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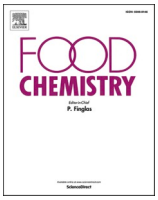

Bioavailability and excretion profile of betacyanins – Variability and correlations between different excretion routes

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ABSTRACT

The present study addresses the knowledge gap in betalain bioavailability, transformation and excretion. Analysis of renal and fecal excretion profiles in humans after consumption of beetroot revealed very low bioavailability (renal recovery of 0.13 %) and fast elimination of pigments (renal elimination rate constant of 0.16 h^{-1}), while the majority of betalains underwent severe depletion during GI transit, evidenced by decarboxylation, deglucosidation and dehydrogenation. Betacyanin metabolite levels in human urine were positively associated with those in stools ($p < 0.05$), indicating significant impact of pigment metabolism in the gut on their bioavailability. In addition, the current study revealed large inter-individual and compositional variabilities of pigment after colonic fermentation compared with systemic metabolism, likely attributed to the increasing complexity of intestinal environment with diverse gut microbiota. To conclude, intestinal uptake and systemic metabolism of betacyanins are intimately associated with their intestinal biotransformation, with gut microbiota serving as a crucial factor.

1. Introduction

There is emerging *in vitro* and *in vivo* research emphasizing the health-promoting properties of betalain pigments including anti-lipidemic (Reddy et al., 2005; Wróblewska et al., 2011), anti-hyperglycemic (Dhananjayan et al., 2017), anti-tumorigenic (Zhang et al., 2013), and hepato-/neuro-/cardio-protective effects (Allegra et al., 2015; Lugo-Radillo et al., 2020). Betalains, comprising a group of hydrophilic and nitrogenous compounds, are mainly found in plants of the order *Caryophyllales* (e.g., beet, cacti, and amaranth) and are divided into red-violet betacyanins and orange-yellow betaxanthins (Khan and Giridhar, 2015). As the dominant pigments in red beetroot, betacyanin glucosides, especially (iso)betanin, have been shown to possess comparable antioxidant capacity to anthocyanins and carotenoids (Cai et al., 2003; Gliszczyńska-Świgło et al., 2006). They were also found to be active in biological systems to scavenge free radicals and attenuate inflammatory response, with betaxanthins (e.g., vulgaxanthin, indicaxanthin) demonstrating a lower superoxide scavenging potency compared to betacyanins (Fernando et al., 2022, 2023; Wang et al., 2022b).

There is some evidence from *in vivo* trials on the efficacy of betanin supplementation in challenge models; for example, betanin administration has shown to alleviate paraquat-induced renal inflammation in rodents (Tan et al., 2015) and isoproterenol-induced myocardial damage in rats with acute myocardial infarction (Yang et al., 2016). These beneficial outcomes were primarily attributed to the suppression of pro-inflammatory signalling and subsequent attenuation of downstream inflammatory markers e.g., cytokines and inducible nitric oxide synthase. Further beneficial effects of lowering blood lipids and heart function improvement were observed after intervention with betacyanin-rich drinks in healthy humans (Archana et al., 2015) and in rodents (Raish et al., 2019), respectively.

Despite evidence on bioefficacy *in vivo*, the bioavailability of betacyanins is considered very low which may be a bottleneck to improve physiological functions, similar to polyphenols (Khan, 2016; Sorrenti et al., 2020). The bioavailability of bioactive compounds, incorporating terms of bioaccessibility, intestinal absorption, distribution, metabolism and elimination, could be heavily and differentially impacted by a

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variety of intrinsic (e.g., physiological conditions) and extrinsic factors (e.g., dose, food matrix, exposure duration). It is generally evaluated *in vivo* by analyzing betalain contents in the physiological fluids of subjects (e.g., plasma, urine) following oral administration of betalainic foods. The urinary recovery fraction of betacyanins was documented to be *ca.* 0.21 % in the post-prandial window of 24 h (Frank et al., 2005; Wiczkowski et al., 2018). According to a limited number of pharmacokinetic studies (Tesoriere et al., 2004; Frank et al., 2005; Wiczkowski et al., 2018; Sawicki et al., 2020), the native glucosides of betacyanins, (iso) betanin, were discovered as the prominent contributor to the pigments in physiological fluids. Nevertheless, Sawicki et al. (2018) (7-week trial) was an exception with intervention of fermented beetroot juice which reported a higher proportion of metabolites than native pigments in plasma and urine. While betaxanthins (e.g., indicaxanthin), with relatively high bioavailability, were mostly absorbed untransformed, their systemic metabolism has yet to be researched (Tesoriere et al., 2004; Allegra et al., 2014). With high susceptibility to decomposition, betalains are proposed to undergo sequential biotransformation before and after absorption under physiological conditions (Sawicki et al., 2018). There is little information available on the human urinary pharmacokinetics of betalain derivatives after acute consumption, which could be imperative in understanding the metabolomic fate of the pigments.

Escaping small intestinal absorption, the bulk of betacyanins is anticipated to transit through the GI tract while closely interacting with mucosal epithelia and gut bacteria before excretion via feces. A previous *in vitro* study demonstrated the immunomodulatory as well as radical scavenging activity of (iso)betanin on stimulated intestinal Caco-2 cells, implying their great potential to mitigate intestinal inflammation (Wang et al., 2022b). Furthermore, recent *in vivo* research has begun to explore the microbiota-modulatory effect of betacyanin-rich food. Gut microbiota serves an indispensable role in energy harvest, nutrient metabolism, and epithelial development, with further coordination to immunological and neurological functions and disorders (Hakansson and Molin, 2011; Mailhe et al., 2018). Song et al. (2016) observed a lower ratio of Firmicutes to Bacteroidetes phyla in high-fat diet-challenged rats after a 14-week supplementation with red pitaya-derived betacyanins compared with the control. Our recent findings documented a marked decline in the abundance of *Bacteroides fragilis* and an enrichment in *Akkermansia muciniphila* during a 2-week intervention with red beetroot juice, which suggests a positive contribution of betacyanin-rich food to the gut and systemic health via microbiota modulation (Wang et al., 2022a).

Mutually the gut microbiota and fermentative environment exert a decomposing effect on pigments, with increasing strength from proximal to distal intestine due to the ascending microbial density. For a profound assessment of the beneficial potential of betacyanins to the gut, it is pivotal to analyze their presence and composition in the intestinal digesta. Previous studies on *in vitro* simulative digestion have reported a significant loss of betalains during the intestinal digesting phase with approximately 29–38 % betalains remaining (Tesoriere et al., 2008; Vieira Teixeira da Silva et al., 2019; Gómez-Maqueo et al., 2020; Wang et al., 2020; Montiel-Sánchez et al., 2021; Sánchez-Recillas et al., 2022). The fecal excretion of betanin *in vivo* was initially explored by Krantz et al. (1980) who indicated recovery fractions of 3 % in rat feces after a single oral dose, whereas a significantly higher recovery of betanin (41 %) was determined *ex vivo* after incubating the pigment with dissected large rat intestine (Reynoso et al., 1999). However, due to limitations with detectability of betalains and their metabolites, details regarding betalain degradation and metabolism during intestinal processing are scarce.

The current study investigated the bioavailability and biotransformation of betacyanins, the main betalain group, based on i) renal and fecal excretion profiles in humans following consumption of beetroot juice, and ii) intestinal betacyanin degradation profiles in pig digesta following administration of beetroot supplemented feed. In addition, the human data were analyzed for inter-individual variability in responsiveness as well as correlation between differentially transformed betacyanins excreted through the renal and fecal routes.

2. Experimental methods

2.1. Materials and reagents

The beetroot concentrate for human trial intervention was kindly provided by Active Edge Nutrition Ltd. (Hartley Wintney, UK), while the red beetroot powder for animal trial was supplied by Wholefoods Ltd. (Ramsgate, UK). All the sampling materials were purchased from Alpha Laboratories Ltd. (Eastleigh, UK), HyStool (Longniddry, UK), Sarstedt (Nuremberg, Germany), and MedDX Solution Ltd. (Hereford, UK). The Strata-X polymeric SPE cartridges (200 mg, 6 mL) for sample processing were purchased from the Phenomenex Ltd. (Macclesfield, UK), whilst organic solvents (LC-MS and UHPLC-MS grades) for sample processing and chromatographic analysis were obtained from Fisher Scientific (Loughborough, UK). Betanin standard was obtained according to the separation and purification procedures described in Fernando et al. (2022).

2.2. Human intervention study and samples

For the investigation of betacyanin absorption and excretion, samples of human urine and stool from a previous human study were analyzed (Wang et al., 2022a), with the ethics approval by the Research Ethics Committee of University of Leeds (AREA 20–058). The study involved 18 volunteers with a mean age of 29.1 $\pm$ 6.1 years and a mean body mass index of 22.5 $\pm$ 2.4 kg m^{-2}, who were generally healthy, beetroot tolerable, tobacco abstained, and without pregnancy/lactation. The previous study focused on examining the modulation of human gut microbiome following the ingestion of red beetroot juice over a 14-day period. The current work is concentrating on further in-depth analysis of betacyanin bioavailability and intestinal metabolism, with the number of participants in this trial comparable to those in other bioavailability studies (Mullen et al., 2008; Wiczkowski et al., 2018).

With the consent obtained, participants were asked to perform the intervention and sampling procedures out of the campus at their premises due to covid restriction guidelines, and were therefore provided with full guidance, explanation and instruments required for the study. After fasting overnight, they were requested to consume a portion of red beetroot juice (250 mL) with two pieces of white bread in one sitting on the first day of the intervention (D1). No other food or beverage was to be consumed in the following 3 h except for water if required for hydration. The food intake of individuals on the day of urine sampling was recorded in detail via a food diary to report food type, approximate amount and time of ingestion. Urine samples were collected before (baseline, BSL) and after the juice consumption at 6 time intervals (0–2 h, 2–4 h, 4–6 h, 6–8 h, 8–12 h and 12–24 h) by participants for the kinetic measurement; and the total volume of urine at each time point was measured and recorded using graduated containers.

During the remainder of the intervention period, participants resumed their habitual diets while consuming half of the daily portion of beetroot juice with lunch and dinner, respectively. Following the instructions, stools were sampled by participants before juice consumption (BSL), after 3 days (D3) and after 14 days (D14) of consumption. The diet pattern of individuals was evaluated via weekly food frequency questionnaires. The systolic and diastolic blood pressures (SBP & DBP) of participants were also measured weekly to provide an insight of the beetroot juice effect on blood pressure if any.

2.3. Betacyanin analysis in urine samples of the human study

Urine samples were aliquoted, centrifuged (3600*g*, 8 min, 4 °C) and frozen at −80 °C, prior to being submitted to a Labconco freeze dryer (USA) (0.2 mmHg, −55 °C) until complete dryness. The residues were

resuspended in water (1 mL), sonicated (10 min, 4 °C), and centrifuged (3600*g*, 8 min, 4 °C) to obtain 10 times concentrated urine samples. Based on Sawicki et al. (2017) with modifications, the pigment compounds were separated from the urine matrix via solid-phase extraction (SPE) using Strata-X reversed-phase cartridges (33 μm, 200 mg, 6 mL) filled with modified co-polymer of styrene divinylbenzene. After conditioning and equilibrating the cartridge with methanol and water, respectively, the aqueous samples (1 mL) were loaded and passed through the sorbent in a dropwise speed. The cartridges were washed with 0.1 % formic acid (*v/v*) before eluting the retained betacyanins with methanol. The organic solvent in the eluents was evaporated via a Genevac centrifugal concentrator EZ-2 (low b.p mode) followed by freeze drying to achieve complete removal of solvent. The solid samples were resuspended in water (100 μL) and centrifuged (22000*g*, 20 min, 4 °C) for chromatographic analysis. The red beetroot juice was diluted and filtered through a 0.2 μm PTFE syringe filter prior to analysis.

Betalain compounds in urine samples and juice were identified and quantified by a 2020 Shimadzu high-speed LC followed by a 2020 quadrupole electrospray ionization source (ESI-MS). The method was based upon the Shimadzu application guide on betalain analysis (Borzymowska-Reszka and Spółka Jawna, 2011). Separation was performed by reverse phase HPLC with a Gemini C18 column (250 mm × 4.6 mm I. D., 5 μm, 110 Å, Phenomenex, U.K.) at 40 °C. The eluent gradient was established by a binary mobile phase of 2 % formic acid (*v/v*) as solvent A, and LCMS-grade methanol as solvent B. With discrete sample injection volume (10 μL) and flow rate (0.95 mL min^{-1}), the proportion of solvent B was increased with time from 5 to 25 % (0–15 min); to 70 % (15–19 min), and was held until 21 min before returning to the initial 5 % (21–26 min). Betacyanin compounds were characterized based on corresponding molecular masses determined by ESI-MS at positive ion mode with nebulizer gas flow rate of 1.5 L min^{-1} and desolvation line temperature of 250 °C, and the *m/z* values, absorbance and retention time were compared with those reported in Sawicki et al. (2018) and Nemzer et al. (2011). All betalains were quantified according to calibration curve of purified betanin and expressed as betanin equivalent.

2.4. Betacyanin analysis in human stool samples

An aqueous supernatant of human stool (15 %, *w/v*) was acquired from 100 mg of ground fecal sample following horizontal vortexing, sonication, and centrifugation (20000*g*, 8 min, 4 °C) in 0.5 % formic acid (*v/v*). From the aqueous fecal matrix, betacyanins and corresponding derivatives were separated via the SPE method described in Section 2.3 with an additional washing step at the end using methanol/formic acid solvent (95:5, *v/v*). The eluents were subject to the Genevac concentrator and subsequent freeze drying to obtain solid samples. After resuspending in water (60 μL), the samples were centrifuged (22000*g*, 10 min, 4 °C) and prepared for chromatography submission.

Compared with betacyanin analysis in urine, pigments in stool samples were examined via UHPLC method as established in Wang et al. (2022a) with improved sensitivity towards metabolic compounds. Briefly, pigments in the processed fecal samples were analyzed by a Thermo-Vanquish UHPLC combined with TSQ Quantiva MS (Thermo Scientific, UK). The chromatography was performed with a Kinetex XB-C18 column (100 mm × 2.1 mm, 2.6 μm) using the same solvent phases as used in the LC-MS method in Section 2.3. At the flow rate of 200 μL min^{-1} and injection volume of 5 μL, the proportion of solvent B was increased from 5 to 25 % (0–2 min), then to 95 % (2–5 min), and was held for 5 min before reducing to 5 % (10–15 min). The heated ESI-MS was performed at the spray voltage of 3500 V, sheath gas flow rate of 35 arb, and vaporizer temperature of 275 °C. Quantification (in betanin equivalent) and identification of betacyanins were achieved by comparing the *m/z* values of precursor ions in positive ionization as well as retention times with previously published data (Nemzer et al., 2011; Khan and Giridhar, 2015; Sawicki et al., 2020).

2.5. Pig digesta samples

To further understand the progressive transformation of pigments during intestinal digestion, digesta samples obtained from a study on pigs fed diets supplemented with red beetroot were analyzed (Adekolurejo et al., 2023). The animal trial was approved by the Animal Welfare and Ethical Review Committee of University of Leeds (N0. 070510HM). The pig feeding trial randomly allocated 36 pigs weaned on day 28 (Large-White × Landrace × Duroc, 7.6 ± 0.7 kg, 12 per group) to post-weaning diets comprising either 0, 2 or 4 % of beetroot powder (BSL, RBR2, RBR4) for 14 days. The control diet was formulated to meet the nutritional requirement for piglets (National Research Council, 2012) and the supplemented diets were prepared on-site by manual addition of pulverized red beetroot. At the end of the feeding period, the animals were euthanized by captive bolt and exsanguination with the process being covered under the Animals (Scientific Procedures) Act 1986. Intestinal samples including digesta (8 per group) were collected. In this study, the content and profile of betacyanins was analyzed in digesta samples from small intestine (i.e., ileum) and from colon of individual pigs in the indicated groups.

2.6. Analysis of betacyanins in feed and pig digesta

The processing of feed and pig digesta samples resembled that of human feces. In brief, the feed of each group (200 mg) and digesta in ileum and colon segments (100 mg) were extracted twice each using water/methanol/formic acid (84.95/15/0.05, *v/v/v*), undergoing vortexing, sonication and centrifugation (21000*g*, 10 min, 4 °C). The feed extracts were filtered through PTFE membranes (0.2 μm) for the submission to chromatography. In terms of digesta, the combined aqueous supernatant of each sample (*ca.* 960 uL) was subject to SPE processing and drying as described in section 2.3. Eventually the reconstituted digesta pigments were centrifuged and analyzed using the UHPLC-MS method as described in section 2.4, except with a prolonged solvent gradient for an improved resolution of chromatograph, i.e., rise of solvent B from 5 % to 25 % (0–5 min) then to 95 % (5–10 min) followed by a plateau (10–20 min) and a decline to 5 % (20–22 min) until the end (32 min). The native and metabolized betacyanins were identified based on product *m/z* data and quantified in betanin equivalent as described earlier.

2.7. Data analysis and statistics

Statistical analysis was performed using SPSS Statistics 26.0, Excel, and R-4.1.2. Urinary excretion data were analyzed under non-compartmental model conditions and presented as mean with standard error of mean (SEM). The cumulative renal excretion of betacyanins within a 24 h window was reflected by the area under renal excretion rate curve ($AURC_{0\text{-}24}$) and calculated by linear trapezoidal rule. The renal elimination rate constant (k_{re}) was estimated at the descending region of each curve via Log-linear regression model. Data normality was evaluated by a Shapiro-Wilk test, while the group comparisons employed the one/two-way analysis of variance (ANOVA) combined with Dunnett post hoc test, as well as the non-parametric Kruskal-Wallis test at the confidence interval of 95 %. Computations of permutation test, observation diagnostics, and projection to latent structures-discriminant analysis (PLS-DA) were achieved using *mixOmics* and *ropls* packages in R (Thévenot et al., 2015; Rohart et al., 2017). Relationships concerning betacyanin variables, and dietary, anthropometric, and lifestyle factors were further assessed using SPSS Statistics 26.0, with performance of data transformation, normalization, Pearson and Spearman's rank correlation. Data visualization was achieved on GraphPad Prism 9.0, R-4.1.2 and ChemDraw 17.0.

3. Results

3.1. Human study compliance and profile of red beetroot juice

As reported in Wang et al. (2022a), all participants completed the study without withdrawals nor critical deviation from the protocols, thus all data obtained were included in the analysis of urinary and fecal excretion of betacyanins. The diet patterns of participants suggested a marked inter-individual variability ($p < 0.05$) but minor for intra-individual ($p > 0.05$) regarding the approximate ingestion amount and frequency of six food categories (distance matrix is shown in Fig. S2).

The betacyanin content in red beetroot juice (208 ± 7 μmol per day portion) was dominated by (iso)betanin (86.7 %) and neobetanin (9.3 %), with the rest being contributed by other degraded forms of pigments such as (iso)betanidin and 17-decarboxy-(iso)betanin. Principal betaxanthins in red beetroot, such as vulgaxanthins and miraxanthins, were not detectable in the particular batch of beetroot juice, thus subsequent sample preparation and analysis were optimized specifically for detection of betacyanins.

3.2. Urinary excretion profile and kinetics of betacyanins

During the 24 h post-prandial window after beetroot juice consumption, the area under the renal excretion rate curves ($AURC_{0\text{-}24}$) of individual compounds was determined, from which the recovery fraction (*fe* %) to the administered dose was estimated and is presented in Table 1. A representative chromatogram of urinary excreted betacyanins is shown in Fig. S1.

In the present study, the cumulative amount of urinary excreted betacyanins was calculated as 275.8 ± 2.8 nmol on average, occupying 0.13 % of those in the single dose on D1, while betacyanins were not detected in any forms in baseline urine (BSL). Among the excreted pigments in urine, there were 11 betacyanin compounds identified in the present study (8 native, 3 metabolites) which were majorly composed of (iso)betanin (**1/2**)[1](64.1 %), (iso)betanidin (**3/4**) (22.3 %), and 2,17-bidecarboxy-neobetanin (**9**) (7.1 %). The $AURC_{0\text{-}24}$ of (iso)betanin, as principal betacyanins found in beetroot juice and urine, were significantly higher than (iso)betanidin which further outstripped neobetanin (**5**), 17-decarboxy-(iso)betanin (**6/7**), 15-decarboxy-betanin (**8**), 2,17-bidecarboxy-betanin (**11**), and 2,15,17-tridecarboxy-neobetanin (**10**) ($p < 0.05$). The maximum value of k_{re} appeared in (iso)betanin while the minimum in (iso)betanidin and 2,17-bidecarboxy-neobetanin ($p < 0.05$). However, the results indicate an evidently higher *fe* % of (iso)betanidin (18.1 %) compared with (iso)betanin (0.2 %) and other native betacyanins. Note the ratios of $AURC_{0\text{-}24}$ between C15-isomers were consistent with their relative proportions in red beetroot juice except 17-decarboxy-(iso)betanin, and no significant difference was discovered between isomers in this study ($p > 0.05$). At last, there was no gender effect with regards to renal excretion level (female to male ratio of 1.07).

The time course curves of renal excretion rates for different betacyanins are illustrated in Fig. 1 with individual excretion displayed in dots. The compound 2,17-bidecarboxy-betanin was not included in the kinetic and compositional profiles (Figs. 1 & 2) due to its limited detectability in a small number of samples. Betacyanins were rapidly excreted through urine after oral administration of red beetroot juice with white bread matrix. For the majority of compounds (e.g., (iso)betanin, 17-decarboxy-(iso)betanin) and total content of betacyanins, maximum excretion rates were achieved at the post-prandial interval of 2–4 h (i.e., t_{max}) which was followed by a drastic decrease ($p < 0.05$). In contrast, excretion rates of (iso)betanidin and 2,17-bidecarboxy-neobetanin smoothly reached the peak at 4–6 h prior to a gradual decline. Alike previous results of $AURC_{0\text{-}24}$, no significant difference was found between excretion rate curves of stereoisomers in this study. According to Fig. 1C, (iso)betanin showed a markedly greater excretion rate and proportion at the intervals of 0–2, 2–4 and 4–6 h compared with (iso)betanidin and other derivatives ($p < 0.05$). With the convergence of curves at the later time points, the proportion of (iso)betanin was gradually outweighed by the others, and eventually the dominance of (iso)betanidin from 8 to 12 h onwards. Observed from the distribution of subjects, greater variance was displayed at 0–2, 2–4, and 4–6 h compared to other observation points, the detailed analysis of inter-individual variation is described in section 3.3.

3.3. Inter-individual variation and stratification based on urinary excreted betacyanins

The PLS-DA scores (Fig. 2A-B and S3B) derived from the urinary pharmacokinetic data have effectively revealed the metabolic trajectory of betacyanins and inter-individual variation of urinary excretion profiles. Performance of the PLS-DA model was validated by a permutation test, suggesting 4 predictive components with Q2Y value of 0.123. Fig. 2A demonstrated the covariance between urine samples grouped by 6 time points as a function of inclusive native and metabolic betacyanins. With ellipse drawn at confidence level of 95 %, a perturbation in metabolic and excretion profiles was manifested after beetroot juice intake that was amplified during 2–6 h followed by a restoration to nearly BSL condition during 6–24 h. The plot was in line with excretion curves in Fig. 1 in terms of the increasing betacyanin quantity during 0–4 h and growing dispersion of metabolites during 4–24 h after juice intake.

Inter-individual variation in renal excretion was reflected by the minimal *fe* (%) value of 0.08 % and maximum of 0.19 % among the subjects. To improve the demonstration of variability, subjects were classified into low (n = 4), medium (n = 6), and high excreters (n = 8) based on their total amount of renal excretion with the inter-group difference ($p < 0.05$). The PLS-DA score plot (Fig. 2B) showed the proximity between excretion profiles of low and medium excreters but a larger variance in high excreters. In association with Fig. 2C, the augmentation in $AURC_{0\text{-}24}$ from low to high excreters was mainly contributed by the increases in (iso)betanin (116.0 vs. 208.1 nmol) and (iso)betanidin (37.9 vs. 78.6 nmol), whereas the composition regarding native betacyanins and metabolites remained similar between groups. Inter-individual variation was further elucidated via the subject-based proximity matrix (Fig. S3A), indicating a relatively large deviation in the metabolic profiles of subject 1 and 11 from the others. The subject-based profile of renal excretion is presented in histogram in Fig. S3C.

3.4. Characterization of betacyanin catabolites in human feces

By employing the UHPLC-MS technique, there were in total 14 betacyanin derivatives identified in human fecal samples with corresponding *m/z* values and composition as listed in Table 1. It is noteworthy that the fragment ions of (iso)betanin, (iso)betanidin and neobetanin were also detected at miniature ion intensity under study conditions. Twelve compounds were characterized by comparing the retention time and product ion mass with Sawicki et al. (2018), comprising (iso)betanin (**1/2**), (iso)betanidin (**3/4**), neobetanin (**5**), 17-decarboxy-betanin (**6**), 2,17-bidecarboxy-neobetanin (**9**), 2,17-bidecarboxy-(iso)betanin (**11/12**), 2,15,17-tridecarboxy-neobetanin (**10**), 17-decarboxy-neobetanin (**15**) and 2,15,17-tridecarboxy-betanin (**16**). Compound **14** with *m/z* 345 $[MH]^+$ and relatively early retention time was identified as 17-decarboxy-betanidin based on Nemzer et al. (2011). Correspondingly, compound **13** with *m/z* 343 $[MH]^+$ was proposed to be 17-decarboxy-2,3-dehydro-betanidin based on Wybraniec et al. (2016). An example chromatogram of the betacyanin metabolites in human feces was exhibited in Wang et al. (2022a). The existence of other catabolites cannot be excluded considering the limitations in detection scope and sensitivity of this study.

[1] The bold numbers in brackets were linked to the compound numbers in Table 1.

Table 1
Urinary and fecal excretion profiles of betacyanin derivatives in human trial. Data of AURC and k_{re} are presented as mean ± SEM (n = 18). Different letters indicate significant differences between compounds within individual parameters ($p < 0.05$).

No.	Compounds	m/z $[MH]^+$	Presence	Urinary excretion parameters [#]			Fecal excretion composition (%) [‡]	
				$AURC_{0\text{-}24}$ (nmol) [*]	k_{re} (h^{-1})	*fe* (%)	D3	D14
1	Betanin	551	Juice, urine, stool	86.29 ± 4.86 (31.3) ^{a}	0.29 ± 0.01 ^{a}	0.10	0.79 ^{ac}	1.81 ^{ac}
2	Isobetanin	551	Juice, urine, stool	90.65 ± 4.63 (32.9) ^{a}	0.26 ± 0.01 ^{a}	0.09	0.43 ^{a}	0.55 ^{a}
3	Betanidin	389	Juice, urine, stool	37.06 ± 3.15 (13.4) ^{b}	0.06 ± 0.02^{b}	9.74	1.59 ^{a}	3.68 ^{a}
4	Isobetanidin	389	Juice, urine, stool	24.57 ± 2.47 (8.9) ^{bd}	0.08 ± 0.02^{b}	8.39	2.33 ^{a}	3.80 ^{a}
5	Neobetanin	549	Juice, urine, stool	3.83 ± 0.27 (1.4) ^{c}	0.19 ± 0.02^{c}	0.03	0.00 ^{a}	0.08 ^{ac}
6	17-Decarboxy-betanin	507	Juice, urine, stool	2.51 ± 0.14 (0.9) ^{c}	0.16 ± 0.01 ^{cd}	0.09	10.57 ^{ac}	13.20 ^{abc}
7	17-Decarboxy-isobetanin	507	Juice, urine	3.58 ± 0.26 (1.3) ^{c}	0.18 ± 0.02^{c}	1.23	–	–
8	15-Decarboxy-betanin	507	Juice, urine	2.12 ± 0.12 (0.8) ^{c}	0.16 ± 0.02 ^{cd}	0.22	–	–
9	2,17-Bidecarboxy-neobetanin	461	Urine, stool	19.49 ± 1.57 (7.1) ^{d}	0.06 ± 0.01^{b}	–	6.75 ^{bcd}	5.37 ^{abc}
10	2,15,17-Tridecarboxy-neobetanin	417	Urine, stool	5.52 ± 0.53 (2.0) ^{c}	0.11 ± 0.02 ^{bd}	–	22.20 ^{abc}	15.78 ^{abc}
11	2,17-Bidecarboxy-betanin	463	Urine, stool	0.24 ± 0.02 (0.09) ^{c}	N/A	–	2.28 ^{a}	8.89 ^{ac}
12	2,17-Bidecarboxy-isobetanin	463	Stool	–	–	–	1.35 ^{a}	2.98 ^{ac}
13	17-Decarboxy-2,3-dehydro-betanidin	343	Stool	–	–	–	8.92 ^{bcd}	11.44 ^{abc}
14	17-Decarboxy-betanidin	345	Stool	–	–	–	24.12 ^{bd}	17.86 ^{bc}
15	17-Decarboxy-neobetanin	505	Stool	–	–	–	0.74 ^{ac}	0.86 ^{a}
16	2,15,17-Tridecarboxy-betanin	419	Stool	–	–	–	17.94 ^{d}	13.64^{b}
Total betacyanins in urinary elimination				275.85 ± 2.83	0.16 ± 0.01	0.13	–	–
Total concentration of fecal betacyanins (nmol/g stool)				–	–	–	12.18 ± 4.56	19.92 ± 8.59

[#] Abbreviations – $AURC_{0\text{-}24}$, area under the urinary excretion rate curve (0–24 h); k_{re}, urinary excretion rate constant; *fe* (%), eliminated fraction in urine respect to dose.
[*] Proportion of individual compounds (%) respect to total excreted amount in urine was displayed in brackets.
[‡] The compositional difference was absent between D3 and D14 groups.

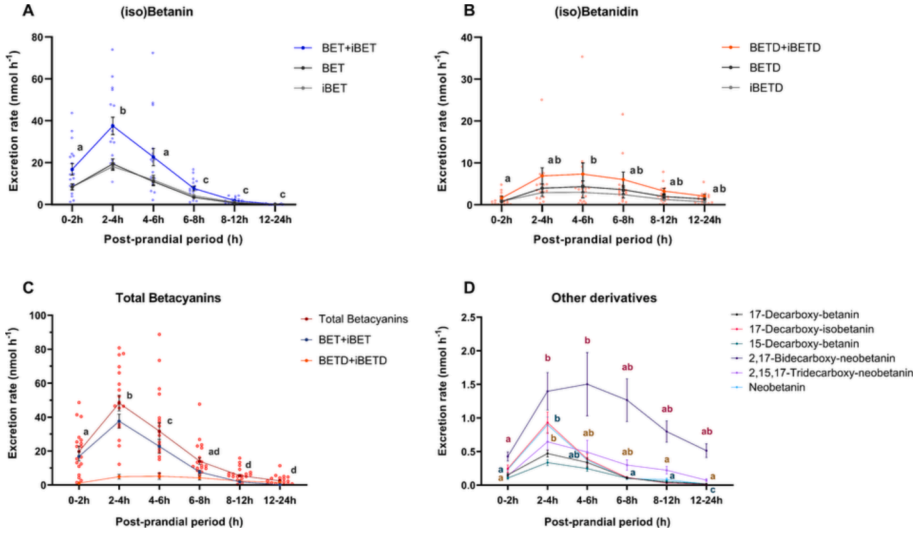

Fig. 1. Urinary excretion profiles of native betacyanins and their metabolites against time intervals following acute beetroot juice consumption, including (A) betanin and isobetanin (BET, iBET), (B) betanidin and isobetanidin (BETD, iBETD), (C) total betacyanins, and (D) all other betacyanins. Data are presented as mean ± SEM (n = 18) and excretion level of individuals is displayed in dots. Significant difference of excretion rate across the time is indicated in different letters ($p < 0.05$). Meanwhile in (D), the time-dependent difference of 2,17-bidecarboxy-neobetanin is indicated in red letters; 2,15,17-tridecarboxy-neobetanin in yellow and the remainders in blue. Note that inter-compound difference at each time point is not compared. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Like urinary excretion, native betacyanins and catabolites were absent from BSL stools. Betacyanin concentrations in D3 and D14 stool samples were 12.2 ± 4.6 and 19.9 ± 8.6 nmol/gram of stool, respectively, from which the proportion of catabolites (77–84 %) has apparently outweighed that of native betacyanins (16–23 %). The compositional profile of D3 was dominated by 17-decarboxy-betanidin, 2,15,17-tridecarboxy-betanin and 2,15,17-tridecarboxy-neobetanin, where the first two exhibited remarkably higher fractions compared to native components ($p < 0.05$). At D14, the previous three compounds showed a decrease in fractions, instead the rise in proportions of 17-decarboxy-betanin, 2,17-bidecarboxy-(iso)betanin and 17-decarboxy-2,3-dehydro-betanidin of D14 resulted in a relatively balanced distribution of catabolites. However, it is important to note that there was no significant difference from any inter-group comparisons in terms of individual ($p > 0.05$) or total betacyanin content ($p = 0.07$). Due to the large individual heterogeneity in fecal excretion profiles, the non-parametric hypothesis tests did not provide an explicit interpretation for the divergence among betacyanins in the current study.

3.5. *Inter-individual variation of fecal excreted betacyanins and stratification*

Betacyanin profiles of intestinal excretion were discriminated by PLS-DA models from the perspectives of intervention duration and individual compounds. As illustrated in Fig. 2D, the greater dispersion of D3 samples compared to D14 has revealed a larger variation among

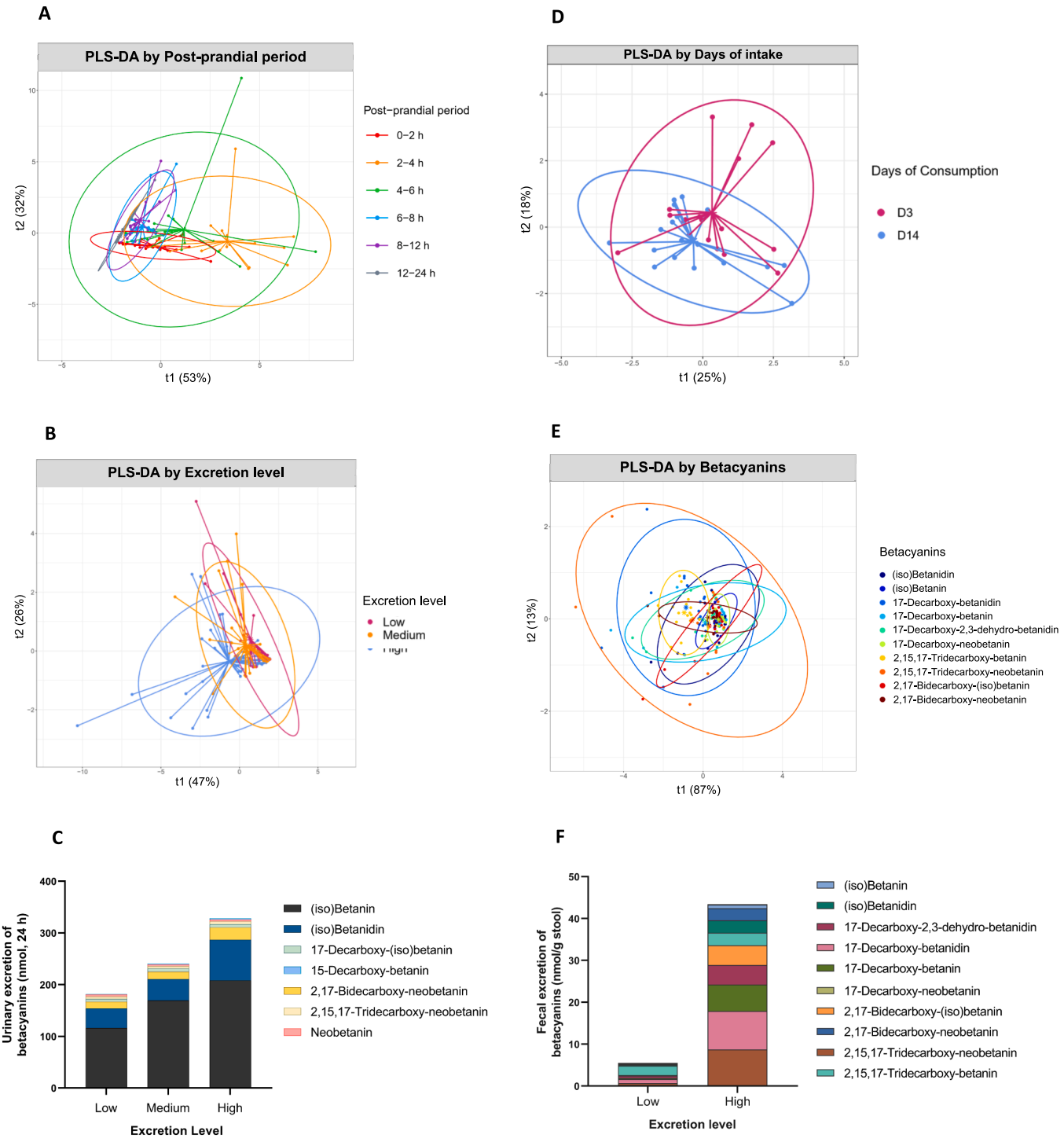

Fig. 2. Variability in urinary and fecal excretion profiles of betacyanins and subject stratification according to excretion level. Urinary excretion was illustrated by the PLS-DA score plots at (A) different time intervals after beetroot juice consumption, and at (B) each stratified excretion level. Fecal excretion was demonstrated in PLS-DA plots grouped by (D) different days after beetroot juice ingestion, and by (E) individual betacyanin derivatives. These are accompanied with the exhibition of compositional histogram of betacyanin metabolites in (C) urine and (F) stool samples from low/(medium)/high excreters.

profiles of the former regardless of the averagely lower pigment level. Yet, the convergence of most samples implied some degree of resemblance in the feature of fecal profiles between two groups. The score plot of individual compounds (Fig. 2E) was in accord with the significant difference demonstrated in Table 1, showing a highly scattered distribution of 2,15,17-tridecarboxy-neobetanin and 17-decarboxy-betanidin, whereas 2,15,17-tridecarboxy-betanin, as one of the dominant catabolites, displayed a relatively clustered ellipse hence lower variance.

Significant inter-individual variation was observed when comparing fecal betacyanin profiles with urinary extraction profiles (Fig. S3A and S4A), which was reflected by the overall greater z-scores in proximity matrix with apparent deviations of subject 11 and 12. Stratification was also carried out based on the corresponding betacyanin level in stools, into low (n = 13) and high excreters (n = 5) with significant difference (p = 0.01). It was evidenced from Fig. 2F that fecal betacyanin

composition of low excreters was predominated by 2,15,17-tridecarboxy-betanin, while that of high excreters comprised larger proportions of 17-decarboxy-betanidin, 2,15,17-tridecarboxy-neobetanin, as well as minorly transformed betacyanins such as 17-decarboxy-betanin and 2,17-bidecarboxy-(iso)betanin. The fecal excretion profile of individual subjects in the relative composition (%) was presented in Fig. S4C.

3.6. Betacyanin composition and variance in pig digesta

Results illustrated a drastic decline of betacyanin levels during digestion in the pig intestinal tract, which, in ileum and colon digesta, were reduced to 0.6–2.3 % and 0.2–0.6 % of the content in feed, respectively (Fig. 3). The inter-group difference was also recognized between ileum and colon contents regarding their (iso)betanidin and total betacyanin levels ($p < 0.05$). However, comparison of other metabolites was not available considering their limited occurrence in different digesta samples. Despite the significantly higher betacyanin level in the feed of RBR4 than RBR2, there was no difference in individual or total pigment levels between RBR2 and RBR4 digesta hence the dose-dependency in bioaccessibility was not reflected in this particular trial. Meanwhile, an increasing degree of fragmentation along the digestion was revealed by the compositional profile. The feed pigments were predominated by betacyanin glucosides especially (iso)betanin. Aglycones (i.e., (iso)betanidin) were the major pigments in ileum digesta, whereas composition in colon digesta became more diverse including multiple decarboxylates. Regardless of the dose, the striking difference in betacyanin profiles of feed, ileum and colon contents, along with the rising variability in this particular order, were further depicted in Fig. 3B. No betalains were detected in the BSL samples.

3.7. Correlation between urinary and intestinal excreted betacyanins of human and lifestyle factors

While the transformation pathways of betacyanin molecules were summarized in Fig. 4, the tendency and strength of relationship between urinary and fecal excreted pigments were examined via correlation analysis. The normality test suggested the normal distribution pattern of urinary betacyanins therefore application of the Pearson correlation, meanwhile non-parametric Spearman rank correlation was used on stool data.

The correlation matrix in Fig. 5A has demonstrated an overall positive relationship among betacyanin compounds in renal and fecal excretion routes, which was the most pronounced among urinary betacyanins. Strong positive correlation was discovered among three pairs of C15 stereoisomers in terms of renal excretion ($p < 0.01$). Furthermore, (iso)betanin and neobetanin were intimately associated with their decarboxylated products, which was carried down to the derivatives with further degradation ($p < 0.05$). It is also interesting that a significant relationship was absent between (iso)betanidin and (iso)betanin in the present study, which was found, instead, between the (iso)betanidin and decarboxylated neobetanins. With regard to fecal excretion, the positive tendency in correlation was evidently attenuated compared with renal excretion, and the significant relationship was mainly observed among the more fragmentized betacyanins. Similar to urine, interconnections were discovered between (iso)betanidin and the decarboxylates of (iso)betanin and neobetanin in stools. Other prominent correlations mainly appeared among the decarboxylated forms of (iso)betanin and neobetanin ($p < 0.05$), whereas some mild negative associations were observed on betanin and 17-decarboxy-neobetanin ($p > 0.05$). Eventually, there were 11 out of 13 compounds showing positive contribution to the total betacyanin level in stool. Moving on to the matrix of urinary-fecal betacyanins, it revealed an overall negative relationship of (iso)betanin content in urine with the majority of derivatives as well as total betacyanin level in feces. Moreover, the study reported a positive correlation between urinary and fecal (iso)betanidin ($p < 0.05$). The other strong relationships mainly involved the urinary pigments of neobetanin, 15-decarboxy-betanin, and 2,15,17-tridecarboxy-neobetanin. Eventually, from the current investigation, there was no association between renal and fecal excretion regarding the total betacyanin levels.

Associations between betacyanin excretion and the factors of diet, anthropometric, and lifestyle were presented in Fig. 5B-C. With regard to diet components, intake of meat and fish was discovered to be positively correlated with urinary and fecal betacyanin contents ($p < 0.05$). The increase in consumption of staple, fruits, polyphenol-rich and dairy beverages were also linked to a potential rise in the excretion level of native betacyanins. The vegetable intake, however, demonstrated an inverse relationship to the excretion of neobetanin derivatives in both urine and stool. With regard to other factors, results suggested exercise and frequent defecation as positive contributors to betacyanin excretion in this study.

4. Discussion

The current study, for the first time, compared and contrasted the betacyanin profiles from renal and intestinal excretion routes with presentation of inter-individual variability following 14-day consumption of red beetroot juice. The analysis is providing evidence of direct associations between betacyanin compounds in the two types of discharge, allowing a deeper insight into the interconnections between bioavailability and bioaccessibility of betalain pigments.

The results confirmed the low urinary excretion rates thus bioavailability of betacyanins as observed in Wiczkowski et al. (2018), which was accompanied by their time-dependent alteration and inter-individual variability. Urinary excretion rate profiles of most compounds, after reaching maximum at 2–4 h, followed the first-order elimination kinetic, namely the removal of a constant proportion per unit of time. Secondary peaks were not observed from the profile, indicating a decreasing rate of their intestinal uptake and/or systemic formation following the ingestion. It is presumably caused by a constant dissipation of pigments, and the reducing number as well as diameter of

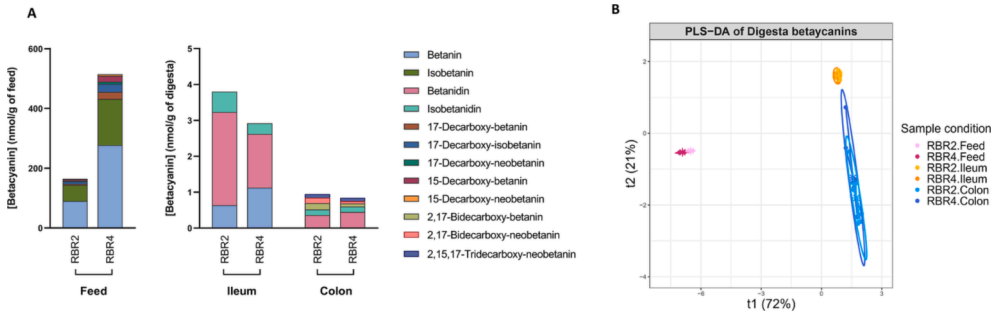

Fig. 3. Compositional profile and variation of betacyanins in ileum and colon digesta of pigs, as illustrated in (A) the histogram of betacyanins in feed and digesta samples, and (B) the PLS-DA plot grouped by dose and intestinal location, respectively.

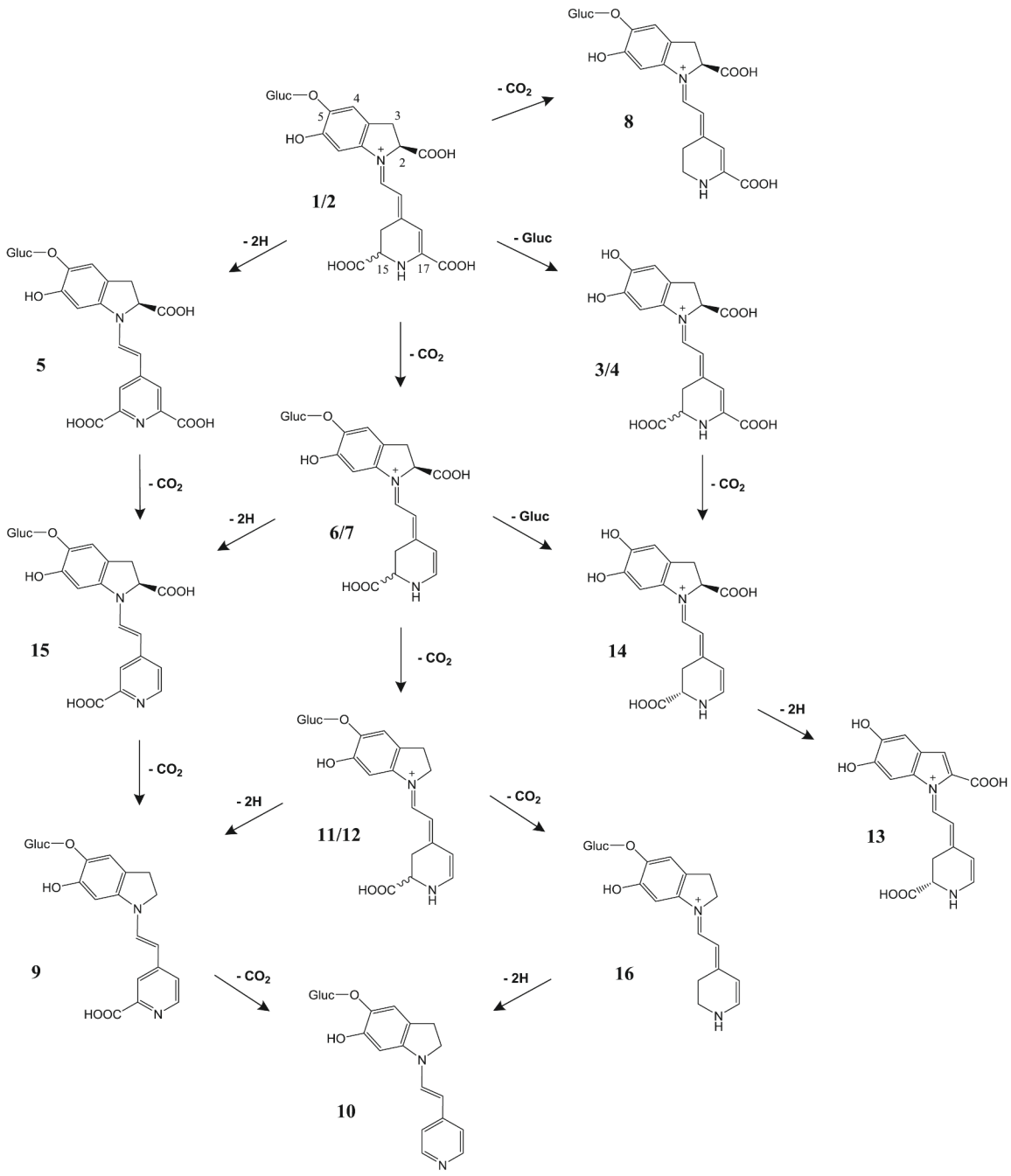

Fig. 4. Proposed mechanistic pathway of betacyanin transformation and catabolite formation. The numbers of compounds are linked to those in Table 1. The figure was adapted from Sawicki et al. (2018) and Nemzer et al. (2011) according to the analytical results of the present study.

paracellular passages from jejunum to colonic epithelia (Smith et al., 2012; Camilleri, 2021). The urinary pharmacokinetics of total betacyanin was previously studied by Frank et al. (2005) and Wiczkowski et al. (2018) in healthy volunteers following a single oral administration of red beetroot juice. Higher values of *fe* % (0.28 % and 0.30 %, respectively) and lower k_{re} (both 0.07 h^{-1}) were reported, in comparison to the current work. There were also earlier studies, Kanner et al. (2001) and Tesoriere et al. (2004) addressing the bioavailability of (iso)betanin, indicating the *fe* % values of 0.5–0.9 % and 3.7 %, respectively. The discrepancies between studies are plausibly explained by multiple factors, specifically the dose and matrix of ingested compounds, individual physiological characters, and sample treatment and analytical methods. Contrary to Wiczkowski et al. (2018) who carried out a human intervention with beetroot juice only, the ingestion of juice with white bread in this work likely resulted in a lengthened gastric retention time of subjects and buffered release of pigments from food matrix, which potentially caused a more severe betacyanin decomposition and a deferred t_{max} (2–4 h in this study vs. 0–2 h in reference). The undesirably low *fe* % of betacyanins could in theory be attributed to the limited bioaccessibility, poor intestinal absorption efficiency, and powerful first-pass metabolism of betacyanins in the human body prior to their further distribution and metabolism in systemic circulation. The betacyanin loss during liver metabolism was also noted by Sawicki et al. (2020), indicating an approximate 23 % reduction of the average pigment level after liver detoxification compared with that in the portal plasma.

In part agreement with Wiczkowski et al. (2018), our results indicate the overall dominance of (iso)betanin (64.1 %) in urinary betacyanin

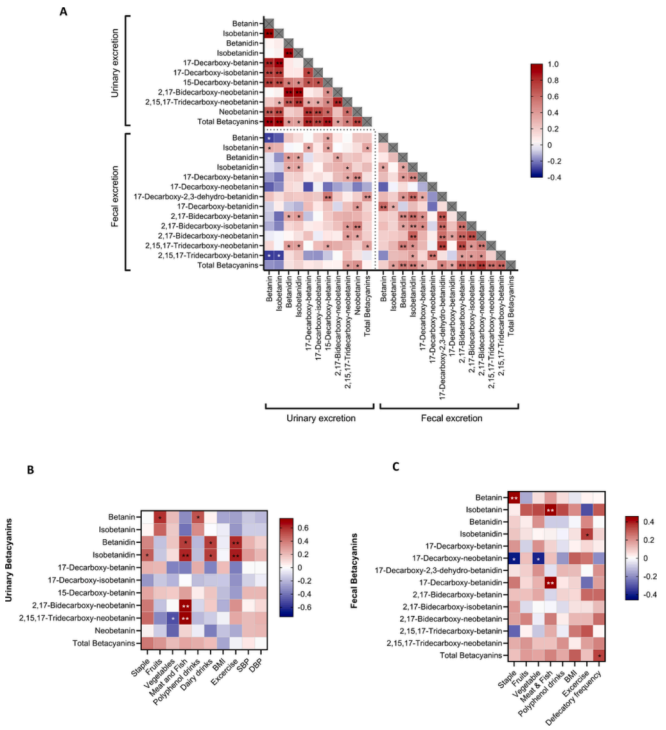

Fig. 5. Correlation matrices between (A) urinary and fecal excretion of betacyanins, (B) among urinary excreted betacyanins and dietary/anthropometric/lifestyle factors, and (C) among fecal betacyanins and dietary/anthropometric/lifestyle factors. Correlations with strong evidence are marked by stars: * $p < 0.05$, ** $p < 0.01$. Note the pigment correlations in urine were analyzed via Pearson rank correlation test while the rest was of Spearman rank test.

composition with an overtaking by (iso)betanidin at the later stage of excretion. Three betacyanin metabolites were identified in urine which were lacking in beetroot juice, and therefore were likely generated during hepatic and/or kidney detoxification. It supported the hypothesis that (iso)betanin tends to maintain the glucosylated structure during phases of intestinal absorption and liver metabolism. Relevant *in vivo* evidence was also exhibited in the animal trials of Takahashi et al. (2017) and Sawicki et al. (2020), reporting (iso)betanin as the principal constituents of betacyanins in portal and systemic plasma, and urine. According to the *in vitro* cell culture evidence, betanin was deduced to adopt passive paracellular diffusion as the major route of intestinal trans-epithelial transport, therefore insignificant mechanism of glucosyl hydrolysis. This was reinforced by the non-saturable diffusion kinetics of betanin absorption and the lack in evidence of involvement of epithelial transporters, although the contribution of active transport could not be precluded (Tesoriere et al., 2013; Wang et al., 2022b). However, the absorption mechanisms of other betacyanins still remain largely unclear.

As suggested by previous studies, betacyanin composition in urine was intimately linked to the digestive stability, hence the uptake and metabolism were susceptible to alteration under varied pigment type, dose and food matrix (Sawicki et al., 2018; Sawicki et al., 2020). Present data indicate that the maximum excretion rate of (iso)betanin was reached before that of (iso)betanidin, informing potentially higher uptake and distribution rates of the former. Additionally, the relatively large k_{re} values of (iso)betanin and decarboxylated derivatives implied the efficient metabolism and elimination, hence rapid clearance of the compounds from plasma in recruited subjects. Aglycones, to the contrary, possessed evidently greater *fe* % than (iso)betanin, which also displayed a larger contribution to the total pigments in urine (22.3 %) compared with that to pigments in beetroot juice (0.3 %). The differing kinetic behaviors of aglycones and other metabolites (e.g., 2,17-bidecarboxy-neobetanin) from (iso)betanin were likely caused by their generation from the systemic metabolism of native (iso)betanin. It is also proposed that the deglucosidation of non-absorbed (iso)betanin during digestion might help maintaining and prolonging the intestinal exposure to aglycones hence their bioaccessibility in the distal GI tract (0.48–1.49 nmol/gram of stool).

Overall, the urinary excretion data exhibited in this study informed a notably poor bioavailability of betacyanins in comparison to several flavonoids such as epicatechin (*fe* % of 1.9–6.2 %) and cyanidin 3-*O*-glucoside (1.0–6.7 %) (Scalbert and Williamson, 2000; Pérez-Jiménez et al., 2010). It echoed with the findings of cell culture studies showing lower intestinal trans-epithelial permeability of betanin ($0.4–3.2 \times 10^{-6}$ cm s^{-1}) compared to dietary phenolic compounds ($2–8 \times 10^{-6}$ cm s^{-1}) (Tesoriere et al., 2013; Rastogi and Jana, 2016; Hithamani et al., 2017; Wang et al., 2022b). This is heavily attributed to the physicochemical nature of betacyanins, specifically their relatively large molecular weight and hydrophilicity that pose inverse impact to their absorption efficiency and bioavailability (Liveri et al., 2007; Wang et al., 2022b).

On account of the exceptionally low fraction of urinary excretion, the bulk of pigments was speculated to undergo intestinal excretion, experiencing severe fragmentation and potentially interacting with gut mucosa and microbiota. The fecal excretion of betacyanins were previously explored by Krantz et al. (1980) via an animal study which recovered 3 % of betanin from rat feces in the post-prandial 24 h. Another attempt was made by Vieira Teixeira da Silva et al. (2019) *in vitro* despite that pigment was not detected in samples post colon fermentation. The present study has addressed the acute betacyanin composition in human feces and potential alteration following a longer-term ingestion of red beetroot juice.

There were five betacyanins detected in stool yet absent in beetroot juice and urine, suggesting their generation mainly in the gut lumen with possible implication of enterohepatic circulation; nonetheless, their intestinal absorption and generation in the circulation cannot be excluded considering the limited sensitivity of urine analysis. In contrast to renal excretion, predominant betacyanins in stools (e.g., 2,15,17-tridecarboxy-neobetanin, 17-decarboxy-betanidin) were of higher degree of decomposition, which was largely driven by the physiological temperature, altered pH, enzymatic activities, and microbial catabolism during the transit in GI tract particularly distal intestine. The significant loss of native betacyanins during the phase of small intestine was demonstrated by Tesoriere et al. (2008) and Gómez-Maqueo et al. (2020) using the *in vitro* digestion model. In this study, an increasing variation of betacyanin compounds from ileum to colon digesta of pigs further denoted the richness and diversity of gut bacteria as the crucial factors responsible for the intensity and specificity of catabolic reactions of pigments, such as species of genera *Bacteroides* and *Bifidobacterium* involved in deglucosidation (Bokkenheuser et al., 1987; Hasegawa, 2004).

Adapted from results in the present study and earlier bioavailability research (Nemzer et al., 2011; Sawicki et al., 2018), betacyanins were speculated to undergo sequential or simultaneous degradative pathways as illustrated in Fig. 4. The degree and pattern of biotransformation were proposed to be principally determined by their chemical scaffolds. The betacyanin derivatives recognized were primarily derived from (iso)betanin with different extents of deglucosidation, decarboxylation and dehydrogenation. The higher level of aglycones in urine compared to other metabolites implied deglucosidation as a dominant degradation route of pigment. The glucuronic acid- and sulfate-conjugated pigments were not detected in urine and fecal samples therefore lacking evidence on Phase II metabolism. The detected catabolites in stool are likely to experience further fragmentation through the fission of aromatic and heterocyclic rings in the lower GI tract (Kawabata et al., 2019), which was unable to be traced in this study. Meanwhile the breakdown of betacyanins could be extrinsically attenuated by ingesting a higher dose of pigment or fiber-rich whole beetroot thanks to the physicochemical preservation exerted by the outermost compounds as well as the food

matrix (Tesoriere et al., 2008; Wiczkowski et al., 2018; Sawicki et al., 2020).

The study further depicted the overall positive relationship between excreted pigments which partly evidenced the proposed degradation mechanism. The positive tendency was gradually weakened from the relationship between urinary and urinary, to fecal and fecal, and to urinary and fecal betacyanins, suggesting the increasing variability and unpredictability of pigment metabolism in the corresponding excretion route. Given the strong associations among (iso)betanin-derivatives in urine and among catabolites in stool (Fig. 5), the results indicated a straight influence of betacyanin level and conversions in the intestine to their bioavailability and systemic metabolic profile, with the serial decarboxylation and deglucosidation as the dominant degradation mechanisms. Noteworthy, the association between (iso)betanin and (iso)betanidin was discovered in human stool only but absent in urine. Linking to the positive correlation between urinary and fecal (iso) betanidin, it was hypothesized that slower elimination of aglycones in renal excretion was dominantly contributed by their prolonged intestinal absorption instead of deglucosidation of (iso)betanin during systemic metabolism. Considering (iso)betanin as the source of other metabolites and catabolites, higher uptake of (iso)betanin was accompanied with decreased intestinal excretion of betacyanins. Concurrently an increment in luminal (iso)betanin level thus the bioaccessibility could potentially improve absorption efficiency of pigments, as conceded by previous research (Gerardi et al., 2020; Sawicki et al., 2020).

Significant intra- and inter-individual variations were observed from the betacyanin profiles in urine and stool from the perspectives of observation times, excretion level, and pigment composition. The inter-individual variation was assessed in some of the other bioavailability studies (Lampe and Chang, 2007; Wiczkowski et al., 2018; Nishioka et al., 2021), from which the response range of phytochemicals may be located for exploring the bioactivities in further research. Current work discovered a time inconsistency between the peak urinary excretion rate (2–4 h) and maximal metabolic perturbation (4–6 h) of betacyanins. The delay in maximal perturbation has possibly resulted from the ingestion of habitual meals by the subjects after 3 h of juice consumption that likely complicated the digesta matrix and induced enzyme and hormonal fluctuations. Subsequent regression in metabolic profiles (6–24 h) has followed a similar downward trend as in the urinary excretion of pigments.

According to individual excretion level, the study stratified the participants into low/(medium)/high groups representing their innate abilities to uptake and metabolize pigments. The absorption, metabolism and excretion of betacyanins can be modulated by a variety of influencers with crucial determinants such as the genetic variation and health status (intrinsic), habitual diet pattern and lifestyle of individuals (extrinsic). Evidence in Fig. 5B-C confirmed the significant impacts of participants' exercise and intake of meat and fish on the bioavailability and bioaccessibility of betacyanins. The betacyanin profile in stool exhibited more considerable variability than urinary excretion, implying the growing complexity and divergence of luminal environments between individuals from proximal to distal intestine, which prevailed over the variation of pigment metabolism in the systemic milieu. Gut microbiota is undoubtedly one of the most pivotal confounders to the significant heterogeneity of betacyanin profiles in stools.

With high chance of interacting with dietary nutrients, a symbiotic gut microbiota is extensively involved in the intestine-mediated metabolism, enterohepatic circulation and first-pass effect, hence the bioavailability of compounds in humans (Liang et al., 2015; Zhang et al., 2021). It is assumed that gut microbiota primarily influences the bioavailability of betacyanins by catalyzing their biotransformation, e.g., via deconjugation and reduction, thereby altering their physicochemical features and pharmacokinetics in the human body (Cussotto et al., 2021). Moreover, the gut microbiota was found to participate in gene regulation of epithelial transporters as well as the secretion of bile acids that could potentially influence the absorption and metabolic trajectory of pigments (Klaassen and Cui, 2015; Bielik and Kolisek, 2021; Zhang et al., 2021). Mutually, the impact of beetroot pigment to human gut microbiota has been increasingly explored by recent studies (Capper et al., 2020; Wang et al., 2022a). Previous results revealed a positive correlation between betacyanins in stool and the relative abundance of genera *Coprococcus* and *Bifidobacterium*, meanwhile the content of short chain fatty acids (SCFAs) in feces was markedly increased after red beetroot juice consumption. It is noted that *Bifidobacterium* and *Coprococcus* are well-known fermentative taxa contributing to the SCFAs production, and their function in the depletion of betacyanins is inferable. Most of the relationships among betacyanins were exhibited by 2,17-bidecarboxy-(iso)betanin and 2,15,17-tridecarboxy-betanin with their correlated taxa showing specific biological activities (Wang et al., 2022a). It is therefore essential to link the intestinal excreted betacyanins to different biological markers (e.g., mucosal microbiome) to further our comprehension towards the health benefits of this pigment group.

However, there are several limitations to the current work and its interpretation which need refinement in future research. For example, the recovery fraction of betacyanins from fecal excretion could be determined via a single-dose trial and/or *in vitro* fermentation experiments. Furthermore, the demonstration of systemic metabolomic signature is recommended by analyzing the excretion profiles with plasma pharmacokinetics of betacyanins (Li et al., 2021), which was infeasible in the present study due to covid restrictions. Given that this is pilot work exploring the betacyanins in fecal excretion and their correlation with bioavailability, future research should continue broadening the scope of metabolite characterization and enhancing the detectability of fragments, with further emphasis on the effects of dose and food matrix as well as fecal excretion profiles of betaxanthins.

5. Conclusion

As the abundant pigment in red beetroot, betacyanins are increasingly investigated for their bioactivities and bioavailability. However, their excretion via intestinal route has rarely been researched. The present study has exhibited and contrasted the betacyanin profiles between renal and fecal excretions of human volunteers following ingestion of beetroot juice. The results demonstrate a notably poor bioavailability and rapid renal elimination of betacyanins. The (iso) betanin-dominant urinary profile revealed a modest biotransformation of pigments during the acute intestinal uptake and systemic metabolism. Meanwhile the major proportion of betacyanins move along the GI tract, being subjected to severe fragmentation including deglucosidation and decarboxylation which was coherently supported by the pigment metabolite profiles in both pig digesta and human feces. As interpreted by the positive correlation between urinary and intestinal excreted betacyanins, bioavailability hence systemic bioactivity of pigments is primarily determined by their bioaccessibility and biotransformation in the gut. Moreover, with participation of diverse gut bacteria in the host, the intestinal transformation of betacyanins appeared to be more intricate and individual-specific compared with systemic metabolism. The present results should greatly advance our knowledge in the metabolomic fate of betacyanin pigments regarding the mechanisms underlying their absorption, metabolism and excretion, contributing to future research on their systemic efficacy and gut-related benefits.

Statement of consent

The human trial (AREA 20-058) was performed in compliance with the Human Tissue Act 2004, and the consent regarding sample usage and publication was acquired from each individual before the study commencing. The conductance of pig study (N0. 070510HM) was according to the Animal (Scientific Procedures) Act 1986.

CRediT authorship contribution statement

Yunqing Wang: Conceptualization, Methodology, Formal analysis, Investigation, Project administration, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Opeyemi O Adekolurejo:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Binying Wang:** Formal analysis. **Katie McDermott:** Writing – review & editing. **Thuy Do:** Writing – review & editing, Supervision. **Lisa J Marshall:** Methodology, Writing – review & editing, Supervision. **Christine Boesch:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137663.

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