



Comprehensive analysis of gut microbiome and anthocyanin metabolomics uncover effects of long-term mulberry and chokeberry anthocyanin intake in a C57BL/6J mouse model

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ABSTRACT

The health benefits of anthocyanins arise from direct absorption and gut microbial metabolism, which can vary with the glycoside structure depending on the microbial enzyme affinities. In this study, we investigated the effects of mulberry and chokeberry anthocyanin intake on anthocyanin-derived metabolites and gut microbiota composition using an *in vivo* model. Mulberry and chokeberry are rich in cyanidin-3-O-glucoside and cyanidin-3-O-galactoside, respectively, and the composition of anthocyanin metabolites in plasma and feces depends on the glycoside moiety. Vanillin sulfate and methyl gallic acid were detected in feces after mulberry intake, whereas homovanillic acid sulfate was identified following chokeberry intake. The plasma levels of protocatechuic and hippuric acid were also higher in the mulberry group. Long-term cyanidin glycoside intake increased gut propionic and butyric acid levels, which could potentially contribute to a favorable metabolic and colonic environment via gut microbiota modulation. The intake of both extracts increased taxa associated with beneficial health effects.

1. Introduction

Anthocyanins, a subclass of flavonoids, are natural pigments found in various plants, particularly in berries. They occur as glycosides of flavylium salts, with structural variations depending on their hydroxylation and methylation patterns (Faria et al., 2014). The composition and glycosidic forms of anthocyanins vary by berry type. Our recent study showed that mulberry fruit (*Morus alba* L.) contains cyanidin-3-O-glucoside, and chokeberry fruit (*Aronia melanocarpa*) contains cyanidin-3-O-galactoside as the primary anthocyanin (Kim et al., 2020; Kim & Lee, 2017). Mulberry is a rich source of anthocyanins, which have been associated with anti-inflammatory effects and

potential health benefits (Zhang & Tsao, 2016). Anthocyanins (i.e., petunidin glycoside) promote an anti-inflammatory effect by inhibiting secretion of pro-inflammatory cytokines through blocking nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), which may help restore mucosal homeostasis in inflamed intestines (Zhang et al., 2017).

The health benefits of anthocyanins may arise from the absorption of intact anthocyanins and/or anthocyanin metabolites in the human body. However, anthocyanin bioavailability ranges from 0.26 to 1.8% (Borges et al., 2007; Edirisinghe et al., 2011; Marczylo et al., 2009). Therefore, investigating the anthocyanin metabolite composition after anthocyanin consumption is important. The low bioavailability of anthocyanins and

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their low recovery from feces and urine can be attributed to the degradation of anthocyanins in the colon. Various bacteria in the colon, such as *Bacteroides* spp., *Enterococcus casseliflavus*, *Eubacterium* spp. and *Clostridium* spp., degrade anthocyanins into phenolic acids using enzymes such as β -glucosidase, α -galactosidase, and α -rhamnosidase (Eker et al., 2019; Rowland et al., 2018). The gut microbiota hydrolyze anthocyanins into anthocyanin aglycones by cleaving sugar moieties using microbial enzymes. Anthocyanin aglycones are subsequently catabolized by the gut microbiota into various phenolic acids (e.g., protocatechuic acid, vanillic acid) (Chen et al., 2017). However, the composition of anthocyanin metabolites may vary depending on the glycoside moiety, as differences in microbial glycosidase specificity can influence the extent and rate of deglycosylation, thereby affecting downstream microbial catabolism and the resulting phenolic acid profiles.

Anthocyanin intake can significantly impact gut microbiome composition, depending on the anthocyanin glycosides consumed. In a previous study, cyanidin and peonidin derivatives induced the growth of *Bifidobacterium* and *Lactobacillus* spp. and suppressed the growth of *Bacteroides* and *Clostridium histolyticum* (Zhang et al., 2016). Cyanidin-3-O-glucoside and delphinidin-3-O-glucoside induced the growth of *Bifidobacteria* and *Lactobacilli* (Flores et al., 2015). *Clostridium histolyticum* is one of the most common pathogenic microbes associated with inflammatory bowel disease (Holmes et al., 2011). While malvidin-based anthocyanins increase the abundance of *Lactobacillus*, *Helicobacter*, *Alistipes*, and *Bacteroides*, they reduce the relative abundance of *Alloprevotella* in the gut microbiota of oxidatively stressed mice (Zheng et al., 2021).

Short-chain fatty acids (SCFAs), a group of fatty acids with carbon chain lengths of fewer than six atoms produced via fermentation by the gut microbiota, have been demonstrated to regulate the intestinal immune system and enhance the integrity of the intestinal barrier (Morrison & Preston, 2016; Tao et al., 2017). Blueberry and blackberry anthocyanins were shown to reduce elevated SCFA levels in mice fed a high-fat diet, restoring them toward normal levels (Du et al., 2023). Anthocyanin intake may influence the regulation of SCFA production; however, research on the relationship between SCFA production and gut microbiome is lacking.

To elucidate the mechanisms underlying the health benefits of anthocyanins and their metabolites, interactions among anthocyanins, their metabolites, and the gut microbiota should be considered. Furthermore, the relationships between glycoside-dependent differences in metabolite profiles, gut microbiota, and potential health benefits of anthocyanins remain unclear. In this study, we investigated the *in vivo* metabolite profiles of purified anthocyanin-rich extracts obtained from mulberry and chokeberry, which are characterized by different predominant cyanidin glycosides (glucoside vs. galactoside). In addition, the impact of anthocyanin metabolites on the gut microbiota structure and their potential role in modulating microbial dynamics were investigated.

2. Materials and methods

2.1. Chemicals and reagents

Authentic anthocyanin standards (cyanidin-3-O-glucoside, $\geq 96\%$; cyanidin-3-O-galactoside, $\geq 97\%$; cyanidin-3-O-rutinoside, $\geq 96\%$; pelargonidin-3-O-glucoside, $\geq 96\%$; cyanidin aglycone, $\geq 96\%$) were purchased from Extrasynthese (Genay, France). SCFA standards (acetic, propionic, isobutyric, butyric, 2-methylbutyric, isovaleric, and penta-noic acids) and protocatechuic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Isotope-labeled standards (acetic acid- d_4 , propionic acid- d_6 , butyric acid- d_7 , and cinnamic acid- d_5) were purchased from CDN Isotopes (Quebec, Canada). Nanopure water was prepared using a Milli-Q Direct 8 system (Merck Millipore, Billerica, MA, USA).

2.2. Purification of anthocyanins from mulberry and chokeberry fruit for *in vivo* study

Mulberry (*Morus Alba* L.) and chokeberry (*Aronia melanocarpa*) fruits were harvested in 2022 from commercial farms in Buan, Jeollabuk-do, Korea, and Geochang, Gyeongsangnam-do, Korea, respectively. The harvested mulberries and chokeberries were freeze-dried, and anthocyanins were extracted as previously described, with minor modifications (Kim et al., 2020). After grinding, anthocyanins were extracted from mulberry and chokeberry powders using ethanol at a 1:20 (w/v) ratio. The mixture was centrifuged at $4000\times g$ for 15 min at 4°C . The supernatant was collected, and the residue was re-extracted using the same process. The supernatants were filtered using filter paper (Whatman No. 41, Maidstone, UK). The filtrates were concentrated using a rotary evaporator (n-1300, EYELA, Tokyo, Japan). The concentrates were then purified via a Sephadex LH20 column. The anthocyanins in the extracts were eluted with 0.1% acetic acid in 70% aqueous ethanol solution. The eluents were dried using a rotary evaporator and dehydrated via lyophilization. Dried extracts were passed through a Sep-Pak C18 solid-phase extraction (SPE) cartridge (Waters, Milford, MA, USA). Non-anthocyanin phenolic compounds retained on the SPE cartridge were removed by washing with ethyl acetate. The anthocyanins were eluted with 0.1% formic acid in ethanol. Finally, the eluent, containing 0.1% formic acid in ethanol, was dried in a rotary evaporator. The purified extracts were re-dissolved in water and stored at -80°C until use in the mouse assay.

2.3. Determination of anthocyanin contents of mulberry and chokeberry extracts by HPLC

One hundred milligrams of dried mulberry and chokeberry extracts were dissolved in methanol. Anthocyanin composition was analyzed using high-performance liquid chromatography (HPLC; Agilent 1260 Infinity II LC system, Agilent Technologies, Santa Clara, CA, USA), following a previously reported method with minor modifications (Kim et al., 2020). The purity of anthocyanins in the mulberry and chokeberry extracts was determined by quantifying total anthocyanin content using external calibration curves and normalizing to the mass of the extract. Detection was performed at 520 nm. The retention times and ultraviolet-visible spectra of the identified anthocyanins were compared with those of authentic standards. Individual anthocyanins (cyanidin-3-O-glucoside, cyanidin-3-O-galactoside, cyanidin-3-O-rutinoside, pelargonidin-3-O-glucoside, and cyanidin aglycone) were quantified using external calibration curves. All samples were analyzed in triplicate ($n = 3$).

2.4. Animals and diets

Thirty healthy male C57BL/6 mice (5 weeks old, 18 ± 2 g) were procured from Dooyeol Biotech (Seoul, Korea). Mice were raised under controlled temperature and humidity conditions with a 12 h light-dark cycle. The mice were acclimatized for one week. Oral administration commenced at six weeks of age (day 42), corresponding to the onset of puberty. Given that mice typically reach adulthood at around 10 weeks of age, the eight-week intervention spanned both the pubertal transition and early adulthood (Taft et al., 2006). Based on the age correlation reported by Dutta and Sengupta (2016), this duration corresponds to approximately 15.8 human years, indicating a relatively long-term exposure in this model. The animals were divided into three groups ($n = 10$ per group): control (oral administration of water), ME (mulberry extract), and CE (chokeberry extract). The ME and CE groups received mulberry and chokeberry extracts, respectively, at a dose of 125 mg/kg body weight (BW) once daily by oral administration for 8 weeks. This dose corresponds to approximately 600–750 mg/day for a 60 kg adult, based on standard dose conversion methods, and falls within a range reported to be safe and well tolerated in human studies (Hassellund

et al., 2012; Yan et al., 2016). Equal volumes of water were administered to the control group. All the mice were fed AIN-76A as the basal diet. The feces of the three groups were collected after 0, 2, 4, and 8 weeks to determine metabolite profiles and microbiome composition. At the end of the study period, anesthesia was induced by intraperitoneal injection of a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture, administered according to the body weight of the animals. After confirming adequate anesthesia, mice were euthanized by cervical dislocation. Plasma samples were then collected and used for subsequent analyses. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University (A2022014), and conducted in accordance with the institutional guidelines and the national regulations of the Republic of Korea (Laboratory Animal Act and Animal Protection Act), as well as the internationally accepted principles for the care and use of laboratory animals, including the NIH Guide for the Care and Use of Laboratory Animals.

2.5. Determination of metabolites in feces and plasma

The metabolite profile of the feces was determined based on a previously described method with minor modifications (Zhou et al., 2020). Fecal samples were extracted with cold methanol, and an internal standard (cinnamic acid- d_5) was added, followed by vortexing for 5 min. The mixture was then centrifuged at $25,029\times g$ for 15 min at 4°C . The resulting supernatant was collected and dried using a vacuum centrifuge. The dried extract was reconstituted in 100 μL of methanol.

The metabolite profile of the plasma was determined based on previously published methods with minor modifications (de Ferrars et al., 2014; Lee & Mitchell, 2012). One hundred microliters of plasma was mixed with an equal volume of 1% formic acid in water, and an internal standard (cinnamic acid- d_5) was added. The extracts were loaded onto an Oasis HLB SPE cartridge (1 cc, 30 mg, Waters) and eluted with 3 mL of 1% formic acid in methanol. The eluent was dried using a nitrogen evaporator. The residue was reconstituted with 50 μL of 1% formic acid in water.

The metabolite profiles of the feces and plasma were determined using ultra-HPLC (UHPLC) (Agilent 1290 Infinity II LC system, Agilent Technologies) coupled with a quadrupole time-of-flight mass spectrometer (X500R QTOF, SCIEX, Framingham, MA, USA). The metabolite of anthocyanin was separated on a Poroshell C18 column (2.1 mm $\times$ 150 mm, 1.9 μm , Agilent Technologies) at 30°C . The ultra-high performance liquid chromatography (UHPLC) method followed the protocol described previously (Lee et al., 2012). The injection volume was 5 μL , and the flow rate was 0.4 mL/min. The flow rate and gas temperature of the drying gas were 8.0 L/min and 225°C , respectively. The nebulizer gas pressure and fragmentor voltage were 45 psi and 125 V, respectively.

Cyanidin-3-O-glucoside was quantified using its corresponding calibration curve generated from authentic standard. All other individual anthocyanins were quantified using the cyanidin-3-O-glucoside calibration curve and are therefore reported as cyanidin-3-O-glucoside equivalents. For anthocyanin-derived phenolic acids, relative quantification was performed using a protocatechuic acid calibration curve, and the results are expressed as protocatechuic acid equivalents. Cyanidin-3-O-glucoside, cyanidin-3-O-galactoside, and protocatechuic acid stock solution were diluted to a series of concentrations (1, 50, 100, 250, 500, 1000 $\mu\text{g/mL}$) with 1% formic acid in water. For plasma and fecal samples, peak areas were normalized to the internal standard, cinnamic acid- d_5 prior to quantification. The relative quantification normalization approach has been used in previous untargeted metabolomics studies (Fan et al., 2019), with additional normalization using an internal standard.

2.6. Determination of short-chain fatty acid content in feces

The SCFA content in mouse feces was determined based on a

previously described method with minor modifications (Han et al., 2018); 30 mg fecal samples were mixed with 1 mL of diethyl ether. A working solution of the internal standard mixture (acetic acid- d_4 , propionic acid- d_6 , and butyric- d_7 acid) was added to the extract to achieve a final concentration of 30 ng/g. The mixtures were shaken for 15 min at 4°C and centrifuged at $15,000\times g$ for 10 min at 4°C . The resulting supernatant was collected. The SCFA composition in the feces was determined using gas chromatography (7890A GC, Agilent Technologies) coupled to a mass-selective detector (Agilent 5977 B, Agilent Technologies). SCFAs in feces were separated on a HP-FFAP capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness, Agilent Technologies). The initial column temperature was 70°C and held for 2 min, followed by a ramp up to 180°C at a rate of 10°C/min , and then finally increased to 200°C at a rate of 5°C/min and maintained at this temperature for 5 min. The flow of helium (99.999%) and split ratio were 1 mL/min and 10:1, respectively. The transfer line, quadrupole, ionization source, and inlet temperature were set to 280, 150, 230, and 250°C , respectively. The electron energy for the ionization of SCFAs was 70 eV. The SCFAs in the samples were quantified in selected ion monitoring (SIM) mode and identified by comparing their mass spectra and retention times with those of authentic standards.

The selected ions used as quantifier and qualifier ions of the SCFAs and internal standards in the SIM mode are summarized in Table S1. Peak areas of selected quantifier ions (m/z) were used to quantify the SCFAs. Quantification of isobutyric acid, 2-methyl butyric acid, isovaleric acid, and pentanoic acids was performed using the peak area ratio of each compound to butyric acid- d_7 as the internal standard.

2.7. Determination of microbiome composition different with anthocyanin sources

The genomic DNA was extracted from 0.25 g of the sample following the manufacturer's protocol using a QIAamp PowerFecal Pro DNA Kit (QIAGEN). The quality and quantity of gDNA were evaluated using a NanoDrop spectrophotometer and gel electrophoresis, respectively. For the Illumina MiSeq sequencing library, the gDNA was amplified by targeting the 16S rRNA gene V4-V5 region fused with the Illumina Nextera XT V2 barcode kit. Amplification of the V4-V5 regions of the 16S rRNA gene was conducted by polymerase chain reaction (PCR) using the bacterial-specific primer, 518F (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCA GCA GCG GTA AN-3') and 927R (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCC GTC AAT TCN TTT RAG T-3'). The PCR-amplified products were purified using AMPure XP beads (Beckman Coulter, High Wycombe, UK). The final product was qualified and quantified using a NanoDrop spectrophotometer and gel electrophoresis. Paired-end (2 $\times$ 300 bp) sequencing was performed using an Illumina MiSeq platform (Illumina, San Diego, CA, USA) by Macrogen (Seoul, Korea).

A total of 48 samples generated 7.0 Mb paired-end reads. Raw read sequences were trimmed using the Cutadapt program and those containing uncorrectable primer sequences were removed from the analysis. The reads were merged, denoised, and clustered according to amplicon sequence variants (ASVs) using the DADA2 software package. Representative ASV sequences were assigned to the bacterial taxonomy using the Silva 132 reference database through the feature-classifier classify-sklearn with a 0.5 minimum support threshold. Unassigned ASVs and those assigned to the chloroplasts and mitochondria, which were considered eukaryotic sequences, were removed. All sample sequences were rarefied to a depth of 11,000 for microbial diversity analysis. Shannon's diversity index and Simpson's evenness were used to evaluate microbial richness and evenness, respectively. Principal coordinate analysis was performed to assess the differences in the composition of bacterial communities among the samples using unique fraction metric (UniFrac)-weighted distance matrices. All analyses were performed using the Quantitative Insights into Microbial Ecology 2 (QIIME 2) pipeline (2021.11) and visualized using R packages, including

ggplot2. To predict the microbial pathways of the fecal microbiota, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUST2) was conducted.

2.8. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 23 (version 23.0; SPSS Inc., Chicago, IL, USA). Significant differences among mulberry cultivars and metabolite contents were determined via one-way analysis of variance (ANOVA), followed by a post-hoc test and Duncan's multiple range test at $p < 0.05$. Principal component analysis (PCA) and a heatmap of anthocyanin metabolites identified by UHPLC-electrospray ionization (ESI)-quadrupole time-of-flight (QTOF) in feces were obtained using XLSTAT (2022.03, Microsoft Excel Addin software, New York, USA). A paired t -test was conducted to compare the microbial alpha diversity between three groups (control, chokeberry, and mulberry) at four weeks (w0, w2, w4, and w8) using the `stat_compare_means` of the `ggpubr` package in R. ANOSIM was used to evaluate microbial community composition among the groups for beta diversity analysis. Linear discriminant analysis effect size (LEfSe) was conducted to identify specific ASVs or predicted microbial pathways that could explain the variation between groups. Specifically, ASVs or genera that were significantly different between chokeberry and mulberry and considered taxa with anthocyanin effects were identified using both t -test and LEfSe analysis. To determine how the gut microbiome was altered by anthocyanin intake and related to the metabolites of anthocyanin, Pearson's correlation analysis was performed. Only ASV counts were filtered, retaining only those present in at least 50% of the samples. These filtered ASVs were then transformed using the centered log-ratio (CLR) transformation, which adds a small pseudocount (10^{-6}) to zero values. For the metabolomic data, we auto-scaled metabolite concentrations to ensure comparability across features with different magnitudes. Features with near-zero variance were excluded from further analysis to ensure statistical robustness. Pearson's correlation analysis was performed separately for each time point (2, 4, and 8 weeks) and dietary intervention group (chokeberry and mulberry), comparing them to the control group. This analysis was conducted for each subgroup to capture the dynamic shifts in the diet-microbe-metabolite axis. Network analysis based on these correlations ($p < 0.1$) was conducted and visualized using the `ggnetwork` package. To investigate microbial ASVs that potentially mediate the effects of anthocyanin intake on metabolite profiles, we performed causal mediation analysis. For ASV-metabolite pairs showing a preliminary correlation ($p < 0.2$), we estimated the average causal mediation effect (ACME) and the average direct effect (ADE) using the quasi-Bayesian Monte Carlo method with 100 simulations. The significance of the mediation effect was determined based on the ACME p -value.

3. Results & discussion

3.1. Determination of purified extract from mulberry and chokeberry

The anthocyanin compositions of the purified mulberry and chokeberry extracts are presented in Table S2. In the mulberry extract, cyanidin-3-O-glucoside (1.09 mg/mL) was the major anthocyanin, representing approximately 83.8% of total anthocyanins. In the chokeberry extract, cyanidin-3-O-galactoside (1.64 mg/mL) was the major anthocyanin, representing approximately 93.2% of total anthocyanins. In addition to cyanidin-3-O-glucoside, the mulberry extract contained cyanidin-3-O-rutinoside (0.09 mg/mL), pelargonidin-3-O-glucoside (0.02 mg/mL), and cyanidin aglycone (0.10 mg/mL). The chokeberry extract also contained cyanidin-3-O-glucoside (0.03 mg/mL) and cyanidin aglycone (0.09 mg/mL), in addition to cyanidin-3-O-galactoside.

The anthocyanin purities of the mulberry and chokeberry extracts, determined using external calibration curves of authentic standards, were 96.2% and 93.3%, respectively. These anthocyanin-enriched

extracts were used for the *in vivo* study. Minor non-anthocyanin components were also detected, including quercetin-3-O-rutinoside, based on UHPLC-QTOF-MS analysis. However, these compounds were present at extremely low concentrations and were not detected after dilution for *in vivo* studies.

3.2. Identification of anthocyanin metabolites in plasma and feces of mice that consumed anthocyanin extracts for 8 weeks

Anthocyanin metabolites in mouse plasma and feces were identified by UHPLC-(ESI)-QTOF based on their precursor and fragment ion patterns. A total of eleven metabolites were detected following 8 weeks of dietary intake of mulberry and chokeberry anthocyanins (Table 1). All compounds were characterized with a mass error of <0.5 ppm. A representative MS/MS spectrum of anthocyanin metabolites in mouse plasma and feces is shown in Fig. S1.

Six and eight anthocyanin-derived metabolites were identified in the plasma and feces, respectively, of mice administered mulberry and chokeberry anthocyanins for 8 weeks. Cyanidin hexosyl hexoside exhibited a pseudomolecular ion at m/z 611.1607 and a fragment ion at m/z 287.0550, indicating the formation of cyanidin aglycone via the loss of 324.1056 Da, corresponding to two hexose units. Both cyanidin-3-O-galactoside and cyanidin-3-O-glucoside showed a pseudomolecular ion at m/z 449.1078 and generated a fragment ion at m/z 287.0550, consistent with the cleavage of a galactose or glucose moiety (162.0528 Da). These compounds were further confirmed by comparing their retention times and MS/MS fragmentation patterns with those of authentic standards.

Protocatechuic acid showed a pseudomolecular ion at m/z 153.0193 and a fragment ion at m/z 109.0295 following the loss of CO_2 (43.9898 Da). Identification was confirmed using authentic standards. Vanillic acid sulfate exhibited a pseudomolecular ion at m/z 246.9918 and a characteristic fragment ion at m/z 167.0350, corresponding to the loss of a sulfate group (79.9568 Da). Homovanillic acid sulfate showed a pseudomolecular ion at m/z 261.0074.

Hippuric acid was identified with a pseudomolecular ion at m/z 178.0510 and a fragment ion at m/z 77.0392, resulting from the loss of a $\text{C}_3\text{H}_4\text{NO}_3$ moiety. This fragmentation is consistent with a previous report (Penner et al., 2010). Hippuric acid is recognized as a common metabolite of flavonoids (Pero, 2010). Phenylacetylaminic acid exhibited a pseudomolecular ion at m/z 192.0666. Dihydroquercetin presented a pseudomolecular ion at m/z 303.0510 and a fragment ion at m/z 285.0405, indicative of dehydration (loss of H_2O , 18.0105 Da), in agreement with the findings of Abad-García et al. (2009).

3.3. Quantification of anthocyanin metabolites in plasma and feces of mice that consumed anthocyanin extracts for 8 weeks

The concentrations of anthocyanin metabolites in the plasma of mice that were fed mulberry and chokeberry extracts for 8 weeks are presented in Table S3. The total plasma metabolite concentration was highest in the mulberry group (132.2 nmol/L), followed by the chokeberry (50.3 nmol/L) and control (7.1 nmol/L) groups. These results suggest that intake of mulberry extract, characterized by a high cyanidin-3-O-glucoside content, leads to greater systemic exposure to anthocyanin-derived metabolites compared to chokeberry extract, which is rich in cyanidin-3-O-galactoside. Previous studies have indicated that cyanidin-3-O-glucoside and its metabolites may exert biological effects at nanomolar concentrations *in vivo*, supporting the physiological relevance of the plasma metabolite levels observed in this study (Fornasaro et al., 2016).

In the control group (i.e., animals not administered anthocyanin extracts), most metabolites detected in the mulberry and chokeberry groups were not observed. Only protocatechuic acid and hippuric acid were detected. These compounds are known flavonoid degradation products (Lin et al., 2016; Pero, 2010). The basal diet (AIN-76A)

Table 1

Identified anthocyanin metabolites in mouse plasma and feces.

RT (min)	Compound	Molecular Formula	Ionization Form	Theoretical <i>m/z</i>	Error (ppm)	Fragment <i>m/z</i>	Plasma	Feces
2.0	Cyanidin hexosyl hexoside	C ₂₇ H ₃₁ O ₁₆	M ⁺	611.1607	−0.08	287	O	
2.9	Vanillic acid	C ₈ H ₈ O ₄	[M-H] [−]	167.0350	−0.12			O
3.5	Methyl gallic acid	C ₈ H ₈ O ₅	[M-H] [−]	183.0299	0.05			O
5.4	Cyanidin-3-O-galactoside*	C ₂₁ H ₂₁ O ₁₁	M ⁺	449.1078	−0.20	287	O	
5.7	Protocatechuic acid*	C ₇ H ₆ O ₄	[M-H] [−]	153.0193	0.07	109	O	O
6.4	Vanillic acid sulfate	C ₈ H ₈ O ₇ S	[M-H] [−]	246.9918	−0.09	167	O	O
6.6	Cyanidin-3-O-glucoside*	C ₂₁ H ₂₁ O ₁₁	M ⁺	449.1078	−0.07	287	O	
7.5	Homovanillic acid sulfate	C ₉ H ₁₀ O ₇ S	[M-H] [−]	261.0074	−0.50			O
13.4	Hippuric acid	C ₉ H ₉ NO ₃	[M-H] [−]	178.0510	0.11	77	O	O
17.1	Phenylacetylaminocetic acid	C ₁₀ H ₁₁ NO ₃	[M-H] [−]	192.0666	−0.31			O
20.7	Dihydroquercetin	C ₁₅ H ₁₂ O ₇	[M-H] [−]	303.0510	−0.49	285		O

contained corn starch and corn oil as the primary sources of carbohydrate and fat, respectively. Corn grain has been reported to contain multiple phenolic compounds, including quercetin, as identified by UHPLC-(ESI)-ion trap mass spectrometry (Razgonova et al., 2022). Therefore, the low levels of protocatechuic acid and hippuric acid in the control group likely reflect background dietary phenolics from the corn-based components of the diet. However, the concentrations of protocatechuic acid and hippuric acid in the control group accounted for only 5.3–11.2% and 13.7–26.1%, respectively, of those observed in the mulberry and chokeberry consumption groups, indicating that anthocyanin extract intake was the primary contributor to the observed increases. Nevertheless, the lack of direct chemical analysis of the basal diet remains a limitation, and background dietary phenolics should be considered when interpreting baseline metabolite levels.

Cyanidin hexosyl hexoside (8.1 nmol cyanidin equivalents/L) was exclusively detected in the plasma of mice that were administered mulberry extract. This observation is consistent with a previous study reporting the presence of cyanidin diglucoside in mulberry (Jin et al., 2017) although the compound was not identified in chokeberry (Wu et al., 2004). Accordingly, the cyanidin hexosyl hexoside detected in plasma is likely derived from the mulberry extract. The plasma concentration of protocatechuic acid in the mulberry group (53.3 nmol/L) was 19.0-fold higher than that of the control group (2.8 nmol/L) ($p < 0.05$). Similarly, the chokeberry group (25.1 nmol/L) exhibited a 9.0-fold increase compared with the control ($p < 0.05$). In addition, the mulberry group showed a 2.1-fold higher level of protocatechuic acid than the chokeberry group. Protocatechuic acid has been reported to exhibit stronger antioxidant activity than Trolox and butylated hydroxytoluene (BHT) (Li et al., 2011). Therefore, the consumption of cyanidin-3-O-glucoside, the predominant anthocyanin in mulberry, may be associated with the higher circulating levels of antioxidant-related metabolites compared with cyanidin-3-O-galactoside from chokeberry. Vanillic acid sulfate was uniquely detected in the plasma of the mulberry group. Vanillic acid, a known metabolite of cyanidin-3-O-glucoside, has been previously identified in rats (Han et al., 2021) and its sulfate conjugate, a product of phase II metabolism, has been reported in humans (Czank et al., 2013). Hippuric acid, a well-established end-product of anthocyanin, amino acid, and fiber metabolism, is formed in the liver from benzoic acid and glycine (Del Bò et al., 2010; Jokioja et al., 2021). The plasma hippuric acid concentration in the mulberry group (31.5 nmol/L) was significantly higher than in the chokeberry (16.5 nmol/L) and control (4.3 nmol/L) groups ($p < 0.05$). Previous studies have linked elevated serum hippuric acid levels with long-term improvements in glucose and insulin metabolism (de Mello et al., 2017). Overall, these findings suggest that the glycoside form of anthocyanins may influence their metabolic fate *in vivo*, resulting in distinct plasma metabolite profiles and concentrations. The higher systemic exposure to anthocyanin-derived metabolites observed in the cyanidin-3-O-glucoside-rich mulberry group, compared to the cyanidin-3-O-galactoside-rich chokeberry group, may reflect differences in their metabolism; however, further studies, including direct

enzymatic assays, are required to elucidate the underlying mechanisms. These differences may contribute to variations in physiological responses and warrant further investigation.

The metabolite profiles in feces following 8 weeks of anthocyanin extract intake are shown in Table S4. Total metabolite concentrations were elevated in both mulberry and chokeberry groups (8.2–10.4 nmol/g), compared with the control group (0.7–3.1 nmol/g). Protocatechuic acid was the predominant metabolite, accounting for 28–60% of the total, regardless of the source of anthocyanin. Vanillic acid was detected in both mulberry and chokeberry groups, with higher levels observed in the mulberry group (0.8–1.2 nmol/g) than in the chokeberry group (0.2–0.8 nmol/g). However, its fecal concentration fluctuated depending on the duration of anthocyanin consumption. Vanillic acid has been shown to promote the growth of weaned piglets by reducing thio-barbituric acid reactive substance levels, a marker of oxidative stress (Hu et al., 2022). Similarly, methyl gallic acid levels were higher in the feces of the mulberry group (0.7–1.6 nmol/g) compared with the chokeberry group (0.3–0.9 nmol/g). This compound, previously identified as an anthocyanin metabolite from grapes (Forester & Waterhouse, 2008), has been reported to reduce the viability of human colon cancer cells (Forester et al., 2014), suggesting its potential role in reducing the risk of colorectal cancer. The fecal protocatechuic acid content was also higher in the mulberry group (4.2–5.7 nmol/g) than in the chokeberry group (2.9–3.6 nmol/g). Homovanillic acid sulfate was identified in both mulberry (4.2–5.7 nmol/g) and chokeberry (2.9–3.6 nmol/g) groups. This metabolite is a conjugated product of flavonoid degradation (Rechner et al., 2002). Interestingly, hippuric acid levels in the feces of the chokeberry group (1.5–2.8 nmol/g) were higher than those in the mulberry group (0.7–1.5 nmol/g), suggesting differences in microbial or hepatic metabolism between the two anthocyanin sources. Overall, the fecal metabolite analysis revealed distinct time-dependent accumulation patterns of anthocyanin-derived metabolites between mulberry and chokeberry groups, indicating broader differences in metabolic processing.

In the present study, certain anthocyanin derivatives and phenolic acids were quantified as equivalents of cyanidin-3-O-glucoside and protocatechuic acid, respectively, due to the lack of commercially available authentic standards for all identified metabolites. This semi-quantitative approach is commonly used in metabolomic studies of polyphenols (Kim et al., 2020). However, this method has inherent limitations, as MS response factors may vary among compounds, potentially leading to over- or underestimation of their actual concentrations. Nevertheless, the combined plasma and fecal metabolite profiling allows for an integrated assessment of anthocyanin metabolic fate under consistent *in vivo* conditions.

3.4. Principal component analysis and heatmap based on anthocyanin metabolite contents in feces

The PCA loading and score plots shown in Fig. 1A and B, respectively, are based on anthocyanin metabolite contents in mouse feces.

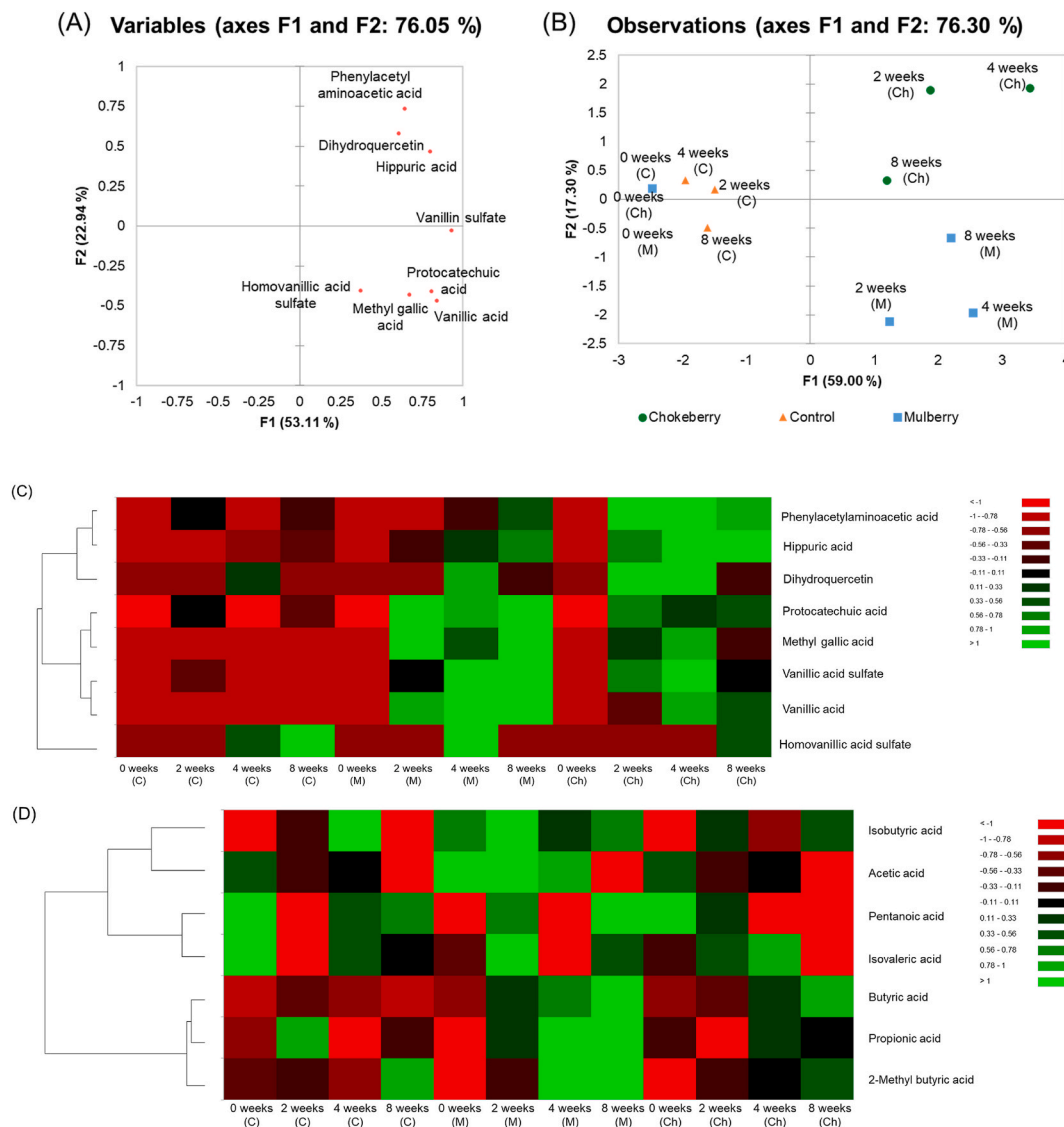


Fig. 1. PCA loading plot (A), score plot (B), and heatmap visualization and hierarchical clustering of changes in the control (C), mulberry (M) and chokeberry (Ch) anthocyanin metabolites (C) and short chain fatty acids (SCFAs) (D) identified in mice feces.

The first two principal components (PC1 and PC2) explained 76.05% of the total variance, contributing 53.11% and 22.94%, respectively. In the PCA score plot, clear separation is observed between the fecal samples of mice that were administered mulberry and chokeberry extracts. Specifically, samples from the chokeberry group are distributed in the upper region of the PCA plot, whereas those from the mulberry group are located in the lower region. This separation reflects the relative abundance of specific metabolites: hippuric acid and dihydroquercetin are more abundant in the feces of the chokeberry group, whereas protocatechuic acid and vanillic acid are more prominent in the mulberry group.

In Fig. 1C, a heatmap and hierarchical clustering of anthocyanin metabolite profiles in fecal samples are displayed. The clustering pattern confirms group-specific metabolite trends, with vanillic acid and vanillic acid sulfate showing higher intensities in the mulberry group, and hippuric acid and dihydroquercetin showing higher levels in the chokeberry group.

The proposed metabolic pathway of cyanidin-3-O-glucoside shown in Fig. 2, is based on the identified metabolites. Initially, cyanidin-3-O-glucoside undergoes hydrolysis to form cyanidin aglycone, which can subsequently be methylated to yield peonidin aglycone. Peonidin

aglycone is further degraded to homovanillic acid (Nurmi et al., 2009), which is then converted into vanillic acid. Both homovanillic and vanillic acids can undergo sulfation by sulfotransferases (SULT), to produce homovanillic and vanillic acid sulfates, respectively.

In a parallel pathway, cyanidin aglycone is degraded to dihydroxyphenylpropionic acid (Nurmi et al., 2009), which may undergo α -oxidation to form dihydroxyphenylacetic acid. This intermediate is subsequently converted to protocatechuic acid (Racova et al., 2020), a phenolic compound known for its high intestinal absorption (Selma et al., 2009). Protocatechuic acid has demonstrated anti-atherogenic properties by inhibiting NF- κ B activation and reducing the expression of vascular inflammatory markers such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Del Rio et al., 2013; Wang et al., 2010). Additionally, it has been shown to suppress colon cancer cell proliferation in Caco-2 cells (Forester & Waterhouse, 2010).

Hydroxybenzoic acid, a downstream degradation product of protocatechuic acid, may be further converted to gallic acid via a multistep pathway (Valanciene et al., 2020), followed by methylation to form methyl gallic acid. Alternatively, hydroxybenzoic acid may be metabolized into benzoic acid, which conjugates with glycine to form hippuric

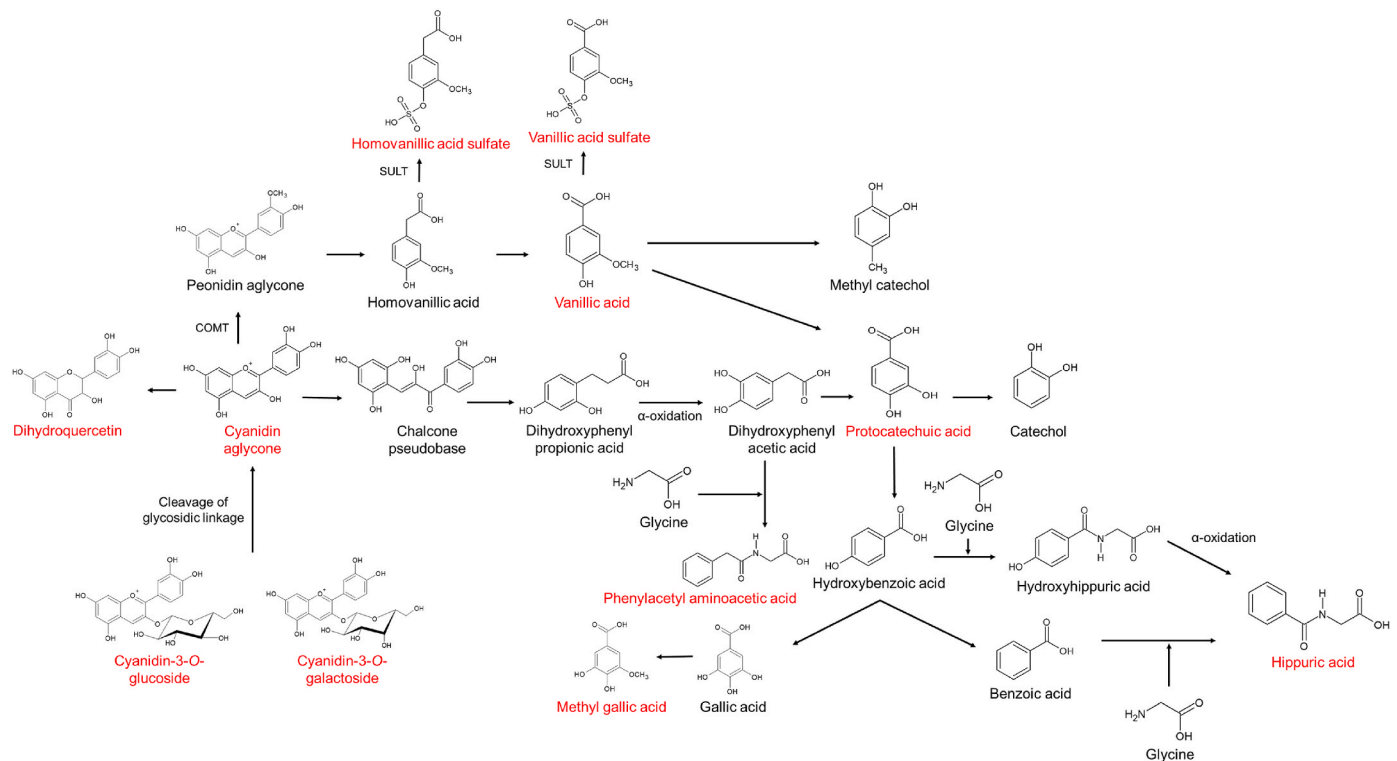


Fig. 2. Proposed metabolic pathway of cyanidin-3-O-glucoside based on the identified metabolites. COMT and SULT mean catechol-O-methyltransferase and sulfotransferase, respectively. Red indicates metabolites identified in the plasma and feces of mice.

acid (Wikoff et al., 2009). Furthermore, hydroxybenzoic acid can be conjugated to hydroxyhippuric acid (Xiong et al., 2016), which may subsequently undergo α -oxidation to yield hippuric acid. The multivariate analysis provides a global overview of source-dependent differences in fecal metabolite patterns based on quantitative metabolite data.

3.5. Dependence of changes in SCFA composition on anthocyanin glycoside consumption for 8 weeks

The SCFA contents in mouse feces are summarized in [Table S5](#). Acetic acid was the predominant SCFA, regardless of the anthocyanin source. In [Fig. 1D](#), SCFA profiles are visualized using a heatmap with hierarchical clustering. Notably, after 8 weeks of cyanidin glycoside consumption, propionic acid levels increased by 1.04- to 1.53-fold compared with the control group, depending on the anthocyanin source. In a previous study, propionic acid was produced during fermentation of purified blueberry anthocyanins in an *in vitro* gut model ([Wu et al., 2020](#)), suggesting that gut microbiota can convert dietary anthocyanins into SCFAs. Propionic acid has been shown to stimulate the release of appetite-regulating hormones such as peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) in the colon ([Joseph et al., 2019](#)). In addition, propionic acid acts as a ligand for free fatty acid receptors 2 and 3 (FFAR2 and FFAR3), which play roles in energy homeostasis and immune modulation. These mechanistic insights imply that microbially derived propionic acid may contribute to the regulation of food intake and metabolic health ([Yan et al., 2018](#)) although further *in vivo* studies are required to confirm its anti-obesity potential.

Similarly, butyric acid concentrations increased by 1.30- to 1.60-fold in the cyanidin-fed groups compared with the control group. A previous study using a simulated digestion model reported that anthocyanins from *Lycium ruthenicum* Murray increased fecal butyric acid levels compared with the blank control (Yan et al., 2018). Butyric acid is a key energy substrate for colonocytes and has been shown to support colonic epithelial integrity, reduce inflammation, and enhance insulin sensitivity by promoting energy expenditure (Gao et al., 2009; Yan et al.,

2018). Therefore, the long-term intake of cyanidin glycosides modulates the intestinal SCFA profile, which may provide insights into host-microbe metabolic interactions under physiological conditions.

To identify the impact of anthocyanin intake on SCFAs and the gut microbiota, we conducted a correlation-based mediation analysis (Fig. S2). This analysis revealed a time-dependent shift in the microbial mediation of specific SCFAs. For instance, Ruminococcaceae species initially mediated 2-methylbutyric acid levels in both anthocyanin-supplemented groups. However, by week 8, a robust mediation of propionic acid by *Staphylococcus* and *Roseburia* emerged predominantly in the mulberry group, showing the highest ACME estimates and mediation proportions. Notably, a high ACME value (8.38) coupled with a significant ADE (−6.3) indicates that the mulberry diet suppresses *Staphylococcus* both directly and indirectly via propionic acid production. The simultaneous involvement of 2-methylbutyric acid suggests that the total pool of branched-chain fatty acids (BCFAs) and SCFAs—likely generated through cross-feeding between *Roseburia* and other commensals—acts synergistically to maintain a colonization-resistant environment against *Staphylococcus* (Culp & Goodman, 2023; Wang et al., 2014).

3.6. Changes in the gut microbiota composition depending on anthocyanin glycoside consumption for 8 weeks

From 48 samples, 7.0 Mb of raw reads were generated, and a total of 632 ASVs were obtained. To observe the effects of anthocyanins on the gut microbiome, we treated mice with chokeberry extract, mulberry extract, or water as control, collected feces, and conducted 16S rRNA gene sequencing using the Illumina MiSeq platform. Firmicutes (average relative abundance of 70.0%) and Bacteroidetes (25.4%) were dominant in the mouse gut, followed by Deferribacteres and Actinobacteria. In the feces of all 48 mice, *Faecalibaculum* (32.7%), *Bacteroides* (15.9%), and *Lactobacillus* (6.0%) were identified (Fig. 3A and B). At the species level, uncultured bacterium of *Faecalibaculum*, *Bacteroides*, and Muribaculaceae were predominant species (Fig. 3). The comparison of gut

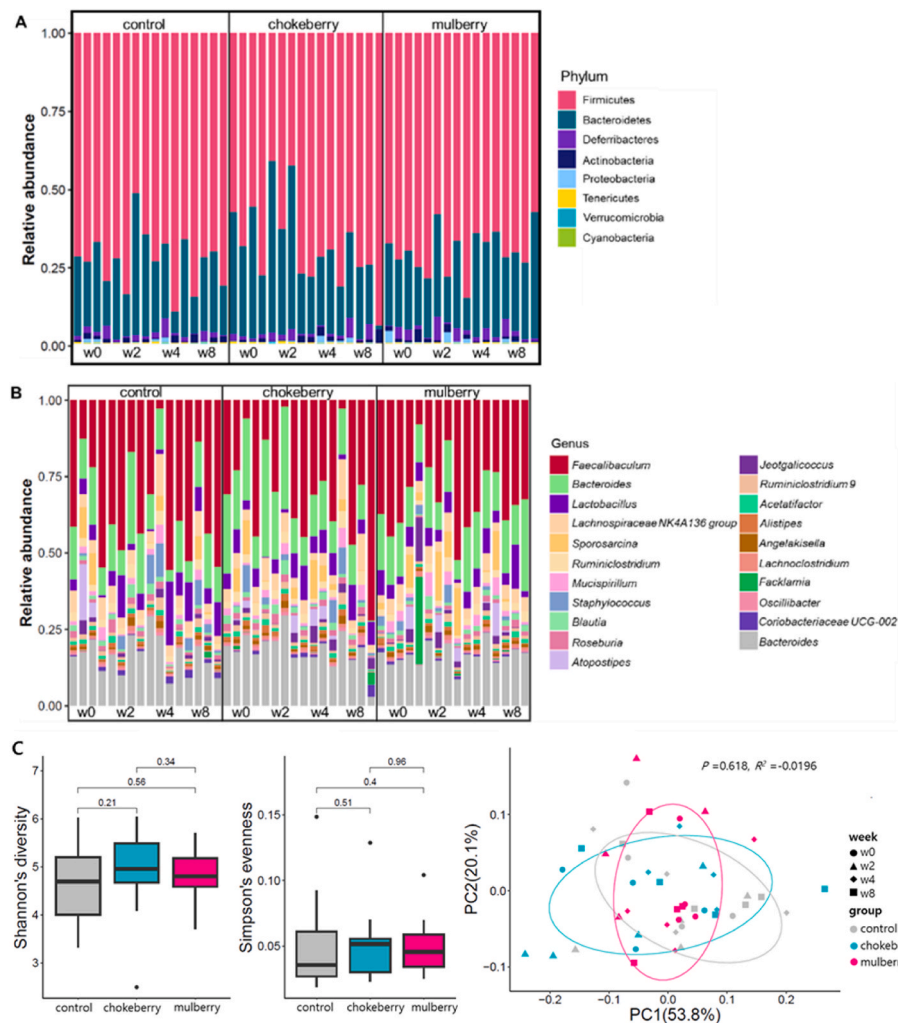
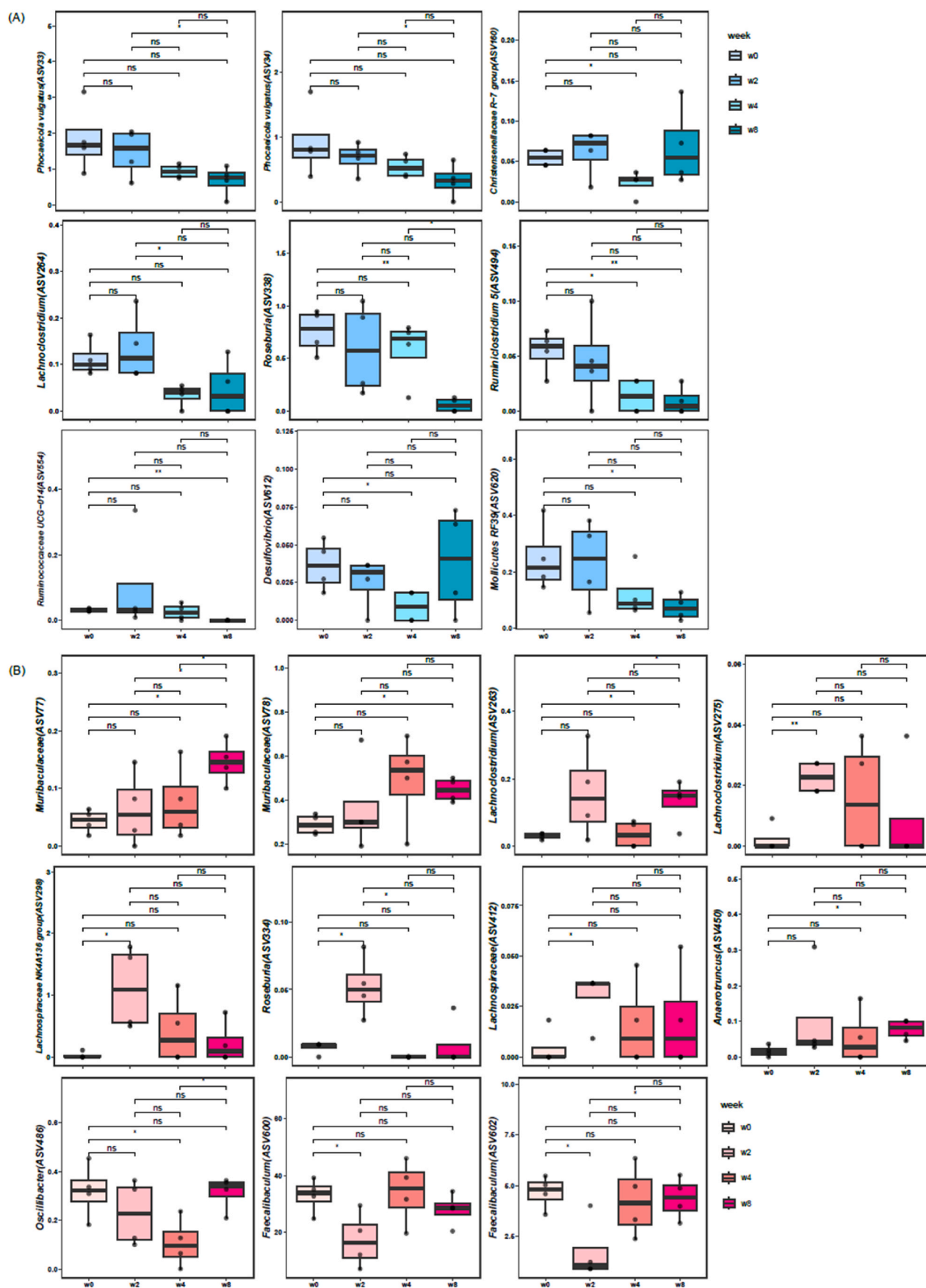


Fig. 3. Microbial composition in the mice gut at the phylum (A) and genus (B) level. Characterization of the microbial diversity in the gut microbiome of control, chokeberry and mulberry groups (C). Biodiversity was calculated using Shannon's diversity and Simpson's evenness indices. Principal coordinate analysis (PCoA) plot of gut microbial communities across all samples using the Unifrac weighted distance matrix. The statistical significance of alpha diversity was tested using the paired *t*-test, and PCoA was evaluated using the analysis of similarities (ANOSIM) test.

microbial diversity, including alpha diversity based on rarefied ASVs table and beta diversity based on Unifrac weighted distance matrices, showed no differences among the three groups and sampling times (week) (Fig. 3C). Although PCoA revealed no significant global shift in the microbial community composition ($p > 0.05$), these findings suggest that anthocyanin supplementation contributed to the maintenance of overall ecological stability within the gut microbiome.

A LEfSe analysis was conducted to determine the effects of anthocyanin intake on the gut microbiome. First, we compared the significantly different ASVs across the three groups at each sampling time point (Fig. S3). The relative abundance of ASVs assigned to *Roseburia* was higher in all samples in the chokeberry and mulberry groups, and those of Ruminococcaceae and Lachnospiraceae belonging to Firmicutes were higher in the chokeberry group at week 2. After 4 weeks of chokeberry extract consumption, *Sporosarcina* (ASV121) was more abundant than in the other groups, and ASVs belonging to *Phocaeicola vulgatus* and Muribaculaceae of the Bacteroidetes phylum were more abundant in mice consuming mulberry extract. Notably, six ASVs were identified as having significant differences during the 8 weeks of mulberry consumption, including *Ruminiclostridium* of Firmicutes, *Alistipes* and Muribaculaceae, which showed a high abundance of Bacteroidetes even at week 4. Subsequently, we compared significantly increased or decreased ASVs in the same group according to the sampling time point

(Fig. 4). The relative abundance of ASVs belonging to *P. vulgatus*, *Ruminiclostridium* 5, and *Mollicutes RF39* decreased in the chokeberry group (Fig. 4A). After eight weeks of chokeberry intake, the relative abundance of *Lachnospirillum* (ASV264), *Roseburia* (ASV338), *Ruminiclostridium* 5 (ASV494), Ruminococcaceae UCG-014 (ASV554), and *Mollicutes RF39* (ASV620) decreased below baseline levels. In the mulberry intake group, the relative abundance of Muribaculaceae (ASV577 and ASV 578) increased, showing a significantly high abundance by week 8 (Fig. 4B). Two ASVs belonging to *Lachnospirillum* increased at weeks 2 and 8. Conversely, mulberry intake decreased two *Faecalibaculum* ASVs after two weeks. The other five ASVs also showed significant differences during two, four, or eight weeks of mulberry consumption. Interestingly, these results present a contrast to previous reports, which observed a depletion of both *Lachnospirillum* and *Roseburia* following the administration of blueberry and mulberry anthocyanin extracts in aging male mice (Li et al., 2023). This inconsistency may be generated by differences in the chemical profiles of the anthocyanin sources used, the baseline gut environment of the animal models, or the specific intervention periods. Also, statistical differences of specific ASVs at various time points demonstrated that the impact of anthocyanin is precisely targeted toward key metabolic drivers rather than entire microbial community. This suggests that anthocyanin-mediated modulation is a highly selective process.



In addition, we performed PICRUST2 analysis and found more pathways in the group that consumed chokeberry and mulberry for 2 weeks than that in the control group (Fig. 5A). Interestingly, an increase in the fatty acid biosynthesis [ko00061], biosynthesis of ansamycin [ko01051], and primary [ko00120] and secondary bile acid biosynthesis [ko00121], which were mentioned as beneficial pathways related to type-2 diabetes, were observed in the gut microbiome of the mulberry

intake group. We further identified microbial MetaCyc pathways predicted by PICRUST2 that exhibited differential abundance between the control and mulberry groups at weeks 2, 4, and 8. In the early phase of mulberry intake, an enrichment in fundamental energy metabolism (TCA) and biosynthesis (HEME) was observed in the mice gut. The control group demonstrated an enrichment in pathways associated with the synthesis of purine and degradation of specific carbohydrates

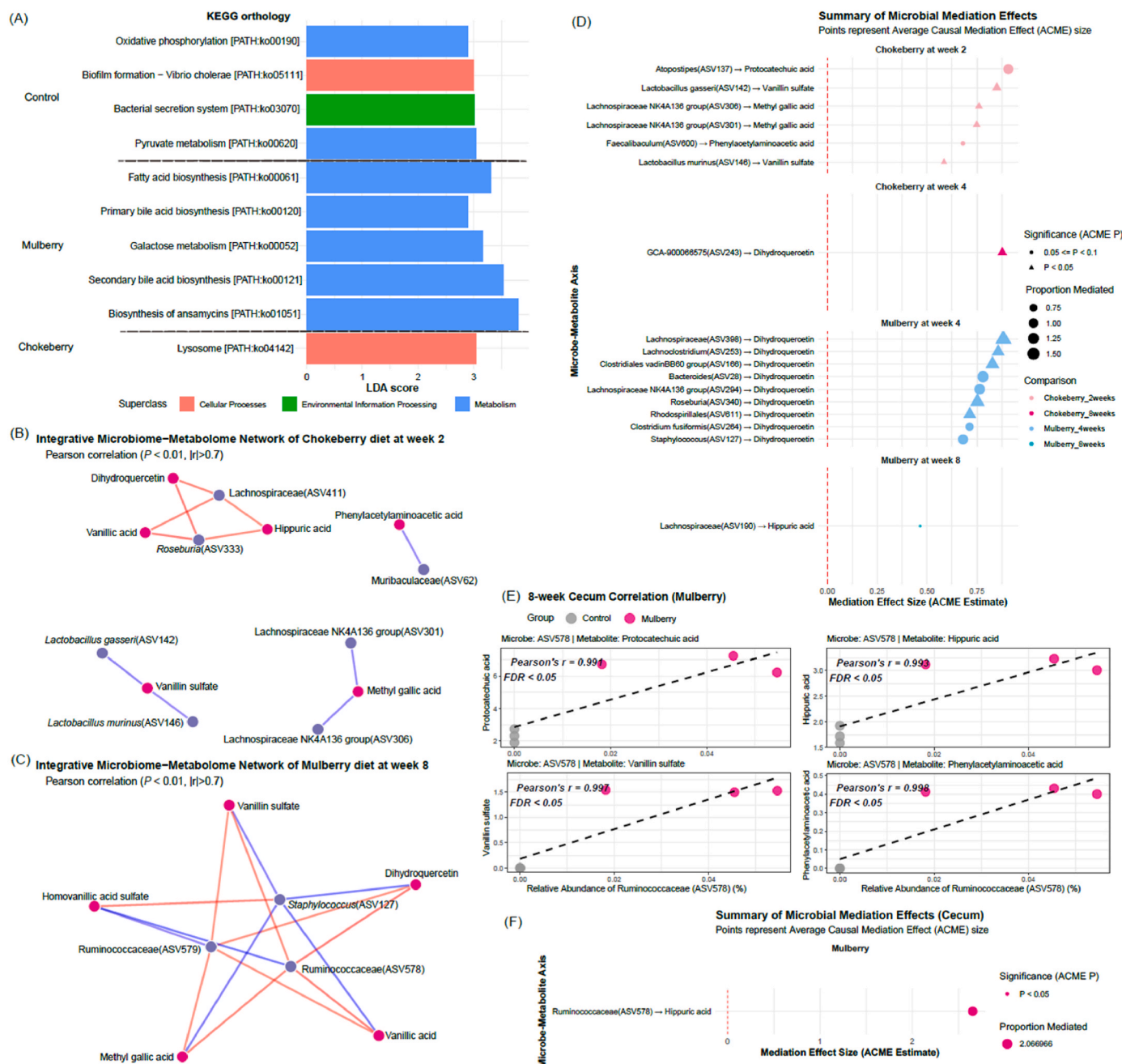


Fig. 5. Bar graph for significantly different functional KEGG Orthology (KO) of the mice gut microbiome predicted by PICRUST2 (A). Integrative microbiota-metabolite interaction networks induced by dietary interventions for the chokeberry group at week 2 (B) and the mulberry group at week 8 (C). Each node represents either a bacteria (ASV level, purple) or a detected metabolite (pink). Edges indicate significant Pearson's correlation coefficients ($P < 0.01$ and $|r| > 0.9$), with red and blue lines indicating positive and negative correlations, respectively. Forest plot illustrating the causal mediation analysis of anthocyanin intake-fecal microbe-fecal metabolite interactions (D). Scatter plot showing the Pearson's correlation analysis between the relative abundance of Ruminococcaceae (ASV578) and cecal metabolites after mulberry intake (E). The relative abundance displays raw abundance, but statistical significance was determined using transformed data. Forest plot illustrating the causal mediation analysis of anthocyanin intake-cecal microbe-cecal metabolite interactions (F). In panels D and F, each point represents an Average Causal Mediation Effects (ACME) estimate, with shapes and colors indicating significance based on the ACME p-value. The point size is proportional to the Proportion Mediated, and the vertical dashed red line at zero indicates the absence of a mediation effect. Positive ACME values present that dietary intervention increases metabolite levels through the enrichment of the corresponding ASVs.

(lactose), whereas the mulberry group showed a shift to P108_PWY in the later weeks. Conversely, significant variation was observed in the chokeberry intake group. These alterations indicate a mulberry-induced shift in the gut microbiome, favoring pathways that are more aligned with active energy metabolism in the early weeks, suggesting a potent immediate impact of the intervention on microbial function.

To elucidate the functional relationship between the gut microbiota and metabolites derived from anthocyanin intake, we performed a Pearson's correlation analysis, followed by a causal mediation analysis. In the longitudinal fecal analysis, *Roseburia* (ASV333) in the chokeberry group at week 2 exhibited a strong positive, but not significant correlation with vanillic acid and dihydroquercetin ($r > 0.99$, $FDR < 0.1$; Fig. 5B). The elevation of vanillin sulfate at week 2 was mediated by *Lactobacillus* species, including *L. gasseri* (ASV142), and *L. murinus* (ASV146), while methyl gallic acid was mediated by ASVs belonging to the Lachnospiraceae NK4A136 group. In the mulberry intake group, shifts in dihydroquercetin, methyl gallic acid, vanillic acid, and vanillin sulfate at week 8 were consistently associated with two specific bacteria, including *Staphylococcus* (ASV127) and Ruminococcaceae (ASV578, ASV579), which showed a strong correlation ($|r| > 0.99$, $p < 0.01$; Fig. 5C). Further mediation analysis confirmed that the elevation of dihydroquercetin was mediated by several bacteria in the anthocyanin intake group (Fig. 5D). Notably, Lachnospiraceae (ASV398) acted as a significant mediator of dihydroquercetin production in the mulberry intake group (ACME $p = 0.04$, Proportion Mediated; Prop. Med. = 1.72), suggesting that the increase in this metabolite induced by mulberry is heavily dependent on the enrichment of this specific bacteria. Previous studies have reported an association between dihydroquercetin and Lachnospiraceae, showing that dihydroquercetin administration increases the number of Lachnospiraceae or reduces its richness (Filippovich et al., 2025; Wang et al., 2022). Interestingly, the causal mediation analysis for anthocyanin glycosides and the gut microbiota exhibited lower effect sizes (including ACME_Est and Prop. Med.) compared to those observed for SCFAs. This gap in results suggests that the primary physiological impact of dietary anthocyanins is likely exerted through their downstream fermentation products rather than the glycoside forms. However, the consistent intake of chokeberry for two weeks and mulberry for four weeks demonstrated a potential to modulate the gut microbial environment. These findings imply that the health benefits of an anthocyanin-rich diet are derived from a time-dependent remodeling of the gut ecosystem, which eventually leads to the production of functional metabolites, such as SCFAs, that inhibit pathogens.

Additional analysis of both anthocyanin intake groups on the cecal data indicated a general shift in the microbiota-metabolite landscape (Fig. S4). Five ASVs, including *Alistipes* (ASV82), *Lachnoclostridium* (ASV253), Lachnospiraceae UCG-006 (ASV325), *Roseburia* (ASV338), and *Ruminiclostridium* (ASV526) were strongly correlated with various metabolites. Also, both correlation and mediation analyses consistently showed the near-perfect association between Ruminococcaceae (ASV578) and several metabolites, including hippuric acid (Fig. 5E and F). ASV578 showed a remarkably strong positive correlation with hippuric acid ($r = 0.997$, $p < 0.001$) in the mulberry group. Although the scatter plots represent raw abundances for intuitive visualization, the statistical significance was rigorously determined using transformed data to account for compositionality. The high Prop. Med. values (many >50%) across various time points and samples strengthen our hypothesis that the microbiota is a functional bridge between diet and the host metabolic environment. Hippuric acid is a co-metabolite of the gut microbiota and host and is also produced by gut microbial metabolism of polyphenol (Pan et al., 2025). It is also known to show an inverse relationship with obesity and a positive association with several bacteria, including Ruminococcaceae and Rikenellaceae families (Ejtahed et al., 2020).

The metabolite composition of anthocyanins differs according to their glycoside form. In addition, the long-term intake of anthocyanins

changes the gut microbiota. Therefore, long-term intake of different anthocyanin glycosides may uniquely modulate the gut microbiota, likely driven by distinct metabolite profiles derived from their specific glycosidic structures.

4. Conclusion

In this study, cyanidin-3-O-glucoside and cyanidin-3-O-galactoside were extracted and purified from mulberry and chokeberry. Eleven cyanidin glycoside metabolites were identified in the plasma and feces of the mice, with a mass error of less than 0.5 ppm. Six and eight metabolites were identified in plasma and feces, respectively. Vanillic acid sulfate and methyl gallic acid were identified in the feces of mice that consumed the mulberry extract for 8 weeks, and homovanillic acid sulfate was identified in the feces of mice that consumed the chokeberry extract for 8 weeks. The metabolite profiles of cyanidin-3-O-glucoside and cyanidin-3-O-galactoside were different. In particular, the hippuric acid and dihydroquercetin contents in the feces of the chokeberry group were higher than those in the mulberry group. However, the protocatechuic and vanillic acid contents in the feces of the mulberry group were higher than those of the chokeberry group. Furthermore, plasma metabolite levels, including those of protocatechuic and hippuric acids, were higher in the mulberry group than in the chokeberry group. Mulberry and chokeberry intake induced no significant changes in the entire gut microbial community structure, however, led an increase in *P. vulgatus* and Muribaculaceae, which are associated with beneficial effects for patients with type-2 diabetes. Strong mediation of key bacteria and metabolites, including both SCFA and anthocyanin glycoside, provided deep understanding of how dietary effects of anthocyanin shape the functional output of the gut microbiota. Therefore, the long-term consumption of anthocyanins may induce a change in microbiome composition as a result of anthocyanin metabolism by the microbiome and could potentially help in the management of type-2 diabetes.

CRediT authorship contribution statement

Inhwan Kim: Writing – original draft, Methodology, Investigation. **Han Na Oh:** Writing – original draft, Methodology, Investigation. **Bong Ki Park:** Writing – review & editing, Writing – original draft, Investigation. **Joon-Kwan Moon:** Writing – review & editing, Resources. **Bokyung Lee:** Writing – review & editing, Resources. **Woo Jun Sul:** Writing – review & editing, Conceptualization. **Jihyun Lee:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fbio.2026.108949>.

Data availability

Data will be made available on request.

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