

1 **Supplemental material**

2 **Phenazines modulate gastrointestinal metabolism and microbiota**

3 Wenjing Peng^{1#}, Hui Li^{2#}, Xiaole Zhao¹, Bing Shao^{1, 2*}, Kui Zhu^{1*}

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5 ¹ National Center for Veterinary Drug Safety Evaluation, College of Veterinary Medicine, China
6 Agricultural University, Beijing, 100193, China.

7 ² Beijing Key Laboratory of Diagnostic and Traceability Technologies for Food Poisoning, Beijing
8 Center for Disease Prevention and Control, Beijing, 100013, China.

9 *Corresponding authors. E-mail address: shaobingch@sina.com (B. Shao), zhuk@cau.edu.cn (K.
10 Zhu).

11 [#] These authors contributed equally to this work.

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37 **Materials and Methods**

38 **Materials**

39 The standard of pyocyanin (99%) was purchased from Cayman Chemical (Michigan, United
40 States). Liver microsomes of rat, swine, human and chicken were obtained from Meilunbio, CHI
41 Scientific Inc., Gentest, and Primetox, respectively. β -Nicotinamide adenine dinucleotide phosphate
42 (NADPH) and uridine-5'-diphosphoglucuronic acid (UDPGA) were purchased from Sigma-Aldrich
43 (Hamburg, Germany). HPLC water was purified through a Milli-Q ultrapure system (Millipore,
44 Bedford, MA). LC-MS grade acetonitrile and methanol were purchased from Merck (Darmstadt,
45 Germany). HPLC-grade acetonitrile and formic acid were supplied by Fisher Chemicals (Fair Lawn,
46 NJ, USA). Tris-HCl was acquired from Phygene Life Sciences Co. Ltd. (Fuzhou, China).

47 **Animals**

48 Wistar rats (body weight 200 ± 20 g) and C57BL/6J mice (male, two-month-old were purchased
49 from Beijing Boehringer Ingelheim Vital Technology Co. Ltd. (Beijing, China), male Bama minipigs
50 (20 ± 2 kg) bought from Animal Breeding Center of Liulilihe Kexing Experimental Beijing (Beijing,
51 China). Before the experiment, all animals were acclimatized for one week in laboratory conditions
52 with standardized temperature ($23^{\circ}\text{C} \pm 2^{\circ}\text{C}$), humidity ($55\% \pm 10\%$), 12 h light/dark cycle
53 conditions, and were fed a standard diet and given water ad libitum. All animals experimental
54 procedures followed the National Institute of Health and the ARRIVE guidelines. Housing and care
55 of the animals met the National Research Council guidelines and were ensured by trained

56 veterinarians. The laboratory animal usage license number is SYXK-2018-0038, certified by Beijing
57 Association for Science and Technology.

58 **Quantification of PYO in plasma**

59 Plasma samples were determined according to our previously published analytical method [1]. The
60 HPLC-MS apparatus (Shimadzu, HPLC/MS-8060, Kyoto, Japan) was equipped with an ACQUITY
61 BEH C₁₈ column (2.1 × 100 mm, 1.7 μm; Waters, MA, USA) at an oven temperature of 35 °C and a
62 flow-rate of 0.3 mL/min. The parameters of the ion source were set as follows: nebulizing gas flow:
63 3 L/min; heating gas flow: 10 L/min; drying gas flow: 10 L/min; interface temperature: 300°C;
64 desolvation line temperature: 250°C; and heat block temperature: 400°C. The mobile phases were
65 acetonitrile (A) and 0.1% formic acid water (B). The gradient conditions were as follows: 0–5.0 min,
66 5% A to 30% A; 5.0–5.1 min, 30% A to 50% A; 5.1–7.1min, 50% A to 70% A; 7.1–11.1 min, 70%
67 A to 100% A; 11.1–13 min, 100% A for 2 min; 13.0–14.0 min, returned to 5% A; 14.0-16.0 min, 5%
68 A for 2 min, and the injection volume was set to 5 μL. The data was analyzed using WinNonlin v8.1
69 software via non-compartmental analysis.

70 **Microbiome analysis**

71 Total genome DNA from samples (livers and contents of duodenum, jejunum, ileum, cecum,
72 colon, and rectum) was extracted using cetyltrimethylammonium bromide/sodium dodecyl sulfate
73 (CTAB/SDS) method. DNA concentration and purity was analyzed on 1% agarose gels. Then DNA
74 was diluted to 1 μg/μL using sterile water. The V3–V4 hypervariable regions of the bacteria 16S
75 rRNA gene were amplified with primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-
76 GGACTACNNGGGTATCTAAT-3'). The PCR reactions were conducted using the following

77 program: 1 min of denaturation at 98 °C, 30 cycles of 10 s at 98 °C, 30 s for annealing at 50 °C, 30 s
78 for elongation at 72 °C, and a final extension at 72 °C for 5 min. All PCR reactions were carried out
79 with 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 µM of forward
80 and reverse primers, and about 10 ng template DNA. The resulted PCR products were extracted from
81 a 2% agarose gel and further purified using the Qiagen Gel Extraction Kit (Qiagen, Germany).
82 Purified amplicons were pooled in equimolar and sequencing libraries were generated using TruSeq®
83 DNA PCR-Free Sample Preparation Kit (Illumina, USA). The library quality was assessed on the
84 Qubit®2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system (Agilent, USA).
85 Finally, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads
86 were generated at SinoBioCore Biological Technology Co. Ltd. (Beijing, China).

87 Raw fastq files were demultiplexed using the barcode sequence with the exact barcode matching
88 parameter using div-utils (<https://github.com/jameslz/div-utils/blob/master/div-utils> version: 0.0.1-
89 r1-dirty). Quality-filtering was applied using Trimmomatic v.0.36 [2] with the following criteria: (i)
90 bases off the start and end of a read below a threshold quality (score < 2) were removed. (ii) the
91 reads were truncated at any site receiving an average quality score < 15 over a 4 bp sliding window,
92 discarding the truncated reads that were shorter than 36 bp. Paired-reads were merged using
93 USEARCH fastq_mergepairs command (version 11.2.64, <http://drive5.com>) [3] with the default
94 parameters. Reads which could not be merged were discarded. The merged reads with more than 2
95 nucleotide mis-match in primer matching were removed. The primer sequences were removed from
96 the merged reads. Zero-radius Operational Units (ZOTUs) were generated using USEARCH
97 UNOISE [4] algorithm. The chimeric sequences were removed in the denoising procedure. The data

98 were analyzed using GraphPad PRISM 8 (GraphPad Software, San Diego, CA). All data are
99 expressed as the mean $\pm$ SEM.

100 ***In vitro* metabolism of PYO incubated with liver microsomes**

101 PYO was separately incubated with liver microsomes of rats, chickens, swine and human under
102 the same conditions. For the phase I metabolism experiment, the incubation buffer were mixed in 49
103 μ L of 0.05 M Tris-HCl buffer (pH 7.4), consisted of 100 μ L of 2 mg protein/mL liver microsomes, 1
104 μ L of 5 mg/mL PYO, and 50 μ L of 1 mM NADPH with the total volume of 200 μ L. After 2 h of
105 incubation at 37 °C in a metabolic shaker, the reaction was terminated by adding 200 μ L of ice-cold
106 acetonitrile. For the phase II metabolism experiments, the incubation mixture contained 150 μ L
107 supernatant of phase I metabolism mixture, 100 μ L of 2 mg protein/mL liver microsomes, 25 μ L of
108 3.8 mM UDPGA, and 25 μ L of 0.05 M Tris-HCl buffer (pH 7.4) with the total volume of 300 μ L.
109 The reaction was terminated by adding 300 μ L of ice-cold acetonitrile after 2 h of incubation at
110 37 °C in a metabolic shaker. Parallel controls include the absence of NADPH or UDPGA and
111 incubation without PYO, respectively [5]. All experiments were conducted in triplicate. All the
112 incubated mixture were voluted after terminated, and then centrifugated at 14,000 rpm at 4 °C for 15
113 min. The supernatant was collected and evaporated to dryness under nitrogen gas. The residue was
114 reconstituted in 200 μ L (phase I samples) and 300 μ L (phase II samples) 50% acetonitrile (50:50,
115 v/v), and analyzed by UPLC-Q/TOF-MS for the identification of the metabolites.

116 **Preparation of samples for metabolism in vivo**

117 For urine samples, 800 μ L of acetonitrile was added to 200 μ L volume of urine, and vortexed for 5
118 min, centrifugated at 14,000 rpm at 4 °C for 10 min, the supernatant was collected and evaporated to

dryness under nitrogen gas and the residue was reconstituted in 500 μ L 50% acetonitrile (50:50, v/v), and analyzed by UPLC-Q/TOF-MS. For feces samples, 1 mL of acetonitrile was added to 0.3 g of feces. The mixture was vortexed for 5 min and ultrasonic extracted for 10 min, then centrifuged at 14,000 rpm at 4 °C for 10 min. The organic layer was transferred into a polypropylene tube, and the extraction process was repeated for 3 times as described above. Afterward, the three supernatants were pooled and evaporated to dryness in a water bath at 40 °C under a gentle flow of nitrogen. The residue was redissolved in 1 mL of acetonitrile/water (50:50, v/v), then the solution was diluted with 5 mL pure water, and loaded onto an Oasis[®] HLB cartridge (3 cc, 60 mg), which was preconditioned with 3 mL methanol and 3 mL water. The cartridge was washed with 3 mL water, and finally the analytes were eluted from the cartridge with 3 mL acetonitrile [5, 6]. The eluate was evaporated to dryness under nitrogen in a 40 °C water bath and finally reconstituted in 0.5 mL of acetonitrile/water (50:50, v/v). The resolutions were analyzed by UPLC-Q/TOF-MS after centrifugation at 14000 rpm at 4 °C for 20 min.

UPLC-Q/TOF conditions

The metabolites were identified by the SCIEX Triple TOF[™] 5600+ system (SCIEX Co., USA) coupled to a processing software of MetabolitePilot[™] (SCIEX Co., USA). The separation was performed on an ACQUITY UPLC BEH C₁₈ column (100 mm $\times$ 2.1 mm, 1.7 μ m; Waters, USA) using a gradient elution program consisting of mobile phase A (acetonitrile) and mobile phase B (0.1% formic acid). The gradients were as follows: 0–5.0 min, 5% A to 95% A; 5.0–7.0 min, 95% A to 100% A; 7.0–15.0 min, 100 % A for 2 min; 15.0–15.10 min, returned to 5% A; 15.10-18.0 min, 5% A for 3 min. The injection volume was 10 μ L, and the flow rate was 0.3 mL/min. The column

temperature was maintained at 40°C. The mass spectrometer was equipped with an electrospray ionization source (ESI) operated in the positive ionization mode. The optimized MS parameters were set as follows: ion spray voltage floating (ISVF), 5500 V; the turbo spray temperature, 550 °C; nebulizing gas (GAS1), 50 psi; heating gas (GAS2), 50 psi; curtain gas (CUR), 35 Psi; declustering potential (DP), 80 V. Data were acquired from 100 to 800 Da. Low-energy data were acquired at a collision energy of 10 eV, and high-energy data were acquired using ramped collision energy in a range from 20 to 50 eV.

Results for metabolism *in vivo* and *in vitro*

MS/MS spectrum interpretation of PYO

The elemental compositions and corresponding mass errors for the fragment ions of PYO were predicted by the MetabolitePilotTM software, based on these, the structures of the fragment ions were proposed in **Fig. 2A and B**. PYO can be added hydrogen to form the adduct ([M+H]⁺) ion at *m/z* 211.0866 (C₁₃H₁₁N₂O⁺, -0.1 ppm), and underwent a neutral loss of CH₃ to form *m/z* 196.0631 (C₁₂H₈N₂O⁺, 0.1 ppm), subsequently lost CO to become the main fragment *m/z* 168.0682 (C₁₁H₈N₂⁺, -0.1 ppm) or lost O to generate the fragment ion at *m/z* 181.0760 (C₁₂H₉N₂⁺, 0 ppm), and the fragment ion *m/z* 183.0917 (C₁₂H₁₁N₂⁺, -0.4 ppm) was formed by losing CO from *m/z* 211.0866. The fragmentation pathway for PYO is proposed and illustrated in **Fig. 2B**. The fragments *m/z* 196, 181, 183 and 168 are the typical debris, they will be regarded as the primary guidance for subsequent structure elucidation of the metabolites of PYO.

Structural interpretation for metabolites of PYO in liver microsomes

Liver microsomes have been extensively utilized in drug metabolism studies for their lower cost, good reproducibility, and simple preparation [7]. We first dissected the metabolism of PYO in liver microsomes, and the data was analyzed by MetabolitePilotTM and PeakView software. A total of 14 metabolites including 11 phase I metabolites (M1~11) and 3 phase II metabolites (M12~14) were detected in rat, chicken, swine, and human liver microsomes (**Supplementary Table S1**). The accurate extracted ion chromatograms (EICs) and accurate MS/MS spectra of the metabolites were exhibited in **Supplementary Fig. S4** and **S5**. The chemical structures of the metabolites were clarified in the below sections by using their chromatographic behavior, accurate mass measurements, and basic rules of drug metabolism.

Metabolite M1 (1-Phenazinol) showed a protonated molecular ion $[M+H]^+$ at m/z 197.0708, which was 14 Da less than the protonated PYO at m/z 211, indicating it was demethylated from PYO.

Metabolite M2 was eluted at a retention time of 3.50 min and showed the $[M+H]^+$ ions at m/z 213.0684 ($C_{12}H_9N_2O_2^+$, -4.7 ppm), its typical fragment ions were m/z 198, 185, 169, which was 2 Da increases than the ions at m/z 211 of PYO, m/z 197, m/z 183, m/z 167, respectively. The debris m/z 198 was 16 Da less than its parent ion (M2, m/z 213), suggesting m/z 198 was formed by losing an element of O, the fragment m/z 185 was 28 Da less than M2 and showed a loss of CO. Then the fragment m/z 169 was reduced by another 16 Da on the basis of fragment m/z 185, suggesting that a more loss of O. Thus, metabolite M2 (1,4-Phenazindiol) contains two O, suggesting it was oxidized from M1 after methylated.

180 **Metabolite M3** was eluted at a retention time of 3.55 min and showed the $[M+H]^+$ ion at m/z
181 215.0809 ($C_{12}H_{11}N_2O_2^+$), which was 18 Da more than M1, indicating M3 was internal hydrolyzed
182 from M1.

183 **Metabolite M4** was eluted at a retention time of 1.008 and 1.55 min and showed the $[M+H]^+$ ion
184 at m/z 229.1174 ($C_{13}H_{13}N_2O_2^+$), which was 18 Da more than m/z 211 of PYO, indicating that internal
185 hydrolysis was happened in M4 and the occurrence of isomers of mono- hydrolyzed PYO
186 metabolites, consistent with the typical fragment ions m/z 183, 168.

187 **Metabolites M5** showed a protonated molecular ion $[M+H]^+$ at m/z 225.1021, which was 14 Da
188 more than the protonated PYO at m/z 211, indicating that metabolite M5 might be the methylated
189 product of PYO. There were multiple methyl binding sites on the benzene ring, in which the
190 neighboring and opposite site of the hydroxyl group on the benzene ring are somewhat more active,
191 therefore, there were two sites for methyl substitution with two retention times correlating with
192 actual chromatographic retention times, consistent with the retention time of 4.39 and 4.97 min.

193 **Metabolites M6** showed the molecular ion $[M+H]^+$ at m/z 227.1176, its typical fragment ions
194 were m/z 212, 197, 168. Debris m/z 212 was 15 Da less than M6, indicating a neutral loss of CH_3 ,
195 and a more of CH_3 was lost successively to turn into debris m/z 197, then the fragment m/z 168 was
196 formed by dropping the CO. Therefore, there were two CH_3 in the metabolite M6, which 2 Da more
197 than M5, suggesting that metabolite M6 was the methylated and hydrogenated product, consistent
198 with two retention times (3.99 and 4.54 min) compared to metabolite M5.

199 **Metabolite M7** exhibited the protonated molecule $[M+H]^+$ at m/z 227.0806 ($C_{13}H_{11}N_2O_2^+$, -0.9
200 ppm), and was 16 Da more than PYO at m/z 211.0866, indicating that mono-oxidization happened in

201 the metabolite M7. Fragment ions at m/z 212, 184, and 199 were 1 Da higher than the ions at m/z
202 211, m/z 183 and m/z 197 of PYO, respectively, suggesting CH_3 was firstly dropped to generate the
203 debris m/z 212, subsequently, CO was lost to become the fragment m/z 199, finally, a neutral loss of
204 OH happened to form the ion m/z 184. The cracking pathway was displayed in the MS/MS spectra.
205 There were several eluted times for M3 because multiple methyl binding sites existed on the benzene
206 ring for oxidization.

207 **Metabolite M8 and M9** showed the molecular ion $[\text{M}+\text{H}]^+$ at m/z 241.0963 and 241.0652, and
208 eluted at 4.1 and 3.03 min, respectively, which were both 30 Da higher than that of PYO, and 16 Da
209 more than M6, 14 Da more than M7, suggesting methylation on the base of M7 or demethylation to
210 carboxylic acid occurred in PYO. From the MS/MS spectra, the typical fragment ions of M8 and M9
211 were m/z 226, 211, 183 and m/z 225, 209, 197, 169 respectively. M8 lost a CH_3 to form the debris
212 m/z 226, and dropped another CH_3 to become the debris m/z 211 subsequently. Therefore, there were
213 two CH_3 in M8, which was tentatively determined as methylated product based on M7, that was, M8
214 was methylated and oxidated from PYO. For metabolite M9, the fragment ion m/z 225 was 16 Da
215 less than M9, suggesting that M9 lost a O to form the debris m/z 225, and dropped another O to form
216 the debris m/z 209 successively, indicating that metabolite M9 was demethylated to carboxylic acid
217 from PYO. There were two free hydroxyl groups in metabolites M8 and carboxyl site in M9, while
218 the polarity of the carboxyl site is greater than that of the hydroxyl group, thus M9 eluted earlier than
219 M8, consistent with the actual chromatographic retention time.

220 **Metabolites M10 and M11** showed the molecular ion $[\text{M}+\text{H}]^+$ at m/z 239.1176 and 239.0809,
221 which was 28 Da higher than PYO, suggesting the loss of O then N-acetylation or demethylation and

222 N-acetylation happened. From the MS/MS spectra analysis, the typical fragment ions of M10 and
223 M11 were m/z 223, 180 and m/z 211, 193, 183, 168, respectively. Metabolite M10 lose a O to get the
224 fragment m/z 223, then broke off the C=N bond to form the fragment m/z 180, therefore M10 was
225 identified as the dehydroxylated and then N-acetylated product. The M11 lost a C and O to formed
226 the debris m/z 211, subsequently dehydroxylated to generated the debris m/z 193 successively, or lost
227 another CO to formed the debris m/z 183. Therefore, M11 was the demethylated and N-acetylated
228 product from PYO, and eluted at 2.28 and 4.64 min, respectively

229 **Metabolite M12** was eluted at 2.88 min with molecular ion m/z 387.1184 ($[M+H]^+$,
230 $C_{18}H_{17}N_2O_7^+$), which was 176 Da higher than m/z 211 of PYO, and the fragment ion at m/z 211, 196,
231 183, 168 were consistent with fragment ions of PYO, implying that metabolite M12 was the
232 glucuronide conjugate of PYO (PYO-1-GlcA).

233 **Metabolite M13** was eluted at 2.48 min with molecular ion m/z 373.1036 ($[M+H]^+$,
234 $C_{18}H_{17}N_2O_7^+$), which was 176 Da higher than m/z 197 (M1), indicating that it was the glucuronide
235 conjugation of M1. The typical fragment ions were observed at m/z 197, which is the product after
236 breaking the glucuronic acid (GlcA).

237 **Metabolite M14** showed the molecular ion $[M+H]^+$ at m/z 403.1123 with six different retention
238 times from 1.01 min to 2.48 min. They were 176 Da higher than M6 and M7, suggesting they were
239 the glucuronide conjugate of M6 or M7. For the fragment ion analysis, the typical fragment ions
240 were m/z 227, 199, 184, consistent with the fragments of M7. Therefore, M14 was tentatively
241 determined as the glucuronide conjugation of M7. The diverse eluted times suggesting the
242 occurrence of isomers, consistent with M7.

243 These results demonstrated that there was species diversity of metabolites in different liver
244 microsomes, such as N-oxidation (M3), methylation (M6) and N-acetylation (M10, M11) were not
245 found in swine or chicken liver microsomes, while metabolite M9 was detected in swine and
246 chickens liver microsomes. Meanwhile metabolites (M1-M2, M4-M5, M7-M8 and M12-M14) were
247 observed in the all species (rat, human, swine, and chicken liver microsomes) (**Supplementary**
248 **Table S1**). On the base of these results, the proposed metabolic pathways of PYO in liver
249 microsomes (**Supplementary Fig. S6**) were demethylation, methylation, oxidation, and glucuronide
250 conjugation.

251 **Structural interpretation for in vivo metabolites of rats**

252 Given the most amounts of metabolites in rat liver microsomes, we utilized rats to dissect the *in*
253 *vivo* of metabolism by treating rats with PYO at the dose of 20 mg/kg body weight. And 10
254 metabolites were identified in urine and feces (**Supplementary Table S2**), their accurate EICs and
255 MS/MS spectra were showed in **Supplementary Fig. S7 and S8**, respectively.

256 **Metabolite M15** was observed at 3.08 min with a deprotonated molecule ion $[M+H]^+$ at m/z
257 285.0758 ($C_{15}H_{13}N_2O_4^+$), which was 84 Da higher than PYO and with the main ions at m/z 270, 242,
258 225, 197. Fragment ion m/z 270 was 15 Da lower than metabolite M15, suggesting a CH_3 group lost.
259 Ion m/z 242 was 43 Da lower than M15, indicating an acetyl group was dropped, successively OH
260 was lost to become the fragment ion m/z 225, or formic acid was dropped to form the fragment ion
261 m/z 197. Therefore, N-acetylation and demethylation to carboxylic acid had occurred in metabolite
262 M15 after hydrogenation base on PYO.

263 **Metabolite M16** was observed at 1.94 min with a deprotonated molecule ion $[M+H]^+$ at m/z
264 461.1079 ($C_{21}H_{21}N_2O_{10}^+$), which was 176 Da higher than M15. And the main ions at m/z 285, 270,
265 242, 225, 197, consistent with the fragment ions of M15, suggesting that M16 was the glucuronide
266 conjugated product of M15.

267 **Metabolite M17** exhibited the protonated molecule $[M+H]^+$ at m/z 344.0754 ($C_{16}H_{14}N_3O_4S^+$),
268 which was 103 Da higher than the protonated M8 and M9 (m/z 241.0963 and 241.0652). The typical
269 fragments were ions at m/z 298, 266, 238, 283, 227, the debris m/z 298 derived from the loss of the
270 methylthio group of cysteine, and the debris m/z 283 developed from deoxidation and
271 decarboxylation. Metabolite M8 oxidated and methylated from PYO and possessed two free
272 hydroxyl groups, while M9 possessed a free carboxyl group which cysteine conjugated to. Thus,
273 M17 was the cysteine conjugated metabolite of M9.

274 **Metabolite M18 and M19** eluted at 5.03 min and 5.60 min with molecular ion at m/z 478.2938
275 and m/z 480.3458 respectively. Metabolite M19 was 2 Da more than metabolite M18, and the debris
276 m/z 339 was also 2 Da more than the fragment m/z 337 of metabolite M19, indicating that M19 was
277 the hydrogenated product of M18. However, M18 and M19 were remained ambiguous.

278 Compared with the metabolites in liver microsomes, only five metabolites were existed both in rat
279 and in liver microsomes. The difference between *in vivo* and *in vitro* may be due to the diverse
280 metabolic enzymes and substrates on the inside and outside, the number of enzymes involved in drug
281 metabolism was very limited in the liver microsomes, for instance, cysteine conjugation was
282 produced for the abundant substrates and enzymes *in vivo* [7]. Compare to the previous study [8],
283 four metabolites were found in urine of mice through intraperitoneal injection (IP) with the dose of

284 40 mg/kg, while only the metabolite at m/z 373 was detected in our study, demonstrating that
285 metabolic pathway was different between oral administration and intraperitoneal injection. Our data
286 showed PYO could be metabolized by liver and gut microbiota in urine and feces, respectively, and
287 all the metabolites retained the nitrogen-containing tricyclic structural skeleton of phenazines. Given
288 that the majority of metabolites of PYO were eliminated in 0–24 h according to the concentration in
289 plasma and few metabolites was detected in 24–48 h, we quantified metabolites by absolute peak
290 intensity using the excreta of rats in the 0–24 h. The results showed that the metabolite M15 was the
291 primary product in liver and finally excreted with urine, while M1 was the primary metabolite
292 produced by intestinal microbiota, and excreted with feces. Altogether, these studies indicated that
293 PYO could be metabolized by liver and intestinal microbiota, and all metabolites retained the
294 nitrogen-containing tricyclic structural skeleton of phenazines.

295 **Tables**

296 **Table S1** The metabolic kinetic parameters of PYO in male swine at oral administration with dose of
 297 5 and 1 mg/kg body weight. The results were presented as mean $\pm$ SD (n = 5).

Parameters	PYO	
	5 mg/kg BW	1 mg/kg BW
T _{max} (h)	0.65 $\pm$ 0.285	0.6 $\pm$ 0.137
C _{max} (ng/mL)	22.106 $\pm$ 20.872	0.761 $\pm$ 0.857
AUC _{last} (ng·h/mL)	53.177 $\pm$ 11.117	3.839 $\pm$ 1.432
Lambda _z (1/h)	0.14 $\pm$ 0.016	0.065 $\pm$ 0.052
HL_Lambda _z (h)	4.988 $\pm$ 0.568	26.186 $\pm$ 33.728
AUCINF_obs (ng·h/mL)	54.586 $\pm$ 11.033	9.178 $\pm$ 9.09
Cl_F_obs (L·kg/h)	94.771 $\pm$ 19.834	201.605 $\pm$ 167.605
MRTlast (h)	4.942 $\pm$ 0.728	9.132 $\pm$ 3.345
AUC_%Extrap_obs (%)	2.69 $\pm$ 0.827	40.66 $\pm$ 23.058
Vz_F_obs (L)	689.038 $\pm$ 19.789	3569.898 $\pm$ 1250.038

298 Note: T_{max}, time to peak concentration; C_{max}, maximum concentration after intravenous
 299 administration; AUC_{last}, area under the curve from the time of dosing to the last measurable
 300 concentration; Lambda_z, the elimination rate constant; HL_Lambda_z, elimination half-life;
 301 AUCINF_obs, area under the concentration-time curve from 0 to t the last blood collection time point;
 302 Cl_F_obs, apparent total body clearance; MRTlast, mean retention time; AUC_% Extrap_obs, area
 303 under the first moment of the plasma concentration-time curve extrapolated from time to infinity as a
 304 percentage of total AUC; Vz_F_obs, apparent volume of distribution.

305 **Table S2** Summary of PYO metabolites in liver microsomes of rats, chickens, swine, and human.

Metabolite s	Formula	Adduct ion	Mass (<i>m/z</i>)	Retention Time (min)	Error (ppm)	Liver microsomes				Major fragments	Metabolic type
						rat	human	swine	chicken		
M1	C ₁₂ H ₉ N ₂ O ⁺	[M+H] ⁺	197.0708	3.56	-2.1	√	√	√	√	169, 168, 179	I
M2	C ₁₂ H ₉ N ₂ O ₂ ⁺	[M+H] ⁺	213.0684	3.5	-4.7	√	√	√	√	198, 180, 169, 168	I
M3	C ₁₂ H ₁₁ N ₂ O ₂ ⁺	[M+H] ⁺	215.0809	3.55	0.1	√	√	nd	nd	200, 187, 182, 171	I
M4	C ₁₃ H ₁₃ N ₂ O ₂ ⁺	[M+H] ⁺	229.0973	1.008, 1.55	0.9	√	√	√	√	212, 183, 168	I
M5	C ₁₄ H ₁₃ N ₂ O ⁺	[M+H] ⁺	225.1021	4.39, 4.97	-1	√	√	√	√	210, 182, 197	I
M6	C ₁₄ H ₁₅ N ₂ O ⁺	[M+H] ⁺	227.1176	3.99, 4.54	-2.4	√	√	nd	nd	212, 197, 179, 168	I
M7	C ₁₃ H ₁₁ N ₂ O ₂ ⁺	[M+H] ⁺	227.0806	2.18	-0.9	√	√	√	√	212, 184, 199	I
M8	C ₁₄ H ₁₃ N ₂ O ₂ ⁺	[M+H] ⁺	241.0963	4.1	-2.3	√	√	√	√	226, 211, 183	I
M9	C ₁₃ H ₉ N ₂ O ₃ ⁺	[M+H] ⁺	241.0652	3.03	-3.0	nd	nd	√	√	225, 209, 197, 169	I
M10	C ₁₅ H ₁₅ N ₂ O ⁺	[M+H] ⁺	239.1176	2.28	-1.4	√	√	nd	nd	223, 180	I
M11	C ₁₄ H ₁₁ N ₂ O ₂ ⁺	[M+H] ⁺	239.0815	4.64	-0.2	√	√	nd	nd	211, 193, 183, 168	I
M12	C ₁₉ H ₁₉ N ₂ O ₇ ⁺	[M+H] ⁺	387.1183	2.88	-0.9	√	√	√	√	211, 196, 183, 168	II
M13	C ₁₈ H ₁₇ N ₂ O ₇ ⁺	[M+H] ⁺	373.1036	2.48	1.4	√	√	√	√	197, 179	II
M14	C ₁₉ H ₁₉ N ₂ O ₈ ⁺	[M+H] ⁺	403.1123	1.01, 1.69	-2.5	√	√	√	√	227, 199, 184	II

306 √ : detected; nd: not detected.

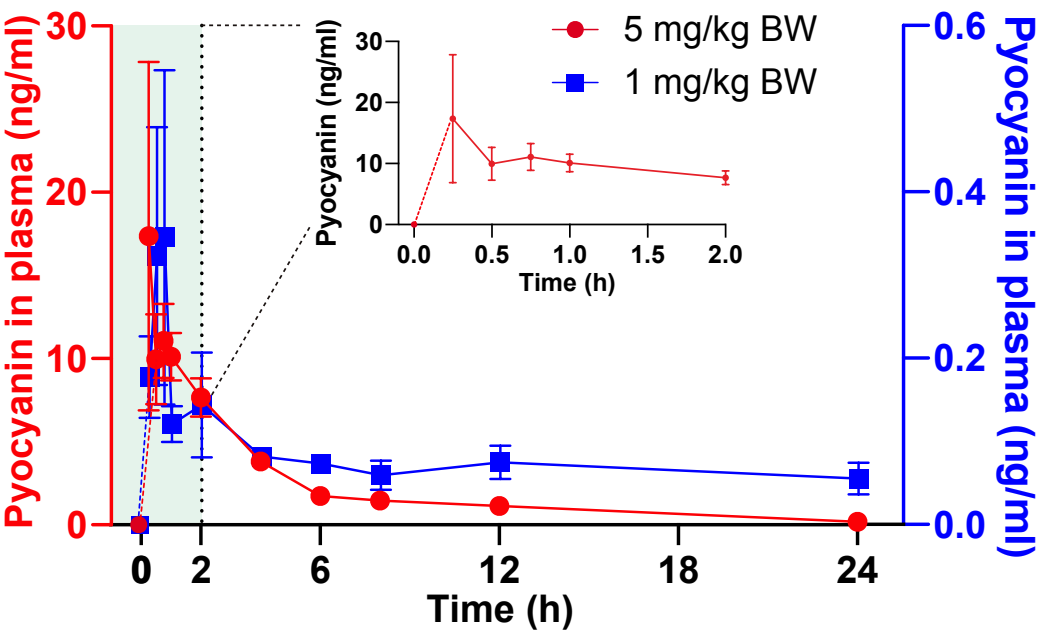
307 **Table S3** Summary of PYO metabolites in rats.

Metabolites	Formula	Adduct ion	Mass (<i>m/z</i>)	Retention Time (min)	Error (ppm)	Male		Female		Major fragments	Metabolic type
						urine	faeces	urine	faeces		
M1	C ₁₂ H ₉ N ₂ O ⁺	[M+H] ⁺	197.0710	2.57	0.2	√	√	√	√	179, 151, 123	I
M7	C ₁₃ H ₁₁ N ₂ O ₂ ⁺	[M+H] ⁺	227.0817	2.19	-1.6	√	√	√	√	184, 212, 135	I
M8	C ₁₄ H ₁₃ N ₂ O ₂ ⁺	[M+H] ⁺	241.0930	1.56	-0.3	√	nd	√	nd	226, 211, 183	I
M12	C ₁₉ H ₂₁ N ₃ O ₇ ⁺	[M-H+NH ₄] ⁺	403.1391	2.56	4.1	nd	√	nd	√	167, 153, 181, 283	II
M13	C ₁₈ H ₁₇ N ₂ O ₇ ⁺	[M+H] ⁺	373.1028	2.32, 2.49	0.9	√	nd	√	√	197, 180	II
M15	C ₁₅ H ₁₃ N ₂ O ₄ ⁺	[M+H] ⁺	285.0758	3.08	-1.1	√	nd	√	nd	242, 197, 168	I
M16	C ₂₁ H ₂₁ N ₂ O ₁₀ ⁺	[M+H] ⁺	461.1079	1.94	-0.6	√	nd	√	nd	285, 270, 242	II
M17	C ₁₆ H ₁₄ N ₃ O ₄ S ⁺	[M+H] ⁺	344.0248	2.52	-3.2	√	nd	√	nd	298, 166, 227	II
M18			478.2938	5.03	-0.7	√	nd	√	nd	337, 181	II
M19			480.3458	5.60	0.4	√	nd	√	nd	339, 181	II

308 √ : detected; nd: not detected.

Table S4 Primer sequences used in the study.

Primers	Forward/ Reverse	Sequences
<i>β-actin</i>	Forward sequence	CATTGCTGACAGGATGCAGAAGG
	Reverse sequence	TGCTGGAAGGTGGACAGTGAGG
<i>il-1β</i>	Forward sequence	TGGACCTTCCAGGATGAGGACA
	Reverse sequence	GTTTCATCTCGGAGCCTGTAGTG
<i>il-6</i>	Forward sequence	TACCACTTCACAAGTCGGAGGC
	Reverse sequence	CTGCAAGTGCATCATCGTTGTTC
<i>il-8</i>	Forward sequence	GGTGATATTCGAGACCATTTACTG
	Reverse sequence	GCCAACAGTAGCCTTCACCCAT
<i>il-10</i>	Forward sequence	CGGGAAGACAATAACTGCACCC
	Reverse sequence	CGGTTAGCAGTATGTTGTCCAGC
<i>tnfα</i>	Forward sequence	GGTGCCTATGTCTCAGCCTCTT
	Reverse sequence	GCCATAGAACTGATGAGAGGGAG
<i>ifnγ</i>	Forward sequence	CTTGAACCCTGTTCGTATGCTGG
	Reverse sequence	TTGGTGCAGGAATCAGTCCAGG
<i>nlrp3</i>	Forward sequence	ATTACCCGCCCCGAGAAAGG
	Reverse sequence	TCGCAGCAAAGATCCACACAG
<i>zo-1</i>	Forward sequence	GTTGGTACGGTGCCCTGAAAGA
	Reverse sequence	GCTGACAGGTAGGACAGACGAT
<i>occludin</i>	Forward sequence	TGGCAAGCGATCATACCCAGAG
	Reverse sequence	CTGCCTGAAGTCATCCCACTC
<i>muc1</i>	Forward sequence	AGTGCCTCTGACGTGAAGTCAC
	Reverse sequence	GGGAGGGAACTGCATCTCATTC
<i>muc2</i>	Forward sequence	ACTGCACATTCTTCAGCTGC
	Reverse sequence	ATTCATGAGGACGGTCTTGG



311

312 **Fig. S1** Metabolic kinetic analysis in plasma of male swine. Swine were oral administrated with
313 PYO at the dose of 5 and 1 mg/kg body weight (n=5).

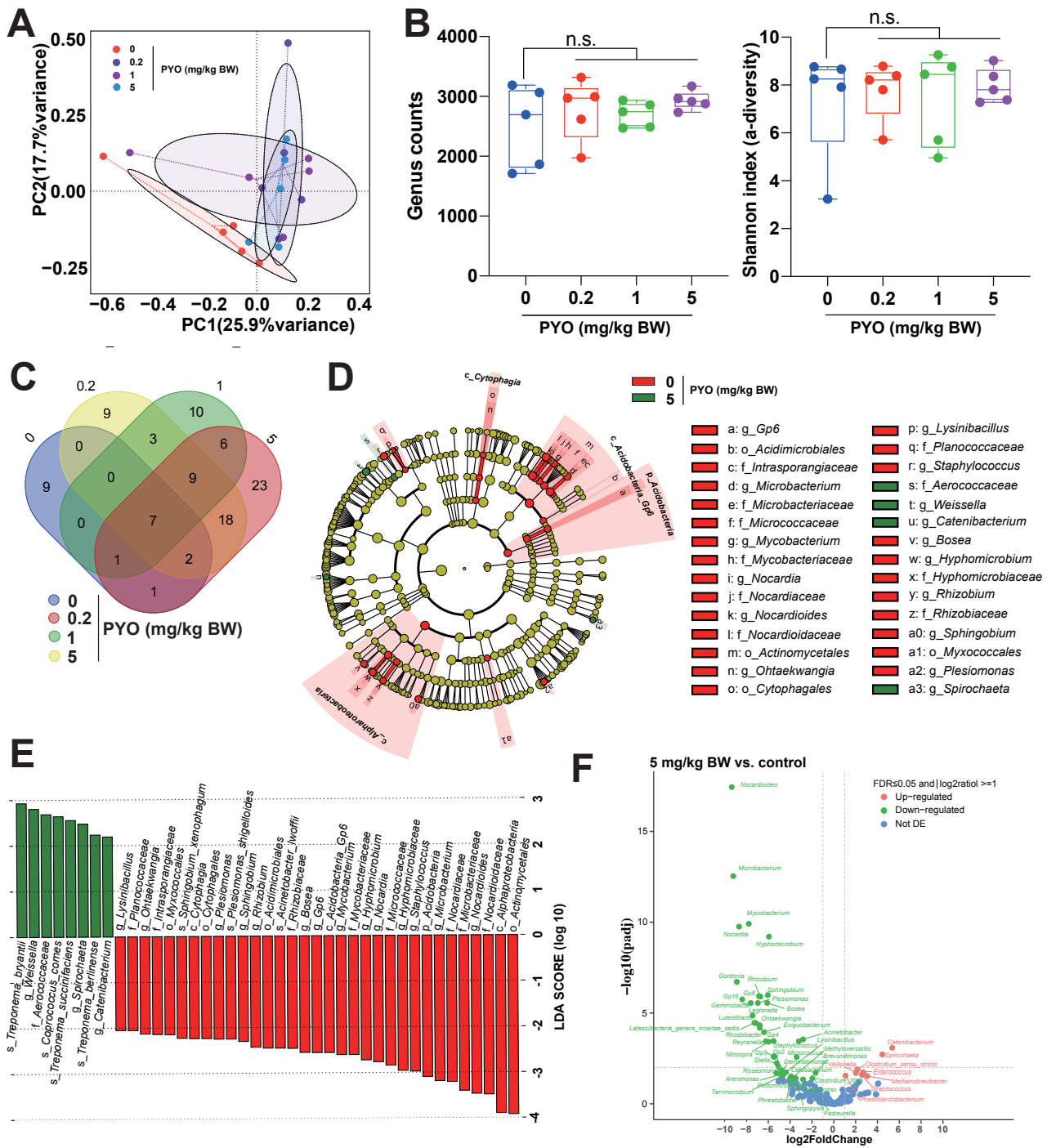


Fig. S2 PYO induced intestinal microbiota dysbiosis in swine. Swine were oral administrated with PYO at the dose of 0.2, 1, 5 mg/kg body weight (n = 5), data were shown as the mean $\pm$ SEM.

(A) Principal-coordinate analysis (PCoA) of intestinal microbiota based on UniFrac. Each symbol represents one sample.

319 (B) Comparison of microbial genus counts and α -diversity (as assessed by the Shannon index) based
320 on the genus profiles in the contents of swine.

321 (C) Venn diagrams based on genera, showing substantial overlap of OTUs shared or unique in the
322 gut microbiota of PYO treated groups as compared with the controls (0) (blue circle), compared with
323 the control group, PYO was dose-dependent to alter the intestinal microbiota for the less shared
324 OTUs (10 out of 76) in high does (red circle).

325 (D, E) LEfSe analysis of duodenal microbiota between the CMC-Na group (0) and high dose of PYO
326 group (5mg/kg body weight). (D) Cladogram displays the taxonomic tree of differentially abundant
327 taxa, and (E) histogram represents the LDA scores of bacteria with significant differential abundance
328 between the compared groups, identified by different colors. *Features with LDA score >2.0.

329 (F) Volcano plots of duodenum showing significance of differences in microbiome composition vs.
330 fold change from baseline at CMC-Na in swine.

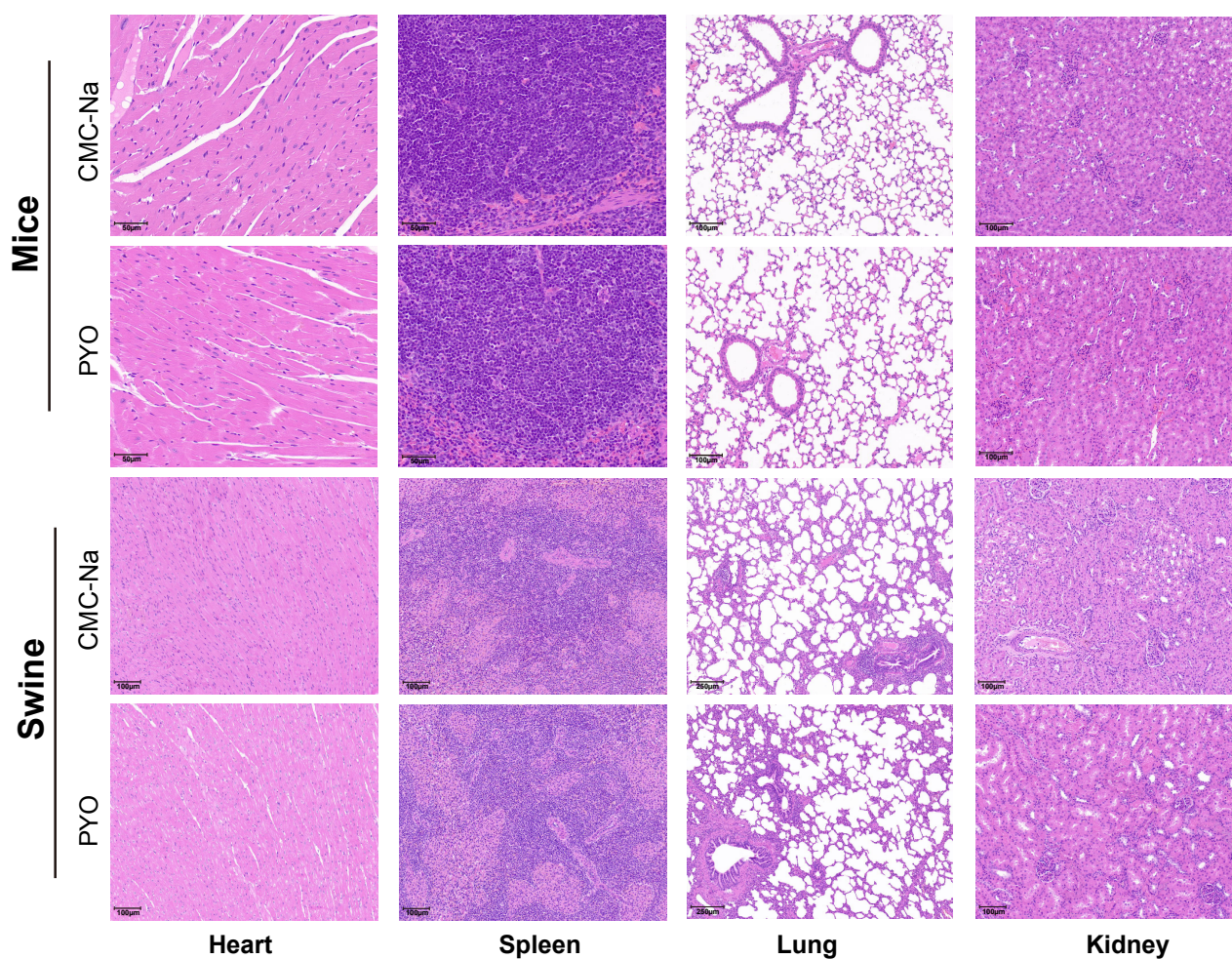


Fig. S3 PYO induced no damage to other organs in mice and swine at the dose of 50 mg/kg and 5 mg/kg body weight respectively. (H&E staining of histological heart, spleen, lung, and kidney sections, scale bars, 50 mm, 100 and 250 μm).

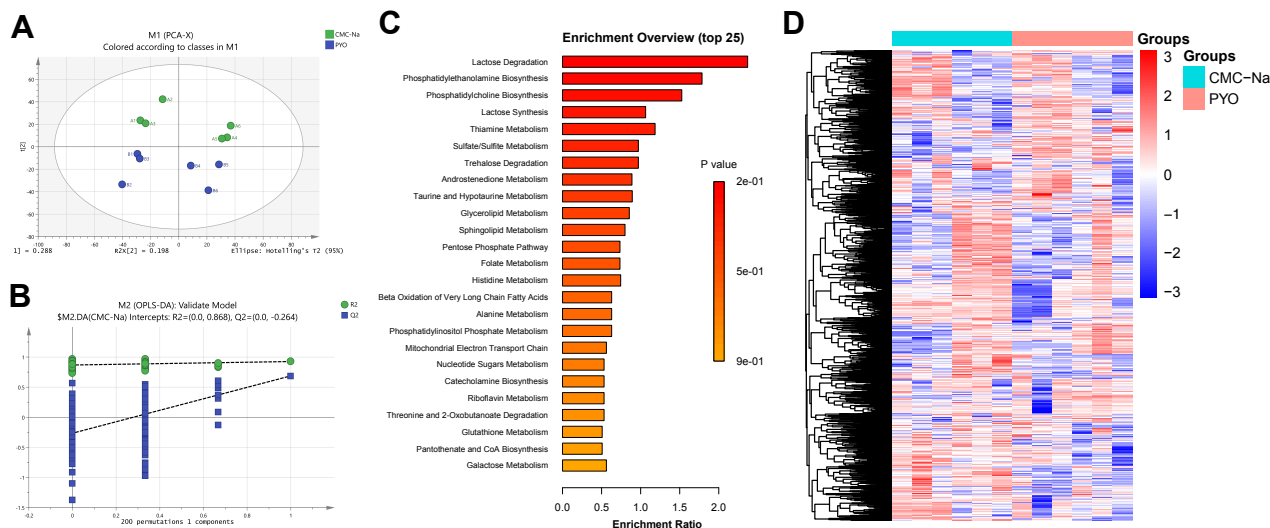


Fig. S4 PYO induced metabolic disorder in liver in mice. C57BL/6J mice were oral administrated with PYO at the dose of 50 mg/kg body weight (n = 6).

(A) Principal component analysis of liver extracts from PYO and Control group.

(B) Orthogonal partial least squares discriminant analysis of mice liver metabolome.

(C) Enrichment analysis of the identified differential metabolites involved in PYO induced liver damage.

(D) Cluster heat map visualization of all the metabolites contained in each sample. Rows: metabolites; columns: samples. Color key indicates metabolite expression value: blue: lowest; red: highest.

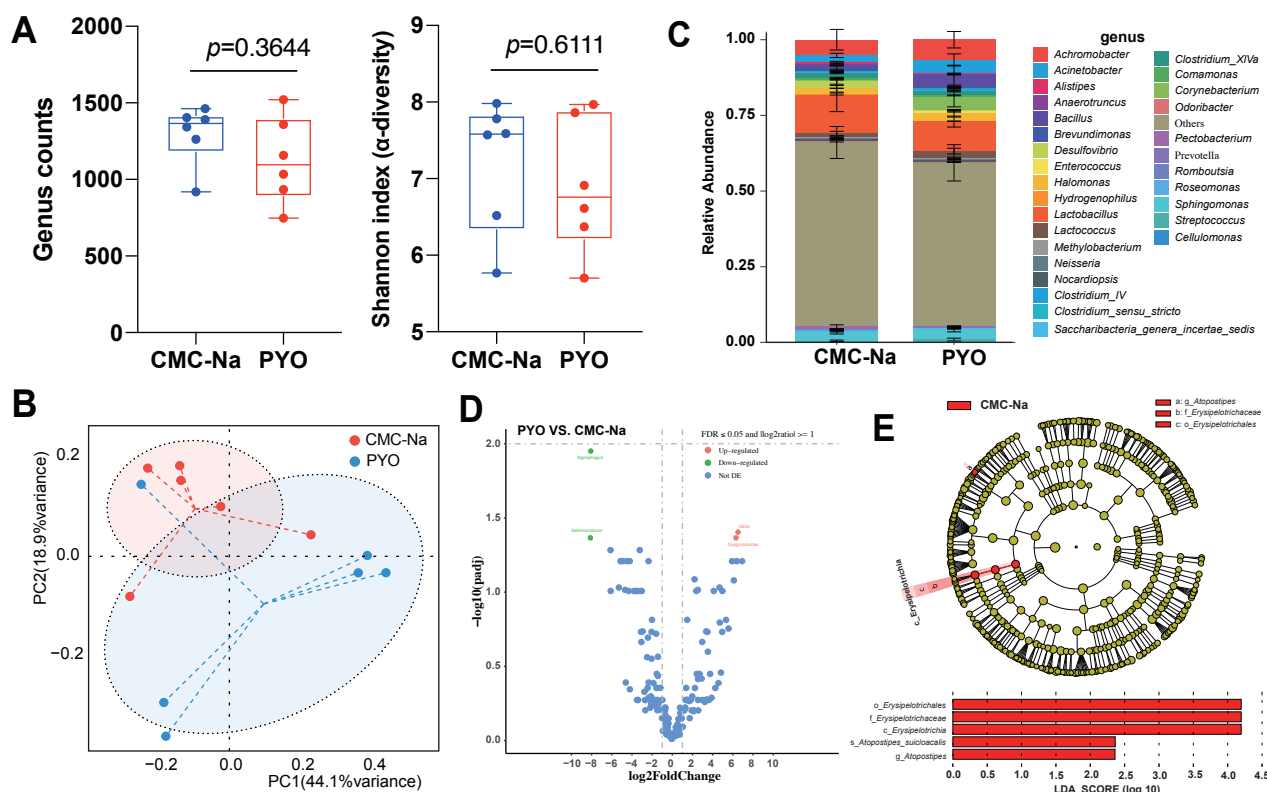


Fig. S5 PYO had little effect on liver microbiome composition in mice. C57BL/6J mice were oral administrated with PYO at the dose of 50 mg/kg body weight (n = 6), data were shown as the mean $\pm$ SEM.

(A) Comparison of microbial genus counts and α -diversity (as assessed by the Shannon index) based on the genus profiles in the two groups.

(B) Principal-coordinate analysis (PCoA) of liver microbiota based on UniFrac. Each symbol represents one sample.

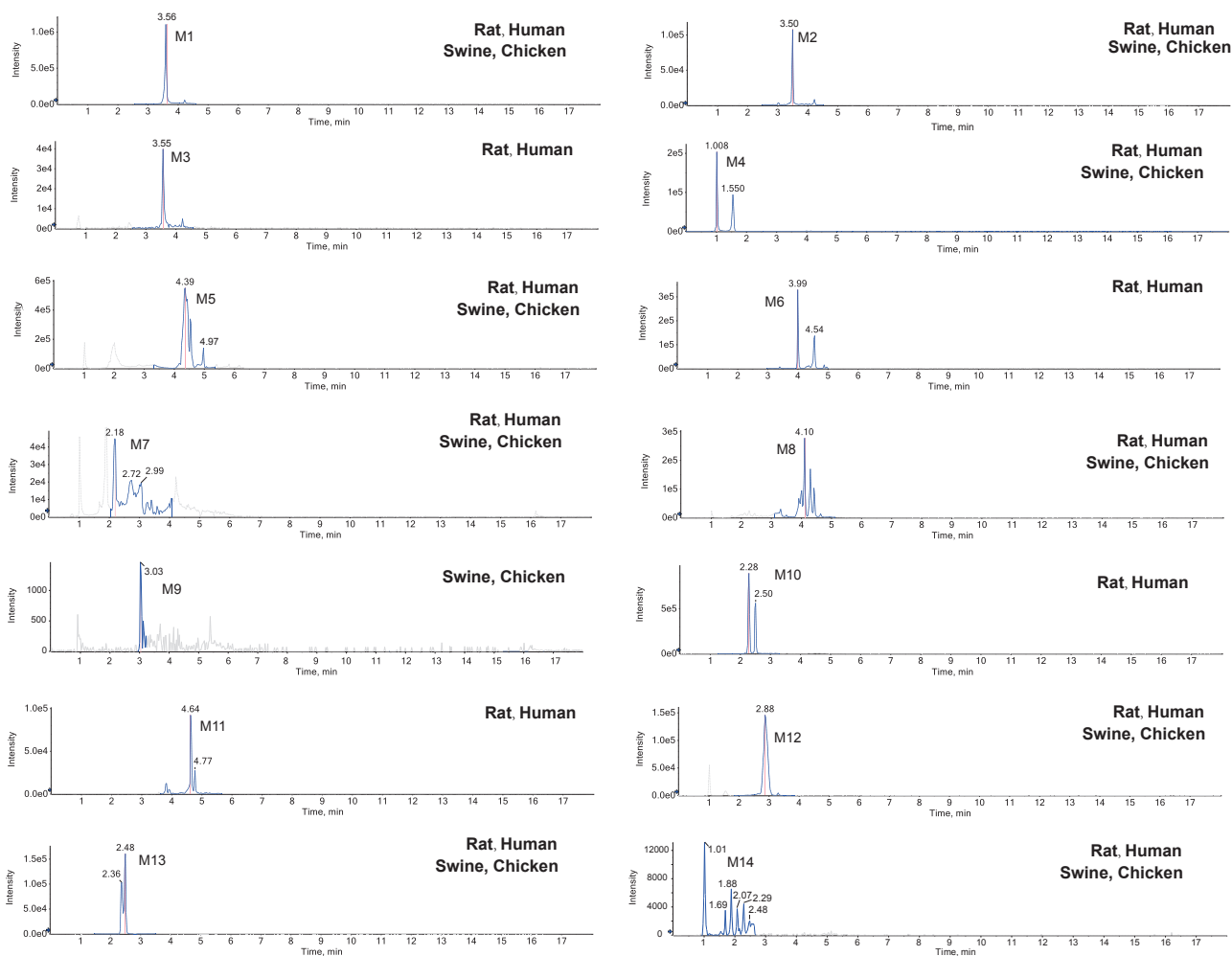
(C) Relative abundance of top 30 genus in liver in the two groups.

(D) Volcano plots showing significance of differences in microbiome composition vs. fold change from baseline at CMC-Na.

(E) LefSe analysis between the CMC-Na groups and PYO group, cladogram displays the taxonomic tree of differentially abundant taxa, and histogram represents the LDA scores of bacteria with

357 significant differential abundance between the compared groups, identified by different colors.

358 *Features with LDA score > 2 . (n=6, mean with SEM).



359

360 **Fig. S6** Accurate EICs of PYO metabolites from the incubated rat, human, swine and chicken liver
 361 microsomes (n=4).

362 M1-11 are metabolites from phase I systems, M12-14 are metabolites from phase II systems.

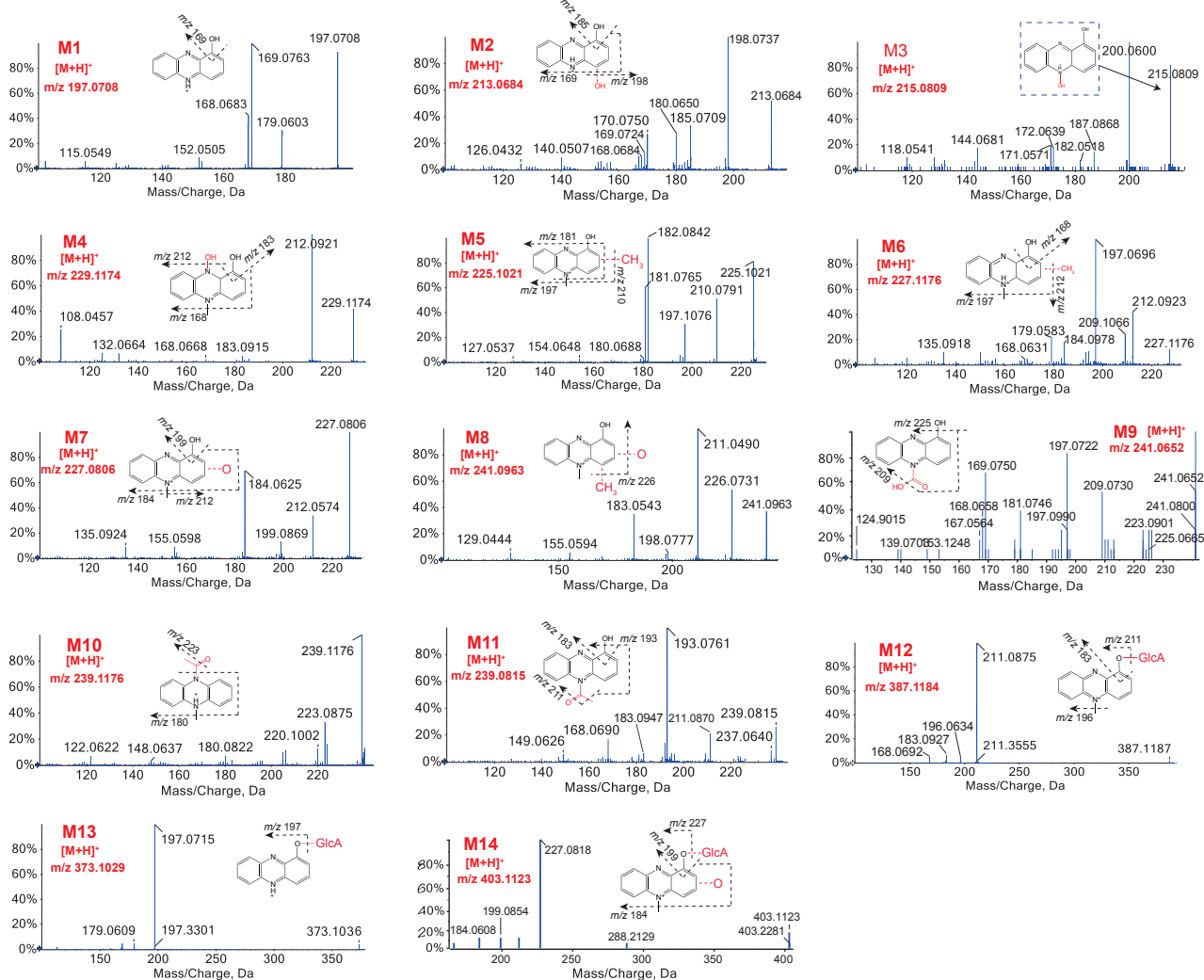


Fig. S7 MS/MS spectra of the metabolites of PYO detected in human and livestock liver microsomes (n=4).

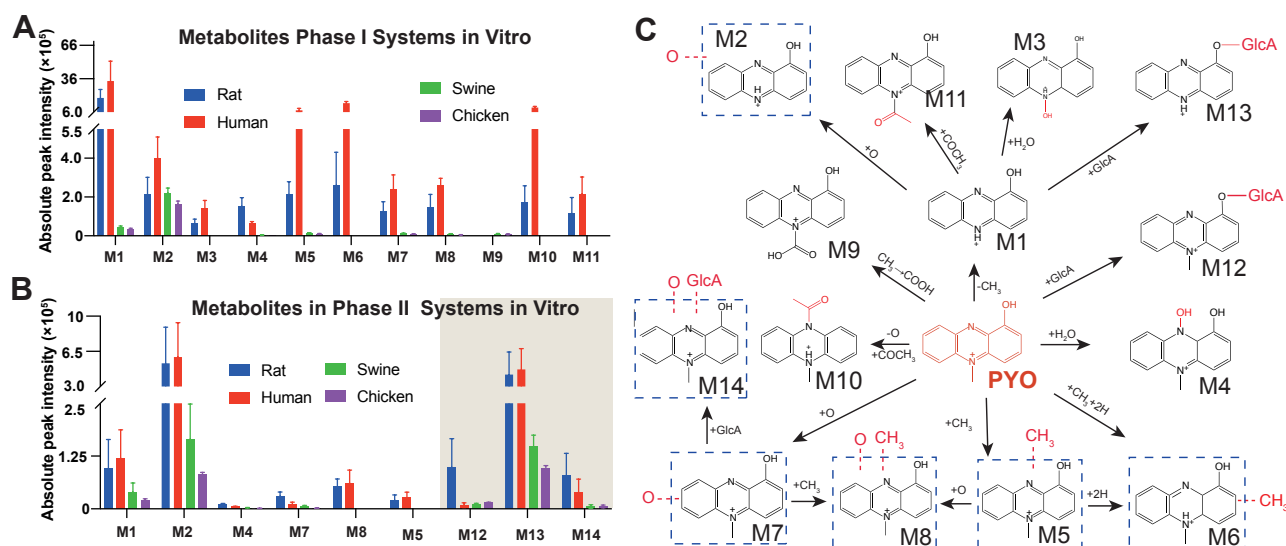
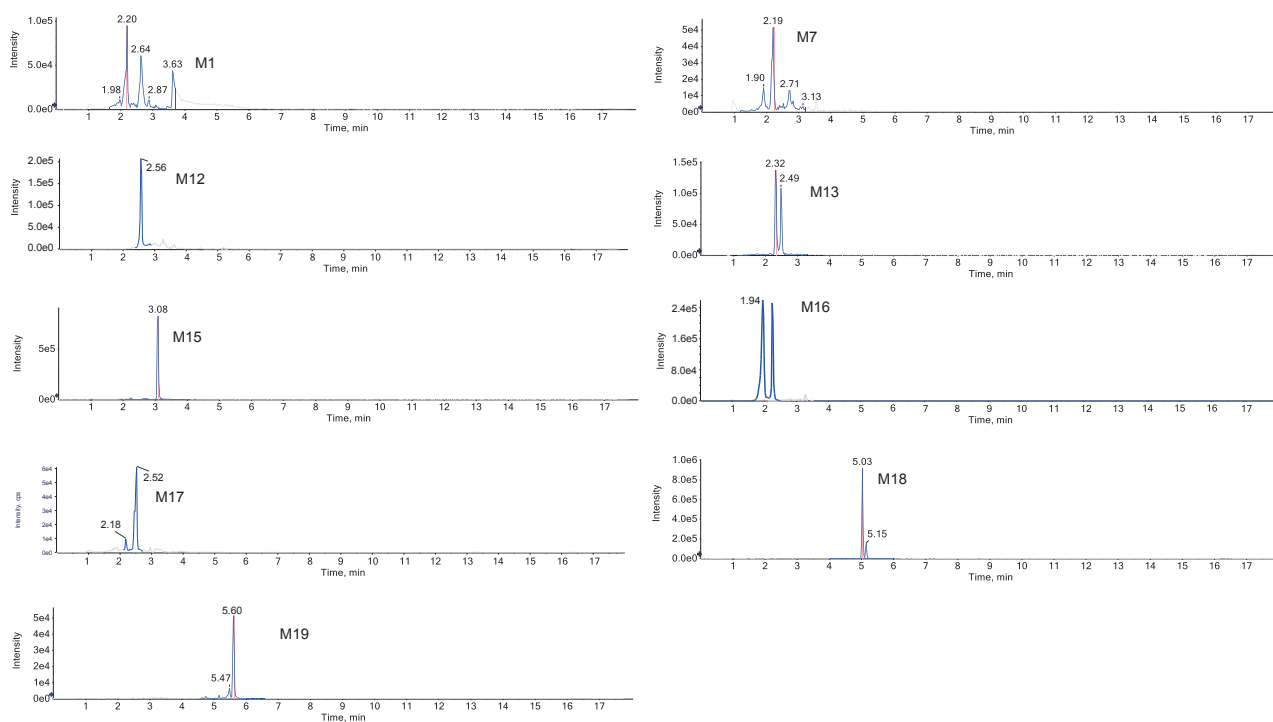


Fig. S8 The metabolism of PYO in liver microsomes of livestock and human (n=3).

(A) Phase I (M1-11) metabolites. Under phase I systems, demethylation (M1), oxidization (M2), methylation (M5, M6), N- acetylation (M10) were the most abundant metabolites.

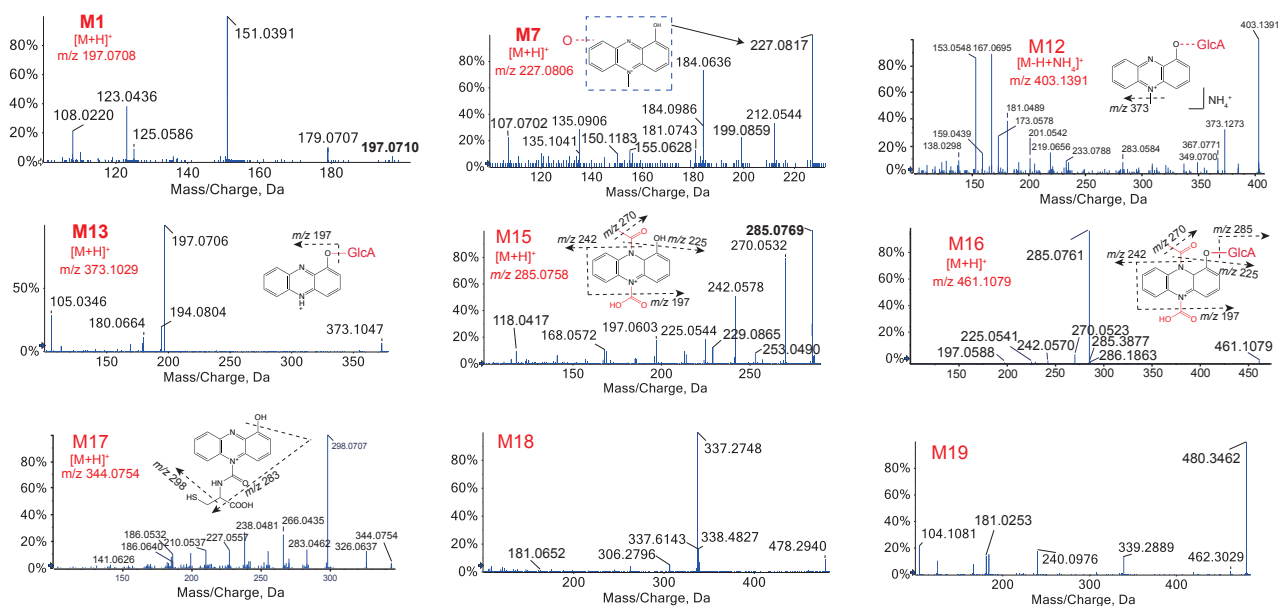
(B) All metabolites found in phase II systems, and M12-14 were the phase II metabolites (n=3). Data were shown as the mean $\pm$ SEM.

(C)The proposed metabolic pathways of PYO in liver microsomes.



373

374 **Fig. S9** Accurate extracted ion chromatograms (EICs) of PYO metabolites in urine and feces of rats.



375

376 **Fig. S10** MS/MS spectra of PYO metabolites in urine and feces of rats.

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