is positively or negatively associated with metabolite abundance. These associations also do not prove direct microbial–metabolite interactions, microbial regulation of host lipid metabolism, or causality. Further targeted and mechanistic studies are needed.

4. Discussion

In this study, shotgun metagenomics and untargeted serum metabolomics were used to characterize changes associated with chronic low-dose phlorizin intake in healthy SPF C57BL/6J mice. The use of healthy animals reduced confounding from pre-existing disease and allowed these changes to be examined under relatively intact physiological conditions. Oral administration of phlorizin at 30 mg/kg/day for 28 days caused no overt intestinal or major-organ injury and was not associated with detectable changes in tissue SGLT protein expression or overall glucose tolerance, although 24-h urinary glucose excretion increased modestly after the washout period. Phlorizin intake was also associated with higher gut microbial diversity, altered community composition, and changes in serum metabolites, particularly those related to arachidonic acid and other PUFA-derived lipid pathways. Together, these findings indicate that chronic low-dose phlorizin exposure in healthy mice was accompanied by coordinated microbial and metabolic changes that were not readily explained by changes in SGLT protein expression; however, whether these effects involve SGLT-independent mechanisms remains to be determined.

We next examined whether the microbial changes could be explained by tissue injury or impaired intestinal integrity. Histopathological examination showed no overt injury in the intestine, heart, liver, or kidney. Villus–crypt architecture was preserved, and the PAS-positive mucin area was comparable between groups. Because mucus barrier disruption can alter microbial selection pressures and the spatial organization of host–microbiota interactions^[26], the observed changes in microbial community structure were unlikely to result primarily from overt intestinal damage. However, histology and PAS staining alone do not fully assess intestinal barrier function. Subtle changes in intestinal permeability, tight-junction organization, mucus composition, or epithelial turnover cannot be ruled out and require further investigation. Serum creatinine, creatine kinase, lactate dehydrogenase, and fasting blood glucose were unchanged, with no clear indication of overt renal dysfunction, cardiac injury, or altered fasting glucose regulation. In contrast, serum ALT, AST, and BUN were significantly increased. Although these values remained within the assay reference ranges and were not accompanied by histopathological abnormalities, the increases may still be biologically relevant. They could reflect mild physiological adaptation or early subclinical stress, although the present data do not allow these possibilities to be distinguished. Further safety studies using additional doses, longer treatment periods, and more sensitive organ-specific endpoints are therefore needed.

Phlorizin is best known as an inhibitor of SGLT-mediated glucose transport^[27]. But transporter abundance and transporter activity aren't the same thing — functional inhibition can occur without any detectable change in total protein expression^[28]. SGLT1 participates in intestinal glucose uptake and glucose-stimulated incretin secretion^[29], while renal SGLT1 and SGLT2 contribute to the reabsorption of filtered glucose^[30]. In this study, chronic low-dose phlorizin was not associated with detectable changes in intestinal SGLT1, renal SGLT1/2, or cardiac SGLT1 protein expression, suggesting that under these conditions, phlorizin did not significantly regulate SGLT protein abundance in the tissues examined. OGTT and urinary glucose measurements were performed after a 24-hour washout period and served as indirect

indicators of SGLT-related glucose handling^[31]. Glucose excursion curves and AUC values were comparable between groups, indicating no measurable change in overall glucose tolerance, and urinary glucose concentration also did not differ between groups. Total 24-hour urinary glucose excretion was, however, modestly higher in the phlorizin group. Because total urinary glucose excretion depends on several factors — urine volume, individual variability, and metabolic-cage exposure, among others^[32] — this finding alone does not demonstrate persistent inhibition of renal SGLT activity. Taken together, neither the protein expression data nor the indirect functional data show a clear or sustained pattern of SGLT inhibition following phlorizin treatment. The accompanying microbiota and serum metabolome changes therefore cannot simply be attributed to SGLT downregulation or suppressed SGLT functional activity. This does not rule out transient transporter inhibition shortly after dosing, nor does it establish that the observed effects are independent of SGLT activity. Distinguishing between these possibilities will require direct measurement of fractional glucose excretion, renal glucose threshold, intestinal glucose absorption, and transporter kinetics.

Previous studies have linked oral phlorizin to microbiota alterations in diverse pathological settings, including obesity-associated endotoxemia^[10], insulin resistance^[33], high-fat diet-induced redox imbalance^[34], D-galactose aging^[23], colitis^[29], maternal metabolic disorders^[35], viral infection^[11], and neurobehavioral abnormalities^[25]. In vitro fermentation studies have also suggested that phlorizin may modulate microbial communities in a host-independent manner^[36]. In disease models, shifts in the microbiota may be secondary consequences of reduced inflammation, improved barrier function, or changes in host metabolism rather than direct responses to phlorizin exposure^[37] [38]. By using healthy SPF mice, this study mitigated some confounding factors associated with diseases, allowing for an examination of phlorizin-related changes under relatively intact physiological conditions.

Phlorizin intake was associated with higher Shannon and Simpson diversity indices, while Chao1-estimated richness stayed about the same — pointing to a shift in community evenness more than in the number of taxa present. Greater community evenness has been associated with greater functional stability in experimental microbial ecosystems^[39]; however, diversity metrics alone do not establish a healthier or more stable gut microbiota, as their interpretation depends on community composition, functional context, and temporal dynamics^[40]. At the taxonomic level, phlorizin-treated mice showed enrichment of *Akkermansia*, *Alistipes*, and *Ligilactobacillus*, alongside lower relative abundances of several *Bacteroides*, *Duncaniella*, and *Muribaculum* taxa. *Akkermansia muciniphila* has been linked to epithelial barrier support and metabolic homeostasis^[36], including in a human supplementation study^[38], and some *Alistipes* species have been associated with short-chain fatty acid production and reduced inflammation in certain settings^[41]. These associations vary by species, strain, diet, and host background, so the present findings are best read as phlorizin-associated taxonomic shifts — not proof that the community became more beneficial overall.

In vitro fermentation work suggests phlorizin can alter microbial composition and metabolites even without a host^[33]. Combined with our findings in healthy mice, this raises the possibility that phlorizin or its metabolites interact with the gut microbiota without requiring the correction of pre-existing disease or SGLT

expression changes. But we did not test this directly: intestinal phlorizin/metabolite concentrations were not measured, and host-driven changes in nutrient availability, bile acids, motility, or immune signaling may also have influenced the microbial shifts. Functional annotation pointed to differences in lysine degradation, glycerolipid metabolism, aminoacyl-tRNA biosynthesis, and carbon metabolism, indicating that the two microbial communities differ in encoded metabolic potential as well as taxonomy. This reflects gene presence, not actual transcription, translation, or flux, so these pathways are candidates for follow-up rather than direct evidence of altered microbial activity.

Untargeted metabolomics showed group-level differences in circulating metabolites, with arachidonic acid (AA) metabolism as the most enriched pathway. AA feeds into several bioactive lipid mediators — prostaglandins, leukotrienes, HETEs — via cyclooxygenase, lipoxygenase, and cytochrome P450 routes^[26]. Several eicosanoid- and PUFA-related metabolites, including 12-HEPE, 17-HDoHE, and 6-trans-12-epi-leukotriene B4, showed lower normalized abundance in phlorizin-treated mice. Some of these mediators have known roles in inflammation and metabolism: 12-HETE is linked to macrophage activation, leukocyte adhesion, β -cell dysfunction, and insulin resistance^[42], and LTB4 is a potent leukocyte chemoattractant tied to inflammatory bowel disease, metabolic syndrome, and atherosclerosis^[43]. Still, this doesn't show phlorizin is anti-inflammatory — the mice were healthy, no inflammatory challenge was given, and we didn't measure inflammatory endpoints directly. Not all of these metabolites are simply pro-inflammatory, either. 17-HDoHE, a DHA-derived intermediate, feeds into specialized pro-resolving mediators^[44], so its lower abundance isn't necessarily good or bad. Confirming metabolite identities and their physiological relevance will require targeted lipidomics with authentic standards, plus measurement of downstream pro-inflammatory and pro-resolving mediators.

The integrated multi-omics analysis showed that the composition of gut microbiota was closely associated with the serum metabolomic profile. Several potential mechanisms may connect gut microbial activity to lipid metabolism: short-chain fatty acids could influence inflammatory transcription processes and enzymes related to AA metabolism^[15]; microbial bile acids may signal through nuclear receptors and G-protein-coupled receptors involved in lipid regulation^[45]; additionally, the effects of the microbiota on fatty-acid desaturation might alter AA availability and downstream mediator production^[46]. However, as we did not directly measure SCFAs, secondary bile acids, FADS1 expression, intestinal exposure to phlorizin, or downstream pro-resolving mediators, these proposed mechanisms remain speculative. Therefore, the observed covariation between the microbiome and metabolome warrants further investigation to explore the potential link between gut microbial remodeling and AA-related lipid metabolism.

A few limitations are worth noting. The associations we observed among gut microbiota, serum metabolome, and multi-omics features are correlational, so the data cannot establish whether microbiota remodeling is necessary for the metabolomic changes, or whether the two are simply independent correlates of phlorizin exposure. Clarifying this would require interventional work — fecal microbiota transplantation, microbiota depletion, gnotobiotic mice, or colonization with specific candidate taxa. The experimental

design also has some inherent constraints. Only male C57BL/6J mice were examined, at a single dose and duration, and the sample size was modest; together, these limit the power to detect subtle associations and leave open whether the findings would hold in females, other strains, different doses, or longer treatment. Serum ALT, AST, and BUN were significantly associated with phlorizin treatment; although values stayed within reference ranges and H&E staining showed no overt tissue changes, a mild subclinical hepatic or renal association cannot be excluded, and whether this scales with dose remains untested. SGLT function was assessed only indirectly (protein expression, OGTT, urinary glucose), without capturing transporter kinetics or intestinal/systemic phlorizin exposure, so the microbiota changes cannot be confidently linked to direct gut luminal exposure. Intestinal barrier integrity relied on histology and PAS staining alone, and the omics data reflect predicted functional potential and relative abundance rather than confirmed activity, so key metabolite identities require targeted validation. Finally, as the mice were healthy and not challenged with inflammation, and pro-resolving mediators were not measured, any anti-inflammatory relevance of these changes remains to be tested in disease models.

In conclusion, chronic low-dose phlorizin intake was well tolerated in healthy mice and was associated with changes in gut microbiota and the serum metabolome, particularly regarding AA- and other PUFA-derived lipid pathways, without indications of intestinal injury or detectable changes in SGLT protein expression. Indirect functional assays did not reveal a clear pattern of SGLT inhibition, suggesting that these effects may not be solely attributed to SGLT downregulation. The observed covariation between microbiota and metabolome may indicate a possible link between gut microbial remodeling and arachidonic acid metabolism, but this requires further investigation. These findings offer a basis for future studies exploring the interactions between dietary polyphenols, host physiology, and microbiota.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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