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Original Research Article

Mulberry leaf supplementation inhibits skatole deposition by regulating gut microbiota and upregulating liver cytochrome P450 1A1 expression in finishing pigs

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ABSTRACT

Skatole, a strong fecal odor substance, is generated through microbial degradation of tryptophan in the animal hindgut. It easily accumulates in adipose tissue and affects meat quality. In this study, the effect of mulberry leaf supplementation on skatole in finishing pigs was studied. In a 35-day trial, 20 finishing pigs (barrows and gilts) were fed with a basal diet or basal diet with 6% mulberry leaves. Growth performance of the pigs ($n = 10$) was automatically recorded by a performance-testing feeder system and 8 pigs in each treatment were slaughtered and sampled for the remaining tests. Skatole and short-chain fatty acids were detected using HPLC and gas chromatography, respectively. Fecal microbiota were analyzed using 16S rRNA gene sequencing. The metabolomics analysis of feces and serum was performed with UHPLC-MS/MS. The major cytochrome P450 (CYP) enzymes that catalyze skatole degradation in the liver were tested by using RT-PCR and Western blot. Effects of major bioactive compounds in mulberry leaves on the *CYP* genes were verified in the hepatic cell line HepG2 in an in vitro test ($n = 3$). In finishing pigs, mulberry leaf supplementation had no significant effect on the average daily gain, average daily feed intake, and feed conversion ratio ($P > 0.05$), but reduced skatole levels in feces, serum, and backfat ($P < 0.05$), and increased acetic acid levels in feces ($P = 0.027$). Mulberry leaf supplementation decreased the relative abundance of the skatole-producing bacteria *Megasphaera* and *Olsenella* ($P < 0.05$). Indole-3-acetic acid, the intermediate that is essential for skatole production, was significantly reduced in feces by mulberry leaf supplementation ($P < 0.05$) and was positively correlated with skatole content in feces ($P = 0.004$). In pigs treated with mulberry leaves, liver *CYP1A1* expression was increased ($P < 0.05$) and was negatively correlated with skatole content in backfat ($P = 0.045$). The in vitro test demonstrated that mulberry leaf polyphenols and polysaccharides could directly stimulate *CYP1A1* expression in hepatic cells. These findings suggest that mulberry leaf supplementation reduces skatole production and deposition in finishing pigs by regulating the gut microbiota and promoting skatole degradation in liver.

1. Introduction

Skatole (3-methylindole) is a fecal-odor-causing substance that is produced by the microbial degradation of tryptophan in the animal gut. It is the main substance causing the unpleasant odor of pork and one of the main odor substances emitted by swine manure during pig farming (Ma et al., 2021). Skatole can be perceived by humans at very low concentrations. For example, skatole concentrations of as low as 26 ng/g in fat are easily detected

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by the human nose (Annor-Frempong et al., 1997), and the olfactory threshold of skatole in air is reported to be less than 0.0004 μg/m^3 (Rosenfeld et al., 2007). Skatole produced in the gut is partly excreted through feces and partly absorbed by the gut. The absorbed skatole is transported to the liver through the portal vein, then rapidly degraded by cytochrome P450 (CYP) enzymes and excreted through urine (Lanthier et al., 2007). Skatole that is not degraded in the liver is further transported to the peripheral blood and deposited in the body. Skatole accumulates easily in adipose tissue due to its lipophilic characteristics, thus causing a reduction in pork quality (Meinert et al., 2017; Morlein et al., 2012; Rowe et al., 2014). Therefore, decreasing the production and deposition of skatole in pigs is of great importance for the environment and pork quality.

Mulberry, an important cash crop in China, has been used as silkworm feed and as a traditional Chinese medicine since ancient times. Mulberry leaves contain numerous active components, such as polyphenols (PP), polysaccharides (PS), alkaloids (AL), flavonoids, and steroids, and have been reported to have various physiological activities, including antidiabetic, antioxidant, antihyperlipidemic, and antiatherosclerotic activities (Chan et al., 2016). The application of mulberry leaves in pig diets has also been reported. We previously reported that the inclusion of mulberry leaves in feed could improve meat quality by decreasing serum triglyceride and cholesterol and increasing inosinic acid and intramuscular fat content in muscle in finishing pigs (Li et al., 2012). Other studies reported that in finishing pigs, the mulberry leaf diet improves antioxidative capacity; inhibits lipid oxidation; increases polyunsaturated fatty acid content in muscle (Liu et al., 2021); and decreases the shear force, cooking loss, and drip loss of muscle (Zeng et al., 2019). Some compounds in mulberry leaves are also beneficial to pigs. For example, mulberry leaf PS can improve the ecology of the gut microbiota, reduce diarrhea rate, and improve growth performance in early-weanling pigs (Zhao et al., 2015). Mulberry leaf flavonoids can improve growth performance, meat tenderness, and polyunsaturated fatty acid content in finishing pigs (Liu et al., 2022). Song et al. (2023) recently found that mulberry leaf extract could reduce odorous substances in feces of weaned piglets. Although the application of mulberry leaves in pig feed has been reported, the effects of mulberry leaves on skatole deposition in finishing pigs have not been reported. In the present study, we explored the effects of mulberry leaves on skatole in finishing pigs and investigated their underlying mechanism by using 16S rRNA sequencing, metabolomics analysis, gene expression analysis, and correlation analysis. We aim to explore new ways to reduce skatole deposition in finishing pigs and to expand the functional application of mulberry leaves.

2. Materials and methods

2.1. Animal ethics statement

All the experimental procedures applied in this study were in accordance with the guidelines of Farm Animal Welfare Requirements: Pigs (T/CAS 235-2014) promulgated by China Association for Standardization and approved by the Ethics Committee of the Zhejiang Academy of Agricultural Sciences (authorization code number: 2022ZAASLA77). All animal experiments were compliant with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020).

2.2. Preparation of mulberry leaves and mulberry leaf extracts

Mulberry leaf powder was supplied by Zhejiang Cathaya International Co., Ltd. (Hangzhou, China) and was prepared as follows: mulberry leaves were dried naturally, crushed through an 80-mesh screen, and stored in a dry environment at room temperature before use. Some of the mulberry leaf powder was extracted to prepare PP, PS, and AL for the cell experiment according to previous methods (Ma et al., 2019; Wu et al., 2013) with some modifications. Firstly, mulberry leaf powder was boiled with 1:50 (wt/vol) ultrapure water, and the supernatant was filtered and concentrated with a rotary evaporator. Ethanol was slowly added to the concentrate to a final concentration of 75%. The mixture was then centrifuged at 12,000 × g at room temperature for 10 min. The supernatant was concentrated with a rotary evaporator, and then dried through lyophilization to obtain mulberry leaf PP. The precipitate was dissolved in hot water, and protein was removed by using isoamyl alcohol:chloroform solution (1:4, vol/vol). Then, the redissolved solution was dried via lyophilization to obtain mulberry leaf PS. For the extraction of total AL, mulberry leaf powder was extracted with 80% ethanol at 1:50 (wt/vol) and 60 °C for 2 h. The supernatant was filtered, and ethanol was removed by using a rotary evaporator. The supernatant was collected through centrifugation and then adsorbed with cation exchange resins for 24 h. After being rinsed with ultrapure water, the resins were eluted with 0.5 mol/L ammonia, and the eluent was collected for the removal of ammonia by using a rotary evaporator. The concentrated eluent was dried through lyophilization to obtain mulberry leaf AL. The total PP content in PP extract was 65.5% as detected by using the Folin–Ciocalteau method (Chamorro et al., 2012). The total PS content in the PS extract was 62.7% as detected by using the phenol–sulfuric acid method (He et al., 2017). The total AL content in the AL extract was 76.6% as detected via the Reinecke salt colorimetry method (Li et al., 2008).

2.3. Experimental animals, diets, and design

The pigs used in this experiment were a crossbreed with approximately 30% Chinese native pig genotypes bred by our team. A total of 20 healthy pigs (10 barrows and 10 gilts) aged at 150 ± 10 days with an average body weight (BW) of 75.66 ± 7.89 kg were selected. Pigs in each gender were blocked by BW into 2 blocks with similar BW, and pigs in each block were randomly assigned to 2 dietary treatment groups in a completely randomized block design resulting in 10 animals (5 barrows and 5 gilts) per treatment. All pigs were raised in the same environmentally controlled fattening house. Pigs in the same treatment were kept in 1 pen equipped with an Osborne FIRE performance testing feeder system (Osborne, USA) that could automatically record the BW and feed intake of every single pig. Pigs were fed with a basal diet (CON group) or basal diet with 6% mulberry leaves (MB group) for 35 days. The diets were formulated in accordance with the nutrient requirements of swine (meat-type pig) recommended by the National Standards of the People's Republic of China (GB/T 39235-2020). The composition and nutritional levels of the experimental diets are shown in Tables 1 and 2. The test methods of nutrient levels of the diets are supplied in the Supplementary materials. The pigs were allowed to eat and drink freely during the experiment and were vaccinated in accordance with the requirements of the farm. The BW and feed intake of pigs were automatically recorded by using the Osborne FIRE performance testing feeder system. The average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated (n = 10 per treatment).

2.4. Sample collection and processing

At the end of the experiment, pigs were slaughtered humanely after electrical stunning in a commercial slaughterhouse. In order to save the cost of subsequent tests, 8 pigs (4 barrows and 4 gilts) in

Table 1
Ingredients of the diet (%, as-fed basis).

Ingredient	CON group	MB group
Corn	68.19	68.10
Soybean meal	12.81	11.90
Wheat bran	16.00	11.00
Mulberry leaf powder	0.00	6.00
Limestone	0.30	0.30
Premix [1]	2.70	2.70
Total	100.00	100.00

CON = basal diet; MB = mulberry leaf diet.
[1] Provided per kilogram of complete diet: 5500 IU vitamin A; 700 IU vitamin D_3; 25 IU vitamin E; 2.5 mg of vitamin K_3; 1.2 mg of vitamin B_1; 4.5 mg of vitamin B_2; 3.0 mg of vitamin B_6; 0.020 mg of vitamin B_{12}; 25 mg of nicotinic acid; 15.0 mg of D-pantothenic acid; 0.5 mg of folic acid; 0.065 mg of biotin; 50 mg of Fe (as $FeSO_4 \cdot H_2O$); 50 mg of Zn (as $ZnSO_4$); 15 mg of Cu (as $CuSO_4 \cdot 5H_2O$); 10 mg of Mn (as MnO_2); 0.3 mg of I (as $Ca[IO_3]_2$); 0.3 mg of Se (as $Na_2SeO_3 \cdot 5H_2O$); 3200 mg of Ca (as limestone).

Table 2
Nutrient levels of the diet (%, as-dry matter basis).

Item	CON group	MB group
Gross energy, MJ/kg	15.60	16.01
Digestible energy, MJ/kg	13.59	13.51
Crude protein	14.04	13.96
Crude fiber	2.80	3.70
Ether extract	3.01	2.62
Ash	4.51	4.75
Calcium	0.52	0.53
Total phosphorus	0.49	0.42
Neutral detergent fiber	12.46	14.47
Lysine	0.72	0.68
Methionine	0.14	0.17
Cystine	0.21	0.21
Threonine	0.55	0.52
Tryptophan	0.19	0.21

CON = basal diet; MB = mulberry leaf diet.
Digestible energy values are calculated values while other nutrient levels are analyzed values.

each treatment group were randomly selected for sample collection under the premise that the sample size was sufficient for statistical analysis. The samplers were blinded to the animal experimental design. The blood, rectal feces, liver, backfat, and longissimus dorsi muscle of the selected pigs were sampled. Blood was kept at room temperature for 3 h to allow clotting, and then centrifuged at 3500 × *g* at 4 °C for 10 min to obtain serum. The serum samples were stored at −80 °C. Part of longissimus dorsi muscle samples were measured for meat color (CIE L*a*b*), drip loss, mill loss, cooking loss, and intramuscular fat content according to the method in Technical Specification for Determination of Pork Quality (NY/T 821-2019) promulgated by the Ministry of Agriculture of the People's Republic of China. Other samples were frozen in liquid nitrogen immediately, and then stored at −80 °C. For all samples, the testers were blinded to the experimental design.

2.5. Detection of biochemical indices in serum and muscle

Total protein (TP), albumin (ALB), total cholesterol (TC), triglyceride (TG), nonesterified fatty acid (NEFA), total antioxidant capacity (TAOC), and malondialdehyde (MDA) in serum or muscle (n = 8 per treatment) were tested respectively by total protein quantitative assay kit (A045-2-2), albumin assay kit (A028-2-1), total cholesterol assay kit (A111-1-1), triglyceride assay kit (A110-1-1), nonesterified free fatty acids assay kit (A042-2-1), total antioxidant capacity assay kit (A015-1-2), and malondialdehyde assay kit (A003-1-2) purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) in accordance with the manufacturer's instructions.

2.6. Detection of skatole

Skatole in feces: approximately 0.3 g of fecal sample (n = 8 per treatment) was weighed into a 2-mL centrifuge tube, then 1.5 mL of methanol and 2 steel balls were added. Subsequently, the mixture was homogenized at 60 Hz for 60 s and centrifuged at 13,500 × *g* at room temperature for 5 min. The supernatant was filtered through a 0.45-μm filter and transferred to a headspace vial for testing.

Skatole in serum: aliquots of 1 mL of serum (n = 8 per treatment) were added into 2-mL centrifuge tubes, mixed with 1 mL of diethylether, rigorously vortexed for 1 min, and centrifuged at 13,500 × *g* at room temperature for 5 min. The diethylether phase was separated and dried under a gentle stream of nitrogen at 30 °C, redissolved in 500 μL of methanol, and rigorously vortexed. The samples were further purified by a Microcon 30 kDa centrifugal filter device (Millipore, USA) via centrifugation at 13,500 × *g* at room temperature for 10 min. A 400 μL aliquot of the extract was diluted with 400 μL of acetonitrile:water solution (60:40, vol/vol). The diluted extract was filtered through a 0.45-μm filter and transferred to a headspace vial for testing.

Skatole in muscle: longissimus dorsi muscle (n = 8 per treatment) was vacuum freeze-dried and ground into powder. Approximately 0.3 g of muscle powder was weighed into a 2-mL centrifuge tube then 1.5 mL of methanol and 2 steel balls were added, then homogenized at 60 Hz for 60 s, and centrifuged at 13,500 × *g* at room temperature for 5 min. The supernatant was filtered through a 0.45-μm filter and transferred to a headspace vial for testing.

Skatole in backfat: approximately 1 g of backfat (n = 8 per treatment) was weighed into a 2-mL centrifuge tube and microwaved on medium–high for 3 min twice. The melted fat was pipetted into another 2-mL centrifuge tube and weighed, and the tube was swirled for 30 s after the addition of 0.5 mL of hexane. A 1.2 mL aliquot of acetonitrile:water solution (75:25, vol/vol) was added. Then, the mixture was vortexed for 30 s and centrifuged at 13,500 × *g* at room temperature for 5 min. Approximately 0.5 to 1.0 mL of the lower aqueous phase was pipetted into a headspace vial for testing.

An ZORBAX SB-C18 (4.6 mm × 250 mm, 5 μm) column (Agilent, USA) was used for HPLC under the following conditions: column temperature of 40 °C, injection volume of 10 μL, mobile phase of acetonitrile:water (60:40, vol/vol), flow rate of 0.8 mL/min, and FLD detector excitation wavelength of 270 nm and emission wavelength of 360 nm.

2.7. Detection of short-chain fatty acids (SCFA) in feces

Acetic, propionic, butyric, isobutyric, and isovaleric acids in feces (n = 8 per treatment) were tested by using gas chromatography. Approximately 2 g of fecal sample was weighed into a 15-mL centrifuge tube, then 10 mL of ultrapure water was added, and the mixture was vortexed. Then the mixture was centrifuged at 12,000 × *g* at room temperature for 5 min, and the supernatant was separated. An aliquot (2 mL) of the supernatant was mixed with 0.2 mL of 50% sulfuric acid and 2 mL of diethylether in a 10-mL centrifuge tube. The mixture was centrifuged at 12,000 × *g* at room temperature for 5 min. Then, the supernatant was separated for testing. SCFA were tested with a gas chromatograph (Agilent model 7890, Palo Alto, CA, USA) coupled with a flame ionization detector and capillary Nukol column (30 m × 0.25 mm × 0.25 μm, Supelko, Bellefonte, PA, USA). The chromatographic conditions were as follows: injector temperature of 270 °C, detector

temperature of 280 °C, injection volume of 2 μL, splitless mode, and carrier gas of nitrogen at 2.0 mL/min. The column oven was programmed as follows: 100 °C for 1 min, followed by heating to 150 °C at speed of 5 °C/min and holding for 5 min.

2.8. Gut microbiota analysis by 16S rRNA gene sequencing

The microbial genomic DNA of rectal feces ($n = 8$ per treatment) was isolated and purified by using a QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany). The V3–V4 hypervariable regions of the 16S rRNA genes were PCR-amplified by using primers B341F and B806R (B341F: CCTACGGGNGGCWGCAG, B806R: GGACTACHVGGGTATCTAAT). The resulting PCR products were gel purified, quantified, pooled, and sequenced. Sequencing libraries were generated by using a NEBNext Ultra DNA Library Prep Kit for Illumina (NEB, USA). The library was sequenced on an Illumina PE250 platform. The resulting sequences were clustered into operational taxonomic units (OTU) by using UPARSE software at 97% sequence similarity. The taxonomy of each 16S rRNA gene sequence was determined by the RDP Classifier (http://rdp.cme.msu.edu/) with confidence greater than 70%. Data processing and analysis were performed by using the Omicsmart cloud platform (Guangzhou Gene Denovo Biotechnology Co., Ltd., Guangzhou, China).

2.9. Nontargeted metabolomics analysis of feces and serum

For feces ($n = 8$ per treatment), 100 mg of a sample was resuspended with 500 μL of 80% methanol (prechilled at 4 °C) by well vortexing. For serum ($n = 8$ per treatment), 100 μL of a sample and 400 μL of prechilled 80% methanol were mixed in a 1.5-mL centrifuge tube by well vortexing. The following steps were the same for fecal and serum samples. The samples were incubated on ice for 5 min then centrifuged at 15,000 × *g* and 4 °C for 20 min. The supernatant was transferred into a new tube and diluted with distilled water to the final methanol concentration of 53%, then centrifuged at 15,000 × *g* and 4 °C for 20 min. The supernatant was then ready for testing by the UHPLC–MS/MS system.

UHPLC–MS/MS analyses were performed by using a Vanquish UHPLC system (Thermo Fisher Scientific, Germany) coupled with a Hypesil Gold column (100 × 2.1 mm, 1.9 μm) and an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Germany) in Gene Denovo Co., Ltd. (Guangzhou, China). The samples were loaded by using a 17-min linear gradient at a flow rate of 0.2 mL/min. For the positive polarity mode, the eluents were 0.1% formic acid in water (eluent A) and methanol (eluent B). For the negative polarity mode, the eluents were 5 mM ammonium acetate (eluent A) and methanol (eluent B). The elution process was as follows: 2% B, 0–1.5 min; 2%–100% B, 1.5–12.0 min; 100% B, 12.0–14.0 min; 100%–2% B, 14.0–14.1 min; 2% B, 14.1–17 min. The ESI parameters were as follows: spray voltage of 3.2 kV, sheath gas flow rate of 40 arb, aux gas flow rate of 10 arb, and capillary temperature of 320 °C. The MS/MS secondary scans were data-dependent scans, and the scan range was M/Z 100–1500. The raw data files generated by UHPLC–MS/MS were processed by using Compound Discoverer 3.1 (CD3.1, Thermo Fisher Scientific, Germany). Peak intensities were normalized to the total spectral intensity, and peaks were matched with the mzCloud, mzVault, and MassList databases to obtain the accurate qualitative and relative quantitative results.

2.10. In vitro cell test

The hepatic cell line HepG2 was obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Gibco, USA) at 37 °C and 5% CO_2. The cells were incubated in 6-well plates with mulberry leaf PP, PS, or AL (100 or 200 μg/L) for 24 h with 3 replicates per treatment. Then, the culture medium was removed, and the cells were stored at −80 °C before testing.

2.11. Quantitative real-time polymerase chain reaction (qRT-PCR)

The gene expression levels of *CYP* genes were analyzed by using qRT-PCR as previously reported (Sun et al., 2021). The total RNA from pig liver ($n = 8$ per group) or HepG2 cells ($n = 3$ per group) was isolated by using a universal mRNA purification kit (TaKaRa, Japan) and reverse transcribed into cDNA by using a reverse transcription reagent kit with gDNA Eraser (TaKaRa, Japan). The qRT-PCR was performed by using the SYBR Fast qPCR Mix (TaKaRa, Japan) and the StepOne Plus real-time PCR system (Life Technologies, Carlsbad, CA, USA). The amplification conditions were denaturation at 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The gene-specific primers used in qRT-PCR are shown in Tables 3 and 4. The expression level of each target gene was normalized to the housekeeping gene 18S rRNA (for pig liver) or GAPDH (for HepG2 cells) by using the $2^{-\triangle\triangle Ct}$ method.

2.12. Western blot analysis

The TP of liver were extracted with the Minute Total Protein Extraction Kit for Animal Cultured Cells/Tissues (Invent, USA), and protein concentrations were determined by using a BCA protein assay kit (Beyotime, China). Protein extracts were adjusted to the same protein concentration. As a total of 16 samples could not be all loaded into 1 protein gel and 3 replicates for Western blot trial are usually conducted in most studies, the protein extracts from 8 pigs under 1 treatment were mixed into 3 samples by mixing every 2 or 3 extracts into 1 sample. The mixed samples were then denatured with loading buffer at 95 °C for 5 min. Protein samples ($n = 3$) were loaded into each well of a 4%–12% SDS polyacrylamide gel (Invent, USA) then transferred to a nitrocellulose membrane (Invent, USA) by using an IBlot Dry transfer system (Life Technologies, USA). After blocking with SuperBlock T20 blocking buffer (37516, Thermo Fisher Scientific, USA) for 1 h at room temperature, the membranes were incubated with the corresponding primary antibodies at room temperature for 1 h. The membranes were washed 3 times with 1× TBST buffer (20 mmol/L Tris–HCl [pH 7.6], 8 g/L NaCl, and 0.1% Tween-20) for 5 min each time then incubated with the secondary antibody at room temperature for 1 h. The primary antibodies were rabbit CYP1A1 (1324-1-AP, Proteintech, China) and β-actin (ab8227, Abcam, UK). The secondary antibody was Alexa Fluor 790 goat antirabbit IgG H&L (ab175781, Abcam, UK). The membranes were

Table 3
Primers used in qRT-PCR of pig liver.

Gene	Primer	Sequence (5'–3')	Size, bp
CYP1A1	Forward	GGGAAGCCACTCCCATCTAC	72
	Reverse	CCCTTCGTCCTCCCACAGTA	
CYP1A2	Forward	TGAATTTGTGGAGACCGCCT	191
	Reverse	ATAGGGCGCTTGTGATGTCC	
CYP2A	Forward	GCAACCCGGGACACAGATTT	173
	Reverse	CCTGCGGAAAATGCCTTGAA	
CYP2E1	Forward	CTTCGGAGACCGCTTTGACT	185
	Reverse	GCCCCTGCAGGTCCTTAAAT	
18S rRNA	Forward	CCCACGGAATCGAGAAAGAG	122
	Reverse	TTGACGGAAGGGCACCA	

CYP1A1 = cytochrome P450 1A1; *CYP1A2* = cytochrome P450 1A2; *CYP2A* = cytochrome P450 2A; *CYP2E1* = cytochrome P450 2E1.

Table 4
Primers used in qRT-PCR of HepG2 cells.

Gene	Primer	Sequence (5'–3')	Size, bp
CYP1A1	Forward	CAAGGGGCGTTGTGTCTTTG	115
	Reverse	GTCGATAGCACCATCAGGGG	
CYP1A2	Forward	CTTCGCTACCTGCCTAACCC	123
	Reverse	GTCCCGGACACTGTTCTTGT	
CYP2A	Forward	CTCATGAAGATCAGTGAGCGCTAT	195
	Reverse	CCTCATGAAGATCAGTGAGCGCTAT	
CYP2E1	Forward	GAAGCCTCTCGTTGACCCAA	101
	Reverse	ATGAGCGGGGAATGACACAG	
GAPDH	Forward	CCACTAGGCGCTCACTGTTC	105
	Reverse	CGCCCAATACGACCAAATCC	

CYP1A1 = cytochrome P450 1A1; *CYP1A2* = cytochrome P450 1A2; *CYP2A* = cytochrome P450 2A; *CYP2E1* = cytochrome P450 2E1; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

imaged after another 3 washes by using a ChemiDoc touch imaging system (BIO-RAD, USA). Protein expression levels were detected by using ImageLab software.

2.13. Data analysis

Data were expressed as means ± SD or medians with interquartile ranges. Differences in bacterial abundance were analyzed by using Welch's test. Microbial Tax4Fun function predictions were analyzed via the Wilcoxon rank sum test. For phylotype analysis, the alpha diversity of the microbiome was calculated on the basis of OTU level by using mothur (version 1.30.1). Principal coordinate analysis (PCoA), the nonmetric multidimensional scaling (NMDS) analysis of the microbiome, and the partial least squares discriminant analysis (PLS-DA) of the metabolome were performed and visualized by R, and significant differences were evaluated by conducting Adonis analysis. *P*-values were adjusted for multiple comparisons by using the Benjamini-Hochberg correction. The false discovery rate and significance were set at $q < 0.05$. Other statistical analyses were performed by using Student's *t*-test with SPSS, version 16 (SPSS Inc., Chicago, IL, USA). The Pearson correlation coefficient was used for correlation analysis. The $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered extremely significant.

3. Results

3.1. Growth performance, meat quality traits and biochemical indices in serum and muscle

For growth performance, no significant difference in initial BW, final BW, ADG, ADFI, and FCR ($P > 0.05$) between CON and MB groups were observed (Table 5), and none of these results showed differences between the two groups when analyzed separately by sex (Table S1). For meat quality traits of longissimus dorsi muscle, the meat color L* was decreased ($P = 0.018$) in MB group compared to CON group, while the meat color a*, meat color b*, drip loss, mill loss, cooking loss and intramuscular fat did not show significant differences ($P > 0.05$) between the two groups (Table S2). Biochemical indices, such as TP, ALB, TC, TG, TAOC and MDA in serum, and TP, TC, TG, TAOC, MDA and NEFA in muscle, also did not show significant differences ($P > 0.05$) between the two treatment groups (Fig. S1).

3.2. Skatole content in feces, serum, muscle, and backfat

As shown in Table 5, the mulberry leaf treatment significantly reduced the skatole content in feces ($P = 0.011$), serum ($P = 0.018$), and backfat ($P = 0.010$) but had no significant effect on skatole content in muscle ($P > 0.05$). The skatole contents followed the order of feces > backfat > muscle > serum. When analyzed separately by sex, none of these results showed differences between the two groups (Table S1).

Table 5
Effects of mulberry leaf diet on growth performance, skatole and short-chain fatty acids in finishing pigs.

Item	CON	MB	*P*-value
Growth performance ($n = 10$)			
Initial BW, kg	75.68 ± 6.91	75.64 ± 8.40	0.991
Final BW, kg	102.72 ± 5.73	101.36 ± 10.53	0.737
ADG, kg	0.77 ± 0.15	0.73 ± 0.14	0.569
ADFI, kg	2.47 ± 0.23	2.51 ± 0.34	0.773
FCR	3.34 ± 0.89	3.47 ± 0.38	0.704
Skatole content ($n = 8$)			
Feces, μg/g	35.52 ± 3.72	30.06 ± 3.23*	0.011
Serum, ng/mL	30.09 ± 4.70	23.23 ± 4.88*	0.018
Muscle, ng/g	95.15 ± 2.86	99.06 ± 5.55	0.120
Backfat, ng/g	182.44 ± 26.32	146.95 ± 17.06*	0.010
Fecal short-chain fatty acids contents ($n = 8$)			
Acetic acid, mg/g	2.64 ± 0.38	3.25 ± 0.53*	0.027
Propionic acid, mg/g	1.34 ± 0.21	1.43 ± 0.30	0.519
Isobutyric acid, mg/g	0.19 ± 0.04	0.16 ± 0.04	0.228
Butyric acid, mg/g	0.64 ± 0.11	0.71 ± 0.20	0.370
Isovaleric acid, mg/g	0.30 ± 0.07	0.24 ± 0.08	0.159

CON = basal diet; MB = mulberry leaf diet; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio.
The asterisk means significant different from basal diet group, *$P < 0.05$.

3.3. SCFA content in feces

SCFA have been reported to regulate the production of skatole in the gut. Therefore, the SCFA content in feces was measured. As shown in Table 5, mulberry leaf treatment significantly increased the acetic acid content in feces ($P = 0.027$) but did not affect the contents of propionic, butyric, isobutyric, and isovaleric acids in feces ($P > 0.05$).

3.4. Gut microbial community

Given that skatole is produced by microbes, the gut microbial community in rectal feces was analyzed by using 16S rRNA gene sequencing to reveal the responses of gut microbes in finishing pigs to mulberry leaves. Fig. 1A illustrates that the Chao1 and Shannon indices in the MB group were significantly higher ($P < 0.05$) than those in the CON group. These results suggested that the richness and diversity of microbiota in the MB group was increased. PCoA and NMDS analyses at the OTU level (Fig. 1 B) revealed that the gut microbial community of the MB group was separate from that of the CON group, indicating that mulberry leaf supplementation changed the gut microbial structure. Fig. 1C illustrates the genera that were significantly downregulated by mulberry leaf supplementation. The correlations of these genera with skatole and acetic acid in feces were also investigated. Nine genera were significantly downregulated by mulberry leaf supplementation. Two of these genera, namely, *Olsenella* and *Megasphaera*, were skatole-producing bacteria that have been previously reported. *Olsenella* showed a significant positive correlation with skatole in feces. Taxonomy analysis demonstrated that *Olsenella* belonged to family Atopobiaceae in phylum Actinobacteria, and *Megasphaera* belonged to family Veillonellaceae in phylum Firmicutes. Although *Megasphaera* was not significantly correlated with skatole in feces, *Mitsuokella* and *Anaerovibrio*, two genera that also belonged to family Veillonellaceae, showed a significantly positive correlation with skatole in feces. Moreover, *Mitsuokella* and *Anaerovibrio* were significantly and

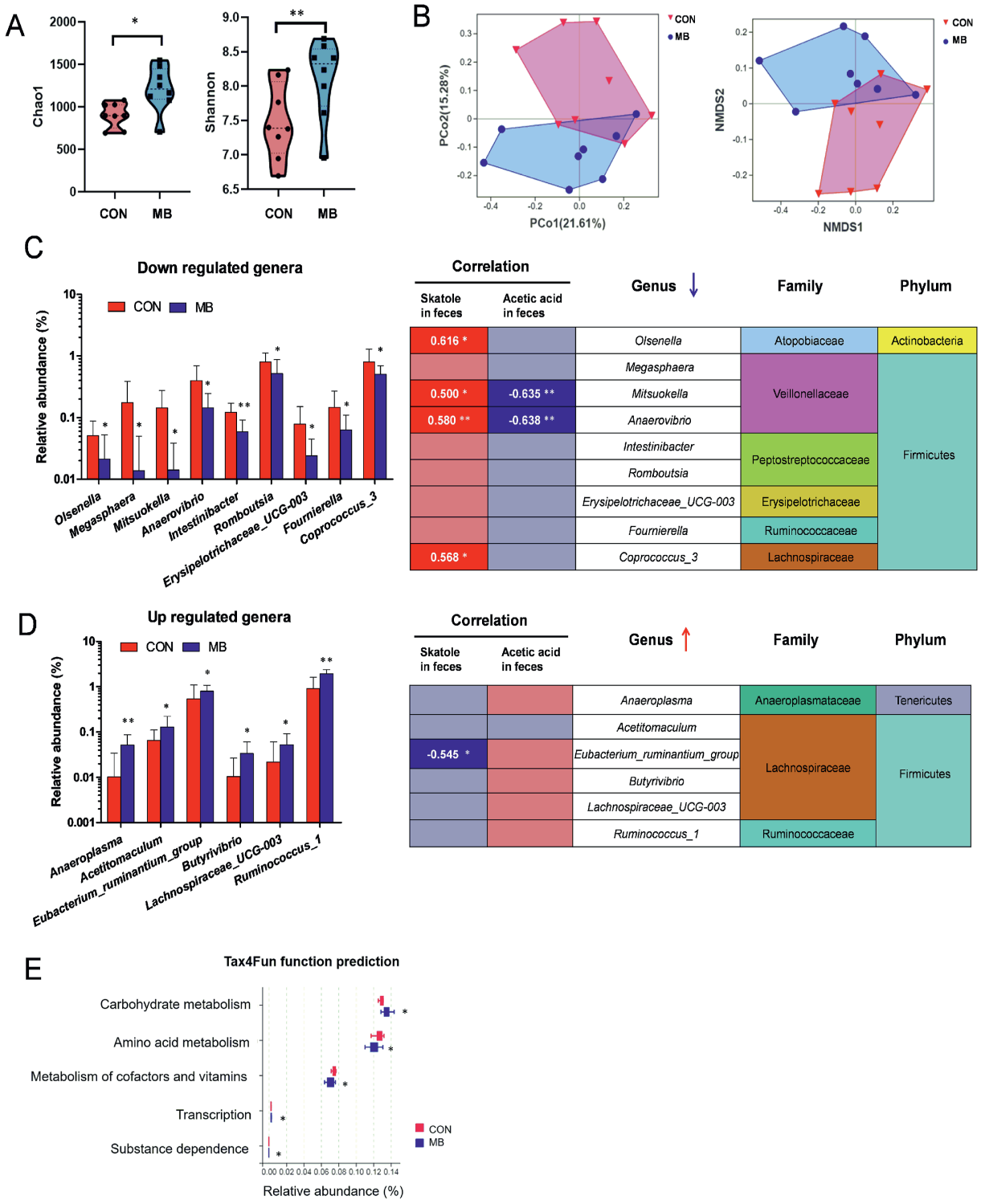

Fig. 1. Effects of mulberry leaf diet on the gut microbiota of finishing pigs. (A) Chao1 and Shannon indices. (B) PCoA and NMDS analyses. (C) Relative abundance, correlation analysis, and taxonomy of genera that were downregulated by the mulberry leaf diet. (D) Relative abundance, correlation analysis, and taxonomy of genera that were upregulated by the mulberry leaf diet. (E) Tax4Fun functional prediction analysis of bacterial operational taxonomic units (OTU). PCoA = principal coordinate analysis; NMDS = nonmetric multidimensional scaling; CON = basal diet; MB = mulberry leaf diet. $n = 8$ for all analyses. The asterisk indicates a significant difference from the basal diet group, $^{*}P < 0.05$, $^{**}P < 0.01$.

negatively correlated with acetic acid in feces ($P < 0.01$), but further research is needed to reveal their role in skatole production. *Coprococcus_3*, a genus belonging to family Lachnospiraceae in phylum Firmicutes, also showed a significant positive correlation with skatole in feces. Fig. 1D displays the genera that were significantly upregulated by mulberry leaf supplementation. Six genera were significantly upregulated by mulberry leaf supplementation. Among these genera, *Eubacterium_ruminantium_group*, a genus belonging

to family Lachnospiraceae, showed a significantly negative correlation with skatole in feces. Tax4Fun function prediction analysis indicated that the relative abundance of the OTU related to carbohydrate metabolism had significantly increased, whereas that related to amino acid metabolism had significantly decreased by mulberry leaf supplementation. Other three predicted functions, such as metabolism of cofactors and vitamins, transcription, and substance dependence, also changed, but the relative abundance of OTU related to these functions were much lower (less than 0.10%).

3.5. Metabolomics analysis of feces

Changes in microbiota are also reflected by changes in metabolites. Feces were subjected to metabolomics analysis to explore the effect of mulberry leaves on the metabolism of microbiota. Fig. 2A shows that in PLS-DA, the samples in the MB group were separate from those in the CON group, indicating the existence of differences between the compositions of the fecal metabolites in the two groups. Compared with the CON group, 91 metabolites were downregulated and 81 metabolites were upregulated in the MB group (Fig. 2B). The KEGG pathway enrichment of these different metabolites showed that the purine metabolism pathway and drug metabolism–cytochrome P450 pathway had significantly changed (Fig. 2C). A heat map of the differential metabolites in the two pathways is provided in Fig. 2D. Tryptophan degradation metabolites, such as indole and indole derivatives, were compared between the two groups to understand the anabolic processes of skatole. As shown in Fig. 2E, indole-3-acetic acid (IAA), the precursor compound of skatole (Jensen et al., 1995a), had significantly decreased under mulberry leaf treatment ($P < 0.05$), whereas 10 other metabolites were not significantly affected ($P > 0.05$). Moreover, IAA showed a significantly positive correlation with skatole content in feces ($P = 0.004$; Fig. 2F).

3.6. Metabolomics analysis of serum

Metabolomics analysis was performed on serum to further investigate the effect of mulberry leaf treatment on metabolites in the blood. Similar to that in the fecal metabolomics analysis, in PLS-DA, the samples of the MB group were distinct from those of the CON group, indicating variations in the composition of serum metabolites between the two groups (Fig. 3A). A total of 48 metabolites in the MB group were significantly changed compared to those in the CON group, of which 10 were downregulated and 38 were upregulated (Fig. 3B). The KEGG pathway enrichment analysis of the differential metabolites (Fig. 3C) showed that the phenylalanine, tyrosine, and tryptophan biosynthesis; protein digestion and absorption; cyanoamino acid metabolism; phenylalanine metabolism; and aromatic compound degradation pathways had significantly changed ($P < 0.05$). A heat map of the differential metabolites in these pathways is provided in Fig. 3D. Indole and indole derivatives with levels that significantly differed between

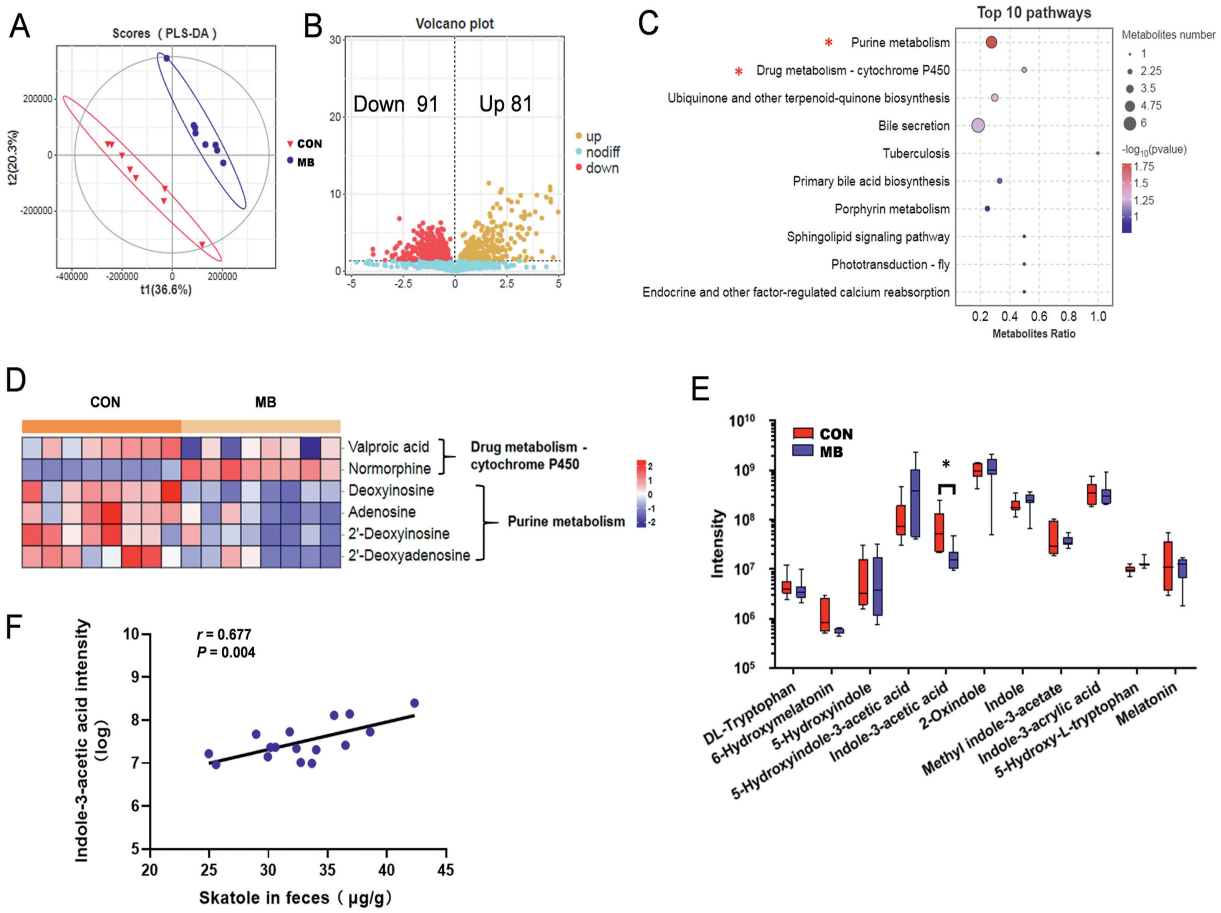

Fig. 2. Fecal metabolomics analysis of finishing pigs. (A) Partial least squares discriminant analysis (PLS-DA), (B) volcano plot of differential metabolites, (C) KEEG enrichment of differential metabolites, and (D) heatmap of differential metabolites in the changed pathways. (E) Peak intensities of metabolites related to indole and indole derivatives. (F) Correlation analysis of indole-3-acetic acid in feces and skatole in feces. CON = basal diet; MB = mulberry leaf diet. $n = 8$ for all analyses. The asterisk indicates a significant difference from the basal diet group, $^*P < 0.05$.

groups are presented in Fig. 3E. DL-tryptophan, indole, and indole-3-lactic acid in the serum of the MB group had significantly increased compared with those in the CON group ($P < 0.05$). Their correlation with skatole content in serum was analyzed to explore the relationship between these indole derivatives and skatole. Fig. 3F shows that DL-tryptophan, indole, and indole-3-lactic acid were all negatively correlated with skatole content in serum ($P < 0.05$).

3.7. Expression of CYP in the liver and hepatic cell line HepG2

The gene expression of major CYP enzymes that were demonstrated to catalyze skatole degradation in the liver was examined to determine whether mulberry leaves have an effect on skatole degradation in finishing pigs. As shown in Fig. 4A, the mRNA expression of *CYP1A1* significantly increased in the MB group ($P < 0.05$), whereas that of *CYP1A2*, *CYP2E1*, and *CYP2A* did not significantly change ($P > 0.05$). CYP can be inhibited by androstenone in intact males, therefore leading to a higher skatole concentration in intact males than in females. In our study, the male pigs were castrated, and gene expression of these CYP did not show differences between sexes (Table S1).

Correlation analysis (Fig. 4B) revealed that the gene expression of *CYP1A1* was negatively correlated with skatole concentration in fat ($P = 0.045$) but was not significantly correlated with skatole in serum ($P > 0.05$). The upregulation of CYP1A1 in pigs under mulberry leaf treatment was also verified at the protein level through Western blot analysis (Fig. 4C). The effects of major bioactive compounds in mulberry leaves on *CYP* genes were tested in vitro by using the hepatic cell line HepG2. As shown in Fig. 4D, PP and PS upregulated *CYP1A1* mRNA expression in HepG2 cells in a dose-dependent manner ($P < 0.01$), and the regulatory effect of PP was considerably stronger than that of PS. By contrast, both doses of AL downregulated *CYP1A1* expression ($P < 0.01$). The expression levels of *CYP1A2*, *CYP2E1*, and *CYP2A* were not significantly affected by treatment with PP, PS, or AL ($P > 0.05$).

4. Discussion

We previously investigated the effects of basal diets containing 10% or 20% mulberry leaves on the growth performance, fat metabolism, and meat quality of finishing pigs in a 65-day trial. We found that in finishing pigs, the 20% mulberry leaf diet reduced ADG and increased FCR, whereas the 10% mulberry leaf diet did not affect ADG and FCR (Li et al., 2012). The 10% and 20% mulberry leaf diets decreased leaf lard percentage and backfat thickness and increased intermuscular fat content (Li et al., 2012). Liu et al. (2021) reported that 3%, 6%, and 9% mulberry leaf diets had no adverse effects on the growth performance of finishing pigs, whereas a 12% mulberry leaf diet decreased growth performance in a 50-day feed experiment. In addition, the serum antioxidant properties were improved linearly with an increase in the amount of mulberry

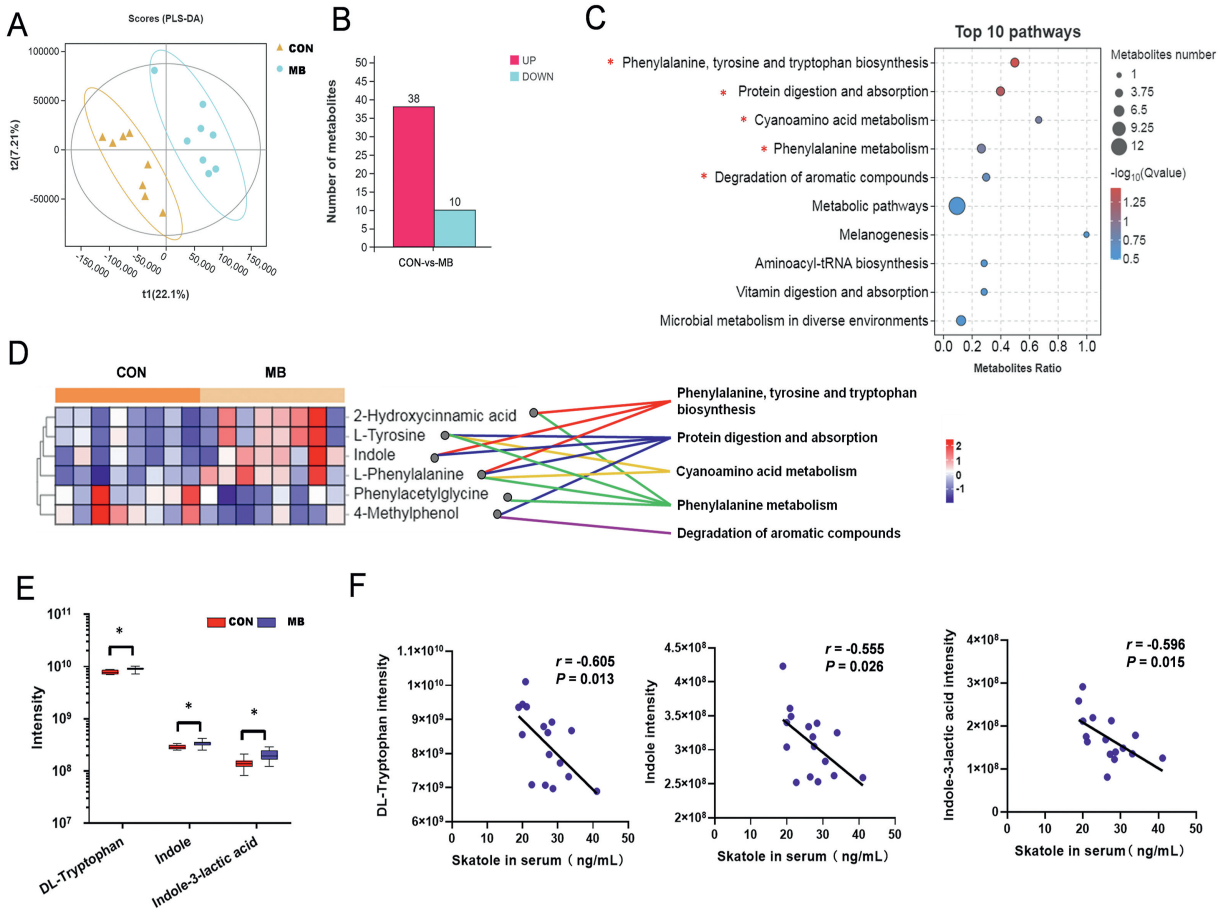

Fig. 3. Metabolomics analysis of the serum of finishing pigs. (A) Partial least squares discriminant analysis (PLS-DA), (B) differential metabolite numbers, (C) KEEG enrichment of differential metabolites, and (D) differential metabolites in the changed pathways. (E) Peak intensities of significantly changed metabolites related to indole and indole derivatives. (F) Analysis of the correlation of DL-tryptophan, indole, and indole-3-lactic acid in serum with skatole in serum. CON = basal diet; MB = mulberry leaf diet. $n = 8$ for all analyses. The asterisk indicates a significant difference from the basal diet group, $^*P < 0.05$.

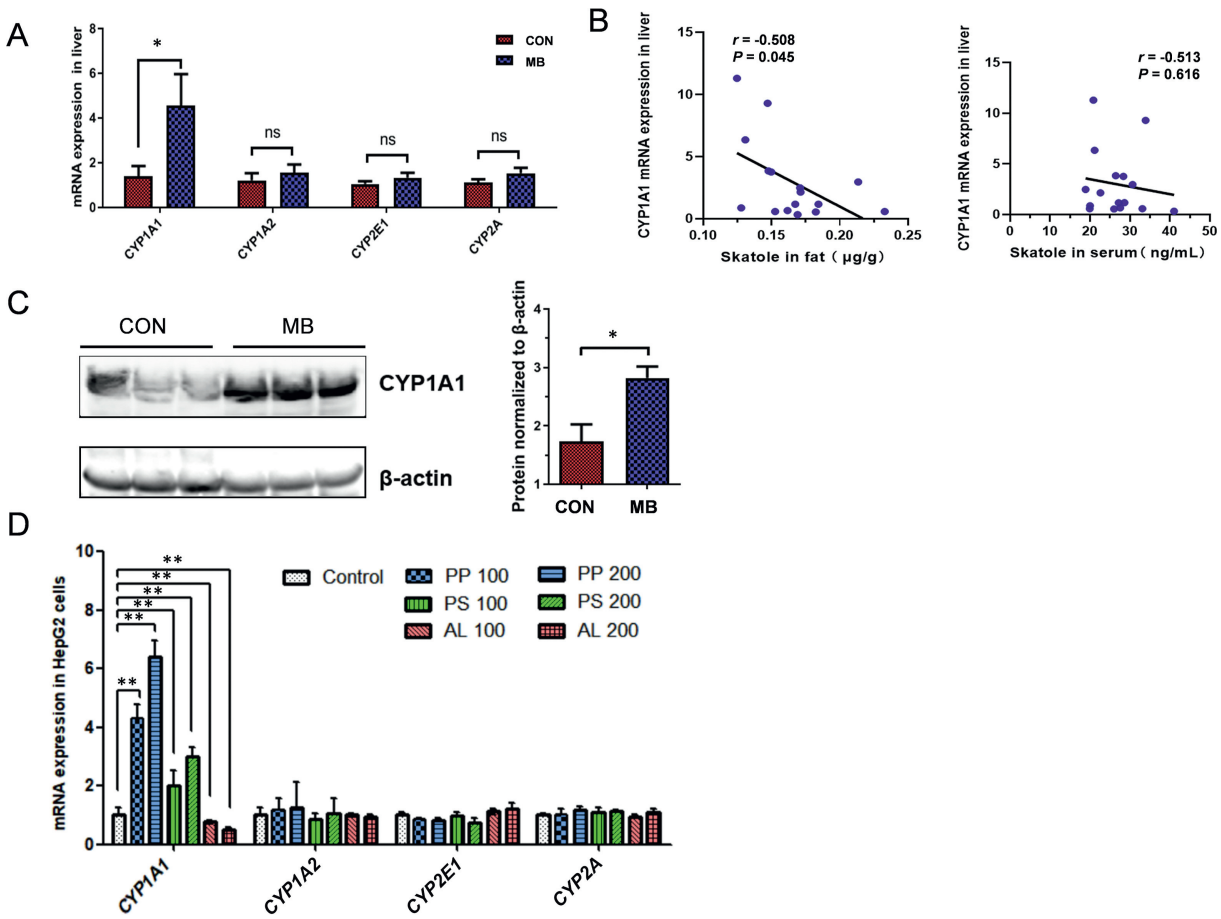

Fig. 4. Cytochrome P450 expression levels in the liver of finishing pigs and the hepatic cell line HepG2. (A) mRNA expression levels of *CYP1A1*, *CYP1A2*, *CYP2E1*, and *CYP2A* in the liver of finishing pigs ($n = 8$). (B) Analysis of the correlation of *CYP1A1* gene expression in the liver with skatole in fat and skatole in serum, respectively. (C) Western blot analysis of CYP1A1 protein level in the liver of finishing pigs ($n = 3$). (D) In vitro test of the effects of polyphenols (PP), polysaccharides (PS), and alkaloids (AL) extracted from mulberry leaves on the mRNA expression levels of *CYP1A1*, *CYP1A2*, *CYP2E1*, and *CYP2A* in the hepatic cell line HepG2 ($n = 3$). CON = basal diet; MB = mulberry leaf diet. The asterisk indicates a significant difference from the basal diet group or control cells, $^*P < 0.05$, $^{**}P < 0.01$.

leaves included in the diet (Liu et al., 2021). In an 85-day trial on finishing pigs, a 15% mulberry leaf diet decreased ADG and increased FCR but improved meat quality by changing myofiber characteristics, enhancing antioxidative capacity, and increasing intramuscular fat content (Zeng et al., 2019). In this study, feeding 6% mulberry leaf in the diet for 35 days had no significant effect on growth performance, but significantly decreased the meat color L* value of longissimus dorsi muscle in finishing pigs. The meat color L* value represents the brightness of the meat. Pale, soft and exudative (PSE) meat has high L* value and low a* value. Our results suggested that mulberry leaf in the diet improved meat color to some extent. Mulberry leaf inclusion did not affect drip loss, mill loss, cooking loss or intramuscular fat of longissimus dorsi muscle. The antioxidative capacity of serum and muscle were also unaffected by a 6% mulberry leaf diet. This may be due to the relatively low content of mulberry leaves in the diet and the shorter feeding time in this study compared to previous studies.

We observed a significant reduction in skatole content in the feces, serum, and backfat of mulberry leaf-treated pigs. Skatole is produced from tryptophan by microbes in the hindgut (Ma et al., 2021). Undigested carbohydrates, such as fibers, PS, and oligosaccharides, have been proven to regulate gut microbiota and reduce skatole production (Bekkelund et al., 2022; Jensen et al., 1995b; Lösel et al., 2006; Vhile et al., 2012). Undigested carbohydrates are thought to inhibit skatole production in two ways: First, skatole-producing bacteria preferentially utilize undigested carbohydrates, thereby reducing the utilization of tryptophan (Jensen et al., 1995b). Second, the increase in SCFA in the hindgut as a result of undigested carbohydrate fermentation reduces the pH of the gut and therefore leads to the bacterial metabolism of tryptophan into indole instead of skatole (Doerner et al., 2009; Vhile et al., 2012). Mulberry leaves are rich in dietary fiber and PS and may reduce skatole production by regulating the microbiota. In this study, mulberry leaf treatment changed the richness, diversity, and structure of the microbiota and decreased the abundance of the skatole-producing bacteria *Olsenella* and *Megasphaera*. To our knowledge, only a few skatole-producing bacteria have been identified thus far. *Lactobacillus*, *Clostridium*, *Olsenella*, and *Megasphaera* are the major known skatole-producing genera (Ma et al., 2021). In genus *Olsenella*, *Olsenella uli* DSM 7084 and *Olsenella scatoligenes* SK9K4 are two known skatole-producing strains that were first isolated from human gingival crevices and pig feces, respectively (Li et al., 2015; Olsen et al., 1991). *Megasphaera* is a genus belonging to family Veillonellaceae. The strain *Megasphaera* sp. TrE9262, which was first isolated from sheep, has been demonstrated to produce skatole (Attwood et al., 2006). However, not all bacteria that belong to the genus *Megasphaera* produce skatole. For example, *Megasphaera* sp. strain DJF_B143, an isolate from the pig hindgut, cannot produce skatole (Li et al., 2016). This situation may

account for the lack of the positive correlation between *Megasphaera* and skatole content in feces. We found that *Mitsuokella* and *Anaerovibrio*, another two genera belonging to family Veillonellaceae, were significantly and positively correlated with skatole in feces. Moreover, they decreased in the MB group and were negatively correlated with acetic acid content in feces. However, further research is needed to reveal their role in skatole production. Although we found that *Coprococcus 3*, a butyrate-producing genus, was also positively correlated with skatole in feces, evidence or literature linking it to skatole production is unavailable. The upregulated genera *Eubacterium ruminantium group*, *Anaeroplasma*, *Butyrivibrio*, and *Ruminococcus 1* are all SCFA-producing fibrolytic bacteria (Koike and Kobayashi, 2009; Liang et al., 2021; Luo et al., 2019; Niu et al., 2015) and may partially account for the increase in acetic acid in feces. The enhanced carbohydrate metabolism and weakened amino acid metabolism found by bacterial function prediction analysis also reflected in the change in gut bacteria. In general, this change was unfavorable to skatole production. Our results are consistent with the recent study of Song et al. (2023), who reported that 0.1% mulberry leaf extract supplementation could increase the number of carbohydrate degrading bacteria *Lactobacillus* and *Bifidobacterium* and reduce the number of nitrogen compound degrading bacteria *Escherichia coli*, thus increasing lactate in feces and reducing the formation of NH_3–N, biogenic amines, and skatole in feces of weaned piglets.

Skatole-producing bacteria convert tryptophan into skatole through a two-step mechanism. During the first step, tryptophan is deaminated into IAA, and during the second step, IAA is further decarboxylated into skatole (Attwood et al., 2006; Doerner et al., 2009; Li et al., 2015). IAA is the essential intermediate for skatole production. In our study, the IAA level in feces from mulberry leaf-treated pigs significantly decreased. Furthermore, fecal IAA level was significantly and positively correlated with fecal skatole level. This correlation suggested that tryptophan deamination, the first step of skatole production, was suppressed by mulberry leaf supplementation. Tryptophan has been proven to be mostly converted into IAA or indole by bacteria (Doerner et al., 2009; Jensen et al., 1995a; Roager and Licht, 2018). Small amounts of other tryptophan metabolites, such as indole-3-lactic acid, may be also detected (Jensen et al., 1995a, b; Roager and Licht, 2018). Low pH benefits indole production, whereas high pH benefits IAA production (Doerner et al., 2009; Vhile et al., 2012). We observed a significant increase in indole in serum and an insignificant increase in indole in feces in pigs treated with mulberry leaves. Indole-3-lactic acid also increased in the serum of pigs treated with mulberry leaves. The increase in indole and indole-3-lactic acid implied that the conversion of tryptophan into IAA in pigs treated with mulberry leaves was reduced. Moreover, the levels of tryptophan in serum were increased in pigs treated with mulberry leaves. As mentioned earlier, skatole-producing bacteria preferentially utilize undigested carbohydrates instead of tryptophan (Jensen et al., 1995b). Therefore, we inferred that the increase in tryptophan in the serum of pigs treated with mulberry leaves may be due to the reduction in tryptophan utilization by bacteria and the consequent increase in tryptophan absorption by the large intestine. The negative correlation of skatole in serum with tryptophan, indole, and indole-3-lactic acid in the serum can be easily understood on the basis of the above analysis.

Skatole deposition in pigs is not only related to skatole production in the gut lumen by bacteria but also to the catabolism of skatole in vivo (Lanthier et al., 2007). The liver is the main metabolic organ for skatole degradation, and CYP is the key enzyme family catalyzing skatole production in the liver (Lanthier et al., 2007; Wiercinska et al., 2012). CYP isoforms, such as CYP1A, CYP2A, and CYP2E1, have been reported to be responsible for the degradation of skatole (Burkina et al., 2019; Liu et al., 2020; Wiercinska et al., 2012). Factors affecting these CYP enzymes can therefore affect skatole accumulation in the body.

In this study, we detected the gene expression levels of four well-known skatole-degrading CYP enzymes, namely, CYP1A1, CYP1A2, CYP2E1, and CYP2A, in the liver of finishing pigs. We found that the expression of *CYP1A1* was significantly upregulated by mulberry leaf treatment. Moreover, *CYP1A1* expression level was negatively correlated with skatole content in backfat but was not correlated with skatole content in serum. This result implied that *CYP1A1* activation affected the deposition, but not absorption, of skatole. Our results suggested that mulberry leaves may promote skatole degradation by stimulating *CYP1A1* expression in the liver of finishing pigs. It is known that boars have higher skatole levels than barrows and gilts because the androstenone secreted by the testicles inhibits the activities of CYP (Lösel et al., 2006; Wesoly et al., 2015; Zamaratskaia et al., 2012). In the present study, barrows and gilts were used as experimental animals because intact male pigs are not used for meat production in China. As castration of boars in some countries is prohibited due to the protection of animal welfare, effects of mulberry leaves on boars need to be studied in the future.

The in vitro test on the hepatic cell line HepG2 further demonstrated that mulberry leaf components, PP, and PS can directly simulate *CYP1A1* expression. Considering that PS are large compounds that are probably not absorbed in entirety by the gut, we speculate that PP are likely the substances that effectively activate *CYP1A1* in vivo.

5. Conclusions

In a 35-day trial on finishing pigs, the 6% mulberry leaf diet did not affect performance but reduced skatole levels in feces, serum, and backfat. Mulberry leaves may reduce skatole production in the gut by decreasing the abundance of the skatole producing bacteria *Olsenella* and *Megasphaera* and reducing the conversion of tryptophan into IAA, the precursor of skatole. Moreover, mulberry leaves may promote skatole degradation by stimulating the expression of *CYP1A1* in the liver. PP in mulberry leaves may be the active compounds with stimulatory effects on *CYP1A1*. The above findings suggest that mulberry leaves could reduce odor emission in pig farming and improve pork quality.

Author contributions

Yuqing Sun: Data curation, Formal analysis, Methodology, Writing-original draft. **Xiaoming Men**: Investigation, Data curation, Formal analysis, Funding acquisition. **Tianbao Lin**: Methodology, Resources. **Bo Deng**: Conceptualization, Resources. **Shi Zhong**: Data curation. **Jinxi Huo**: Writing-review & editing. **Kaipeng Qin**: Methodology, Investigation. **Zhiqiang Lv**: Resources, Funding acquisition. **Ziwei Xu**: Conceptualization, Funding acquisition, Writing-review & editing. **Yougui Li**: Data curation, Conceptualization, Funding acquisition, Writing-review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.05.018.

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