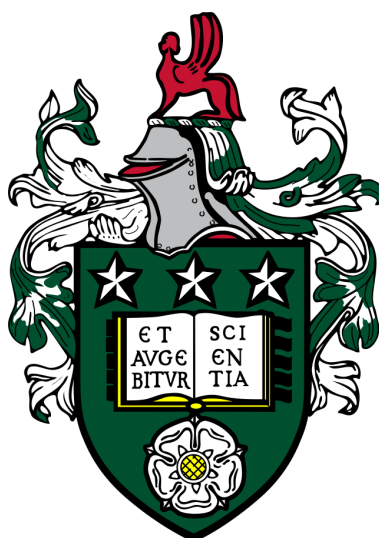




UNIVERSITY OF LEEDS

Intestinal uptake and metabolism of betalain pigments

Investigations in vitro and in vivo



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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. Details of the jointly-authored publications and contribution of each author are outlined on the next page. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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For all the chapters in this thesis, the contributions of the candidate include – conceptualisation, methodology, formal investigation and analysis, data curation, visualisation, writing – original draft, writing – review and editing, and funding acquisition.

For all the chapters in this thesis, the contributions of C. Bösch, T. Do, and L.J. Marshall include – project supervision, academic training, scientific discussion, review and edition of writing, and funding acquisition.

In Chapter III, G.S.N. Fernando and N. Sergeeva contributed with the separation and purification of betalains from plant extracts, and the products were used throughout the cell culture experiments; N. Vagkidis and V. Chechik conducted the EPR experiments. Y.Y. Huan contributed to part of the real-time qPCR measurements by conducting initial analysis regarding crude beetroot extracts.

Chapter IV includes analysis of samples from a recent pig trial conducted by O.O. Adekolurejo and K. McDermott. B. Wang contributed to the processing and chromatographic analysis of pig digesta samples under the guidance of the candidate.

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Abstract

The group of betalain pigments have demonstrated a number of biological properties such as the radical scavenging and anti-inflammation and have been associated with health-promoting effects towards chronic diseases such as T2 diabetes mellitus and cancer. Nonetheless, evidence from limited number of studies revealed their poor bioavailability, while the major proportion are expected to transit through the entire gastrointestinal tract with intensive metabolism. There is yet a dearth of research on both the intestinal uptake and metabolic fate of betalains, as well as their bioactivities at the site of human intestine. Accordingly, this project aims to investigate systematically the bioavailability and metabolic pathway of betalain pigments while exploring their modulatory capacity to the enteric immunity and gut microorganisms.

The assessment of intestinal absorption of betalains employs the differentiated Caco-2 cell model, which reveals a considerably low efficiency in intestinal uptake and migration of the compounds (trans-epithelial fraction of 1.0-2.2%). The linear, dose-dependent kinetics of trans-epithelial transport exhibited by tested betalains (420-1000 μM) suggests passive diffusion as their favoured route during absorption, whose rate and intensity are heavily influenced by physicochemical characters of molecules. The immunomodulatory effect of betalains (5-80 μM) to enterocytes is examined on the stimulated Caco-2 cells with gene measurement of inflammation-associated targets. Current results have proved the strong efficacies of prevalent betalains, especially betanin, in radical scavenging, anti-inflammation and cyto-protection, via their intrinsic reduction potential and regulation of cell signalling, i.e., NF- κ B and Nrf2.

Following the performance of an *in vivo* human study with beetroot juice ingestion, the urinary pharmacokinetics of betacyanins was established via chromatographic analysis, elucidating a poor bioavailability (urinary recovery of 0.13%) and modest systemic metabolism of the pigments (betanin-dominated urinary profile) which concurred with the *in vitro* results of trans-epithelial transport of betalain. As per the faecal metabolic profile, the

major amount of betacyanins is confined in GI tract, severely fragmented into decarboxylates and aglycones prior to the excretion via faeces. The intensity and pathway of pigment metabolism in the gut are crucial determinants of the systemic uptake and excretion of compounds. Furthermore, during the transit through intestinal tract, betalains with other beetroot components induce an alteration of specific taxa in human colonic microbiota following a 2-week consumption of red beetroot juice. Gut microbiome results reveal an acute promotion of *Akkermansia muciniphila* and suppression of *Bacteroides fragilis*, where the former is implicated in mitigation of cardiometabolic and inflammatory bowel diseases, and the latter potentially takes part in the inflammatory diarrhoea. Additionally, the correlation matrices have demonstrated positive associations between the beetroot-sourced betacyanins and metabolites, and the *Bifidobacterium* and SCFAs hence the prebiotic effects.

Findings of the current project could make a substantial contribution to our knowledge on the absorption and metabolism of betalain pigments. In line with prior research, this project gives the insight of several physiological effects of betalains in human gut, highlighting their significance as the promising candidate in mitigating the gut inflammation. Future research is envisaged from both *in vivo* and *in silico* perspectives to validate their efficacy and to analyse in-depth the underlying molecular interactions.

Table of Contents

List of Conference contributions.....	iii
Acknowledgements	iv
Abstract	v
Glossary of Abbreviations and Symbols	x
List of Figures	xii
List of Tables.....	xv
Chapter I – Introduction and Literature Review	1
1.1 Introduction	1
1.2 Sources and chemical structure of betalains.....	3
1.3 Biological effects of betalains	8
1.4 Bioaccessibility of betalains	11
1.4.1 Digestive stability.....	11
1.4.2 Influence of food matrix.....	14
1.5 Pharmacokinetic profile and variability	17
1.6 Trans-epithelial transport and biotransformation	23
1.6.1 Absorption and transport of betalain	23
1.6.2 Betalain transformation	25
1.7 Faecal excretion and gut microbiota.....	27
1.8 Aims and objectives of current thesis.....	29
Chapter II – Experimental Methods	30
2.1 Preliminary betalain processing and analysis.....	30
2.1.1 Reagents and materials.....	30
2.1.2 Betalain extraction from different plant sources	30
2.1.3 LC-MS analysis of plant-derived betalains	31
2.1.4 Spray drying of betalain extracts.....	32
2.1.5 Betalain purification with flash chromatography	32
2.2 Cell culture experiments.....	34
2.2.1 Reagents, materials, and samples	34
2.2.2 Cell culture and maintenance	34
2.2.3 Cytotoxicity assays.....	35
2.2.4 Preliminary tests on Caco-2 cell differentiation.....	36
2.2.4.1 Alkaline phosphatase (ALP) assay.....	36
2.2.4.2 Influence of Caco-2 cell differentiation on intracellular betanin uptake.....	37
2.2.5 Cellular uptake and transport of betalains	38
2.2.5.1 Initial trials with dextrin-coated betanin standard.....	38
2.2.5.2 Intracellular accumulation and trans-epithelial transport of purified betalains	39
2.2.5.3 LC-MS analysis of cell culture samples.....	40
2.2.6 Immunoregulation of betalains in Caco-2 cells.....	41
2.2.6.1 Inflammatory stimulation of Caco-2 cells.....	41
2.2.6.2 Gene isolation and reverse transcription	41

2.2.6.3	Gene measurement of pro-inflammatory and antioxidant markers via qPCR	42
2.2.7	Radical scavenging assays of betalains	44
2.2.7.1	Quantification of intracellular ROS formation.....	44
2.2.7.2	Electron paramagnetic resonance (EPR) and radical assays	45
2.2.8	Statistical analysis	45
2.3	Intervention trials <i>in vivo</i> and analysis	46
2.3.1	Reagents and materials.....	46
2.3.2	Human study and participant screening	46
2.3.3	Intervention design of human study	47
2.3.4	Intervention design of pig trial	49
2.3.5	Chemical analysis of betacyanins in trial samples	49
2.3.5.1	Betalain and polyphenol analysis in beetroot juice	49
2.3.5.2	Betacyanin analysis in human urine samples.....	50
2.3.5.3	Betacyanin analysis in human stool samples	51
2.3.5.4	Betacyanin analysis in feed and pig digesta.....	52
2.3.5.5	Statistical analysis	53
2.3.6	Human gut microbiome and SCFA analysis.....	53
2.3.6.1	DNA extraction from faecal samples	53
2.3.6.2	qPCR amplification and sequencing	54
2.3.6.3	Real-time qPCR of target bacterial species	55
2.3.6.4	SCFAs measurement	56
2.3.6.5	Statistical analysis	56
Chapter III – Intestinal Uptake and Immunomodulation of Betalains towards Caco-2 cell model		58
3.1	Abstract	58
3.2	Introduction	59
3.3	Aims	61
3.4	Method.....	62
3.5	Results	63
3.5.1	Intracellular accumulation and trans-epithelial transport of dextrin-coated betanin	63
3.5.2	Intracellular accumulation and trans-epithelial transport of purified betalains	65
3.5.3	Modulation of NF-κB target cytokines and enzymes by purified betalains	67
3.5.4	Effects of purified betalains on the expression of Nrf2 target enzymes.....	69
3.5.5	Gene modulation by red and yellow beetroot extracts	71
3.5.6	Effects on intracellular oxidative stress and radical scavenging activities.....	72
3.6	Discussion.....	74
3.6.1	Betalain availability to the Caco-2 cell model.....	74
3.6.2	Immunomodulatory effect of betalains.....	77
3.6.3	Radical scavenging activity of betalains	80
3.6.4	Availability of betalains, the bottleneck to immunomodulation.....	81

Chapter IV – Bioavailability and Excretion profile of Betacyanins – Variability and Correlations between different excretion routes	83
4.1 Abstract	83
4.2 Introduction	84
4.3 Aims	85
4.4 Method.....	87
4.5 Results	88
4.5.1 Human study compliance and profile of red beetroot juice.....	88
4.5.2 Urinary excretion profile and kinetics of betacyanins.....	88
4.5.3 Inter-individual variation and stratification based on urinary excreted betacyanins	94
4.5.4 Characterisation of betacyanin catabolites in human faeces	96
4.5.5 Inter-individual variation of faecal excreted betacyanins and stratification.....	97
4.5.6 Betacyanin composition and variance in pig digesta.....	100
4.5.7 Correlation between urinary and intestinal excreted betacyanins of human and lifestyle factors.....	102
4.6 Discussion.....	105
Chapter V – Effect of Two-week Red beetroot juice consumption on Modulation of Gut Microbiota in healthy Human volunteers.....	113
5.1 Abstract	113
5.2 Introduction	114
5.3 Aims	117
5.4 Method.....	117
5.5 Results	118
5.5.1 Subject characteristics and dietary intake.....	118
5.5.2 Presence of betacyanins and their metabolites in juice and in faecal samples	118
5.5.3 Taxonomic diversity and distribution of microbial communities.....	120
5.5.4 Comparison of taxonomic composition before and after beetroot juice consumption	123
5.5.5 Relative abundance of target microbial species	128
5.5.6 SCFA content.....	129
5.5.7 Association between betacyanin catabolites, SCFAs, and microbial profile.....	130
5.6 Discussion.....	133
Chapter VI – General Discussion and Future Perspectives.....	140
6.1 Betalains and their promising evidence from <i>in vitro</i> and <i>in vivo</i> research.....	140
6.2 Absorption, metabolism, and excretion of betalains	142
6.3 Modulatory functions of betalains in the intestine	145
6.4 Future perspectives and social implications	149
Conclusion.....	152
Bibliography.....	153
Appendix	174

Glossary of Abbreviations and Symbols

ADI	Acceptable daily intake	DMEM	Dulbecco's modified Eagle medium
ALP	Alkaline phosphatase	DMPO	5,5-Dimethyl-1-pyrroline-N-oxide
ANOVA	Analysis of variance	DMSO	Dimethyl sulfoxide
AP	Apical	DOPA	3,4-Dihydroxyphenylalanine
ARE	Antioxidant responsive element	DPBS	Dulbecco's phosphate-buffered saline
ATCC	American Type Culture Collection	DPPH	2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl
AUC	Area under the curve	E_A	Activation energy
AUCR	Area under excretion rate curve	EC₅₀	Half maximal effective concentration
a_w	Water activity	ECACC	European Collection of Authenticated Cell Culture
BCA	Bicinchoninic acid	EDTA	Ethylenediaminetetraacetic acid
BET	Betanin	EPR	Electron paramagnetic resonance
BL	Basolateral	ESI	Electrospray ionisation source
BMI	Body mass index	FBS	Fetal bovine serum
BS	Betanin standard (dextrin-coated)	FDR	False discovery rate
BSL	Baseline	fe %	Urinary recovery fraction
bw	Body weight	FFQ	Food frequency questionnaire
CBG	Cytosolic β -glucosidase	GCLC	Glutamate-cysteine ligase catalytic subunit
COX	Cyclooxygenase	GCLM	Glutamate-cysteine ligase modifier subunit
CRC	Colitis-colorectal carcinoma	GPX	Glutathione peroxidase
C_{max}	Peak plasma concentration	GSH	Glutathione
C_T	Threshold cycle number	GST	Glutathione S-transferase
CV	Column volume	HBA	Hydrogen bond acceptor
CXCL	CXC chemokine ligand	HBD	Hydrogen bond donor
DAD	Diode-array detector	HBSS	Hanks' balanced salt solution
DBP	Diastolic blood pressure	HO	Heme oxygenase
DCF	2',7'-Dichlorofluorescein	(U)HPLC	(Ultra-)High performance liquid chromatography
DEPC	Diethyl pyrocarbonate	IBD	Inflammatory bowel disease
DCFH₂-DA	2',7'-Dichlorodihydrofluorescein diacetate	IFN-γ	Interferon- γ

Glossary of Abbreviations and Symbols

IL	Interleukin	PLS-DA	Projection to latent structures-discriminant analysis
IND	Indicaxanthin	p-NPP	p-Nitrophenyl phosphate disodium hexahydrate
iNOS	Inducible nitric oxide synthase	p-NP	p-Nitrophenol
Keap	Kelch-like ECH-associated protein	PTFE	Polytetrafluoroethylene
k_{re}	Renal elimination rate constant	RBR	Red beetroot extract
Log P	Octanol/water partition coefficient	RFI	Relative fluorescence intensity
LPH	Lactase phlorizin hydrolase	RNS	Reactive nitrogen species
LPS	Lipopolysaccharide	ROS	Reactive oxygen species
MANOVA	Multivariate analysis of variance	RT-qPCR	Real-time quantitative polymerase chain reaction
MCT	Monocarboxylate transporter	SBP	Systolic blood pressure
MES	2-(N-morpholino)ethanesulfonic acid	SC	Cytokine-stimulated control
MS	Mass spectrometry	SCFA	Short chain fatty acid
MTT	Thiazolyl blue tetrazolium bromide	SEM	Standard error of mean
NAD(P)H	Nicotinamide adenine dinucleotide phosphate	SGLT	Sodium-dependent glucose transporter
NEAA	Non-essential amino acid	SPD	Spectrophotometric detector
NF-κB	Transcription factor nuclear factor-κB	SPE	Solid-phase extraction
NMDS	Non-metric multi-dimensional scaling analysis	SRB	Sulforhodamine B
NMR	Nuclear magnetic resonance	STD	Standard deviation
NOX	NADPH oxidase	TBE	Tris-borate-EDTA
NQO	NAD(P)H dehydrogenase quinone	TEER	Trans-epithelial electric resistance
Nrf2	Nuclear factor-erythroid-2-related factor 2	t_{max}	Time of reaching peak concentration in plasma/urine
OATP	Organic anion-transporting polypeptide	TNF-α	Tumour necrosis factor-α
OUT	Operational taxonomic unit	tPSA	Topological polar surface area
P_{app}	Apparent permeability coefficients	UGT	UDP-glucuronosyl transferase
PCoA	Principal coordinates analysis	VUL	Vulgaxanthin I
PET	Polyethylene terephthalate	YBR	Yellow beetroot extract

List of Figures

Figure 1-1. Brief biosynthetic scheme of betalamic acid and subsequent products of betalains.	6
Figure 1-2. Degradation profiles of betanin (●) and indicaxanthin (■) along the simulative gastrointestinal digestion <i>in vitro</i> .	13
Figure 2-1. Schematic illustration of the <i>in vitro</i> transwell model for investigating intestinal betalain uptake.	39
Figure 2-2. Schematic illustration of the design of human intervention study.	48
Figure 3-1. Chemical structures of betanin (BET), vulgaxanthin I (VUL) and indicaxanthin (IND).	60
Figure 3-2. Spectrophotometric chromatograph of purified betalain compounds at the detection wavelengths of 536 nm and 486 nm.	62
Figure 3-3. Cellular accumulation and trans-epithelial transport of dextrin-coated betanin standard (BS) across fully differentiated Caco-2 cell membrane	63
Figure 3-4. Detection of Caco-2 cell viability (%) using MTT assay after treatment with increasing concentrations of purified betalains.	65
Figure 3-5. Uptake and trans-epithelial transport of purified BET, VUL and IND across Caco-2 intestinal membrane over 2 h incubation period.	66
Figure 3-6. Effects of BET, VUL and IND (5, 20 and 80 μ M) on mRNA levels of (A) IL-6, (B) IL-8, (C) COX-2, (D) iNOS and (E) NOX-1 following 6 h cytokine stimulation in Caco-2 cells.	68
Figure 3-7. Effects of BET, VUL and IND (5, 20 and 80 μ M) on mRNA levels of (A) NQO-1, (B) HO-1, (C) GCLC, (D) GPX-1, (E) GSTA-1 and (F) GSTP-1 following 6 h cytokine stimulation in Caco-2 cells.	70
Figure 3-8. Effects of red beetroot (RBR) and yellow beetroot (YBR) extracts on the mRNA levels of (A) COX-2 and (B) GCLC in stimulated Caco-2 cell line.	71
Figure 3-9. Radical scavenging capacity of betalains reflected by (A) intracellular ROS	

generation in Caco-2 cells, (B) double integral intensity of EPR signals of DMPO-superoxide adduct, (C) DPPH signal intensity in the presence of betalains, as well as (D) EPR spectra of superoxide and DPPH assays (post 30 min) in the absence (control) and in the presence of 20 μ M purified betalains..... 72

Figure 4-1. MS chromatograms of betacyanins acquired from urine (A) and stool samples (B) in human trial..... 89

Figure 4-2. Urinary excretion profiles of native betacyanins and their metabolites against time intervals following acute beetroot juice consumption 93

Figure 4-3. Variability in urinary excretion profiles of betacyanins and subject stratification according to betacyanin excretion level..... 96

Figure 4-4. Variability of betacyanin excretion profiles in stool and stratification according to betacyanin excretion level 99

Figure 4-5. Compositional profile and variation of betacyanins in ileum and colon digesta of pigs 101

Figure 4-6. Correlation matrices between (A) urinary and faecal excretion of betacyanins, (B) among urinary excreted betacyanins and dietary/anthropometric/lifestyle factors, and (C) among faecal betacyanins and dietary/anthropometric/lifestyle factors..... 104

Figure 4-7. Proposed mechanistic pathway of betacyanin transformation and catabolite formation. 109

Figure 5-1. Profiles of red beetroot juice and the faecal excreted betacyanins after juice ingestion119

Figure 5-2. Alpha diversity indices of human gut microbiome on different days of beetroot juice consumption illustrated with (A) Shannon, (B) Chao, (C) Simpson and (D) phylogenic diversity whole tree indices. 121

Figure 5-3. Beta diversity of gut microbiome on different days of beetroot juice consumption depicted with (A) the PCoA, (B) NMDS, and (C) subject-based matrix of unweighted Unifrac distance (n=18). 122

Figure 5-4. The taxonomic composition and abundance alterations in the BSL, D3 and D14 groups	126
Figure 5-5. Inter-group comparisons expressed in the Log2-fold change of taxa with significantly different abundance between (A) BSL and D3, (B) BSL and D14, and (C) D3 and D14.	127
Figure 5-6. Quantitative changes of the relative species abundance (in Log 2 scale) of (A) <i>Akkermansia muciniphila</i> , (B) <i>Bifidobacterium_uc</i> , (C) <i>Bacteroides fragilis</i> , (D) <i>Lactobacillus acidophilus</i> , and (E) <i>Escherichia coli</i> after day 3 and 14 of juice consumption.	128
Figure 5-7. Spearman's rank correlation matrix demonstrating the relationship of faecal excreted betacyanins with (A) gut microbiota abundance at genus level, and with (B) relative abundance of target species plus SCFA contents.	132
Figure 6-1. Proposed absorption, metabolism, and excretion of betalain pigments following oral administration of betalain-rich matrix.	143
Figure 6-2. Proposed interplay of betalain compounds with molecular signalling pathways in the enterocytes, and with relevant gut bacteria in the intestinal lumen based on the evidence from current study and reference.....	146
Figure 6-3. Simplified chemical structure of beetroot pectin with plausible transformations (e.g., methylation and acetylation), properties of which could greatly impact the alteration trajectory of gut microbiota.....	151

List of Tables

Table 1-1. Mostly studied betalain-containing food sources and their character compounds.	3
Table 1-2. Functional groups of commonly investigated betalains.....	7
Table 1-3. Summary of potential health beneficial activities of betalain compounds and betalain-rich food.....	10
Table 1-4. General influential conditions to the betalain stability	12
Table 1-5. Summarised data of plasma pharmacokinetics and urinary excretion of (iso)betanin and indicaxanthin in human body from the bioavailability studies.	18
Table 1-6. Summarised data of plasma pharmacokinetics and urinary excretion of (iso)betanin and indicaxanthin in rats from the bioavailability studies.	19
Table 2-1. Oligonucleotides of human primers used for qPCR in this study.....	43
Table 2-2. Microbial primer sequences used for qPCR in the present study.	55
Table 3-1. Physicochemical properties of selected betalains.....	74
Table 4-1. Urinary and faecal excretion profiles of betacyanin derivatives in human trial.	92
Table 5-1. SCFA concentrations ($\mu\text{mol/g}$ stool), and the ratio between specific acids in stools at BSL, D3 and D14 following beetroot juice consumption.....	129

Chapter I

Introduction and Literature Review

1.1 Introduction

Betalains, a group of phytochemicals widely recognised as natural food colorant (E162), are abundantly present in beetroot, cactus pear, pitaya, and amaranth. By condensation with alternative moieties, betalains are categorised into red-violet betacyanins and orange-yellow betaxanthins. There is emerging interest in the biological activities of common betalains, such as betanin and indicaxanthin, which would confer significant health benefits to the population. Recent studies have demonstrated the strong efficacy of these compounds in radical scavenging and anti-inflammation with implications for amelioration and prevention of chronic diseases such as diabetes mellitus, hypertension, cancer, and cardiometabolic disorders (Gengatharan et al., 2015; Montiel-Sánchez et al., 2021). *In vitro* studies have demonstrated comparable antioxidant capacity of betalains to ascorbic acid, α -tocopherol, β -carotene and phenolics such as catechin (Cai et al., 2003; Stintzing et al., 2005; Gandía-Herrero et al., 2009; Zhang et al., 2014). Tesoriere et al. (2004a) showed that the onset of LDL oxidation was markedly delayed at the peak plasma concentration of betalains compared with the negative control, suggesting the strong inhibition of lipid peroxidation in the presence of betalains. However, due to the existence of fewer family members and lower prevalence among species, betalains still appear to be less well-researched in comparison to polyphenols and carotenoids.

Despite their desired health benefits, prior literature has documented a notoriously low bioaccessibility and bioavailability of betalain pigments which is regarded as a massive hinderance to their overall health effects. It could be attributed to their poor digestive stability, retention of food matrix, inefficient trans-epithelial permeability, and other metabolic factors. Currently limited research on betalain bioavailability *in vivo* has mainly focused on plasma and urinary pharmacokinetics (Tesoriere et al., 2004a; Wiczowski et al., 2018); there is yet very scarce and inconclusive information on the intestinal absorption and metabolic pathway

of the compounds. Apart from systemic metabolism, betalain pigments remained in the lumen of GI tract are anticipated to endure the progressive metabolisms until excretion, which again has rarely been studied. An extricable challenge is the huge inter-individual variation in metabolic profile of betalains as revealed by Wiczowski et al. (2018) and Sawicki et al. (2018), suggesting a particularly difficult interpretation on the effects of dose and betalain type to the metabolic feature.

Simultaneously, betalains and derivatives that remain in the lumen are believed to pose reciprocal influences on gut function through potential actions on the intestinal tissues and gut bacteria. The gut microbial community is of high diversity and complexity comprising trillions of microorganisms which are intricately associated with the metabolism, immunity and physiology of the host. In recent years, scientific research has devoted increasing emphasis to the mutual interactions between gut bacteria and polyphenol compounds *in vivo*, revealing their positive relations with endogenous probiotics and beneficial by-products (Tzounis et al., 2011; Jakobsdottir et al., 2013). This field has not yet been investigated in the realm of betalains despite that the knowledge is indeed crucial for a profounder understanding of the metabolic fate and holistic health outcomes of this group of pigments.

Given the identified gaps of research, the overall aim of this PhD project is to explore the metabolic fate of dietary betalains from the perspectives of absorption, transformation, and excretion of the molecules, while also investigating the potential biological functions to the human intestine exerted by betalains and betalain-rich matrix.

Preluding by the general physicochemical features and well-recognised biological functions of betalain pigments, the present project commenced with a literature review that primarily summarised the currently available evidence on their availability and metabolism with reference to all the *in vivo* and *in vitro* studies to date examining the bioaccessibility, absorption, biotransformation, and excretion of two common betalain compounds, betanin and indicaxanthin. Potential factors that may impact betalain availability, such as pigment type, dosage, food matrix, and individual heterogeneity are being discussed.

1.2 Sources and chemical structure of betalains

The major edible sources of betalains are restricted in the order of plants *Caryophyllales*, including beetroot (*Beta vulgaris* L.), cactus fruits (*Opuntia* and *Hylocereus*), and leafy amaranth (*Amaranthus*). There are approximately 75 compounds being identified in betalain family. Their composition and concentration vary considerably across different plant species and varieties as tabulated in Table 1-1 (Pavoković and Krsnik-Rasol, 2011; Albano et al., 2015). For example, (iso)betanin, the predominant betalains found in red beetroot, are absent in yellow beetroot which instead contains several vulgaxanthins as the main pigments. The variation in pigment content is further observed at different sections within the same plant, such as the distribution of the highest betalain concentration in the outermost layer of red beetroot pulp (Sawicki et al., 2016). It is also noted that betalain and anthocyanin families possess the analogical chemical properties and functions, which are, however, considered mutually exclusive in the recognised plants (Khan and Giridhar, 2015; Khan, 2016a).

Table 1-1. Mostly studied betalain-containing food sources and their character compounds.

Main Food Source	Distribution	Dominant Betalains	Reference
Beetroot (<i>Beta vulgaris</i> L.)	North and Central America, Europe	(iso)Betanin, Neobetanin, (iso)Betanidin, Vulgaxanthin I/II/IV, Miraxanthin	Gengatharan et al. (2015)
Swiss Chard (<i>Beta vulgaris</i> L. ssp. <i>cicla</i> L.)	North and Central America, Europe	(iso)Betanin, (iso)Betanidin, Vulgaxanthin III & IV,	Khan and Giridhar (2015)
Prickly pear (<i>Opuntia ficus-indica</i> L.)	South and Central America, Mediterranean	Indicaxanthin, Portulacaxanthin I, (iso)Betanin, Vulgaxanthin I/II/III/IV, Methionine-Betaxanthin	Tesoriere et al. (2008); Khan and Giridhar (2015)
Pitaya (<i>Hylocereus polyrhizus</i>)	South Asia	(iso)Betanin, (iso)Betanidin, (iso)Phylloactin, Hylocerenin	Khan and Giridhar (2015)
Amaranth (<i>Amaranthus tricolor</i> L.)	Tropical and sub-tropical zones	(iso)Amaranthine, Methylated arginine-Betaxanthin (iso)Gomphrenin	Cai et al. (2003); Khan and Giridhar (2015)

The principal structure of betalains is a nitrogenous heterocyclic namely betalamic acid. As illustrated in Fig. 1-1, molecular biosynthesis in plant is initiated from enormous accumulation of tyrosine which generates *L*-DOPA, a critical precursor of the entire pathway, after hydroxylation process. Betalamic acid and *L*-DOPA, constituting different moieties of betacyanin molecules, are synthesised by the bifurcate pathways involving oxidation, aromatics cleavage and non-enzymatic cyclisation. Condensation of the carbonyl of betalamic acid with amine groups leads to two major categories of betalains, (I) with cyclo-DOPA plus glucosyl derivatives forming betacyanins (e.g., betanin, phyllocactin), or (II) with amines/amino acids derivatives to generate betaxanthins (e.g., indicaxanthin and vulgaxanthin). They share the chemical scaffold of 1,7-diazaheptamethin with immonium conjugates in the structures. Betacyanins possess the extended π -conjugation system compared with betaxanthins due to the indole group resonance therefore absorb longer wavelength (531-550 nm) and appear in its complementary colour as purple-red, whereas betaxanthins is observed as yellow compounds (458-486 nm). Not only chemical properties, divergence of their biological activities is also resulted from the alteration of glucosyl and/or amino acids groups (Salisbury and Ross, 1991; Khan and Giridhar, 2015). Transition states ($\ddagger$) and electron transfer of the pathway are proposed according to electrophilicity/nucleophilicity of the functional groups. There is also existence of other minor pathways in the plants, such as reverse reactions and conjugations, which are not displayed in Fig. 1-1.

Characterisation of pigment compounds is primarily achieved by mass spectrometry based upon corresponding physicochemical properties conferred by various substituents. The functional groups of some common betalains have been illustrated in Table 1-2. The composition of red beetroot is most profoundly investigated among all the betalainic foods, which contains (iso)betanin and vulgaxanthin I as the predominant compounds (Nemzer et al., 2011; Khan and Giridhar, 2015). Indicaxanthin, as another betalain highlighted in the bioactivity-related studies, is abundantly sourced from prickly pears. The betalain profiles in various plants were explicitly analysed during the preliminary extraction experiment in this project and are presented in Appendix 1&2, comprising of red and yellow beetroots, red and

yellow prickly pears, red pitaya and red amaranth. Nevertheless, pigment characterisation could be complicated by the comparable molecular features (e.g., maximal absorbance ($\lambda_{\max}$) and molecular weight) of the configurational and conformational isomers in a plant species, e.g., 2- and 17-decarboxy-(iso)betanin in red beetroot.

For further identification and purification of the pigments, mass spectrometry is often utilised in conjunction with other techniques such as nuclear magnetic resonance (NMR). Detection of ^1H -NMR provides the precise information of chemical structure and proton interaction with environment. The chemical shift (δ_H) corresponding to 1,7-diazaheptamethin fragment generally appears at *ca.* 2.8, 3.2-3.6, 5.0, 5.8, 6.2, 7.0, and 8.2 ppm (D_2O , 500 MHz, 25 °C). Both δ (ppm) and coupling constant (J) will deviate according to the attachment of different substituents to the core. For instance, the δ_C in ^{13}C -NMR corresponding to the link between glucose and indole of betanin will be moved downfield (i.e., deshielded) when substituting glucose with malonyl moiety (i.e., phyllocactin) (Stintzing et al., 2004; Stintzing et al., 2006; Khan and Giridhar, 2015).

The advancement of betalain identification and purification techniques in scientific research has enabled a more profound investigation on the health significance of this pigment group, especially betanin and indicaxanthin as frequently researched compounds.

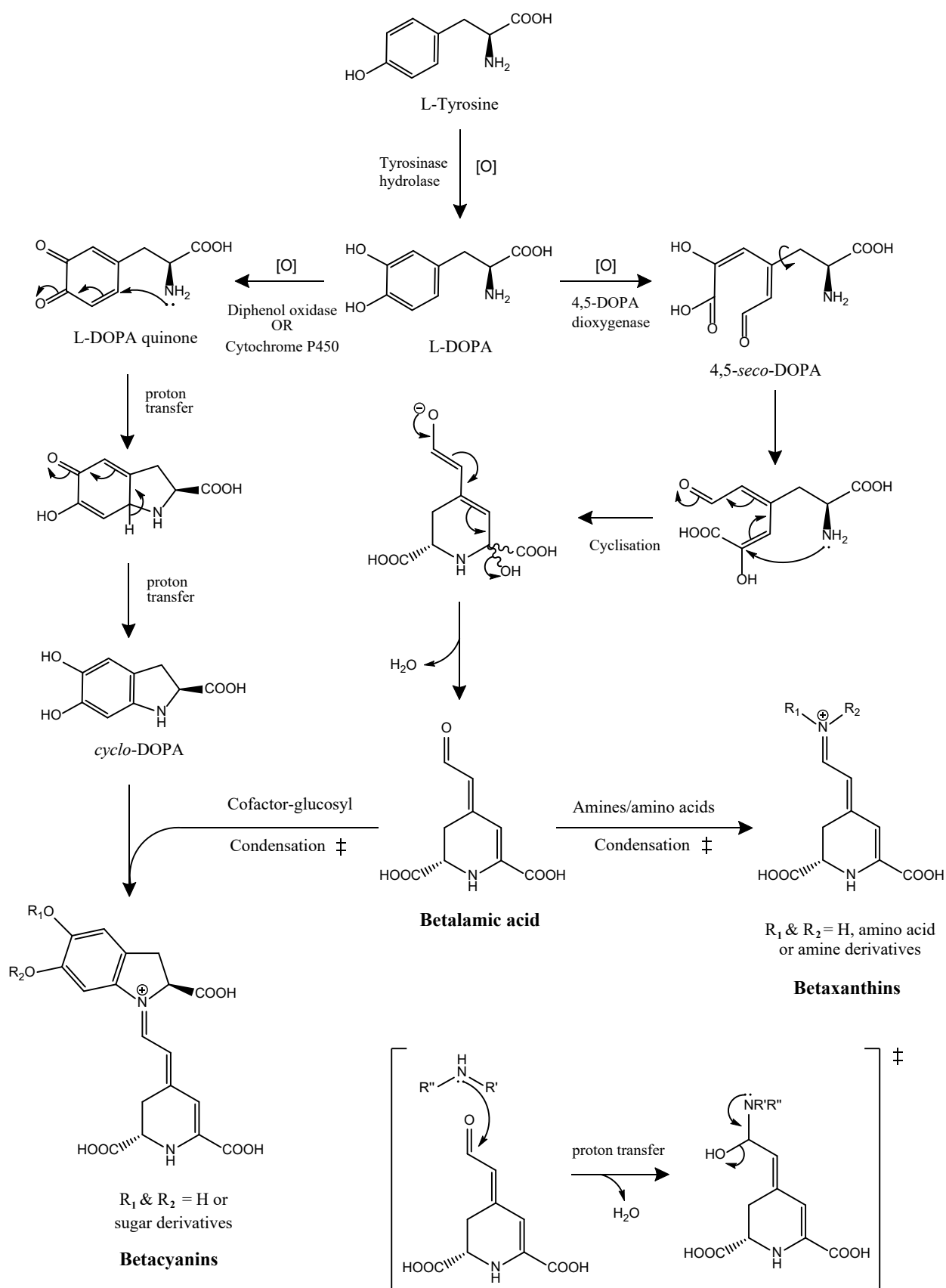
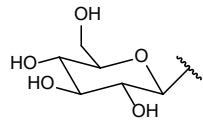
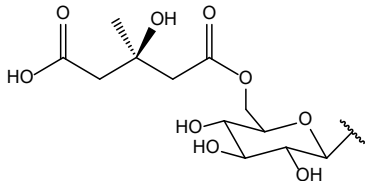
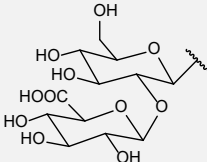
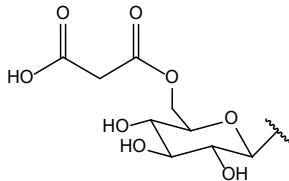
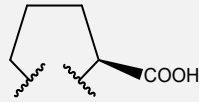
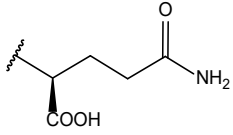
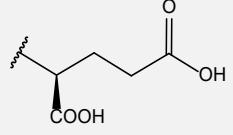
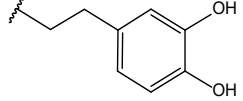


Figure 1-1. Brief biosynthetic scheme of betalamic acid and subsequent products of betalains, adapted from Khan and Giridhar (2015) and Carrillo-López and Yahia (2017).

Table 1-2. Functional groups of commonly investigated betalains. Note the R₁ and R₂ correspond to the substituents of core structure in Fig. 1-1 (Khan and Giridhar, 2015).

Category	Betalain	<i>m/z</i> [MH] ⁺	R ₁	R ₂
Betacyanins	Betanin	551		H
	Betanidin	389	H	H
	Hylocerenin	695		H
	Amaranthin	727		H
	Phyllocactin	637		H
Betaxanthins	Indicaxanthin	309		
	Vulgaxanthin I	340		H
	Vulgaxanthin II	341		H
	Miraxanthin V	347		H
	Ser-/Val-/Phen-/Ile-/Ala-/Thr-/Met-/(methylated) Arg-/aminobutyric acid-betaxanthin	280 – 400	Ser-/Val-/Phen-/Ile-/Ala-/Thr-/Met-/(methylated) Arg amino acids /aminobutyric acid	H

1.3 Biological effects of betalains

Radical scavenging property and antioxidant activity of betalains have been frequently outlined by recent studies. Betacyanins (EC_{50} of 5-9 μ M) mainly exist in their glucosylated form in the plant; the phenol (-OH) and sugar moiety of betacyanins, as well as the π resonance with imino residue in betaxanthins are regarded as efficient electron donors that enable the accommodation of positive charges and generate pre-eminent antioxidant capacity compared with Trolox and ascorbic acid (EC_{50} of 14 μ M) (Cai et al., 2003). The radical scavenging activity of betalains *in vitro* greatly resembles those of anthocyanins and resveratrol which is possibly elucidated by the structure-activity relationship. The compound potency is heavily determined by the number of reducing functional groups, configuration (i.e., steric effect) as well as their stereochemistry. For example, higher capacity is expected from betalains with less stacked glucosides and some degrees of acylation. Other determinants to antioxidant capacity include plant species and post-harvesting treatment. Comparing the data from multiple references, antioxidant activity of beetroot pulp has exceeded that of several whole vegetables but not as high as berry fruits (van den Berg et al., 1999; Pellegrini et al., 2003; Esatbeyoglu et al., 2015). Tesoriere et al. (2004a) and Tesoriere et al. (2008) demonstrated that raw red beetroot offered stronger antioxidant activity than cactus fruits under *in vitro* digestion condition. In term of food treatments, the highest potency was detected from raw beetroot, then beetroot juice, and finally that of steamed beetroot which reflects the heat-sensitive nature of betalain groups. But as recognised by several studies, results of *in vitro* antioxidant assays need to be consolidated by further evidence from *in vivo* cases.

Correspondingly, these characters enable the contribution of betalains to cells and DNA protection (Cai et al., 2003; Gengatharan et al., 2015). By applying betalains to animal model and human trials, other potential functions have been unveiled including amelioration of lipidemic peroxidation, anti-inflammatory, anti-hyperlipidaemia, anti-tumour, hepato-/neuro-/cardio-protection, and diabetes mitigation (Kanner et al., 2001; Gengatharan et al., 2015;

Khan, 2016a). According to immunomodulation-related studies, betalains can act as an interferon to translation and modification of targeted proteins by regulating gene expression of cell signalling mediators, especially those in the transcriptional factor nuclear factor- κ B (NF- κ B) and nuclear factor-erythroid-2-related factor 2 (Nrf2) pathways (Moreno-Ley et al., 2021). Supplement of 15 μ M betanin was able to activate the transcription factor of cell detoxifying signalling (e.g., Nrf2 gene), hence increase the expression of downstream heme oxygenase 1 protein (HO-1) and cellular glutathione (GSH). Induction of this endogenous antioxidant defence system of cells would contribute to the chemopreventive effect in a dose dependent manner (Krajka-Kuzniak et al., 2013; Esatbeyoglu et al., 2014). Previous studies also addressed the anti-inflammatory activity of pigments, reporting their inhibitory effect towards nuclear translocation of the inflammatory mediator (i.e., factor p65) and downstream enzymes (e.g., NO synthase) in tumour cell lines (Reddy et al., 2005; Tesoriere et al., 2014). In addition, the chemopreventive character of betalains was gradually discovered through the investigations on several carcinoma cells such as lymphoma, ovarian and breast cancer cells (Gandia-Herrero et al., 2016). The scientifically recognised health benefits of betalain compounds and betalain-rich food are listed in Table 1-3 with related studies provided. Although some of them are still under repeated and *in vivo* assessments, betalainic food is becoming a popular direction of research in perspective of public health and nutrition.

By contrast, the gap of knowledge is conspicuous particularly with regards to betalain bioavailability and metabolism, which are considered as the prerequisites of the systemic health effects of pigments. Despite potent antioxidant and other *in vitro* biological activities, the overall bio-effectiveness of betalains is likely to be significantly compromised by their poor uptake attributed to the digestive degradation, effects of food matrix, and intrinsic factors of epithelial absorption and metabolism reflected by the large inter-individual variation in the metabolic and excretion profiles of animal and human bioavailability studies of betalains, such as beeturia.

Table 1-3. Summary of potential health beneficial activities of betalain compounds and betalain-rich food, provided with typical studies in the related field.

Beneficial bioactivity	Betalain / Plant source	Study model / Duration	Outcomes	Reference
Scavenging of free radicals	Betanin standard (2-500 μ M)	Isolated neutrophils of healthy human	Inhibited intracellular ROS level	Zielińska-Przyjemska et al. (2012)
	Prickly pear pulp (250 g/day)	Healthy human (2 weeks)	Higher GSH/GSSG ratio; Halved LDL hyperoxides	Tesoriere et al. (2004b)
Antioxidant enzyme regulation	Betanin standard (2-20 μ M)	Liver cell lines (HepG2 and THLE-2)	Increased expressions of Nrf2 and downstream enzymes e.g., NQO-1	Krajka-Kuzniak et al. (2013)
	Betanin standard (1-25 μ M)	Liver cell line (Huh7)	Increased mRNA of Nrf2 and downstream enzymes e.g., HO-1	Esatbeyoglu et al. (2014)
Anti-inflammatory effect	Betanin standard (25 and 100 mg/kg bw *)	Rats with induced kidney inflammation (3 days)	Suppressed activation of NF- κ B and downstream enzymes e.g., iNOS	Tan et al. (2015)
	Indicaxanthin purified from prickly pear (5-25 μ M)	Caco-2 cell line	Suppressed NF κ B pathway mediators and downstream enzymes e.g., NOX-1	Tesoriere et al. (2014)
Hypo-lipidemic effect	Red beetroot juice (400 mL/day)	Healthy human (14 days)	Increased HDL-C/LDL-C ratio	Archana et al. (2015)
	Feed supplemented with red beetroot crisps (0.3, 1, and 3% w/w)	Rats with induced dyslipidemia (4 weeks)	Inhibited cholesterol and triacylglycerols levels in serum and hepatic cholesterol	Wróblewska et al. (2011)
Anti-diabetic effect	Betanin (10, 20 and 40 mg/kg bw/day)	Rats with induced diabetes (30 days)	Positive modulation of glycaemia and insulin; Induced activity of glycolytic enzymes	Dhananjayan et al. (2017)
Anti-tumour effect	Red beetroot extract (25 and 100 μ g/mL/day)	Rats with lung tumour (20 and 18 weeks)	Decreased multiplicity of lung tumour and induced apoptosis of lung cancer cells	Zhang et al. (2013)
Cardio-protection	Red beetroot juice (150 and 300 mg/kg bw/day)	Rats with myocardial ischemia (28 days)	Inhibited cardiac inflammation; Alleviated myonecrosis and improved structural maintenance	Raish et al. (2019)
Hepato-protection	Betanin purified from red pitaya (9.6 mg/day)	Rats (24 weeks)	Mitigated liver inflammation and hepatosteatosis	Lugo-Radillo et al. (2020)
Neuro-protection	Indicaxanthin purified from prickly pear (2 μ mol/kg bw)	Hippocampus of rats (Single dose)	Modulation of glutamate-induced excitation and bioelectric activity in neuron	Allegra et al. (2014)
Gut microbiota regulation	Betalains from red pitaya (200 mg/kg bw/day)	Mice (14 weeks)	Increased <i>g_Akkermansia</i> ; Decreased ratio of <i>Firmicutes/Bacteroidetes</i>	Song et al. (2016)

*bw – body weight.

1.4 Bioaccessibility of betalains

1.4.1 Digestive stability

The poor stability of betalain compounds is regarded as a major bottleneck that compromises their application in food and pharmaceutical industries and research fields. Briefly concluded from Elbandy and Abdelfadeil (2008) and Khan (2016b), betalain possesses optimal stability in the environment of pH 4-7, low temperature, darkness, and absence of oxygen (Table 1-4). The Schiff base hydrolysis of betalain into betalamic acid is promoted beyond pH 4-7 especially at alkaline environment with high water activity (a_w) (Cohen and Saguy, 1983). The shortened π -conjugation during the reaction gives larger energy gap (i.e., shorter wavelength) of radiation absorption, therefore the intensity of its visible spectrum declines. Betalain undergoes thermal kinetic degradation whose transition state involves nucleophilic attack on the immonium nitrogen. The reaction kinetics was found partially reversible with forward activation energy (E_A) of $7.1-8.8 \times 10^4 \text{ kJ mol}^{-1}$ versus the reverse E_A of $0.3-1.5 \times 10^4 \text{ kJ mol}^{-1}$ which indicates the rapidity of regeneration process regardless of thermodynamics (Saguy et al., 1978; Huang and Elbe, 1985). Light-induced degradation of betalain displays the first-order kinetics whose magnitude is proportional to light intensity. The electron excitation from π to π^* orbital during the exposure of λ -specific photons generates by-products including cyclo-DOPA-5-glycoside and betalamic acid. Because of the antioxidant property of betalains, it is well comprehensible that the presence of oxidising agent (e.g., oxygen) can accelerate the dissipation of components, resulting in non-enzymatic browning. Reaction is more likely to involve the change in redox potential rather than occurrence of radical mechanism. Noteworthy, minor transformations in chemical structure could easily result in the overlapping retention time and λ_{max} during characterisation. Therefore mass spectrometry and NMR are considered to be the most efficient methods for identification (Nemzer et al., 2011).

Table 1-4. General influential conditions to the betalain stability (Janiszewska, 2014; Khan, 2016b).

Category	Stabilising Factors	Destabilising Factors
Physical	Darkness	Light
	pH 4 - 7	pH < 4 and/or pH > 7
	Low temperature (storage at 5 °C)	Increase in temperature
	Minimised a_w	Increase in a_w
Chemical	Minimised contact with O ₂ , e.g., by encapsulation, food matrix, presence of antioxidants	Exposure to O ₂ and other oxidising agents
	Inhibition of endogenous degradation enzymes, e.g., PPO	Activated degradation enzymes
	Presence of metal chelating agent, e.g., EDTA, citric acid	Presence of metal ions, e.g., Fe(II), Cu(II), Ni(II)
	High degree of glucosylation and acylation	Less protected core structure

Betalain digestion and absorption profiles are influenced by factors such as dose, food matrix, and individual's physical health. Bioaccessibility is reflected by the amount of soluble betalains available at the sites of absorption. The major bottleneck of improving bioaccessibility is suggested to be the digestive instability of pigments, which has been examined using *in vitro* simulation of the digestion pathway followed by HPLC analysis (Tesoriere et al., 2008; Khan, 2016a). Tesoriere et al. (2008) investigated the bioaccessibility and digestive stability of betaxanthins (indicaxanthin, vulgaxanthin I) and betacyanins (betanin, isobetanin) sourced from betalainic foods – cactus pear, red beetroot, and their derivatives. Compared with betacyanins, two betaxanthins tested have experienced lower degree of thermal degradation during betalainic food digestion. The pKa of indicaxanthin and betanin, calculated by Tesoriere et al. (2013), ranges between 2.0 and 5.4 due to the presence of multiple proton-donating groups, and betanin indicated higher susceptibility to alkaline conditions. At the same time, various betalain compounds showed an inconsistent degree of

decomposition in different phases of digestion. Whereas the most significant loss of (iso)betanin was detected in the small intestine, indicaxanthin was reduced maximally at the gastric and intestinal sites, whilst intense degradation was observed throughout the digestion for vulgaxanthin I. Resembling the trend described in Tesoriere et al. (2008), Gómez-Maqueo et al. (2020) also illustrated a marked reduction of bioactive contents of betanin and indicaxanthin at small intestinal phase with overall higher bioaccessibility of indicaxanthin. However, Sawicki et al. (2020), investigating betalain digestion *in vivo* in rats, showed a remarkably more severe decomposition of betacyanin in the gastric phase compared to *in vitro* results from Tesoriere et al. (2008) and Reynoso et al. (1999). This might be caused by the differing dynamics of *in vitro* and *in vivo* digestion, for example, the continuous gastric secretion during the *in vivo* study Sawicki et al. (2020) versus the static gastric simulating conditions of *in vitro* Tesoriere et al. (2008) and Reynoso et al. (1999).

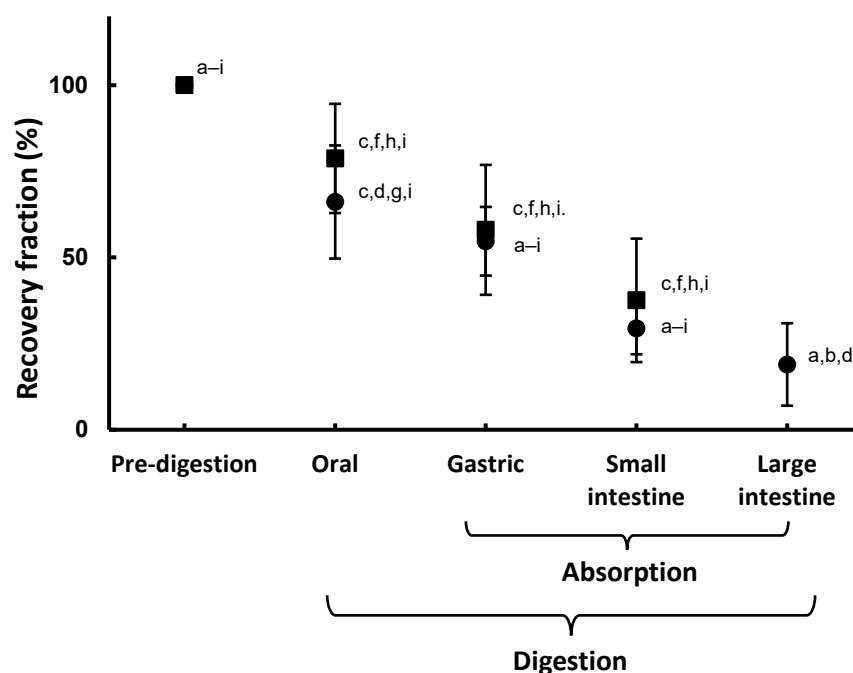


Figure 1-2. Degradation profiles of betanin (●) and indicaxanthin (■) along the simulative gastrointestinal digestion *in vitro*. Plot points were acquired from corresponding references – Krantz et al. (1980)^a, Reynoso et al. (1999)^b, Tesoriere et al. (2008)^c, Vieira Teixeira da Silva et al. (2019)^d, Choo et al. (2019)^e, Gómez-Maqueo et al. (2020)^f, Wang et al. (2020)^g, Sánchez-Recillas et al. (2022)^h, and Montiel-Sánchez et al. (2021)ⁱ.

Overall, in both purified and food-matrix states, indicaxanthin (37.6%) demonstrated the highest *in vitro* bioaccessibility compared to vulgaxanthin I (31.6%) and (iso)betanin (29.4%) before colon fermentation stage. Fig. 1-2 summarises the bioaccessibility data of betanin and indicaxanthin from the relevant research to date. However, difference in the experiment methods between studies should be considered. Also note the scarce number of research managing to address the betalain decomposition at colon fermentation stage.

1.4.2 Influence of food matrix

The influence of food matrix on phytochemical bioaccessibility can be considered from physical and chemical perspectives as previously mentioned. Simulative digestions were performed on betaxanthins (i.e., indicaxanthin, vulgaxanthin I) and betacyanins (i.e., betanin isobetanin) sourced from red beetroot and cactus pear fruit (Tesoriere et al., 2008). According to digestive outcomes at gastric phase, the digestion of betanin incorporated in red beetroot matrix showed an evident mitigation of pigment degradation compared with digesting purified betanin, attributed to the physical and chemical preservation of food matrix on betanin. This effect was minorly observed from cactus pear matrix on indicaxanthin digestion. On the other hand, the epithelial permeability results of Tesoriere et al. (2013) has demonstrated the release of indicaxanthin, with smaller molecular weight and lower water solubility, and was not as greatly restricted by the food matrix as betanin was. Betanin also showed greater transport rate from dietary cactus pear than from red beetroot which was possibly due to the lower fibre content of the former. As a result, food matrix was proposed to cause more significant reduction in the bioaccessibility of betanin compared with indicaxanthin, with net effects of molecular structures and food matrix composition. Food matrix impact on bioaccessibility was further investigated by comparing the stability of betalains in fresh food or product matrices versus their purified status (Tesoriere et al., 2008). In the absence of food matrix, digestion simulations of purified betalains indicated greater loss of betacyanins at gastric phase as well as the accumulation of betalamic acid along the digestion progress. The betalain-preserving capability of natural plant tissues is generally attributed to their chemical protection, oxygen scavenging, and light shielding effects. For

instance, the co-existence of intrinsic polyphenols (e.g., luteolin, quercetin) and vitamins (e.g., A, C & E) as antioxidants helps maintain the reducing environment in the pulp (Neveu et al., 2010). However, the natural character of food matrices as well as their retention effect on betalains are likely to be altered during food processing. The beetroot juice and crunchy slices were selected as variable matrices in a human study carried out by Wiczkowski et al. (2018), whose urinary pharmacokinetics indicated a much faster absorption of betacyanins after juice consumption, with the maximal excretion rate post slice consumption appearing two hours later than that of juice profile.

The scope of betalain bioaccessibility research has been extended from the perspectives of food species, betalain types and processing techniques. A recent *in vitro* study demonstrated the benefits of fermentation treatment in enhancing pigment bioaccessibility in the gastrointestinal tract (Choo et al., 2019). Pulp of red pitaya was fermented with sugar at 25 °C for 8 weeks in a sealed container until strained. The (iso)betanin in fermented red pitaya juice after each digestion phase was found to possess higher retention fraction and minor loss from degradation compared with those in raw pitaya juice. Similar finding was also elucidated by Sawicki et al. (2018) and Sawicki et al. (2020) who employed fermented red beetroot juice in both animal and human trials. The fresh red beetroot pulp was incubated in sugar (1.2%, w/v) and NaCl (1.2%, w/v) for 7 days at 24 °C with lowered pH along the process (Sawicki et al., 2017). Among all the betalain bioavailability studies listed with *in vivo* methodologies (Table 1-5 and 1-6), administration of fermented betalain-rich food has provided several positive responses with regards to pharmacokinetic measurement compared with the cases of non-fermented. However, selection of fermented food product brought challenges to the strict control of compositions and processing methods of samples, especially when food matrix is the major factor to be investigated. Moreover, it is difficult to balance between testing groups regarding the volume of ingested bulk and the effective dose of interested compound. Together with the profiles illustrated in Tesoriere et al. (2004a) and Sawicki et al. (2018), incomparability between results is greatly attributed to the difference in methodology, e.g., dosage, genetic variation and post-harvesting treatment of food, analytical techniques and sensitivity.

The dose effect to bioaccessibility was also attempted by the latest research Sawicki et al. (2020). It was revealed that the administration of higher dose was associated with slower degradation of betacyanins in gastric environment. This could be resulted from the bulk effect, where the increasing amount of betacyanins are shielded from the extreme gastric pH and enzymes by the outermost compounds at higher dose. As a consequence, greater proportion of pigments are able to reach the lower GI tract in their native forms hence corresponding bioactivities. More details of this study were discussed in Section 1.5. Furthermore, there was a subtle difference between the decomposition rate of betanin and isobetanin during the simulated digestion, i.e., a minor gastric but greater intestinal degradation of betanin compared to isobetanin (Choo et al., 2019), which is worth a further research.

1.5 Pharmacokinetic profile and variability

Health benefits of a phytochemical component can be heavily determined by its bioavailability *in vivo*, referring to a fraction of component that enters the systemic circulation while maintaining bioactivity at a specific site (Akbar et al., 2018). Bioavailability of betalains can be demonstrated via plasma pharmacokinetic and urinary excretion curves. Quantification and identification of the pigments in collected physiological fluids were performed after the subjects ingested specific doses of betalain-rich materials (Khan, 2016a). The methodology is most frequently employed in human and animal studies in nutritional and pharmaceutical fields that have attempted to examine the nature of absorption, distribution, metabolism, and excretion of target compounds (Tesoriere et al., 2004a; Wiczowski et al., 2018; Sawicki et al., 2020).

Bioavailability data on the betalain group remain scarce compared to those of polyphenols. Nevertheless, current *in vivo* studies have demonstrated the absorbability of some principal betalains present in the circulation system, e.g., (iso)betanin and indicaxanthin, but in relatively small amounts. Tesoriere et al. (2004a) is one of the pioneering studies that provided an insight into the bioavailability of betanin and indicaxanthin via plasma pharmacokinetics as well as urinary excretion parameters in humans. Betanin and indicaxanthin were detected within a 12 h window following pulp consumption with the time of reaching peak plasma concentration (t_{max}) at around 3 h. The elimination of both compounds displayed first order kinetics. As summarised in Table 1-5, indicaxanthin possessed a higher peak plasma concentration (C_{max}) of nearly 20 times of (iso)betanin, as well as a longer half-life ($t_{1/2}$), following the ingestion of triple molar amount of indicaxanthin in this study. This finding was echoed by Takahashi et al. (2017) who explored the gastric and small intestinal absorption dynamics of rat in terms of purified (iso)betanin and indicaxanthin from red beetroot and cactus pear fruit, respectively. In the window of 30 minutes post intestinal filling, the betalain profiles in the portal and systemic plasma revealed a significantly higher C_{max} of indicaxanthin compared with (iso)betanin; this was accompanied with the evidence that C_{max} of indicaxanthin was reached approximately 3-time faster than (iso)betanin (*ca.* 5 min and 15 min, respectively), which have therefore implied a greater absorption rate as well as intensity of indicaxanthin compound.

Table 1-5. Summarised data of plasma pharmacokinetics and urinary excretion of (iso)betanin and indicaxanthin in human body from the bioavailability studies. Some data were further processed for the purpose of comparison, and the time in brackets indicated the duration of intervention trial.

Reference	Intervention food	Dose	Plasma pharmacokinetics (C_{max})		Urinary recovery (%)	
			(Iso)Betanin	Indicaxanthin	(Iso)Betanin	Indicaxanthin
Watts et al. (1993)	200 mL mixture of beetred liquid with apple & blackcurrant juice	60 mg betacyanin (n=100)	—	—	0.06 – 0.54 (8 h)	—
Kanner et al. (2001)	300 mL processed red beet juice	120 mg betanin (n=4)	—	—	0.5 – 0.9 (12 h)	—
Tesoriere et al. (2004a)	500 g cactus pear fruit pulp	16 mg betanin + 28 mg indicaxanthin (n=8)	$0.11 \pm 0.01 \mu\text{g mL}^{-1}$ ($\text{AUC}_{t=12\text{h}} : 0.25 \pm 0.02 \mu\text{g} \cdot \text{h} \cdot \text{mL}^{-1}$)	$2.13 \pm 0.17 \mu\text{g mL}^{-1}$ ($\text{AUC}_{t=12\text{h}} : 9.01 \pm 0.80 \mu\text{g} \cdot \text{h} \cdot \text{mL}^{-1}$)	3.7 ± 0.2 (12 h)	76.0 ± 3.0 (12 h)
Netzel et al. (2005); Frank et al. (2005)	500 mL commercial red beet juice	362.7 mg betanin equivalent (n=6)	—	—	0.21 – 0.39 (24 h)	—
Clifford et al. (2017)	300 g commercial cooked red beetroot	66 mg betanin (n=10)	Not detected	—	—	—
	250 mL commercial red beetroot juice	194 mg betanin (n=10)	Not detected	—	—	—
Wiczowski et al. (2018)	3.33 mL/kg bw of diluted red beet juice	1.5 mg betacyanin/kg bw (n=12)	—	—	0.21 ± 0.03 (24 h)	—
	0.37 mg/kg bw of red beet crunchy slices + water	1.5 mg betacyanin/kg bw (n=12)	—	—	0.20 ± 0.03 (24 h)	—
Sawicki et al. (2018) *	3.33 mL/kg bw of fermented red beetroot juice (n=24)	0.7 mg betalains/kg bw (n=24)	$7.72 \pm 0.60 \text{ ng mL}^{-1}$ (7 weeks)	—	—	—

“—” indicates the parameter was not taken into account in the cited study

“Not detected” suggests the value was not able to be acquired via the methodology in reference

* Note the pharmacokinetic values from a long-term intervention instead of an acute bioavailability trial.

Table 1-6. Summarised data of plasma pharmacokinetics and urinary excretion of (iso)betanin and indicaxanthin in rats from the bioavailability studies. Some data were further processed for the purpose of comparison, and the time in brackets indicated the duration of intervention trial.

Reference	Intervention food	Dose	Plasma pharmacokinetics (C_{max})		Urinary recovery (%)	
			(Iso)Betanin	Indicaxanthin	(Iso)Betanin	Indicaxanthin
Krantz et al. (1980)	Beetroot extract	2.5 mg betanin (n=8)	Not detected	–	2.7 ± 1.2 (24 h)	–
Watts et al. (1993)	Beetred powder dissolved in water	1 g betacyanin (n=6)	Not detected	–	0.59 ± 0.10 (6 h)	–
Allegra et al. (2014); Tesoriere et al. (2014)	Indicaxanthin extract from yellow cactus pear	0.62 mg indicaxanthin/kg of rat bw (n=15)	–	$67.82 \pm 6.17 \text{ ng mL}^{-1}$ (4 h)	–	21 ± 1.2 (12 h)
Sawicki et al. (2017)	Betalains dissolved in saline	50 mg betalains/kg of rat bw (n=3)	$2.97 \pm 0.06 \text{ } \mu\text{g mL}^{-1} *$ (1 h)	–	–	–
Takahashi et al. (2017)	Purified (iso)betanin from red beet extract	45 mg (iso)betanin/kg of rat bw (n=6)	<i>ca.</i> $0.30 \text{ } \mu\text{g mL}^{-1}$ (0.5 h)	–	–	–
	Purified indicaxanthin from cactus pear juice	45 mg indicaxanthin/kg of rat bw (n=6)	–	<i>ca.</i> $7.30 \text{ } \mu\text{g mL}^{-1}$ (0.5 h)	–	–
Sawicki et al. (2020)	Fermented red beet juice with saline	5 mg betacyanin/kg of rat bw (n=6)	$0.30 \pm 0.05 \text{ } \mu\text{g mL}^{-1}$ ($\text{AUC}_{t=2h} : 0.78 \pm 0.03 \text{ } \mu\text{g} \cdot \text{h mL}^{-1}$)	–	3.04 ± 0.98 (2 h)	–
		20 mg betacyanin/kg of rat bw (n=6)	$5.74 \pm 1.23 \text{ } \mu\text{g mL}^{-1}$ ($\text{AUC}_{t=2h} : 10.74 \pm 1.66 \text{ } \mu\text{g} \cdot \text{h mL}^{-1}$)	–	55.59 ± 15.30 (2 h)	–

“–” indicates the parameter was not taken into account in the cited study

“Not detected” suggests the value was not able to be acquired via the methodology in reference.

* The plasma concentration data was not necessarily maximal since only one time point was measured.

Impact of food matrix on betacyanin absorption was addressed by Wiczowski et al. (2018). Subjects consuming red beetroot in different physical forms, i.e., juice and crunchy slices, have displayed similar renal recovery (%) but diverged excretion kinetics of betacyanins. The release of betacyanins from slices was considerably slower than from juice due to the retention effect of solid, fibre-rich matrix, thus $t_{\max}$ in the excretion profile of slice consumption appeared 2 h later than that of juice. This is also proved by several bioavailability studies of flavonoids where pharmacokinetic $t_{\max}$ after juice intake was evidently shorter than the intake of more complex matrix (Mullen et al., 2008; Brett et al., 2009; Williamson et al., 2018). Prolonged digestion caused by solid matrices also leads to longer exposure of outermost pigment compounds to the digestive environment in the GI tract, hence the generation of metabolites (e.g., aglycones) at higher proportion than juice digestion. Eventually, total amount of urinary excretion of betacyanins was slightly lower in the case of beet slices uptake compared to juice, which was supposedly attributed to the incomplete release of pigments from slice matrix, and severe pigment degradation in the GI tract. However, the excretion difference between groups was statistically insignificant due to the large inter-subject heterogeneity. The ratio of betanin and isobetanin varied between food samples but was highly consistent with the ratio in renal excretion. Comparable evaluations were undertaken by Netzel et al. (2005) and Frank et al. (2005) who assessed the urinary elimination of betalains after oral administration of commercial red beetroot juice. Despite the difference in dose, results of Wiczowski et al. (2018) and Frank et al. (2005) have shown the consistent trend of elimination rate constant and dose-excretion fraction of betacyanins (Table 1-5). The association between food matrix and bioavailability was also targeted by Clifford et al. (2017), who attempted to assess the acute absorption of betalains and nitrate (NO_x) sourced from cooked beetroot and beetroot juice. Unfortunately, betanin concentration was not detected at any time points in plasma thus unavailable information of bioavailability.

From the chemical perspective of food matrix, the most recent body of work investigating betalain bioavailability has demonstrated the dose-dependency of betacyanin degradation, absorption, excretion, and metabolic transformation. For example, Sawicki et al. (2020) performed a gastric administration of two dosages of betacyanin samples on subject

rats (i.e., 5 and 20 mg/kg body weight), as listed in Table 1-6. It is noteworthy that a 4-fold higher dosage has rendered a rise of the urinary excreted fraction by around 15-fold compared to the lower dosage. This is likely a synergistic result of the improved digestive resistance and higher absorption intensity of pigment compounds with possible factors of food matrix preservation and altered metabolic pathway. This tendency was also found similar to Reynoso et al. (1999) where urinary (iso)betanin concentration of rat was increased with higher intake of garambullo fruit (0.5-5.0 g/kg body weight). Furthermore, in contrast to lower dose, administration of high concentration was associated with the increasing ratio of native compounds to metabolites in physiological fluids. As aforementioned in Section 1.4.2, betalain in the more concentrated dose was proposed to experience stronger protection from the food matrix or outermost compounds, leading to a reduced degree of transformation during digestion and metabolism. In other words, the entire biotransformation nature of the compound can be altered by dose and food matrix changes. Therefore, it was speculated according to the previous findings that ingestion of higher dose and proportion of native betacyanins (i.e., glucosidic form) synergistically intensify their absorption level hence bioavailability during the specific intervention. The level of urinary excretion, therefore, was not in linear proportion to the dose of pigment in Sawicki et al. (2020). Associating this evidence with bioactivities of betalains, e.g., antioxidant, radical scavenging, anti-inflammatory properties (Gandía-Herrero et al., 2009; Tesoriere et al., 2014; Ahmadi et al., 2020; Fernando et al., 2021), it offers scientific backing for the enhancement of positive health effect of the pigments from the perspectives of diet and absorption during the functional food development.

Apart from establishing an acute 24 h profile of betalain concentration based on a single dose of betalain-rich food, period of beetroot ingestion was elongated to 6 weeks in Sawicki et al. (2018) who emphasised the monitoring of betalains kinetics and metabolites under the long-term exposure to fermented beetroot juice. Fermented juice was selected for the purpose of producing more observable pigment profile. Nonetheless, the pilot methodology has limited the comparability of the results with other references, and several kinetic parameters of acute experiment were unavailable in this case. Plasma and renal excreted concentrations

of betacyanins peaked in the first and second week of ingestion, respectively. From then on, content in plasma has declined and levelled off ten-fold, while the excreted level was halved. Analytical technique of micro-HPLC-MS/MS with improved resolution and sensitivity allowed the characterisation and quantification of, besides principal compounds, more than 10 other native molecules and metabolites in physiological fluids with various degrees of modification. Individual betalain derivatives was detected at specific time point in specific physiological fluid across 6-week ingestion period. The bioavailability of betaxanthin was suggested to be poor in the study based on the result of vulgaxanthin I which was hardly detected in plasma and urine. Concerning the findings of Tesoriere et al. (2004a) and Tesoriere et al. (2008) about high permeation rate of indicaxanthin, investigations of more betaxanthin and betacyanin compounds are anticipated from various food sources. Other factors to be taken into account will be the physical adaptation of human body to the long-term consumption of dietary betalains, e.g., alteration of gut microbial population and composition, as well as the change of absorption and metabolism-related elements in the intestine.

Regarding the inter-individual variations of phytochemical bioavailability, it is important to locate the variation range of individual's response towards the biologically active plant compounds. Addressed by Wiczowski et al. (2018), the absorption and renal excretion rate of native betacyanins and metabolite varied largely between individuals with vague patterns being captured for the most, eventually rendering a large difficulty in a coherent and thorough evaluation of individual betalain absorption. Genetic polymorphism and dietary habits of individuals are considered as crucial influencers to the pigment uptake, which were expressed in the gastrointestinal pH environment, digestive diseases, and the activity of brush border enzymes (Lampe and Chang, 2007; Cassidy and Minihane, 2017). Variability was also investigated by Frank et al. (2005) but was limited due to low sample size. Wiczowski et al. (2018) has included individual profiles of betacyanin excretion in urine, who has yet confronted similar challenge to other bioavailability studies. There is a large also inextricable heterogeneity in the betalain uptake and excretion levels between subjects that has rendered the conclusion of results extremely difficult. In addition, Sawicki et al. (2018) mentioned the variability of betalain compositions between the plasma of human volunteers and rats, implying the inter-species discrepancy in compound absorption and metabolic pathways.

1.6 Trans-epithelial transport and biotransformation

1.6.1 Absorption and transport of betalain

Many strategies are available for investigating the absorption mechanism of phytochemicals, and they are generally classified as *in vivo*, *in vitro* and *in silico*. Despite the limitations of *in vivo* experiments due to the inter-species and inter-individual variability, the method provides reliable results on phytochemical absorption under physiological conditions. Using the animal model, the fate of (iso)betanin and indicaxanthin in gastric and intestinal phases was explored by Takahashi et al. (2017), who filled the compartmentalised stomach and small intestine of rats with betalain-rich food. A similar method was employed by Sawicki et al. (2020) with intragastric administration of fermented beet juice. Both have provided explicit view of the absorption nature of betalains in rat stomach, which should not be overlooked.

Native pigments were indicated to experience severe decay throughout the GI tract in both studies. The assimilated compounds were congregated in portal vein plasma, from which (iso)betanin was detectable in 5 min after the gastric filling (Takahashi et al., 2017). Following the metabolism in liver, nutrients are transported into the main circulatory system. Betacyanin glucosides, instead of aglycones, were found as the principal constituent being present in the plasma of inferior vena cava of rats after gastric and small intestinal filling. This indicated that pigments may maintain their glucoside structures during absorption and liver metabolism. Glucosides may also remain dominant among the absorbed betacyanins at later circulating stage until urinary excretion. Similarly in Sawicki et al. (2020), betacyanin glucosides were discovered to be more predominantly and rapidly absorbed compared to aglycones. Results were consistent with Tesoriere et al. (2004a) and Tesoriere et al. (2013), suggesting the major transport mechanisms of betacyanins to be passive diffusion with possible involvement of glucose transporters. On the other hand, indicaxanthin concentration in gastric coronary and portal plasma was below the detection limit following the feeding through stomach, implying the negligible gastric absorption of indicaxanthin and different absorption mechanism from (iso)betanin. The distinct research methodology renders difficult

comparison of biological data between studies, particularly regarding the trace concentration of betacyanin metabolites in physiological fluids. But these studies overall contribute to a greater understanding of betalain absorption mechanism.

Meanwhile, methods *in vitro* are often questioned regarding the validity of simulation hence the reasonability of extrapolating results to what is anticipated *in vivo*. Yet the advantages are also obvious in terms of the more rigid control of experimental conditions and data reproducibility. Tesoriere et al. (2013) has targeted to the mechanisms of epithelial transport of betanin and indicaxanthin, simulated by Caco-2 cell line in transwell. The fully differentiated Caco-2 monolayer has partly exhibited the morphology (e.g., microvilli, intercellular junctions) and functions (e.g., enzymes, carriers) of human intestinal epithelium, a physical barrier that compartmentalises the intestinal lumen and internal milieu as simulated by apical (AP) and basolateral (BL) sides, respectively. Thus, examinations of epithelial integrity and betalain cytotoxicity are necessary prior to the cellular absorption and transport experiments.

There are several interesting findings in Tesoriere et al. (2013) that have made contribution to the fundamental understanding of betalain transport mechanism. The trans-epithelial transport of (iso)betanin and indicaxanthin has confirmed the mass balance in AP, BL, and intracellular sites, which did not support the hypothesis of molecular transformation during the movement. Betanin was discovered with significantly higher influx permeability (AP to BL) than efflux (BL to AP) whereas the transport indicaxanthin underwent was relatively non-polarised. Influx transport of indicaxanthin was higher than that of betanin, which was to some extent consistent with bioavailability results in Tesoriere et al. (2004a) and Takahashi et al. (2017). Betalains, especially indicaxanthin, were deduced to experience dominantly the passive diffusion through paracellular route, and this was reinforced by several evidence – I) Influx permeation of both betalains obeyed the diffusion-solubility kinetics, i.e., non-saturation; II) Permeability of betanin was independent to the presence of competitive substrates of potential influx transporter, monocarboxylate transporter (MCT1) and organic anion-transporting polypeptide (OATP2B1), meanwhile competitor addition did

not cause significant variation on indicaxanthin permeability. It should not, however, rule out the possibility of the wrong targets of transporters; III) Influx permeability of both betalains was boosted after treating monolayer with EDTA that loosened the intercellular tight junctions. The other study, Sawicki et al. (2018), has proposed the coexistence of passive and active routes of betalain transport. Uptake was also hypothesised to involve the participation of bilitranslocase, an organic anion transporter present at the epithelium of GI tract, which was based on the cationic property of hydrophilic betalains under the non-basic conditions (Tesoriere et al., 2003).

1.6.2 Betalain transformation

Deduced from the cell culture results in Tesoriere et al. (2013), betanin and indicaxanthin may hardly experience any degree of biotransformation during trans-epithelial transport. According to the excretion profile in Wiczowski et al. (2018), recovery fraction of aglycones in urine within a 24 h period has reached the maximum at around 8 h which was alterable by varying the food matrix. It is worth mentioning that early phase of excretion (0-4 h) was dominated by betacyanin glucosides while the later phase by aglycone forms. It was rationalised as the progressive deglycosidation of betacyanins along the digestion tract. The underlying mechanism is proposed to be catalysed by I) the physiological temperature and changing pH; II) cytosolic β -glucosidase (CBG) expressed in enterocytes; III). β -glucosidase expressed by bacteria in large intestine. To the contrary, compared to the stable absorption of glucosides, only trace amount of aglycones was detected in plasma of human and rat but not urine (Tesoriere et al., 2004a; Takahashi et al., 2017; Sawicki et al., 2018), according to which the glucosyl hydrolysis was suggested to be a non-essential pathway for absorption. Linking to the findings in Sawicki et al. (2020) that absorbed fraction of (iso)betanin to total (iso)betanin in juice was significantly higher than the fraction of (iso)betanidin to total (iso)betanidin in juice, the native betanin compound was assumed to be more easily transported through the gastrointestinal epithelium than their aglycones, nonetheless it might also be attributed to the dose effect.

Detection of aglycones has induced the presumption in Kanner et al. (2001) that absorption mechanism of betacyanins across the epithelial barrier resembled that of quercetin glucoside. There are two hypothetical mechanisms regarding the uptake of glucosyl flavonoids, and corresponding efficiency is greatly determined by the position of glucosides (Besson et al., 2001; Day et al., 2003; De Ferrars et al., 2014; Kaşıkçı and Bağdatlıoğlu, 2016). Major metabolic pathway, in prior to trans-epithelial movement, involves the apical enzyme lactase phlorizin hydrolase (LPH) catalysing glucosyl hydrolysis. Alternatively, glucoside compounds are imported via the sodium-dependent glucose transporter (SGLT1). It is followed by the intracellular deglucosidation process via the CBG enzyme, and re-attachment of sugar moiety at different carbon positions on the aglycone via UDP-glucuronosyl transferase (UGT). This change of hydrophilicity possesses rigid control to the release rate of the compound from enterocyte in the presence of bioactive elements. In other words, import of quercetin is specifically modulated by its luminal release and a cascade of molecular reactions, whose applicability to betalain transport still warrants future investigations.

In the digestive and circulatory systems, betalain is susceptible to multiple modifications such as elimination and conjugation with small molecules, until excretion. Sawicki et al. (2018) analysed the metabolites in physiological fluids (i.e., blood and urine) of human body after consumption of fermented beetroot juice. So far, deglucosidation, decarboxylation, dehydrogenation are the most recognised transformations of the pigments that are significantly promoted by human digestion and metabolism. These reaction pathways are activated sequentially or spontaneously in different compartment which are hardly predictable or controllable. Aglycones are likely decomposed into lower molecule mass in order for better absorption, while the conjugated derivatives with glucuronic acid, methyl and sulphate groups were only measured in trace amount in urine with high inter-individual variability (Takahashi et al., 2017; Wiczowski et al., 2018). Nevertheless, the pigment composition in plasma and urine was reported to be predominated by native (iso)betanin, which reduced the likelihood of betacyanins undergoing similar transformations as flavonoids (Wiczowski et al., 2018; Sawicki et al., 2020).

1.7 Faecal excretion and gut microbiota

Regardless of betalain detection in the physiological fluids by most of previous studies *in vivo*, the evidence of kidney as the principal route of pigment elimination still remains inconclusive. Betalain components rarely have their plasma concentrations above micromolarity, resembling most polyphenols, despite their abundance in the betalainic foods. Proposed by Frank et al. (2005) and Clifford et al. (2017), the low excretion rate of compounds through kidney could be the consequence of low absorption intensity, minor renal excretion pathway, or other non-identified metabolites, and thereby enterohepatic circulation was presumed as an alternative path of betalain excretion. Several studies at early days also attempted to explore the hepatic metabolism of betanin which were, however, largely restricted by the detectability and methodology at the time (Krantz et al., 1980; Watts et al., 1993; Reynoso et al., 1999).

Overall, the evidence from previous research confirmed the GI tract as the main site of betanin absorption and degradation. Considering the low plasma concentration and urinary recovery of betalains, the major proportion of pigments are hypothesised to be excreted through the intestine undergoing successive decay. However, there are very few research to date exploring the biotransformation of betalains in the colonic environment with potential influence from responsible gut bacteria. The faecal excretion of betalains was initially explored by Krantz et al. (1980), demonstrating that only five percent of betanin was recovered from urine and faeces of rats following oral administration. In a more recent study of the related topic, Vieira Teixeira da Silva et al. (2019) reported the non-detectability of betanin content at milli-molar level from the samples of *in vitro* colonic fermentation model. Correspondingly, a research gap was targeted to improve the comprehension of betalain bioavailability and metabolic fate along the course of intestinal digestion and colonic fermentation. Meanwhile, the anti-inflammatory and cyto-protective effects of betalains on several cell lines (e.g., hepatic cells, macrophages) were reported by the literature (Krajka-Kuzniak et al., 2013). These cellular effects are also anticipated during the exposure of

enterocytes to betalain components in the lumen, which will be examined in this project using prevalent betacyanins and betaxanthins.

On the other hand, betalain pigments are expected to encounter and potentially pose influence on the gut microorganisms during their transit through the intestine. There is yet a paucity of knowledge regarding the impact of beetroot pigments to the human gut microbiome. By contrast, the reciprocal interplay between polyphenol metabolism and gut microbial composition has been frequently assessed. Several studies implied the prebiotic character of polyphenols which are frequently referred to as the functions of selectively promoting beneficial taxa and/or subduing pathogens in the gastrointestinal tract and eventually conferring host health (Alves-Santos et al., 2020). There is strong evidence of gut microbial shift mediated by luminal polyphenols and their metabolites, fostering the growth of prebiotic targets (e.g., *Bifidobacterium* and *Akkermansia*) while depleting *Bacteroides* (Jiao et al., 2019; Liu et al., 2020). Polyphenols such as proanthocyanidins can stimulate transmembrane proteins and mucin production, which facilitate the proliferation of mucin-degrading bacteria (e.g., *Akkermansia*) and enhance intestinal barrier function (Anhê et al., 2015). Also given that glucosides of dietary polyphenols are confined in the intestinal lumen, non-absorbed, they are proposed to be good substrates for sugar-fermenting species (e.g., *Roseburia*) with production of energy and short chain fatty acids (SCFAs) (Kawabata et al., 2019). Betalains, which also contain sugar moiety and are poorly absorbed into circulation, are presumed to exert similar positive effects as polyphenols in the gut. Conversely, the structure of bacterial community could substantially determine the profile of degradation and transformation of ingested betalains hence their bioavailability.

This mutual influence between beetroot pigments and human gut microbiota has accordingly become another field to be highlighted in this project therefore the potential health implication to the gut. Nevertheless, as confronted by most *in vivo* studies, inter-individual variability is of a challenge for drawing the conclusion of association between phytochemical metabolism and microbiota alterations considering multiple intrinsic and extrinsic factors involved, such as genetic determinants, health conditions and dietary habits.

1.8 Aims and objectives of current thesis

Based on the available evidence from literature, the perspectives that require further research have been identified. Only a small proportion of betalain compounds are expected to be absorbable through the GI tract, particularly the intestine, where passive diffusion is hypothesised to be the preferred route of their trans-epithelial migration. The majority of pigments, however, is proposed to be excreted through faeces while exhibiting beneficial impacts on the gut environment. This was linked to the objectives of the present research for a deeper comprehension of pertinent topics.

Objectives of this PhD project were:

- i. Investigation at the absorption efficiencies and transport mechanism of prevalent betalains (betanin, vulgaxanthin I and indicaxanthin) during intestinal uptake via *in vitro* cell model.
- ii. Examination on potential immunomodulatory activities exerted by prevalent betalains on the intestinal epithelia using human cell line.
- iii. Characterisation of the metabolic profiles of betacyanins in both renal and intestinal excretions with delineation of inter-individual variability via *in vivo* human bioavailability study.
- iv. Exploration of the modulatory effect of red beetroot juice consumption on gut microbiota in healthy human, with depiction of bidirectional influence between microbiota and betacyanin metabolism via human microbiome trial.

Chapter II

Experimental Methods

Practical investigations played a critical role in the current project. In accordance with the objectives, research experiments were categorised primarily into the sections of cell culture experiments (*in vitro*) and human intervention study (*in vivo*) with throughout performance of chemical analysis. This chapter has expounded the methods of preliminary tests and formal analysis, including the instructions of general principle and method optimisation along the progress.

2.1 Preliminary betalain processing and analysis

2.1.1 Reagents and materials

Freeze dried samples of red and yellow beetroot pulps (*Beta vulgaris* L.) were provided by Whole Food (Ramsgate, U.K.). Freeze dried pulps of red and yellow prickly pear (*Opuntia ficus-indica* L.) were provided by Miguel Hernández University (Elche, Spain). Freeze dried red pitaya fruit (*Hylocereus polyrhizus*) was obtained from Sri Lanka in November 2017, and the freeze dried red amaranth sample (*Amaranthus tricolor* L.) was obtained from China in May 2019.

All the HPLC and LC-MS-grade solvents for betalain extraction and chromatography, i.e., methanol, ethanol and formic acid, were purchased from Fisher Scientific (Loughborough, U.K.).

2.1.2 Betalain extraction from different plant sources

Betalain contents in the red and yellow beetroots, red and yellow prickly pears, red pitaya and red amaranth (0.2 g) were repeatedly extracted using the solvent of H₂O/methanol/formic acid (84.95:15:0.05, v/v/v, 3 × 3 mL). Following the addition of solvent, samples were

vigorously vortexed (1 min), sonicated (15 min, 5 °C), and centrifuged (3500 g, 10 min, 5 °C). The coloured supernatants of each sample were carefully collected and combined, followed by thorough vortex. Extracts were eventually filtered through a 0.2 µm PTFE syringe filter for the purpose of chromatographic analysis. The entire process was conducted with light protection, and extracts were preserved under -20 °C.

2.1.3 LC-MS analysis of plant-derived betalains

High-performance liquid chromatography (HPLC) is the most popular technique applied for phytochemical separation and quantification, which is frequently merged with additional detectors for compound identification, such as diode-array detector (DAD), mass spectrometry (MS) and NMR. The intense colour of betalains is attributed to their conjugated π -system and phenol-like substituents of the nitrogenous cyclic. Condensation of betalamic acid with *L*-DOPA, in the case of betacyanins, results in the extension of structural π -conjugation thus a rise in λ_{max} from 424 nm (i.e., betalamic acid) to 540 ± 9 nm. Betaxanthins are generated by the condensation with primary/secondary amines leading to the λ_{max} of 472 ± 14 nm. Attachment of various sugar moieties or amino acids likely causes the shift of electron density hence subtle changes in absorbance parameters (Khan and Giridhar, 2015).

Different betalain compounds in extract were quantified and characterised by a high-speed LC fitted with a UV-Vis detector (SPD-20A), followed by a single quadrupole MS with an electrospray ionisation source (ESI) (Shimadzu, 2020, Japan). This standard method was based upon the Shimadzu instruction on betalain analysis (Borzymowska-Reszka and Reszka Spółka Jawna, 2011). The reverse phase HPLC was performed by Gemini C18 column (250 mm $\times$ 4.6 mm, I.D. 5 µm, Phenomenex, U.K.) at 40 °C. The eluent gradient was established by a binary mobile phase, i.e., 2% formic acid (v/v) as solvent A, and LC-MS-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 from 5 to 25% (0-15 min); to 70% (15-19 min), and was held for 2 min before returning to the initial 5% (21-26 min). Compounds were identified based on their *m/z* values monitored by ESI-MS in the positive ion mode with

nebuliser gas flow rate of 1.5 L min⁻¹ and desolvation line temperature of 250 °C. Betacyanins and betaxanthins were quantified based on the peak areas of chromatograms at 536 nm and 486 nm, respectively, with the calibration curves established using purified betanin, vulgaxanthin I and indicaxanthin from Section 2.1.5. Results of betalain composition and concentration in different plant species were exhibited in Appendix 1 and 2.

2.1.4 Spray drying of betalain extracts

Powders of red and yellow beetroot extracts were produced for the purposed of cell culture experiments. Pulp samples (1.5 g) underwent two rounds of extraction in the solvent (2 × 25 mL) as described in Section 2.1.2. The extraction solvent at this stage was optimised to 30% ethanol (v/v) in order to minimise the sugar content extracted from the matrix. The combined supernatants were thoroughly mixed. Prior to spray drying, extracts were maximally concentrated by solvent removal using a rotary evaporator (5 °C) and Genevac centrifugal evaporator EZ-2 (Winchester, U.K.). The concentrated crude extracts were filtered through 0.2 µm membrane and submitted to Buchi mini spray dryer (Flawil, Switzerland) with the inlet temperature of 120 °C, 95% aspiration and the flow rate of 8 mL min⁻¹. Powder of extracts was acquired and stored at 4 °C, light- and vapour-shielded.

2.1.5 Betalain purification with flash chromatography

Betalain purification was performed by Dr. Fernando, a member of the research group, for the purpose of biochemical analysis and cell culture experiments; the method was described in details in Fernando et al. (2022). The betanin, vulgaxanthin I and indicaxanthin pigments were isolated from red beetroot, yellow beetroot, and yellow prickly pear, respectively. Briefly, concentrated crude extracts (*ca.* 15 mL) were submitted to a reverse-phase flash chromatography using a KP-C18-HS Biotage SNAP cartridge (particle size 50 µm) mounted on a fully automated Biotage Isorela system (Isorela One, Biotage, Sweden). During the chromatography, samples in cartridge were eluted by a linear-gradient solvent system – water/formic acid (99.9:0.1, v/v) as solvent A and acetonitrile/formic acid (99.9:0.1,

v/v) as solvent B. The elution initiated from 0% of solvent B at 1 column volume (CV) for equilibration, which was then increased to 60% (v/v) at 10 CV. At the flow rate of 50 mL min⁻¹, approximately 20 mL of fraction was collected per tube whose UV-Vis absorbance was monitored at 254 and 280 nm. The identity of betalains in each eluted fractions were determined using HPLC-MS at 536 and 486 nm with *m/z* values, and purity was further assessed using ultra-high-speed LC with evaporative light scattering detector. The component fractions with desired purity were pooled, evaporated using the Genevac centrifugal evaporator, and lyophilised using a Labconco freeze dryer (Kansas, U.S.).

2.2 Cell culture experiments

2.2.1 Reagents, materials, and samples

The Caco-2 cell line, derived from the American Type Culture Collection (ATCC), was purchased from the European Collection of Authenticated Cell Culture (ECACC). Reagents for cell culture, including Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Phosphate-Buffered Saline (DPBS), Hanks' balanced salt solution (HBSS), foetal bovine serum (FBS), non-essential amino acids (NEAA), penicillin and streptomycin, and trypsin EDTA were purchased from Gibco cell culture products, Thermo Fischer Scientific unless specifically stated. Consumables for cell culture were purchased from Corning Inc. (New York, U.S.) and Sarstedt (Nümbrecht, Germany).

Chemicals including thiazolyl blue tetrazolium bromide (MTT), neutral red reagent, dimethyl sulfoxide (DMSO), p-nitrophenyl phosphate disodium hexahydrate (p-NPP), p-nitrophenol (p-NP), diethanolamine, 2-(N-morpholino)ethanesulfonic acid (MES) and sulforhodamine B (SRB) were purchased from Merck Sigma-Aldrich (Dorset, U.K.). All the other reagents were acquired from Invitrogen™ Thermo Fisher Scientific, Bio-Rad (Watford, U.K.), Scientific Laboratory Supplies Ltd. (Nottingham, U.K.), VWR International Ltd. (Poole, U.K.) and Starlab U.K. Ltd. (Blakelands, U.K.). As per previous, organic solvents used (LC-MS and molecular biology grades) were purchased from Fisher Scientific.

The dextrin-coated betanin standard was purchased from Santa Cruz Biotechnology (Dallas, U.S.). Additionally, cell experiments employed the purified betalains from Section 2.1.5 and beetroot extract powders from Section 2.1.4.

2.2.2 Cell culture and maintenance

Caco-2 cell line, isolated from colon adenocarcinoma, is a common model to investigate the barrier function of human intestinal epithelium and trans-epithelial transport of bioactives. It is frequently utilised during *in vitro* screening tests in the fields of pharmaceutical,

toxicological and nutritional research. Exploration of betalain uptake was commenced from the experiments of intracellular accumulation and trans-epithelial movement. The outcome could be an indicator of which being taken up into plasma at the first place while providing an insight into the absorption efficiency and transport mechanisms of the pigments.

Cells were cultivated in full DMEM containing 4.5 mg mL⁻¹ *D*-glucose and 0.11 mg mL⁻¹ pyruvate, which was supplemented with 10% FBS (*v/v*), 1% NEAA (*v/v*), penicillin (50 U mL⁻¹) and streptomycin (50 µg mL⁻¹). The cells were kept under standard conditions (37 °C, 5% CO₂) with medium being changed every two days. At the confluence of *ca.* 90%, cells were rinsed with DPBS (1×), and passaged by 15 min incubation in trypsin EDTA (1×) with tapping. All the experiments used Caco-2 cells between passage number 14 and 20, while aliquots of early-passage cells were stored under liquid nitrogen (-80 °C).

2.2.3 Cytotoxicity assays

Potential cytotoxicity of dextrin-coated and purified betalains to Caco-2 cells was initially determined using MTT and neutral red assays in 24-well plates at the seeding density of 5×10⁴ cells cm⁻². The MTT assay is based on the principle that MTT compound is reduced into MTT-formazan by the NAD(P)H-dependent oxidoreductase enzymes within cell mitochondria. Meanwhile, neutral red assay targets to the ability of viable cells to incorporate eurhodin dye into lysosomes. The amount of released formazan or eurhodin dye from cells is directly proportional to the cell metabolic activity hence the fraction of viable cells.

Medium control and positive control (5% DMSO, *v/v*) were established to indicate the baseline of Caco-2 cell vitality and to validate the cytotoxicity results. After incubation with increasing concentrations of dextrin-coated betanin (5-400 µg mL⁻¹) or purified betalains (1-1000 µM) for 3-6 h, cells were rinsed with DPBS and exposed to MTT reagent (0.4 mM) dissolved in FBS-free DMEM for 3 h at 37 °C in the dark (i.e., MTT assay). Cells were destained by DMSO after removal of medium and washing. In the case of neutral red assay, cells after being stressed with pigments were incubated in the neutral red solution in FBS-

free DMEM ($40 \mu\text{g mL}^{-1}$) and were destained by ethanol/H₂O/glacial acid (50:49:1, v/v/v) after 3 h exposure. Following 10 min shaking on a plate shaker in the dark, absorbance was determined at 590 nm and 540 nm for MTT and neutral red assay, respectively, using Tecan Spark 10MTM (Männedorf, Switzerland). Cell viability was calculated in percentage of the medium control. Considering the intense redness of betanin (λ_{max} of 536 nm), MTT assay was preferred over neutral red to minimise any potential interference from cell incorporated pigments.

2.2.4 Preliminary tests on Caco-2 cell differentiation

Compared to the non-differentiated cell model, Caco-2 cells with proper differentiation provide a better simulation to the intestinal climate in human body from the aspects of morphology (e.g., crypts and villi, tight conjunction) and function (e.g. physical barrier, transport mechanism) (Verhoeckx et al., 2015; Ude et al., 2017), therefore is critical for investigating molecular absorption and permeability to the intestinal barrier. Results of the preliminary tests in this section are exhibited in the Appendix 3 and 4.

2.2.4.1 Alkaline phosphatase (ALP) assay

Intestinal alkaline phosphatase (ALP) is exclusively expressed in villus enterocytes hence was selected as a marker of differentiation progress of Caco-2 cells (Ferruzza et al., 2012). This was accompanied with continuous monitoring of trans-epithelial electric resistance (TEER) (Appendix 3A).

In 24-well plates, ALP assay was performed every two days on the Caco-2 cells from day 3 to day 21 following the cell seeding. On the day of assay, reaction solution was readily made by fully dissolving the p-NPP reagent (2.5 mg mL^{-1}) in a basal buffer comprised of NaCl (150 mM), diethanolamine (75 mM), and Tris-HCl (4 mM) in PBS solvent (Ca^{2+} , Mg^{2+}), with the pH adjusted to 9.4 using HCl (5 M). The study has measured the activities of both suspended (i.e., in cell medium) and adhered (i.e., cellular) ALP enzyme. In the former situation, residual growth medium was incubated with reaction solution (1:3, v/v) in the dark

(30 min, 37 °C) followed by the absorbance detection at 405 nm. In the second case, reaction solution was directly applied to the cell layer after rinsing with DPBS, which was incubated in the dark (30 min, 37 °C) prior to the addition of stopping solution, NaOH (1 M) (3:1, v/v). Under alkaline condition, ALP enzyme catalyses the hydrolysis of p-NPP, generating p-NP in proportion to ALP activity. The p-NP compound was eventually quantified by Tecan Spark at 405 nm based on the p-NP standard curve. ALP enzyme activity was calculated as per Equation 1 (Abcam, 2017),

$$ALP\ activity\ (nmol\ of\ NP/min) = \frac{P \times D}{\Delta T} \quad \text{Eqn.1}$$

where P = amount of p-NP produced per well, ΔT = reaction time, and D = dilution factor.

ALP activity was further normalised to cellular protein measured by bicinchoninic acid (BCA) assay (Pierce™ Thermo Scientific). According to manufacturer's instruction, basal buffer (specific recipe of Na₂CO₃, NaHCO₃, bicinchoninic acid and sodium tartrate in 0.1 M NaOH) was mixed with 4% CuSO₄ solution (w/v) in a ratio of 50:1 (v/v) to obtain the effective reaction buffer, which was added to a clear lysate of cell pellets from triplicate experiments. After 30 min incubation in the dark at 37 °C, absorbance was recorded at 570 nm using Tecan Spark. Protein concentration was determined with reference to a standard curve using bovine serum albumin (30-2000 µg mL⁻¹).

2.2.4.2 Influence of Caco-2 cell differentiation on intracellular betanin uptake

Potential impacts of Caco-2 cell differentiation as well as incubation time on the intracellular betalain accumulation were preliminarily examined. Caco-2 cells at different passages were seeded in 10 cm petri dishes (2.5 × 10⁶ cells/dish). After 7, 14, and 21 days of cell seeding (i.e., 1st, 2nd, and 3rd week of differentiation), dextrin-coated betanin standard in medium (100 µg mL⁻¹) was applied to the cell layer, followed by 2 h and 4 h incubations which simulated the residence period of phytochemicals in the human intestinal lumen. Since the incubation environment (e.g., temperature, oxygen level) was considered a contributor to the betalain loss, as a control, pigment was also incubated in the absence of cells.

The betanin-containing medium was collected before and after incubation period for chemical analysis. The cell layer after incubation was washed and scraped into methanol (800 μ L). Cell pellets were subject to two rounds of methanol extraction via vigorous vortex (1 min), sonication (10 min, 5 $^{\circ}$ C), and centrifugation (2000 g, 5 min, 4 $^{\circ}$ C) (Tesoriere et al., 2013). The supernatants with pigment were combined, completely dried using the Genevac centrifugal evaporator, and reconstituted in water. Samples of medium and cell extracts were filtered through 0.2 μ m PTFE syringe filter for further LC-MS analysis. The detailed method of chromatography was stated in Section 2.1.3.

2.2.5 Cellular uptake and transport of betalains

2.2.5.1 Initial trials with dextrin-coated betanin standard

Intracellular accumulation and trans-epithelial transport of betalains were preliminarily investigated using the dextrin-coated betanin standard (*ca.* 1.63 mg betanin/g of standard) to optimise the window of experimental concentration and incubation duration. For the intracellular uptake, different passages of Caco-2 cells were incubated for 3 weeks until complete differentiation. The cell layer was subsequently treated with dextrin-coated betanin standard (50-200 μ g mL⁻¹), justified by the fact that proximal intestinal epithelium has a great chance of direct exposure to high concentration of betalains. After 3 h incubation, cell layer was washed and scraped into methanol for repeated betanin extraction, as described in Section 2.2.4.2. Supernatants were combined and filtered (0.2 μ m membranes) for the purpose of LC-MS analysis.

Trans-epithelial transport of dextrin-coated betanin (AP-to-BL) was investigated using differentiated Caco-2 cells in the 6-well plate transwell inserts (PET membrane, 0.4 μ m pore size, Sarstedt) at initial seeding density of 1×10^5 cells cm⁻². Complete DMEM was added in both apical (AP) and basolateral (BL) chambers (i.e., symmetric) and was replaced every 2-3 days. Cell membrane integrity was monitored weekly through TEER measurement across the growth area using electrodes and epithelial volt-ohm meter (Millicell ERS-2, Sigma-

Aldrich, U.K.) until full differentiation of the cells after 21 days; only the membranes with TEER > 3000 Ω cm² were selected for trans-epithelial experiment. During the experiment, a pH gradient was established by buffering the HBSS (Ca²⁺, Mg²⁺) in apical compartment to pH 6.5 using MES (2 mM), while basolateral HBSS to pH 7.5 (Deferme et al., 2008). After applying the dextrin-coated betanin standard (200-400 μ g mL⁻¹) to apical chamber, the basolateral solution was collected and replaced at 30 min interval until 3 h. TEER values were recorded at the beginning and the end of experiment to monitor the effect of compound to the membrane integrity. Eventually the collected basolateral solution was processed and submitted to LC-MS.

2.2.5.2 Intracellular accumulation and trans-epithelial transport of purified betalains

Based on the findings of preliminary tests, methods were improved for the subsequent availability assessment of purified betanin, vulgaxanthin I and indicaxanthin. Regarding the intracellular accumulation, differentiated Caco-2 cells were incubated with the three purified betalains (250 μ M) for 2 h in HBSS. In addition to betalain extraction, the accumulated pigment was normalised to the total protein content of lysed cells which was determined by BCA assay as described in Section 2.2.4.1.

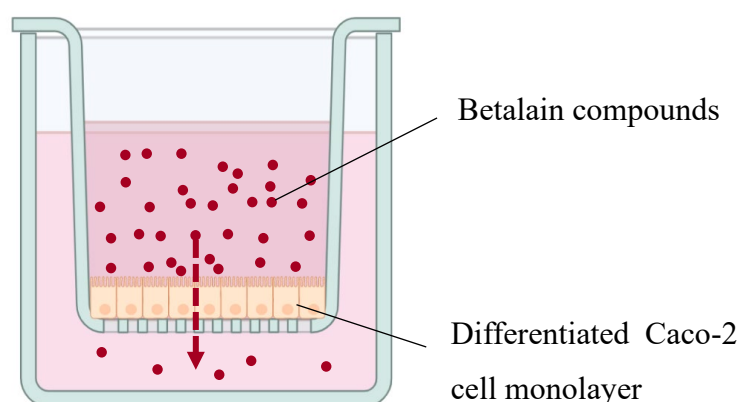


Figure 2-1. Schematic illustration of the *in vitro* transwell model for investigating intestinal betalain uptake.

The transport kinetics of purified betalains (AP-to-BL) was assessed via differentiated

Caco-2 cells in the 12-well plate transwell inserts. Resembling the method aforementioned in Section 2.2.5.1, the three purified betalains (420-1000 μM) were supplied to the apical chamber of transwells and incubated at 37 °C for up to 2 h (Fig. 2-1). Note the concentration ranges were compatible with the pigment level after dietary consumption of beetroot (Sawicki et al., 2018), and were tailored to the instrumental detection limits at the time. As a marker compound of paracellular transport, phenolsulfonaphtalein (5 mM) was also applied to parallel cell membranes in the apical chamber. Moreover, the chapter has evaluated the permeability of compounds through the Caco-2 membrane with a loosened tight junction in parallel wells which was achieved by 5 min incubation of differentiated membrane in trypsin EDTA (0.05%, v/v) before betalain treatment. The basolateral solution was collected and replaced every 30 min in the duration of 2 h, under initial rate conditions. All the betalain-containing solutions were processed and submitted to LC-MS analysis, while phenolsulfonaphtalein was quantified spectrophotometrically at 430 nm by Tecan Spark. The apparent permeability coefficients (P_{app}) were calculated as per Equation 2,

$$P_{\text{app}} = \frac{1}{C_0 \times A} \cdot \frac{V \times dC}{dt} \quad \text{Eqn.2}$$

where V = volume of basolateral solution, A = cell growth area, C_0 = initial concentration of apical betalain, and dC/dt = steady-state flux across the monolayer.

2.2.5.3 LC-MS analysis of cell culture samples

The betalain analysis method for cell culture experiments in Section 2.2.5 was improved from the basis of Section 2.1.3. The HPLC-MS (Shimadzu, 2020) was used again for analysis albeit with an alternative C18 column with smaller particle size (100 mm $\times$ 2.1 mm I.D., 3.5 μm , Phenomenex, U.K.) for a better chromatographic resolution. The chromatography was performed at the temperature of 35 °C, injection volume of 5 μL , and a constant flow rate of 130 $\mu\text{L min}^{-1}$. With the binary mobile phase of (A) 2% formic acid (v/v) and (B) methanol, the proportion of solvent B inclined from 5% to 25% (0-9 min) and to 70% (9-12 min) before returning to the initial 5% (13-16 min). Betacyanins and betaxanthins were detected at the wavelengths of 536 nm and 486 nm, respectively, and their molecular weights were monitored

by ESI-MS in a positive ion mode. Compounds were quantified using the standard curves established with purified betalains.

2.2.6 Immunoregulation of betalains in Caco-2 cells

Intestinal epithelial homeostasis is carefully equilibrated by a network of pro-inflammatory and anti-inflammatory signalling pathways. This could be subtly interfered by some redox-sensitive bioactives, such as polyphenols and betalains, in a dose-specific manner (Esatbeyoglu et al., 2014; Ferrari et al., 2016). The current project has examined the betalain effects on the inflammatory response of Caco-2 cells employing the technique of real-time quantitative polymerase chain reaction (qPCR).

2.2.6.1 Inflammatory stimulation of Caco-2 cells

Confluent Caco-2 cells were stimulated with a cocktail of cytokines comprised of tumour necrosis factor- α (TNF- α , 20 ng mL⁻¹), interleukin-1 β (IL-1 β , 40 ng mL⁻¹), interferon- γ (IFN- γ , 10 ng mL⁻¹) and lipopolysaccharide (LPS, 100 ng mL⁻¹) (Van De Walle et al., 2010) in the presence or absence of purified betanin, vulgaxanthin I and indicaxanthin treatments (5-80 μ M) for 6 h. The concentration range was in alignment with the literature which indicated the effectiveness of betanin and indicaxanthin in radical suppression and immunoregulation at lower concentration (Krajka-Kuzniak et al., 2013; Tesoriere et al., 2014). Cellular effects of red and yellow beetroot matrices were also examined by 6 h treatment with red and yellow beetroot extracts (from Section 2.1.4) at the concentration range of 5-400 μ g mL⁻¹. The negative medium control, cytokine-stimulated control and curcumin control (cytokines + 10 μ M curcumin) were established to validate the inflammatory stimulation and suppression induced by compounds.

2.2.6.2 Gene isolation and reverse transcription

Following the incubation, cells were washed with cold DPBS and lysed with TRIsure reagent (Scientific Laboratory Supplies Ltd.). Total RNA in TRIsure-lysed cell samples was

extracted using chloroform (1:5, v/v of TRIsure). After vortex and centrifugation (12000 g, 15 min, 4 °C), colourless supernatant was carefully collected; and the RNA in which was forced to precipitate by adding icy isopropanol (1:2, v/v of TRIsure), vigorous shaking, incubation (10 min, r.t.p) and centrifugation (12000 g, 10 min, 4 °C). After decanting the liquid, RNA pellets were washed with 75% ethanol (1:1, v/v of TRIsure) followed by vortex and centrifugation (9000 g, 5 min, 4 °C). RNA pellets were air-dried, resuspended in DEPC water and incubated in thermo-block (10 min, 60 °C) for better dissolving. Note the entire process was carried out using solvents in molecular biology grade under RNase/DNase-free conditions to minimise RNA degradation and contamination. The concentration and quality of RNA samples were determined using a NanoQuant plate of Tecan Spark plate reader at 260 and 280 nm.

Reverse transcription from mRNA into cDNA was achieved using the iScript™ cDNA Synthesis Kit (Bio-Rad). At 4°C, the iScript buffer and reverse transcriptase enzyme were added to the diluted samples containing 500 ng of RNA (20:5:75, v/v/v). Mixtures were mounted to a Techne 3Prime thermal cycler (Pennsylvania, U.S.) with programmed heating – 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, and finishing at 4 °C. The acquired cDNA samples were diluted by 20-fold for subsequent qPCR analysis.

2.2.6.3 Gene measurement of pro-inflammatory and antioxidant markers via qPCR

Transcription levels of target genes – interleukin 6 (IL-6), IL-8, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), NADPH oxidase 1 (NOX-1), NAD(P)H quinone dehydrogenase 1 (NQO-1), heme oxygenase 1 (HO-1), glutamate-cysteine ligase catalytic subunit (GCLC), glutathione peroxidase 1 (GPX-1), glutathione S-transferase A1 (GSTA-1) and GSTP-1 were evaluated using the real-time qPCR method. Primers of target genes were designed and blasted using the NCBI database, whose validity and specificity were confirmed by 2% agarose gel electrophoresis (Appendix 5A). In details, mini gel was casted after heating agarose powder in Tris-borate-EDTA (TBE) buffer (0.5 ×) (1:50, w/v) until fully dissolved and adding the SYBR Safe gel stain. The solidified gel was placed in a horizontal electric

chamber filled with TBE buffer. Meanwhile, the qPCR products of newly designed primers and the molecular ladder (25-700 bp) were loaded to individual lanes of gel with dye, followed by gel running at 100 V for 90 min. The bands in gel were eventually visualised in a Bio-rad ChemiDoc Imaging Systems (Bio-rad, California, U.S.) when the product sizes were evaluated according to the ladder. The primer sequences utilised in this chapter are presented in Table 2-1.

Table 2-1. Oligonucleotides of human primers used for qPCR in this study.

Marker	Forward sequence	Reverse sequence	Sequence ID
β -actin	5'- AGAGCTACGA GCTGCCTGAC -3'	5'- AGCACTGTGT TGGCGTACAG -3'	NM_001101.5
IL-6	5'- CGGCCACTCA CCTCTTCAGAA -3'	5'- GGCAAGTCTC CTCATTGAATCC -3'	NM_001371096.1
IL-8	5'- TGGAGAAGTTT TTGAAGAGGGCT -3'	5'- CAACAGACCCA CACAATACATGA -3'	NM_000584.4
COX-2	5'- ATTGACCAGA GCAGGCAGAT -3'	5'- CTCCACAGCA TCGATGTCAC -3'	NM_000963.4
iNOS	5'- ACAGCACATT CAGATCCCCA -3'	5'- GCCGAGATTT GAGCCTCATG -3'	NM_000625.4
NOX-1	5'- GTCTCTTCCT CACCGGATGG -3'	5'- CCCACTACAG ACTTGGGGTG -3'	NM_001271815.2
NQO-1	5'- AGAAAGGATG GGAGGTGGTG -3'	5'- ATATCACAAG GTCTGCGGCT -3'	NM_000903.3
HO-1	5'- CTTCTTCACC TTCCCCAACA -3'	5'- AGCTCCTGCA ACTCCTCAAA -3'	NM_002133.3
GCLC	5'- CAATGGGAAG GAAGGTGTGT -3'	5'- GCGATAAACT CCCTCATCCA -3'	NM_001498.4
GPX-1	5'- CCGGGACTAC ACCCAGATGA -3'	5'- CTTGGCGTTC TCCTGATGCC -3'	NM_000581.4
GSTA-1	5'- CTTCTGCCCCG TATGTCCACC -3'	5'- ACCAGATGAA TGTCAGCCCCG -3'	NM_001319059.2
GSTP-1	5'- GCGGGCAAGG ATGACTATGT -3'	5'- GTCAGCGAAG GAGATCTGGTC -3'	NM_000852.4

During qPCR, the cDNA samples were thoroughly mixed with forward and reverse primers of target genes, and the SybrGreen-based reagent (SensiFast SYBR Green, SLS Ltd.) (20:8:50, v/v/v). Following the enzymatic activation (95 °C, 2 min), mixtures were submitted to 40 thermal cycles (5 sec at 95 °C, 10 sec at 60 °C, 10 sec at 72 °C) using a StepOne Plus

Real-Time PCR System (Applied Biosystems, Nottingham, U.K.). The expression level of target mRNA was reflected by ΔC_T value that indicates the cycle number at which the fluorescence intensity of sample reaches the threshold. The calculation of target gene levels was based upon the Livak ($2^{-\Delta\Delta C_T}$) method with normalisation to the housekeeping gene, β -actin (Livak and Schmittgen, 2001); and the levels were expressed relative to that of cytokine-stimulated controls. Both mRNA and cDNA samples were stored at -80°C , and samples were maintained in ice bath throughout the experiments.

2.2.7 Radical scavenging assays of betalains

2.2.7.1 Quantification of intracellular ROS formation

The generation of reactive oxygen species (ROS) within Caco-2 cells was determined in 96-well plates with the probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA), which is intracellularly deacetylated and oxidised to highly fluorescent 2',7'-dichlorofluorescein (DCF) (Yokomizo and Moriwaki, 2006; Ng and Ooi, 2021). DCFH₂-DA (30 μM) dissolved in PBS with 1% FBS (v/v) was applied to the confluent Caco-2 cells and incubated for 40 min at 37°C . After washing, cells were treated with purified betanin, vulgaxanthin I and indicaxanthin (5-80 μM) for 1 h and subsequently stressed with H₂O₂ (200 μM) for a further hour. The plate was scanned using Tecan Spark with excitation λ of 485 nm and emission λ of 535 nm.

The protein content of each well was subsequently determined by SRB assay (Ng and Ooi, 2021). Briefly, the medium was removed, and the cells were washed with PBS and submerged with 50 μL of 0.004% SRB solution (w/v) in 10% trichloroacetic acid (w/v). Cells were incubated at room temperature for 15 min and washed with 1% acetic acid (v/v). After treating with Tris base (10 mM, 100 μL), cells were incubated for 5 min under shaking conditions. Finally, the plate was scanned using the Tecan plate reader with excitation λ of 540 nm and emission λ of 590 nm. Results of the ROS assay were normalised to the relative protein content of each well and expressed as the relative fluorescence intensity % (RFI) with respect to the value of positive control.

2.2.7.2 Electron paramagnetic resonance (EPR) and radical assays

The experiments below were performed by Dr. Vagkidis and Prof. Chechik at the University of York as part of a research collaboration. Electron paramagnetic resonance (EPR) with spin trapping was used to determine superoxide and DPPH radical scavenging abilities of betalains reflected by the reduction of relative signal strength. EPR spectra were recorded by a JEOL X320 spectrometer (X-band) in glass capillaries (I.D. 0.8 mm). The 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) assay was conducted by mixing ethanol (40 μL) with 30% aqueous ethanol solutions of DPPH (100 μM , 50 μL) and purified betalains (200 μM , 10 μL), providing the working concentration of betalains as 20 μM . Superoxide assay was carried out by mixing the following solutions in 30% aqueous ethanol (v/v) as solvent – hypoxanthine (1 mM, 55 μL), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (1 M, 15 μL) and deferoxamine (10 mM, 10 μL). Individual betalains (200 and 500 μM) or solvent control were subsequently added in a volume of 10 μL . The reaction was initiated by the addition of 2 U mL^{-1} xanthine oxidase (10 μL), achieving 20 and 50 μM as final concentrations of betalains in the mixture.

In both assays, acquisition of EPR spectra was started 1 min after reagent mixing. To improve the accuracy of measurements, signal intensity of the superoxide assay was determined in triplicate by fitting the experimental spectra to the simulated superoxide-DMPO adduct spectra.

2.2.8 Statistical analysis

Data analysis of cell culture experiments was mainly performed using Excel and Graphpad Prism 9.0. The data are displayed as mean values with standard error of mean (SEM) from at least three independent experiments. One-way and two-way analysis of variance (ANOVA) were combined with the Dunnett post-hoc test to determine the existence of significant differences between specific datasets at a confidence interval of 95%. Physicochemical parameters of pigments were estimated using Chem3D and ChemDraw Ultra 14 with AutoDock. Result visualisation was achieved using Graphpad Prism 9.0 and ChemDraw Ultra 14.

2.3 Intervention trials *in vivo* and analysis

2.3.1 Reagents and materials

The red beetroot concentrate (in 30 mL pouches) for human intervention trial was provided by Active Edge Nutrition Ltd. (Settle, U.K.), while the red beetroot powder for the pig trial was supplied by Wholefoods Ltd. (Ramsgate, U.K.). All the materials for sampling were purchased from Alpha Laboratories Ltd. (Eastleigh, U.K.), HyStool (Longniddry, U.K.), Sarstedt (Nümbrecht, Germany), and MedDX Solution Ltd. (Hereford, U.K.).

The purified betanin standard was obtained according to the separation and purification procedures described in Section 2.1.5 (Fernando et al., 2022). Strata-X polymeric SPE cartridges (200 mg, 6 mL) for sample processing were purchased from the Phenomenex Ltd. (Macclesfield, U.K.). QIAamp PowerFecal Pro DNA extraction kit and Gel Extraction Kit were obtained from Qiagen (Manchester, U.K.). The SCFA standards (i.e., acetic, propionic, (iso)butyric, (iso)valeric, and hexanoic acid) were purchased from Sigma-Aldrich (Dorset, U.K.). Organic solvents (i.e., methanol, formic acid, acetonitrile, methyl tert-butyl-ether) of LC-MS and UHPLC-MS grades for sample processing and chromatographic analysis were acquired from Fisher Scientific (Loughborough, U.K.) unless specified.

2.3.2 Human study and participant screening

The present human trial was approved by the University of Leeds Research Ethics Committee (AREA 20-058), for which procedures were carried out in compliance with the Human Tissue Act 2004. In addition, coronavirus-secure guidance of university was put in place during the pandemic. Screening of potential participants was performed with the following inclusion criteria – generally healthy, age between 20-50 years, body mass index (BMI) of 18-25 kg m⁻², and no regular consumption of beetroot-related food/supplements. Participants were excluded if they were intolerant or allergic to beetroot and derivative foods, tobacco users, diagnosed with diabetes, and/or diseases of digestive system/liver/kidney, diagnosed with hyper-/hypotension, having communicable health conditions, alcohol abusive, using pre-

/probiotic supplements or medication, pregnant or lactating. Eventually, 18 healthy participants (13 females, 5 males) were recruited from the University of Leeds; the number was in line with those in earlier studies focused on bioavailability and gut microbiome (Mullen et al., 2008; Wiczowski et al., 2018; Kaczmarek et al., 2019). All participants were fully informed of the study details via information sheet (Appendix 14) and their consent was obtained prior to trial initiation.

2.3.3 Intervention design of human study

Participants were invited onto the University campus to attend an initial visit and undergo anthropometric measurement. They were asked to perform the entire intervention and sampling procedures out of the campus at the premises of covid restriction guidelines, and were therefore provided with full guidance, explanation and instruments required for the study, including the instructions of beetroot drink preparation, urine and stool sampling, blood pressure monitoring and questionnaire completion. Participants were asked to complete the intervention by drinking daily portions of red beetroot juice for 14 consecutive days which contained betacyanins (114.5 ± 3.6 mg), polyphenols (15.6 ± 0.4 mg) and *ca.* 228.5 mg nitrate per portion. To determine the daily dose of beetroot juice, the study followed the manufacturer's suggestions for daily consumption of concentrated juice, and with reference to the literature (Wiczowski et al., 2018). In addition, the nitrate intake of this study complied with the acceptable daily intake (ADI) given by EU Food Safety Authority (3.7 mg/kg bw) to lower the risk of hypotension.

Timeline of the single trial was briefly illustrated in Fig. 2-2. Given the nature of beetroot juice, time restriction and challenge in recruitment, there was no control group for this pilot study. Post-intervention data of individuals on the gut microbiome and other features were compared to corresponding baseline values. Two weeks before the study initiation, participants were asked to abstain from consumption of betalain-containing food as per the food guide. After washout phase, they were asked to dilute and consume the concentrated red beetroot juice (30 mL into 250 mL) for 14 consecutive days. On the first day of intervention

(D1), participants, after fasting overnight, were requested to consume the whole daily portion of red beet juice with two pieces of white bread in one sitting. No other food or beverage was to be consumed in the following 3 hours except water if required for hydration. With acidification using ascorbic acid (*ca.* 1.5 mg mL⁻¹), 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 points was measured and recorded using graduated container. During the remainder of the intervention period, participants resumed their habitual diets while consuming the daily portion of beetroot juice with their lunch and dinner (2 × 125 mL). Following the instructions, stools were sampled by participants before the beetroot juice consumption (BSL), and after 3 days (D3) and 14 days (D14) of juice consumption for the purpose of microbiome classification and betalain characterisation. All the biological samples were immediately stored at -20 °C and transported to -80 °C freezer within two days of sampling for analytical purposes. The compliance and incidence related to juice consumption, sampling, and discomfort were monitored via questionnaires throughout the intervention.

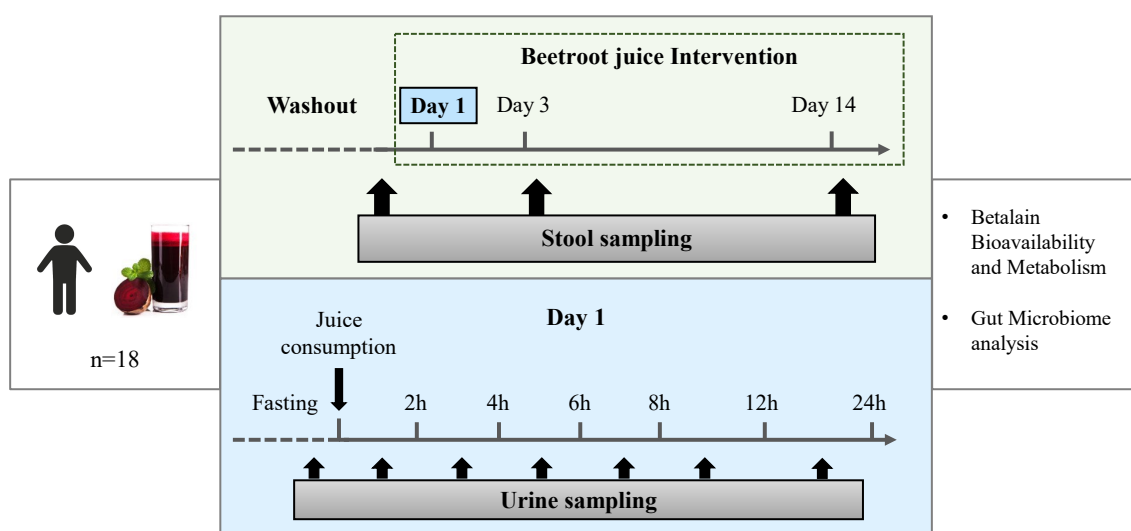


Figure 2-2. Schematic illustration of the design of human intervention study.

The food intake of participants on the day of urine sampling (D1) was recorded in detail via the food diary to report the food type, approximate amount and time of ingestion, meanwhile the diet pattern of individuals in the remaining days was evaluated via weekly food frequency questionnaires (FFQ). Throughout the intervention, participants were asked

to refrain from intense physical activity, as well as excessive intake of polyphenols, caffeine, and alcohol. The systolic and diastolic blood pressures (SBP & DBP) of participants were also self-measured each week to check on potential hypotensive effects of beetroot juice.

2.3.4 Intervention design of pig trial

Digesta samples from a previous intervention trial in weaned pigs were analysed (Adekolurejo et al., 2022, in revision). This animal trial was majorly performed by O. Adekolurejo, another member of the research group, on the pigs weaned at 28 days old (Large-White × Landrace × Duroc, 7.6 ± 0.7 kg). Study was approved by Animal Welfare and Ethical Review Committee of University of Leeds (N0. 070510HM), and the procedures complied with the Animal (Scientific Procedures) Act 1986. During a 14-day feeding trial, 36 piglets in total (12 pigs per group) were randomly assigned to three intervention groups – control (BSL), feed supplemented with 2% (RBR2) and 4% (w/w) of whole red beetroot powder (RBR4). It is noted that the diet was developed from the required nutritional formula, and total feed intake of each group was recorded daily. At the end of the feeding period, 7 pigs were excluded from the trial (2 pigs from each of BSL and RBR2 groups, 3 pigs from RBR4) because of abnormal weight loss and mortality, while the others were euthanised and dissected for sample collections by licensed technical staffs at the university farm. This project has only incorporated the analytical results of betacyanins in digesta samples from small intestine (i.e., the ileum) and from colon of individual pigs in the indicated groups in order to study the progressive transformation of pigments during intestinal digestion.

2.3.5 Chemical analysis of betacyanins in trial samples

2.3.5.1 Betalain and polyphenol analysis in beetroot juice

Native betalain compounds in the beetroot concentrate were analysed via HPLC-MS (Shimadzu, 2020) after dilution, centrifugation (20,000 g, 10 min, 4 °C) and filtration (0.2 µm membrane). Referring to Section 2.1.3, betalain separation was achieved by reverse phase

HPLC via C18 column (250 mm × 4.6 mm, I.D. 5 µm) with eluent system of (A) 2% formic acid (v/v) and (B) methanol. At discrete injection volume (10 µL), flow rate (0.95 mL min⁻¹) and temperature (40 °C), the proportion of solvent B inclined from 5% to 25% (0-15 min) and to 70% (15-19 min) before returning to the initial 5% (21-26 min). Betacyanins and betaxanthins were characterised based on the *m/z* values acquired from ESI-MS in the positive ion mode, and quantified based on chromatograms at wavelengths of 536 nm and 486 nm with standard curves generated from purified betalains. As per the juice analysis, the common betaxanthin in beetroot, vulgaxanthin I, was not detected in the specific batch of juice for human study which was therefore excluded from subsequent analysis.

The polyphenol content in beetroot juice was initially measured by Folin-Ciocalteu assay. However, outcome was found to be significantly interfered by intrinsic betalains, the potent antioxidants and colourant, resulting in an overestimation of polyphenol content (Fernando et al., 2022). Therefore, polyphenols in juice (10-fold dilution) were subsequently quantified by a Thermo-Vanquish ultra-high-performance LC (UHPLC) combined with TSQ Quantiva MS (Thermo Scientific, U.K.). The reverse-phase UHPLC was performed with a Kinetex XB-C18 column (100 mm × 2.1 mm, I.D. 2.6 µm, Phenomenex, U.K.) with binary mobile phases of (A) 0.5% formic acid (v/v) and (B) acetonitrile:water:formic acid (50:49.5:0.5, v/v/v). The fraction of solvent B was gradually inclined from 8 to 18% (0-1.1 min); to 32% (1.1-5.7 min); to 60% (5.7-8.9 min); to 100% (8.9-10.1 min), and was maintained for 1.3 min followed by reversion to 8% (11.4-12.5 min). Compound quantification and identification were achieved by MS in a negative ion mode with spray voltage of 2500 V. The calculation was performed using calibration curves with corresponding standards.

2.3.5.2 Betacyanin analysis in human urine samples

Urine samples from human study were aliquoted, centrifuged (3600 g, 8 min, 4 °C) and frozen at -80 °C, before being submitted to the Labconco freeze dryer (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 the 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 μ m, 200 mg, 6 mL) filled with modified co-polymer of styrene divinylbenzene. After conditioning and equilibrating the cartridge with methanol and water, respectively, the concentrated 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 the Genevac centrifugal evaporator (low boiling point mode, 1 h) followed by freeze drying (0.05 mmHg, -55 °C) to achieve complete removal of solvent. The solid samples were resuspended in water (100 μ L) and centrifuged (22000 g, 20 min, 4 °C) for chromatographic analysis.

Betacyanins and metabolites in concentrated urine samples were identified and quantified using the HPLC-MS (Shimadzu, 2020), with the method aforementioned in Section 2.3.5.1. Betacyanin compounds were characterised based on corresponding molecular masses determined by ESI-MS at positive ion mode with nebuliser gas flow rate of 1.5 L min⁻¹ and desolvation line temperature of 250 °C. 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.

2.3.5.3 Betacyanin analysis in human stool samples

The faecal mass (100 mg) from human trial was extracted twice in 0.5% formic acid (v/v) through horizontal vortex, sonication and centrifugation (20000 g, 8 min, 4 °C), from which the aqueous faecal suspensions (15%, w/v) were obtained. Betacyanins and corresponding derivatives were separated from the suspension matrix via the SPE method described in Section 2.3.5.2, albeit with an additional washing step at the end using methanol/formic acid solution (95:5, v/v). The eluents were subjected to the drying method as described in previous section to obtain solid samples. After resuspending in water (60 μ L), samples were centrifuged (22000 g, 10 min, 4 °C) and prepared for chromatography submission.

Pigments in the processed faecal samples were analysed by the UHPLC-MS which has higher sensitivity and resolution compared with HPLC-MS. Additionally, the eluent gradient was optimised from the HPLC method in Section 2.3.5.1 for more effective elution and separation of the metabolite compounds. Chromatography was performed with the Kinetex XB-C18 column (100 mm × 2.1 mm, I.D. 2.6 µm) using the same solvent phase as urine analysis (Section 2.3.5.2). At the flow rate of 200 µL min⁻¹ and injection volume of 5 µL, 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 declining 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, ion transfer temperature of 325 °C and vaporiser temperature of 275 °C. Quantification (in betanin equivalent) and characterisation of betacyanins were achieved by comparing the *m/z* values of precursor ions in positive ionisation as well as retention times with previously published data (Nemzer et al., 2011; Khan and Giridhar, 2015; Sawicki et al., 2020).

2.3.5.4 Betacyanin analysis in feed and pig digesta

Processing of feed and digesta samples from the pig trial resembled that of human faeces, which was performed by B. Wang (an MSc student) under the guidance. In brief, the feed of BSL, RBR2 and RBR4 groups (200 mg) and the pig digesta in ileum and colon segments (100 mg) were repeatedly extracted (2×) using water/methanol/formic acid solution (84.95/15/0.05, v/v/v), undergoing vortex, sonication and centrifugation (21000 g, 10 min, 4 °C). The feed extracts were filtered (0.2 µm membranes) for the submission to chromatography. In terms of digesta, the combined aqueous supernatant of each sample (*ca.* 960 µL) was subjected to the SPE processing and drying methods as described in the previous section. Finally the reconstituted digesta pigments were centrifuged and analysed using the UHPLC-MS method as mentioned in the previous section, except with a prolonged solvent gradient for an improved analytical resolution, 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). Identification and quantification of native and metabolised betacyanins were mentioned in the previous section.

2.3.5.5 Statistical analysis

Statistical analysis was performed using SPSS Statistics 26.0, Excel, and R-4.1.2. The urinary excretion data were analysed under non-compartmental model conditions and presented as mean with SEM. The cumulative renal excretion of betacyanins within a 24 h window was reflected by the area under renal excretion rate curve (AURC₀₋₂₄) 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 ANOVA combined with Dunnett post hoc test, as well as the non-parametric Wilcoxon rank test and 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, normalisation, Pearson and Spearman's rank correlation. Data visualisation was achieved on GraphPad Prism 9.0, R-4.1.2 and ChemDraw Ultra 14.

2.3.6 Human gut microbiome and SCFA analysis

2.3.6.1 DNA extraction from faecal samples

Faecal specimens from the human study were collected by participants at their respective residences at indicated time points (BSL, D3, D14) during intervention using stool collection bags (Longniddry, U.K.) and DNase/RNase-free tubes. Specimens were stored at -20 °C immediately after sampling and then transported within two days in freezer bags onto the campus for storage at -80 °C.

Following the manufacturer's instruction, microbial genomic DNA of faecal samples was extracted using QIAamp PowerFecal Pro DNA Kit. Briefly, faecal samples (*ca.* 200 mg)

were homogenised and lysed in sodium thiocyanate solution (800 μL , 1-10%, w/w) with the help of fine beads via vigorous horizontal vortex (15 min) and centrifugation (15000 g, 2 min, 20 °C). The supernatant containing nucleic acids was subsequently vortexed and centrifuged (15000 g, 2 min, 20 °C) in aluminium chloride hexahydrate solution (200 μL , 1-10%, w/w) to precipitate non-DNA organic and inorganic materials. Next, supernatant was combined with guanidinium thiocyanate solution (600 μL , 50-70%, w/w) to facilitate the adherence of DNA to a silica-based filter membrane in the subsequent stage, when all the lysate was forced to pass through the membrane via centrifugation (15000 g, 2 min, 20 °C). The DNA bound to the membrane was cleaned twice using ethanol-based liquid (500 $\mu\text{L} \times 2$), which was eventually released and eluted by Tris solution (75 μL , 10 mM). Note the entire process was performed under the RNase/DNase-free conditions, and DNA samples post acquisition were immediately preserved under -80 °C. The purity and quantity of total genomic DNA were determined via the NanoQuant plate of Tecan Spark plate reader (260 nm and 280 nm) and the fragment analyser system (5400 Agilent). Depending on the concentration of individual samples (300-560 ng μL^{-1}), the DNA was diluted to 1 ng μL^{-1} for further analytical procedures.

2.3.6.2 qPCR amplification and sequencing

The 16S rRNA gene (V3 and V4 regions) was amplified via qPCR technique with the specific primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). Thirty thermal cycles were performed at the conditions of 10 sec at 98 °C, followed by 30 sec at 50 °C, 30 sec at 72 °C, and 5 min extension at 72 °C. The quality of PCR products was determined by the performance of electrophoresis on 2% agarose gel, which were subsequently purified using Qiagen Gel Extraction Kit (Qiagen, Germany). The sequencing libraries were established via NEBNext® Ultra™ II DNA Library Prep Kit, which was followed by quality check via a Qubit 2.0 Fluorometer (Thermo Scientific, U.K.) and Agilent Bioanalyzer 2100 system. According to manufacturer's instruction, equimolar PCR products were pooled, end-repaired, tagged and ligated with Illumina adapters. Following the cluster generation, library sequencing process was performed on the Illumina NovaSeq PE250 platform by Novogene Co., Ltd. (Cambridge, U.K.), and the paired-end reads were

assigned to corresponding samples and cleaned for the purpose of statistical analysis.

2.3.6.3 Real-time qPCR of target bacterial species

Real-time qPCR was conducted on genomic DNA of faecal samples to determine the presence of specific bacteria whose primers are listed in Table 2-2. Primers of target species were based on relevant literature, with primer specificity verified by BLAST with NCBI database tool and PCR product size confirmed through 2% agarose gel electrophoresis (previously mentioned in Section 2.2.6.3) (Appendix 5B). Diluted genomic DNA samples were mixed with primers of target species and SybrGreen based reagent following the manufacturer's instruction (20:8:50, v/v/v), and real-time qPCR was performed via the StepOne Plus Real-Time PCR System with 40 thermal cycles of 2 min at 95 °C (enzyme activation), 5 sec at 95 °C, 10 sec at 60 °C, and 10 sec at 72 °C. The threshold cycle values (C_T) of target genes were normalised with corresponding universal gene levels followed by calculation based on the Livak ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001).

Table 2-2. Microbial primer sequences used for qPCR in the present study.

Marker species	Forward sequence	Reverse sequence	Reference
<i>Akkermansia muciniphila</i>	5'- CAGCACGTGA AGGTGGGGAC -3'	5'- CCTTGCGGTT GGCTTCAGAT -3'	Everard et al. (2013)
<i>Bifidobacterium_uc</i>	5'- TCGGGGTGAG TGTACCTTTC -3'	5'- AATTCCAGTC TCCCCTACCG -3'	Park et al. (2020)
<i>Bacteroides fragilis</i>	5'- CTGAACCAG CCAAGTAGCG -3'	5'- CCGCAAACCTTTC ACAACCTGACTTA-3'	Liu et al. (2003)
<i>Lactobacillus acidophilus</i>	5'- TCATGTTGGG ATGCAATGAG -3'	5'- TTTCAAAACT TGTCTGCTG -3'	You and Kim (2020)
<i>Escherichia coli</i>	5'- TGATTTCCGTG CGTCTGAATG -3'	5'- ATGCTGCCGT AGCGTGTTTC -3'	Molina et al. (2015)
<i>Lachnospiraceae_uc</i>	5'- AACAGAGGAG ACAGGTGGTG -3'	5'- GCTTCCCTTT GTTTACGCCA -3'	Park et al. (2020)
<i>Roseburia_uc</i>	5'- CGCAGCAAAC GCAATAAGTA -3'	5'- ACCACTGCTC CGAAGAGAAA -3'	Park et al. (2020)
Universal primers	5'- CGGTGAATA CGTTCYCGG -3'	5'- AAGGAGGTGA TCCRGCCGCA -3'	Suzuki et al. (2000)

2.3.6.4 SCFAs measurement

The analytical method was modified based on De Baere et al. (2013) and Diotallevi et al. (2022). Faecal specimens (100 mg) from human trial were suspended in water (12 %, w/v) and acidified with phosphoric acid (1.5%, v/v) (Sigma-Aldrich, Dorset, U.K.). SCFA content in the faecal suspension (100 µL) was obtained by liquid-liquid extraction twice using methyl tert-butyl ether (2 × 100 µL) through horizontal vortex (5 min) and centrifugation (20000 g, 10 min, 4 °C). The upper organic phase was collected, combined and stored at 4 °C for subsequent chromatographic analysis. The SCFA content in 5 µL of injected aliquot was determined by a combination of Shimadzu HPLC with SPD (Tokyo, Japan). Compounds were separated using Kinetex XB-C18 column (100 mm × 2.1 mm, I.D. 2.6 µm) with mobile phases of (A) NaH₂PO₄/H₃PO₄ buffer (5 mM) and (B) acetonitrile. At flow rate of 0.50 mL min⁻¹ and temperature of 30 °C, fraction of solvent B started from 0% (0-3 min), then was maintained at 30% (3-7 min) before regressing to 0% (7-10 min). SCFAs were quantified at wavelength of 210 nm using calibration curves generated from individual standard.

2.3.6.5 Statistical analysis

Statistical processing of bacterial taxa was performed in QIIME2 software, version 2021.2 (Bolyen et al., 2019). The chimeras-removed data from sequencing were denoised via DADA2 pipeline and annotated to taxa using the Naïve Bayes classifier trained on Greengenes 13_8 99% OTUs sequence (16S rRNA) (McDonald et al., 2012; Bokulich et al., 2018). With yielded information of corresponding abundance, alpha and beta-diversities were processed using QIIME2 software combined with *phyloseq* package in R-4.1.2 (McMurdie and Holmes, 2013), and visualised via R software and Graphpad Prism 9.0. The alpha-diversity indices between categorical variables were compared by non-parametric Kruskal–Wallis tests, while permutational MANOVA method was employed to analyse beta-diversity. Further analysis including differential abundance and Spearman’s correlation matrices was performed among selected variables and taxonomic data using SPSS software and the R packages, *DESeq2* and *MaAsLin2* (Love et al., 2014; Mallick et al., 2021). To lower the

chance of false positives, the differentially populated taxa among groups were evaluated by various models and hypothesis with 0.05 as the significant cut-off false discovery rate (FDR), i.e., Benjamini-Hochberg method. Data of qPCR and SCFAs were analysed and presented as mean with SEM using R, SPSS, and GraphPad Prism 9.0 software. Group comparisons were carried out by non-parametric Wilcoxon signed rank test and one/two-way analysis of variance (ANOVA) combined with Dunnett post hoc or Kruskal–Wallis tests, with significance level of $p < 0.05$.

Chapter III

Intestinal Uptake and Immunomodulation of Betalains towards Caco-2 cell model

3.1 Abstract

There is an emerging interest to betalain pigments owing to their radical-scavenging properties, yet the literature is overall limited regarding their availabilities and cellular effects. Following the preliminary test with dextrin-coated betanin standard, this chapter aims to compare the absorption and transport patterns of three purified betalains – betanin, vulgaxanthin I and indicaxanthin, into intestinal epithelial cells, and to assess their distinct molecular effects on inflammatory and redox-related cell signalling in association with their radical scavenging potencies. Results indicated overall low permeability of three purified betalains with P_{app} of $4.2\text{--}8.9 \times 10^{-7} \text{ cm s}^{-1}$. Higher absorption intensities of vulgaxanthin I and indicaxanthin may be attributed to their smaller molecular size and greater lipophilicity. Transport experiments also demonstrated the first-order kinetics and the absence of betalain biotransformation, which partially supported the hypothesis that passive diffusion is adopted by betalain compounds as a predominant transport route. Regarding the molecular effects, all three betalains showed anti-inflammatory effects ($5\text{--}80 \text{ }\mu\text{M}$), reflected by attenuated transcription of pro-inflammatory mediators such as COX-2 and iNOS. Concomitant augmentations of antioxidant enzymes such as HO-1 were observed but in betanin only. Moreover, betanin uniquely demonstrated a potent dose-dependent radical scavenging activity in EPR and cell-based assays. In conclusion, betanin, vulgaxanthin I and indicaxanthin have differentially contributed to lowering the inflammatory markers and mitigating oxidative stress, implying the potential to ameliorate inflammatory intestinal disease. Compared with two betaxanthins, the greater efficacy of betanin in scavenging radical and promoting antioxidant response might, to some extent, compensate for its poorer absorption efficiency as demonstrated by the differentiated Caco-2 cell model. However, further research is warranted to establish the efficacy of betalains *in vivo*.

3.2 Introduction

Betalain group are well-known food colorants that are mostly derived from plant members of the *Caryophyllales* order, such as beet, cacti, and amaranth. With approximately 75 betalain components characterised from nature to date, their content and composition vary across different plant species and varieties (Khan and Giridhar, 2015). Betalains are categorised into red-violet betacyanins (e.g., betanin, phyllocactin) and yellow-orange betaxanthins (e.g., vulgaxanthin, indicaxanthin), which are determined by the condensation between core carbonyl structure (i.e., betalamic acid) and specific amine moieties (Fig. 3-1). The stability of pigments is maintained at pH 4-7, low temperature (*ca.* 5 °C), darkness, and absence of oxygen, given their susceptibility to a variety of decompositions such as Schiff base hydrolysis and decarboxylation (Watts et al., 1993; Elbandy and Abdelfadeil, 2008; Khan, 2016b).

Radical scavenging and anti-inflammatory activities of some abundant betalains, such as betanin and indicaxanthin, are increasingly highlighted in the scientific literature (Tesoriere et al., 2004a; Belhadj Slimen et al., 2017; Gómez-Maqueo et al., 2019; Gómez-Maqueo et al., 2020). The inflammatory gene expression is largely driven by the transcription factor nuclear factor- κ B (Nf- κ B). Upon stimulation by inflammatory triggers, phosphorylation of inhibitor subunit, I κ B α , is catalysed by I κ B kinase, which enables the accumulation of Nf- κ B dimer in nucleus and binding to respective DNA sequences. This eventually leads to an upregulation of pro-inflammatory cytokines and enzymes, such as COX-2 and iNOS (Pasparakis, 2009; Wardyn et al., 2015). Tan et al. (2015) have demonstrated alleviation of paraquat-induced renal inflammation following betanin administration to rats, attributed to the inhibition of NF- κ B signalling. Allegra et al. (2014) further reported strong suppression of COX-2 (by 88%) and iNOS (75%) following indicaxanthin treatment in a rat model of carrageenan-induced pleurisy. Meanwhile, betanin potentially enhances the cellular defence system via suppressing the oxidant by-products of inflammatory metabolism (e.g., nitric oxide) and interacting with nuclear factor-erythroid-2-related factor 2 (Nrf2), hence promoting cellular

synthesis of phase II detoxifying enzymes that facilitate the maintenance of cellular redox balance (Krajka-Kuzniak et al., 2013). The transcription factor, Nrf2 is a redox sensor orchestrating expression of genes related to the cellular defence system. In the presence of electrophiles and/or bioactives, Nrf2 dissociates from its binding to Keap1 (Kelch-like ECH-associated protein 1), allowing the release and translocation of Nrf2 to the nucleolus. The Nrf2 dimer subsequently binds to antioxidant response element sequences (ARE) in the promoter regions of target genes, increasing the transcription of cell-protective enzymes such as NQO-1 and HO-1 (Jaramillo and Zhang, 2013; Loboda et al., 2016). The subtle regulation of downstream targets of Nf- κ B and Nrf2 signalling by specific betalains, therefore, revealed their influence on the cellular immune response and redox status.

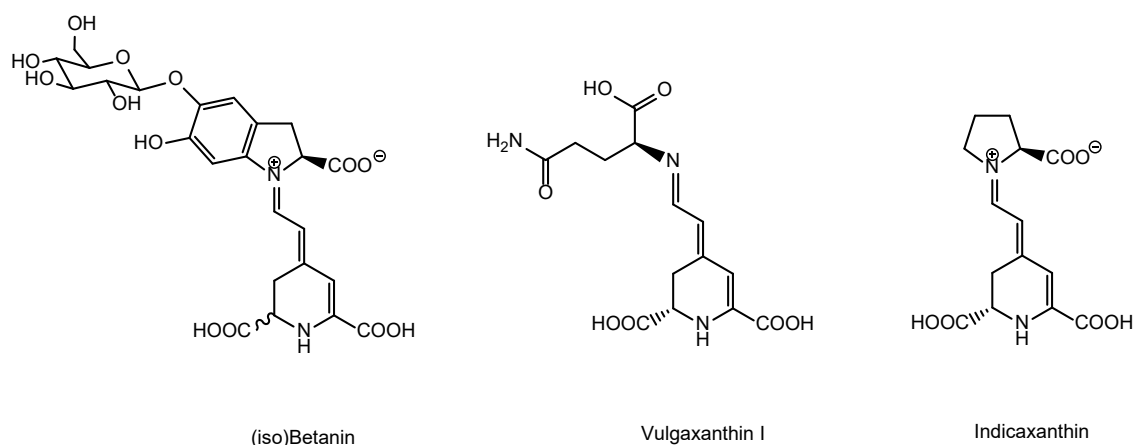


Figure 3-1. Chemical structures of betanin (BET), vulgaxanthin I (VUL) and indicaxanthin (IND).

Resembling betalain group, the anti-inflammatory effect of polyphenols has also been reported by recent studies on the inflamed intestinal Caco-2 cells via the modulation of pro-inflammatory transcription factors and enzymes (Romier et al., 2008; Speciale et al., 2022). It implies the potential contribution of polyphenols in mitigating inflammatory gut disorders, such as inflammatory bowel disease (IBD). Of note, IBD is characterised by the chronic dysregulation of the intestinal immune system associating with an elevated risk of colorectal cancer (Bernstein and Forbes, 2017). Equally pivotal is enhancing the knowledge of inflammation and redox-related cellular effects of betalain molecules, which were examined in this chapter on the cytokine-stimulated intestinal epithelium. In addition, the mitigation in

lipid peroxidation, anti-hyperglycaemia, anti-tumour and hepato-/neuro-/cardio-protection imparted by betalains were also noted by some evidence from *in vivo* animal and human studies, indicating their potential to deliver a variety of health functions (Khan, 2016a; Lechner and Stoner, 2019; Montiel-Sánchez et al., 2021). Nonetheless, betanin and indicaxanthin, as two prevalent compounds, received the greatest attention in the literature, with other members of the betalain family being scarcely explored, such as vulgaxanthin I.

Despite all the positive physiological effects, the bioavailability of betalains is considered a prerequisite to their overall health benefits *in vivo*. Note that bioavailability is commonly defined as the fraction of ingested component that enters the systemic circulation while maintaining the bioactivity at a specific site (Verhoeckx et al., 2015; Akbar et al., 2018). Evidenced by the presence of native and metabolic betacyanins in physiological fluids (Sawicki et al., 2018; Wiczowski et al., 2018), the plasma and renal pharmacokinetic data from human and animal studies show that betanin is inefficiently absorbed and excreted in the urine (urinary recovery of 0.2-3%) (Tesoriere et al., 2004a; Takahashi et al., 2017; Sawicki et al., 2020). For *in vitro* research, the differentiated Caco-2 cell model is widely employed in the realms of pharmacy and nutrition as a simulation of the human intestinal epithelial barrier for assessing the uptake and transport of bioactives. Using this model, Tesoriere et al. (2013) suggested that betanin and indicaxanthin were absorbed predominantly via paracellular passive diffusion at the intestinal epithelia. Yet overall, the research into the betalain availability and transport are still in the early stages compared to phenolic phytochemicals such as anthocyanins (O'Sullivan et al., 2009; Hithamani et al., 2017; Ahmed et al., 2020).

3.3 Aims

The current research aimed to –

- Carry out an *in vitro* investigation of the intracellular accumulation and transport kinetics of three betalains – betanin (BET), vulgaxanthin I (VUL) and indicaxanthin

(IND), via the differentiated Caco-2 monolayer model.

- Compare the potency of three abundant betalains towards radical scavenging as well as the attenuation of inflammation and oxidative stress in intestinal Caco-2 cells via immunomodulation.

3.4 Method

Outcomes of the current chapter were entirely obtained from the *in vitro* cell culture experiments (Section 2.2). Specifically, following the Caco-2 cell preparation and cytotoxicity tests, the cellular absorption and transport of dextrin-coated and purified betalains were examined via cell membranes in a transwell model. The immunomodulatory effects of purified betalains and crude beetroot extracts were investigated on the cytokine-stimulated cells followed by qPCR performance. Radical scavenging assays incorporated the intracellular ROS measurement and EPR-monitored radical quenching reactions. Results of preliminary tests in Section 2.2.4 were presented in the Appendix as specified in the previous chapter. Of note, the purities of BET, VUL and IND for cell experiment use have reached 97%, 79% and 95%, respectively; the chromatogram of which is displayed in Fig. 3-2.

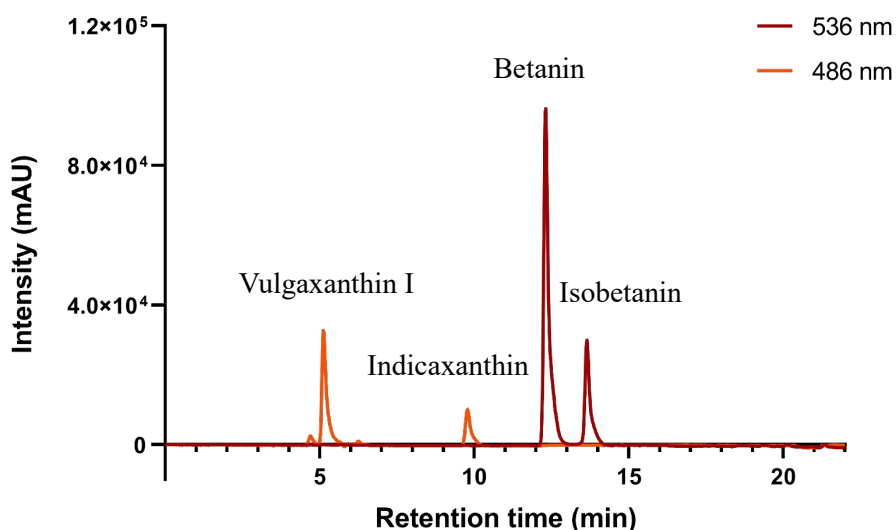


Figure 3-2. Spectrophotometric chromatograph of purified betalain compounds at the detection wavelengths of 536 nm and 486 nm.

3.5 Results

3.5.1 Intracellular accumulation and trans-epithelial transport of dextrin-coated betanin

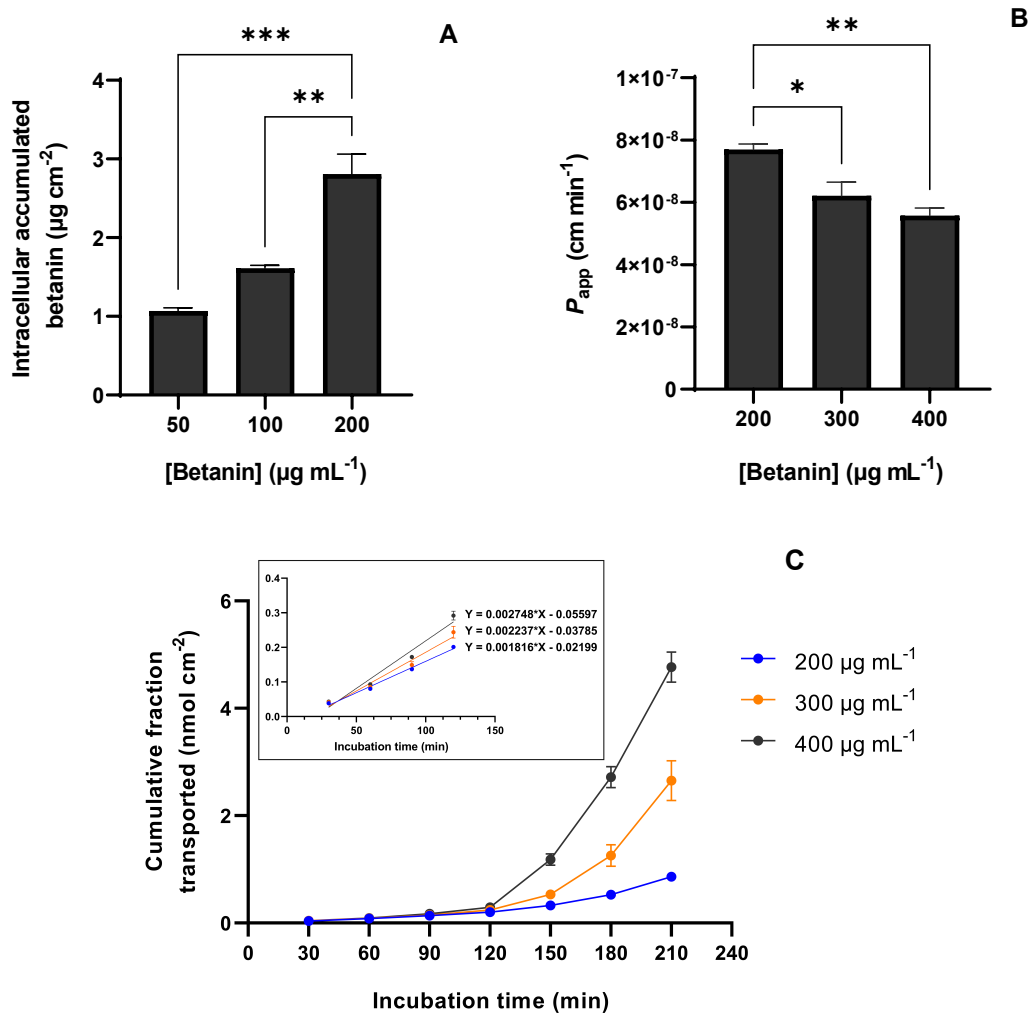


Figure 3-3. Cellular accumulation and trans-epithelial transport of dextrin-coated betanin standard (BS) across fully differentiated Caco-2 cell membrane, illustrated by (A) intracellular concentrations over 3 h incubation period, (B) apparent permeability coefficient (P_{app}) and (C) transport kinetics at increasing apical concentrations of BS, with linear region of transport being displayed at the upper-left corner. Values are mean with SEM of 3 independent cell experiments; several error bars were not observed due to their small magnitude. Significant differences in (A) and (B) are shown as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The influence of betanin treatment (dextrin-coated) on Caco-2 cell viability was preliminarily assessed by MTT and neutral red assays. Compared with the cell viability of negative control (100%) and DMSO positive control ($69.0 \pm 9.8\%$), viability after treatment of dextrin-coated betanin ($0\text{--}400\text{ }\mu\text{g mL}^{-1}$) fluctuated mildly between 92% and 105% with desired reproducibility, suggesting a minor cytotoxicity of the compound towards Caco-2 cells at the specific concentration range and incubation duration (3 h).

The intracellular accumulation and transport kinetics of dextrin-coated betanin standard were examined at increasing concentrations as shown in Fig. 3-3. Results indicated a dose dependent uptake of betanin by Caco-2 cells at the apical concentration of $50\text{--}200\text{ }\mu\text{g mL}^{-1}$, although the linear proportionality was not exactly followed. The transport kinetics of betanin in Fig. 3-3C displayed a linearity in transported amount before incubation time of 120 min (i.e., constant transport rate) followed by an exponential increment (i.e., rising transport rate). This was explained by the gradually impaired cell membrane integrity during compound treatment proved by the significantly lowered TEER values from $3749\text{ }\Omega\text{ cm}^2$ (0 min), to $1814\text{ }\Omega\text{ cm}^2$ (120 min), and to $981\text{ }\Omega\text{ cm}^2$ (210 min). Focussing on the linear region of kinetics, transport rate of betanin showed a dose-dependent increase at the concentration range of $200\text{--}400\text{ }\mu\text{g mL}^{-1}$, which was fitted to the passive diffusion model with non-saturable character. However, it was interesting to find an inverse relationship between compound permeability and applied concentration (Fig. 3-3B), which contradicted the results of cell experiments using purified betalains in the next section; the reason of which will be proposed in the Discussion. Henceforward, the investigation scope was broadened to different betalain components thanks to the pigment purification work achieved by Fernando et al. (2022).

3.5.2 Intracellular accumulation and trans-epithelial transport of purified betalains

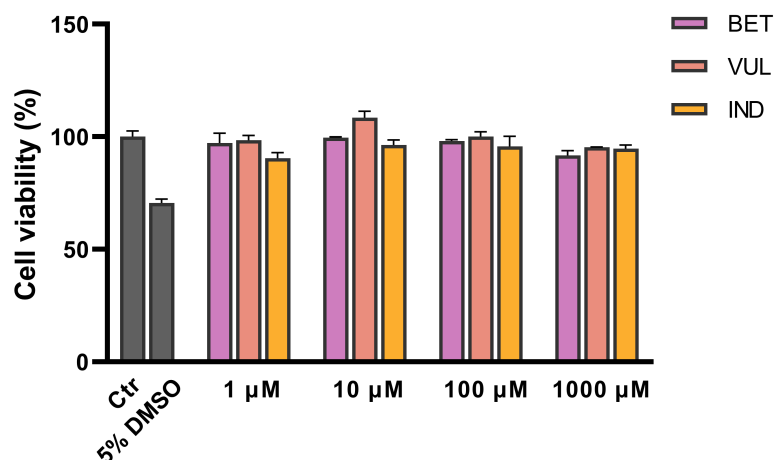


Figure 3-4. Detection of Caco-2 cell viability (%) using MTT assay after treatment with increasing concentrations of purified betalains. Data were presented as mean with SEM of triplicates from independent cell passages.

Resembling the dextrin-coated betanin, the MTT and neutral red assays indicate negligible cytotoxicity of three purified betalain compounds (1-1000 μM) on the employed Caco-2 cell line within 3 h incubation (Fig. 3-4). The results of uptake experiments using differentiated Caco-2 cells (Fig. 3-5) indicate the intracellular accumulation of three purified betalains after 2 h incubation – 0.02 nmol of BET, and 0.03 nmol of VUL and IND per mg of protein. The amounts of VUL and IND incorporated into Caco-2 cells were significantly higher compared to BET, whereas no difference was observed between absorptions of two betaxanthins.

Transport kinetics (AP-to-BL) of purified BET, VUL and IND was monitored over the 2 h incubation period with concentrations ranging from 420-1000 μM . The TEER values determined initially ($1634 \Omega \text{ cm}^2$) and post-incubation ($1556 \Omega \text{ cm}^2$) did not remarkably differ, confirming the integrity of Caco-2 monolayer during the transport experiments. The divergence in P_{app} among betalains was illustrated between different betalains along increasing concentration (Fig. 3-5B). After 2 h incubation with individual betalains (1 mM) in the apical chamber, P_{app} values of BET, VUL and IND in the absorptive direction were obtained as $4.15 \pm 0.18 \times 10^{-7} \text{ cm s}^{-1}$, $6.49 \pm 0.21 \times 10^{-7} \text{ cm s}^{-1}$ and $8.93 \pm 0.52 \times 10^{-7} \text{ cm s}^{-1}$, respectively. The same order was followed at each concentration with significant

difference between three betalains ($p < 0.05$). The poor permeability of three purified betalains was markedly boosted after loosening the tight junction of the Caco-2 membrane (Fig. 3-5C), resembling the behaviour of phenolsulfonaphtalein as a paracellular indicator.

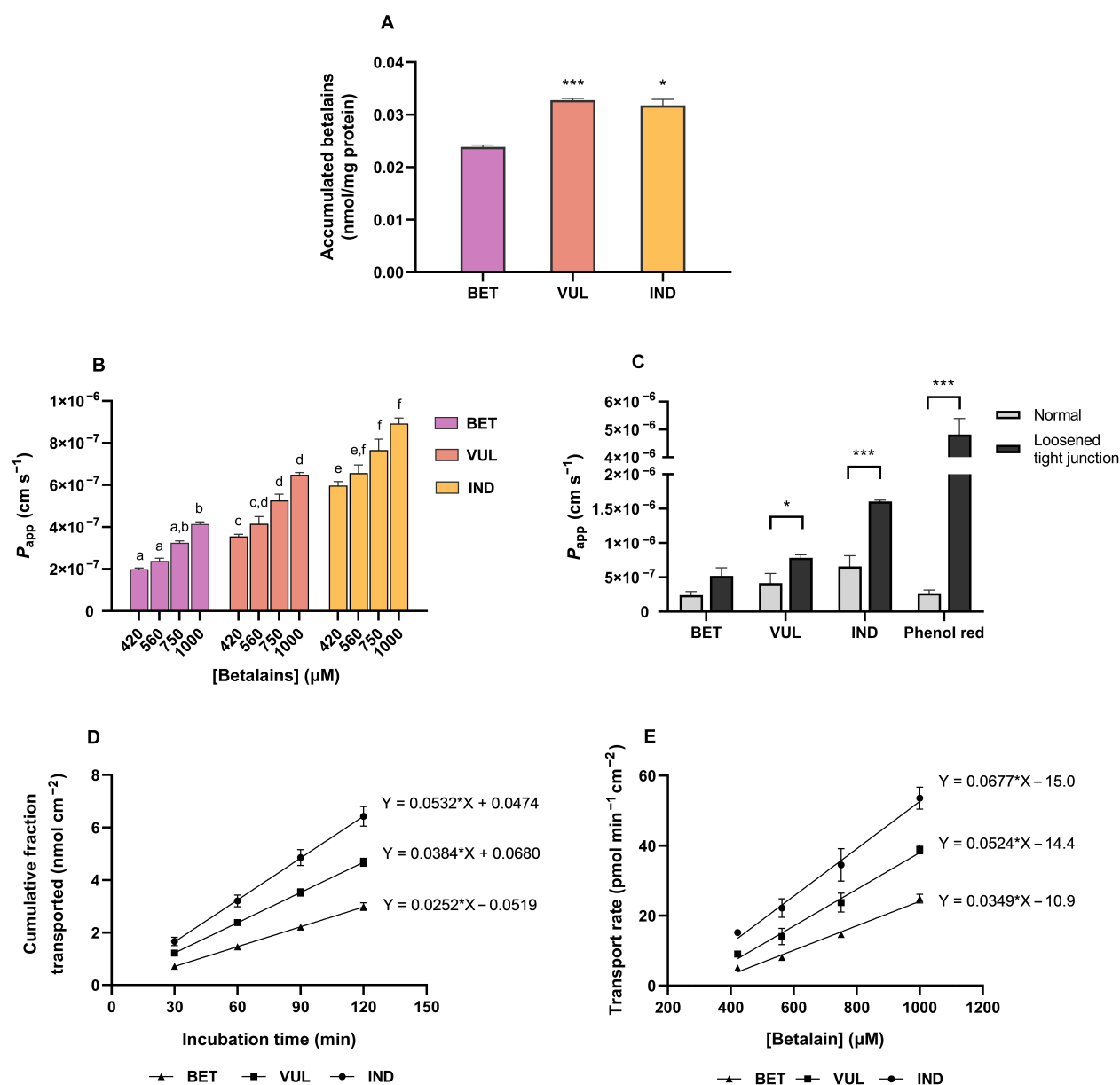


Figure 3-5. Uptake and trans-epithelial transport of purified BET, VUL and IND across Caco-2 intestinal membrane over 2 h incubation period, illustrated by (A) intracellular concentration, (B) P_{app} at normal condition, (C) P_{app} across the membranes with normal vs. loosened tight junction, (D) transport kinetics at apical betalain concentration of 1 mM, and (E) dose-dependent transport rate of three betalains. Values are mean with SEM of 3-4 independent experiments. Significant differences are shown in (A, C) as * $p < 0.05$ and *** $p < 0.001$ comparing betalains to BET (A)

and against controls (C). Inter-compound and inter-concentration differences in (B) are demonstrated as different letters ($p < 0.05$).

Transport kinetics of tested betalains was demonstrated as a function of time and concentration in Fig. 3-5D and E. The proportions of transported BET, VUL and IND within 2 h occupied 1.0%, 1.7% and 2.2% of total amounts applied, respectively, confirming the poor permeability of three betalains tested in the Caco-2 model. Regardless of concentrations, IND displayed the highest transport amount hence transport rate while BET showed the lowest; the order was also observed in the P_{app} of betalains (Fig. 3-5B). The increasing linearity in Fig. 3-5D has indicated a constant transport rate of betalains (AP-to-BL) over time at each concentration. In addition, the transport rate exhibited a first-order relationship with concentration, reflecting non-saturable kinetics of betalain permeation under the experimental conditions (Fig. 3-5E). During the transport process, there was no evidence of enzymatic modulation nor metabolic biotransformation.

3.5.3 Modulation of NF- κ B target cytokines and enzymes by purified betalains

This study has employed IL-1 β combined with TNF- α as the major inducers for the acute inflammatory response in Caco-2 cells, which is of relevance for intestinal inflammatory conditions such as IBD. Compared to the medium control, the generation of IL-6 and IL-8 was markedly augmented after 6 h exposure to the stimulatory cocktail of cytokines. This activation, however, could be strongly suppressed by co-treatment with curcumin (10 μ M), a strong antioxidant and anti-inflammatory compound served as a positive control (not shown). Before treating Caco-2 cells with betalain compounds, their impact on cellular viability was evaluated by the MTT cytotoxicity assay. Cell viability remained above 90% after 6 h incubation with all betalains at the concentration range of 1-100 μ M, suggesting a negligible negative impact of target compounds on cells.

As shown in Fig. 3-6A and B, the incubation with BET, VUL and IND (5-80 μ M) led to significant downregulation of IL-6 and IL-8 expression by up to 50% and 39%, respectively, in comparison to cytokine control (SC). IND efficiently repressed IL-6 expression at all

concentrations, while VUL was the most effective in downregulating IL-8. All three betalains have potently inhibited the expression of both markers at 5 μ M, whereas suppression by VUL appeared to be remarkably stronger at 80 μ M compared to BET and IND ($p < 0.05$).

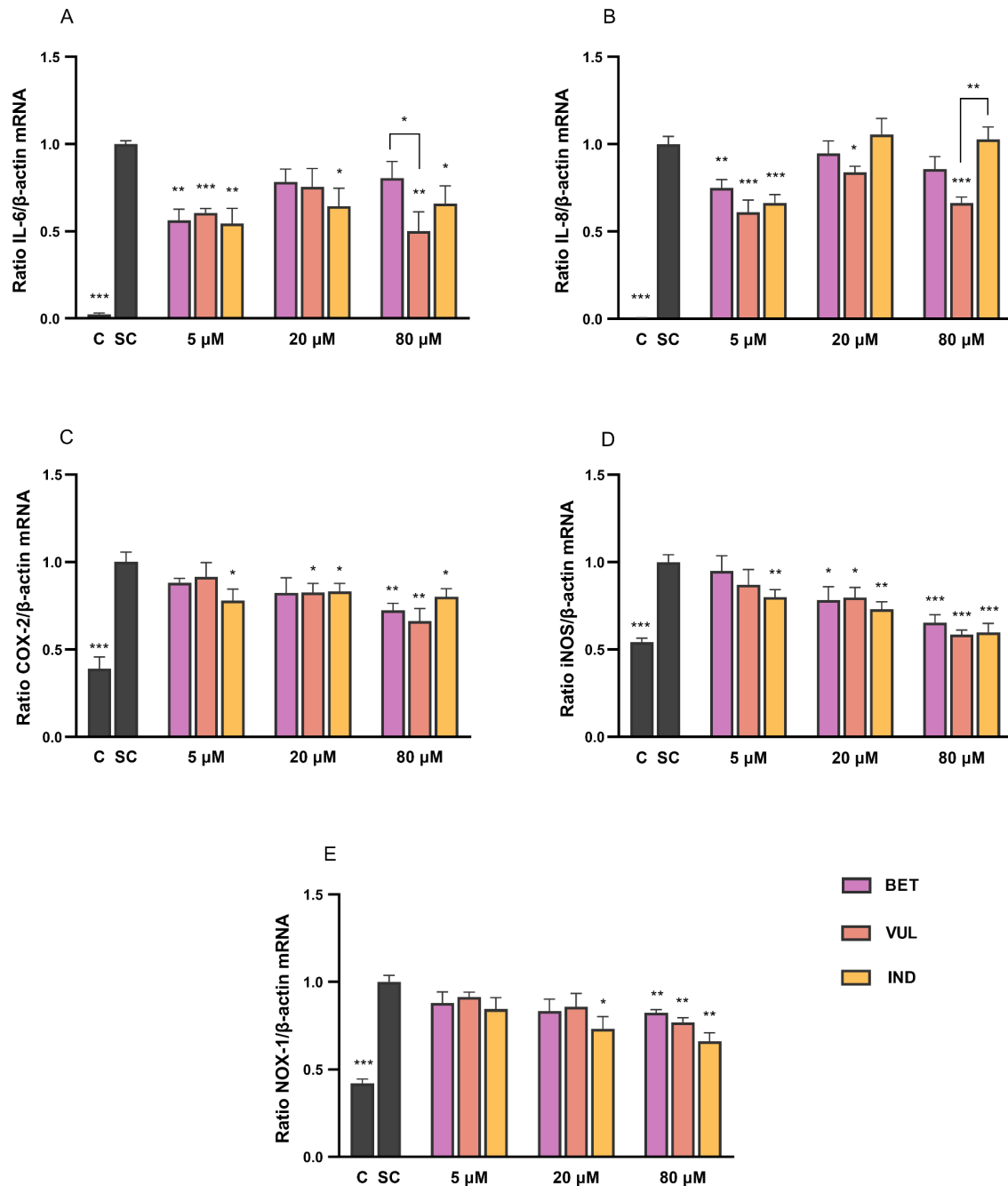


Figure 3-6. Effects of BET, VUL and IND (5, 20 and 80 μ M) on mRNA levels of (A) IL-6, (B) IL-8, (C) COX-2, (D) iNOS and (E) NOX-1 following 6 h cytokine stimulation in Caco-2 cells. C and SC refer to negative medium control and cytokine-stimulated control, respectively. Data are mean with SEM of 3 replicates from independent cell experiments. Non-bridged asterisks indicate significant difference of treatment vs. SC: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As target genes of the NF- κ B signalling pathway, the expression of COX-2, iNOS and NOX-1 were effectively enhanced following the stimulation with IL-1 β and TNF- α cytokines in this study. According to Fig. 3-6C, D, and E, the co-treatment of stimulated cells with BET, VUL and IND (5-80 μ M) has resulted in up to 34% attenuation of COX-2 and NOX-1 mRNA levels, with a greater impact on iNOS (< 42% reduction). The three betalains have featured a dose-dependent inhibition to all target genes except IND to COX-2 expression which, given the absence of dose dependency on IL-6 and IL-8 expression, implied the modulation of pro-inflammatory markers by betalains to be gene-specific. In addition, the two-factor ANOVA analysis suggested the similarity in the variation tendency of gene expression of COX-2, iNOS and NOX-1 as a function of betalain treatments ($p > 0.05$); and at each concentration, their downregulations induced by different betalains were comparable.

3.5.4 Effects of purified betalains on the expression of Nrf2 target enzymes

With the exception of GSTA-1, the expression of the majority of Nrf2 target genes was not remarkably altered in response to cytokine stimulation in this study. Instead, they could be boosted by twofold with the co-treatment of curcumin (10 μ M) as a positive control (not shown). The results of betalain treatment are illustrated in Fig. 3-7, BET has dose-dependently upregulated the expression of HO-1 (< 24%), NQO-1 (< 24%), GCLC (< 33%), GPX-1 (< 23%) and GSTP-1 (< 24%). Meanwhile, the increments of GCLC (< 25%) and GPX-1 (< 26%) were observed after incubation in different concentrations of IND. As the GST isozymes, the modulation of GSTA-1 by betalains was more evident in comparison to GSTP-1 with dose independence. Even at the lowest concentration (5 μ M), all three compounds could compensate for the cytokine-induced downregulation of GSTA-1 although the effect became milder at 20 μ M (Fig. 3-7E). When comparing to BET (< 51%) and IND (< 50%), VUL generally showed more efficient induction of GSTA-1 (< 86%) within the concentration range. With the exception of GSTA-1, the augmentation of selected targets, i.e., potential cytoprotective effect, was mostly observed at 80 μ M of BET.

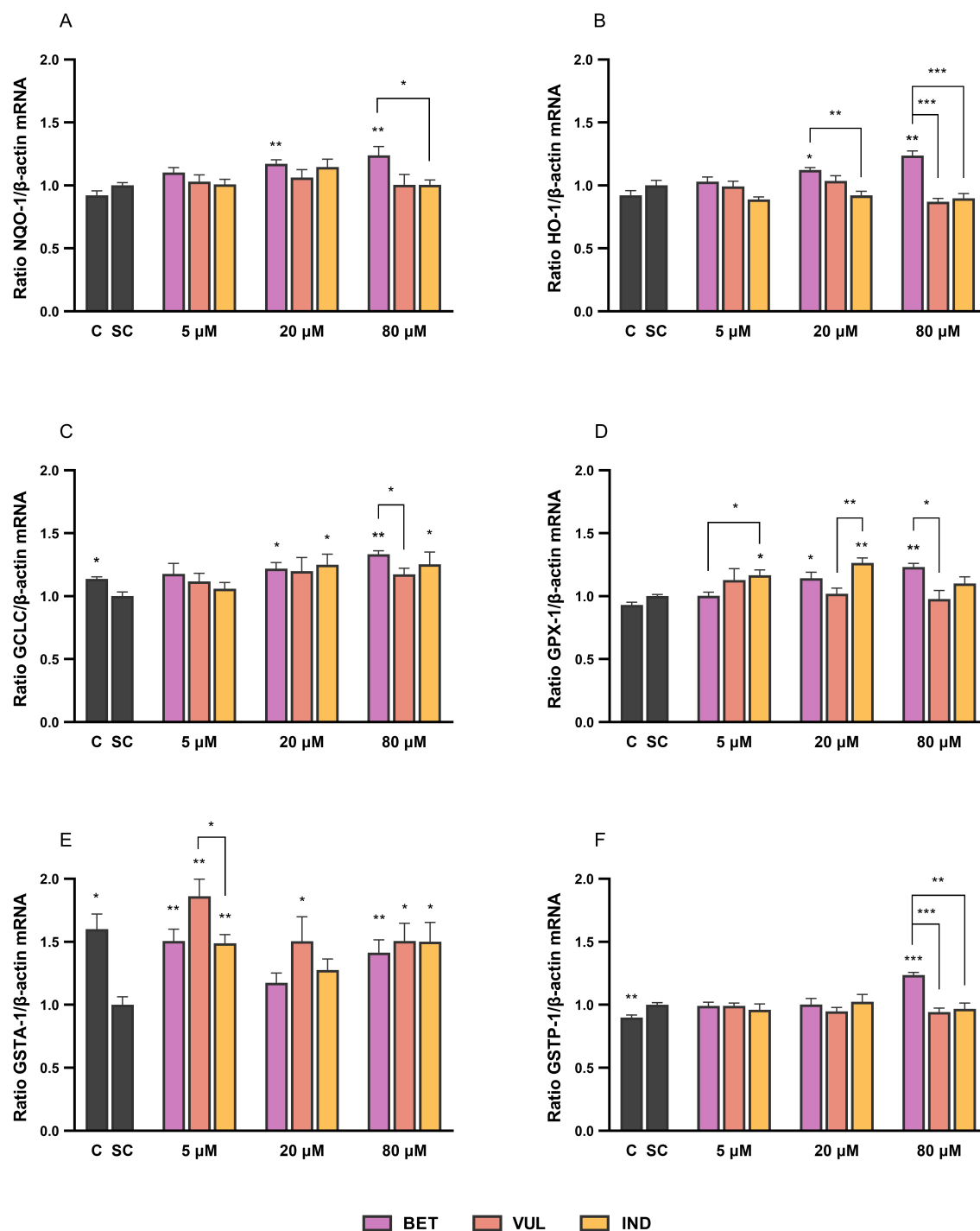


Figure 3-7. Effects of BET, VUL and IND (5, 20 and 80 μ M) on mRNA levels of (A) NQO-1, (B) HO-1, (C) GCLC, (D) GPX-1, (E) GSTA-1 and (F) GSTP-1 following 6 h cytokine stimulation in Caco-2 cells. C and SC refer to negative medium control and cytokine-stimulated control, respectively. Data are mean with SEM of 3 replicates from independent cell experiments. Non-bridged asterisks indicate significant difference of treatment vs. SC: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5.5 Gene modulation by red and yellow beetroot extracts

In Fig. 3-8, inflammatory stimulation of Caco-2 cells has increased the COX-2 mRNA level by 2-fold, whereas prior incubation with curcumin has effectively attenuated the inflammatory response by 60% and augmented GCLC expression by 117% (not shown). Under stimulated condition, both RBR and YBR treatments (10-400 $\mu\text{g mL}^{-1}$) resulted in a dose-dependent downregulation of COX-2 gene expression ($< 73\%$). On the other hand, it was surprisingly discovered that expression level of GCLC was also reduced at higher concentration of beetroot extracts ($< 67\%$) despite the absence of dose dependency. At the same concentration, there was no marked difference between the modulations of RBR and YBR towards both markers.

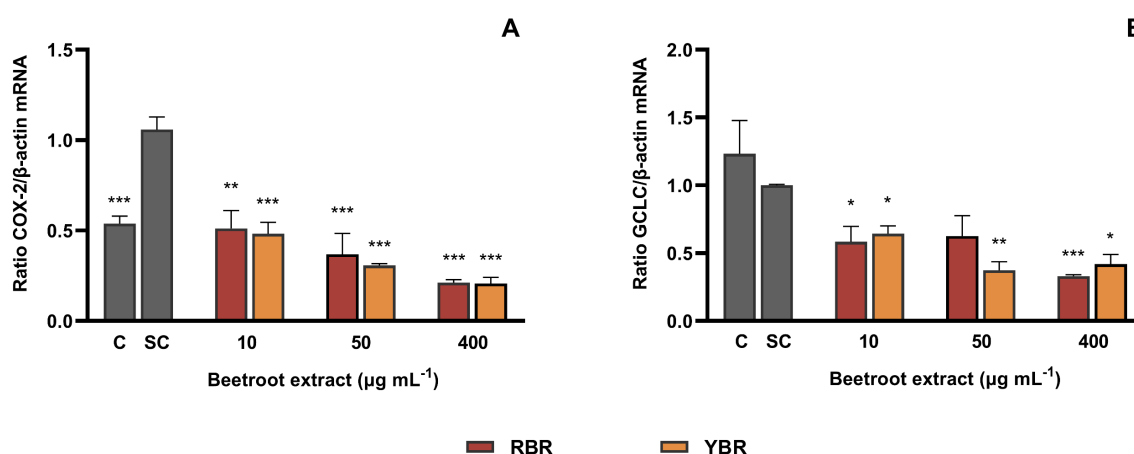


Figure 3-8. Effects of red beetroot (RBR) and yellow beetroot (YBR) extracts on the mRNA levels of (A) COX-2 and (B) GCLC in stimulated Caco-2 cell line. C and SC refer to negative medium control and cytokine-stimulated control, respectively. Data are mean with SEM of 2-4 replicates from independent cell experiments. Asterisks indicate significant difference of treatment vs. SC: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

This experiment was contributed in part by Y.Y. Huan (MSc student) with the guidance. The influence of beetroot extracts on other target genes (e.g., iNOS, HO-1) were also assessed but with fewer replicates due to restrictions in time and materials.

3.5.6 Effects on intracellular oxidative stress and radical scavenging activities

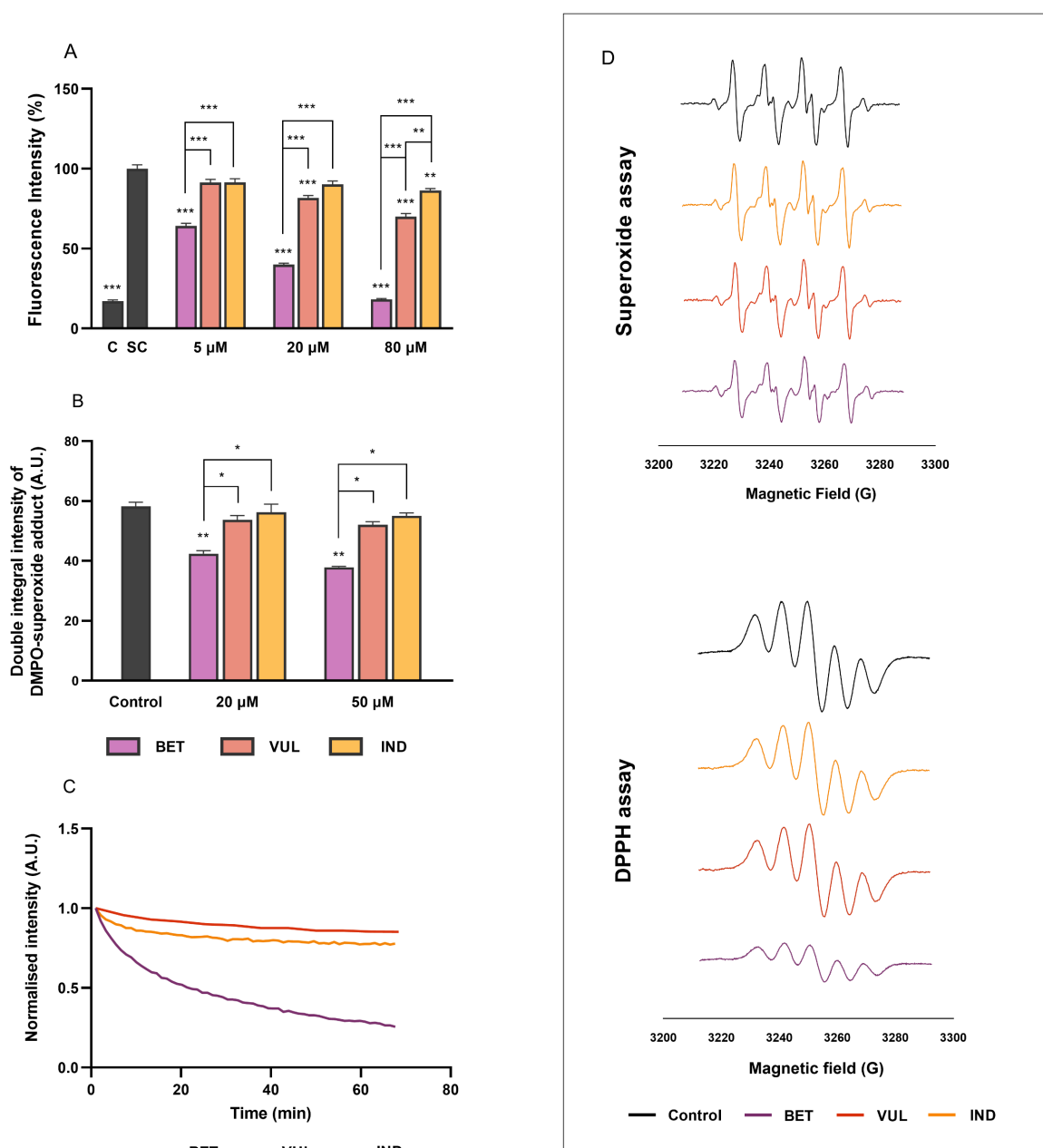


Figure 3-9. Radical scavenging capacity of betalains reflected by (A) intracellular ROS generation in Caco-2 cells, (B) double integral intensity of EPR signals of DMPO-superoxide adduct, (C) DPPH signal intensity in the presence of betalains, as well as (D) EPR spectra of superoxide and DPPH assays (post 30 min) in the absence (control) and in the presence of 20 μ M purified betalains. C and SC refer to negative medium control and H_2O_2 -stimulated control, respectively. Data in A and B are mean with SEM from 3 independent experiments, and non-bridged asterisks indicate significant difference of treatment vs. SC in (A) or Control in (B): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

BET, VUL and IND (5–80 μM) demonstrated potent radical scavenging capabilities by dose-dependent lowering of H_2O_2 -stimulated ROS levels in Caco-2 cells (Fig. 3-9A). The most pronounced suppression of ROS was exhibited by BET throughout the concentration range (< 64%) compared to the two betaxanthins. VUL displayed suppression of ROS generation only at 20 and 80 μM (< 30%), more potent than IND which only showed a 14% reduction at 80 μM .

The direct radical scavenging capacities of all betalains were evaluated in the current study using EPR, with results presented in Fig. 3-9B, C and D. Under spin-trapping conditions, BET (20, 50 μM) demonstrated high scavenging activity towards DPPH and superoxide ($\text{O}_2^{\bullet-}$) radicals. This was reflected by the significant waning in EPR signal intensities in DPPH (< 71%) and superoxide assays (< 35%), suggesting the lowered concentrations of radical products. In contrast to BET, VUL and IND did not exhibit any evident antiradical activity in either DPPH or superoxide assays, highlighting the different potencies of betaxanthins from betacyanins which was in line with previous findings from antioxidant activity assays (Fernando et al., 2022).

3.6 Discussion

3.6.1 Betalain availability to the Caco-2 cell model

The current chapter has investigated the absorption and permeability of selected betalains to intestinal cells determined through the uptake and transport characteristics. The differentiated Caco-2 cells in wells with transwell insert served as a simulation of the compartmentalised environment of human small intestine. It is also noteworthy that the incubation duration resembled the residence period of phytochemicals in the anterior segment of human intestine, and the prolonged incubation did not cause differences in the amount of accumulated betalains (Appendix 4).

Table 3-1. Physicochemical properties of selected betalains. Parameters include molecular weight (MW), number of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA), topological polar surface area (tPSA), and octanol/water partition coefficient (Log *P*). Data were acquired from ChemDraw 3D.

Betalains	MW (g mol ⁻¹)	HBD	HBA	tPSA (Å ²)	Log <i>P</i>
BET	550.48	9	14	246.55	-2.50
VUL	339.29	5	9	173.59	-1.46
IND	308.30	4	7	126.94	-1.12

This divergence in cellular uptake of purified BET, VUL and IND in Fig. 3-5A can be attributed to the physicochemical characteristics of the phytochemical compounds (Rastogi and Jana, 2016). The lipophilic core structure of betalains enabled a relatively low desolvation penalty of molecules when crossing a membrane (Liveri et al., 2007). As shown in Table 3-1, molecular size and weight incline following the sequence of IND, VUL and BET, which are accompanied by increasing counts of hydrogen bond donors and acceptors hence the polar surface area. These factors contribute positively to the water solubility of compounds yet have an inverse relationship with bioavailability. Secondly, the pK_a values for carboxylates on the three betalains are proposed to be low (< 4.5), especially for BET, which are on account of

the vicinity of carboxylates to electron-withdrawing groups such as the positively charged iminium and delocalised π -system (Tesoriere et al., 2013; Medicine, 2019; Rodriguez and Baumgartner, 2020). This helps interpret their susceptibility to deprotonation hence multiple degrees of ionisation in the alkaline environment. The extent of interaction between betalains and lipid bilayer is governed by their lipophilicity and expressed as partition coefficient (Log P). Data in Table 3-1 suggest that IND possesses relatively greater lipophilicity and lower susceptibility to carboxyl ionisation than VUL and BET in the environment of small intestine (pH 6.5), which have, together with smaller molecular size, contributed to the relatively higher absorption of IND. This is also supported by the results from previous studies (Liveri et al., 2009; Hithamani et al., 2017).

The trans-epithelial results suggested the *in vitro* transport of three purified betalains to be on the lower end of the scale compared to several flavonoids and phenolic compounds whose P_{app} located between $2-8 \times 10^{-6} \text{ cm s}^{-1}$, e.g., quercetin, ferulic acid and gallic acid (Rastogi and Jana, 2016; Hithamani et al., 2017). Likewise, P_{app} values appeared to be notably lower than Tesoriere et al. (2013), which might be attributable to the utilisation of higher pH at the apical chamber in this study, therefore possibly shifting the physicochemical properties of pigments and their permeability from the reference. However, the experimental outcomes were consistent with preliminary tests on the influx transport of dextrin-coated betanin standard. Focussing on the initial 2 h window with constant transport rate, the P_{app} measured ($5.6-7.7 \times 10^{-8} \text{ cm s}^{-1}$) was roughly one-tenth of the value of purified BET without dose dependency, which is well conceivable that entanglement of polymeric dextrin at the surface of the Caco-2 membrane may have caused a physicochemical hindrance to the migration of pigment. Moreover, the dramatically lowered TEER value of differentiated Caco-2 layer, i.e., damaged membrane integrity during the treatment of dextrin-coated betanin was also likely attributed to the presence of dextrin which possesses numerous ionisable hydroxyl groups that may cause the redox disturbance at cell membrane under experimental pH (Fig. 3-3). This, again, stressed the importance of employing the purified betalains in further work.

Resembling the intracellular uptake, the physicochemical nature of individual betalains

is reckoned as the determinant of their trans-epithelial permeability (Rastogi and Jana, 2016). The *in vitro* data indicated higher cellular availability of IND compared to BET and VUL, which is in alignment with *in vivo* results demonstrating around 20-fold higher plasma concentration and urinary recovery of IND compared with BET. The considerable difference was likely due to the higher resistance of IND to digestive degradation, greater absorption efficiency and slower elimination rate (Tesoriere et al., 2004a; Allegra et al., 2014; Tsakelidou et al., 2017). In real life, the bioavailability of betalains is further heavily impacted by a variety of intrinsic as well as extrinsic factors.

For VUL and IND, the physicochemical parameters have fulfilled the “rule of 5” by Lipinski (Lipinski et al., 2001), indicating a relatively straightforward passage through the lipid membrane. Based on the phenolsulfonaphtalein result, diffusion of the two betaxanthins (MW < 600 Da) is likely to take place via the paracellular route with the generation of convective molecular flows through the aqueous intercellular space with rate-limiting tight junctions (i.e., pore pathway). The pores are formed in the complex of transmembrane proteins (e.g., claudin), whose sizes are believed to be inversely correlated to their quantity (i.e., pore theory) (Travis and Menzies, 1992; Watson et al., 2001). Therefore the permeation of small molecules is potentially facilitated by the more accessible transport passages due to the sieving effect of paracellular network, which partly explained the higher availabilities of two betaxanthins compared with BET (Ménard et al., 2010). In contrast, the greater size, higher structural complexity and hydrophilicity of BET presumably impede its process of crossing the hydrophobic bilayer region of Caco-2 cells; therefore, a limited absorption efficiency dominated by paracellular diffusion is anticipated. In addition, the lack of evidence on betalain biotransformation in current research has partially supported the hypothesis regarding passive diffusion as the predominant mechanism of betalains crossing the intestinal epithelium (Tesoriere et al., 2013; Takahashi et al., 2017; Sawicki et al., 2020). The possibility of transcellular diffusion, nevertheless, was not precluded (El-Kattan and Varma, 2012). More research is required to determine whether transport enzymes and carriers are involved in the uptake of betalains, particularly for glucosides of betacyanins that may share a comparable transport mechanism as those of polyphenols (Kanner et al., 2001).

Overall, the availability experiments have demonstrated a low absorbability and permeability of betalains in the cell culture model, implying a low oral bioavailability in humans. Future research may focus on the investigation of transport mechanisms considering more physiological *in vitro* cell models, e.g., the employment of a mucus barrier to provide a better simulation of the human body (Verhoeckx et al., 2015).

3.6.2 Immunomodulatory effect of betalains

Despite the low efficacy of betalain uptake and transport through intestinal epithelium, the enterocytes at proximal intestine are likely to be exposed to betalain pigments in the lumen at relatively high concentration following the food ingestion. The current study has further investigated potential effects of different betalains on the modulation of inflammatory signalling in intestinal Caco-2 cells. Upon stimulation by inflammatory triggers, the synthesis and release of a range of pro-inflammatory and immunoregulatory factors lead to the manifestation and amplification of gut inflammation (Pasparakis, 2009; Van De Walle et al., 2010).

The participation of pro-inflammatory mediators, IL-6 and IL-8, is considered indispensable to the maintenance of intestinal homeostasis and immunologic function. They are commonly found overexpressed under gut inflammatory conditions such as Crohn's disease (Ferrari et al., 2016). As a cytokine, IL-6 contributes to the mucosal immune responses and is associated with bowel inflammation and colon necrosis. Meanwhile, the chemotactic IL-8 induces the recruitment of leucocytes in the inflamed region, followed by the secretion of surface antigens and reactive oxygen products (Akira et al., 1990; Baggiolini et al., 1993; Van De Walle et al., 2010). The secretion of these two mediators is modulated by a cascade of intracellular signalling events, particularly with the stimulation of the NF- κ B pathway in intestinal cells.

Results in this chapter have indicated the downregulation of expression levels of IL-6 and IL-8 under inflammatory stimulation, implying the capability of BET, VUL and IND to

interfere with the NF- κ B pathway *in vitro* hence their effectiveness in mitigating the inflammatory status of intestinal epithelium. Effects of IND on IL-6 and IL-8 expression were in partial agreement with Tesoriere et al. (2014), albeit that the lack of dose-dependent response of cytokines plausibly indicated the distinct molecular interference at different betalain concentrations which was also considered to be cell- and gene-specific. It is also noteworthy that the data related to VUL should be interpreted with caution considering its relatively low purity (79%) compared to BET and IND (> 90%), hence the possible contribution of impurities to the results.

Unlike several constitutively expressed isozymes in the families of COX and NOS, COX-2 and iNOS are cytokine-inducible enzymes that are present at elevated levels in an inflammatory environment. COX-2 plays a critical role in regulating the serial reactions from arachidonic acid to prostaglandins and thromboxane, whose secretions are promoted considerably during the active phase of IBD causing tissue oedema (MacDermott, 1996; Needleman and Manning, 1999). The enzyme iNOS catalyses the conversion from *L*-arginine to *L*-citrulline with the generation of nitric oxide (NO) as a by-product, whilst NOX-1 produces $O_2^{\bullet-}$ in the process of NADPH oxidation. Levels of NO and $O_2^{\bullet-}$ that surpass the need for normal physiological activities generally lead to oxidative damage of tissue, inflammatory response and tumour development (Vignoli et al., 2001).

Results of anti-inflammatory properties of betalains were in agreement with other references that stated a marked inhibition of the protein levels and activities of COX-2 and iNOS enzymes by BET (Reddy et al., 2005; Vidal et al., 2014; Tan et al., 2015; Ahmadi et al., 2020). According to Tesoriere et al. (2014), IND (5-25 μ M) dose-dependently inhibited the protein expression of phosphorylated I κ B α (< 80%) and nuclear p65 mediator (< 90%) in NF- κ B pathway which resulted in the decreased synthesis of downstream target COX-2 (< 80%), iNOS (< 90%) and NOX-1 (< 89%). In contrast, the attenuation effects of the three betalains on COX-2 and NOX-1 expression were relatively moderate in the present study, especially at 5 and 20 μ M. The dose-dependent downregulation of COX-2 gene expression caused by betalains was akin to that of NOX-1 and iNOS genes as shown in Fig. 3-6. This could be

elaborated by the interplay between ROS/precursor and the COX-2 pathway which generally creates an inflammatory loop at the transcriptional level (Needleman and Manning, 1999; Chun et al., 2004). Consistent evidence was also found in studies *in vitro* and *in vivo*, indicating that excessively produced ROS and NO from NOX-1 and iNOS catalysis were often accompanied with the enhancement of COX-2 activity hence downstream generation of prostaglandins (Pelletier et al., 1998; Vassalle et al., 2003; Hu et al., 2017).

On the other hand, the Nrf2 regulatory mechanism can be activated by ROS-induced oxidative stress and cellular damage as well as by bioactive compounds, leading to increased levels of enzymes and proteins with antioxidant and detoxifying functions. NQO-1 in epithelial tissues majorly serves a role as the reductase of quinones and superoxide derivatives in the redox cycle for stabilising radicals (Ross and Siegel, 2017). HO-1 enzyme is primarily produced to catalyse the degradation of toxic hemes with co-functions of antioxidant, anti-apoptosis and pro-angiogenesis (Hayes et al., 2015; Loboda et al., 2016).

The glutathione (GSH) system acts as an endogenous antioxidative defence in the intestinal epithelia against diet- and inflammation-induced ROS. A pivotal enzyme in GSH biosynthesis is glutamate-cysteine ligase, a strictly Nrf2-regulated enzyme with catalytic (GCLC) and modifier (GCLM) subunits (Lee et al., 2006). GPXs and GSTs are also prominent contributors to the GSH system, with GPX isoenzymes catalysing H₂O₂ reduction coupled with GSH oxidation, while different GSTs assist in the process of chemical detoxification by conjugating reduced GSH to electrophiles such as carcinogens. Reported in the previous studies, reduced expression and activity of GSTs in the human GI tract are often associated with colorectal cancer (Guo et al., 2002; Bhattacharyya et al., 2014; Loboda et al., 2016).

The effects of betalain on Nrf2-target genes, as presented in this work, are partly explained by Esatbeyoglu et al. (2014) who demonstrated moderate Nrf2 transactivation by BET (15 µM) in Huh7 cells that could contribute to the augmentation of downstream enzymes (e.g., HO-1). Krajka-Kuzniak et al. (2013) presented a dose-dependent increase in both

mRNA levels and enzyme activities of NQO-1 and various GST enzymes in human hepatocytes following treatment with BET (2-20 μ M). Nonetheless, the significant increases of NQO-1 and GSTM expression (*ca.* 60% and 100% increase in the baseline) at 20 μ M BET in Krajka-Kuzniak et al. (2013) were not observed in the current study, which could be due to the cell-specific differences in responsiveness.

Apart from purified betalains, the results have confirmed the strong anti-inflammatory character of RBR and YBR extracts on stimulated Caco-2 cells reflected by their potent suppression of COX-2 expression. The results, however, also demonstrated their inhibitory effects on GCLC expression, in contrast to the impact of purified betalains. The divergence in the cellular effects of beetroot extracts from that of purified compounds has manifested their immunomodulatory activities via distinct interactions with cell signalling, implying a great interference of other intrinsic compounds in beetroot matrices, e.g., phenolics, nitrates, and sucrose, to the cellular inflammatory response. As the principal ingredients in the dietary beetroots, their physiological effects should be holistically evaluated along with betalain pigments in the context of beetroot consumption.

3.6.3 Radical scavenging activity of betalains

The overexposure of the human intestine to ROS and toxic secondary metabolites is readily associated with increased cytokine production with subsequent impacts on DNA stability, resulting in cellular damage and inflamed tissues which may lead to adenocarcinoma in the colon (Bhattacharyya et al., 2014; Halliwell, 2015). Mutually, intracellular ROS levels can be elevated via inflammatory and/or chemotactic induction, e.g., H₂O₂ in this study. The magnitude of cellular ROS inhibition by BET in this study (< 64%) were consistent with Esatbeyoglu et al. (2014) who demonstrated dose-dependent radical quenching property of BET in a similar concentration range (1–10 μ M). The potency of BET was also found to resemble that of several well-known antioxidant flavonoids, e.g., quercetin and curcumin (Yokomizo and Moriwaki, 2006).

The betalain-induced modulation of intracellular ROS production is likely a combination of different molecular events, including suppression of inflammatory signalling and activation of Nrf2-signalling. Downregulation of targets driven by pro-inflammatory signalling, such as iNOS and NOX-1, leads to the reduced superoxide and subsequent radical generation in the cellular environment (Bhattacharyya et al., 2014; Moreno-Ley et al., 2021), while the Nrf2 pathway promotes the transcription and synthesis of proteins and enzymes associated with cellular redox regulation (e.g., HO-1 and NQO-1), thereby contributing to the redox balance. Furthermore, the intrinsic reduction potential of betalains plays an essential role in radical scavenging properties which varies across different compounds following the structure-activity relationship (Gliszczyńska-Świgło et al., 2006). The π -system resonance and multiple deprotonations of betalain structures under basic conditions render them more susceptible to electron donation, therefore radical stabilisation (Belhadj Slimen et al., 2017). The stronger radical scavenging properties of BET compared to VUL and IND are possibly due to electron and proton transfers facilitated by the phenol substituent in BET (Butera et al., 2002; René et al., 2010). Meanwhile, in the case of VUL and IND, direct quenching of radicals was negligible as observed from intracellular ROS and EPR data, accordingly immunomodulation was considered the major contributor to their redox-regulating ability. Based upon the betalain availability results demonstrated in Sections 3.5.1 and 3.5.2, betalains can be incorporated into and transported across the Caco-2 monolayer, during which they are likely involved in the modulation of cellular events.

3.6.4 Availability of betalains, the bottleneck to immunomodulation

The anti-inflammatory and antioxidant features of tested betalains indicated their potential to prevent or mitigate intestinal inflammatory condition with cellular exposure to a concentration range of 5-80 μ M. The comparable molecular effects were observed with several polyphenols (e.g., cyanidin-3-glucoside) and carotenoids (e.g., lycopene). For instance, *in vitro* studies have documented the anthocyanin-mediated downregulation (40-50 μ M) of apical IL-6 (70-90% reduction) and tripled HO-1 expression in an inflamed Caco-2 cell model. While a further *in vivo* study showed the alleviation of colitis in mice after

anthocyanin administration (Kuntz et al., 2015; Ferrari et al., 2016; Le Phuong Nguyen et al., 2018; Gan et al., 2019). There was also evidence of the synergistic cytoprotective impact of coexisted bioactives (e.g., anthocyanin with lycopene) in light of immunoregulation and scavenging radicals (Phan et al., 2019). Noteworthy, the dietary matrix of beetroot is a rich source of betalains, phenolics and nitrates, in which the constructive and destructive interferences of components to their biofunctions are speculated.

However, the results of this chapter have provided an insight into the low transport efficiency of BET, VUL and IND being uptaking into the circulatory system despite the limitations of the Caco-2 cell model as an *in vitro* simulation. Current results are aligned with those of Sawicki et al. (2018) and Wiczowski et al. (2018) who described low plasma concentrations and urinary recovery fraction *in vivo* following normal consumption of a betalain-rich diet. Apart from the restricted kinetics of trans-epithelial transport, betalain uptake is also shown to be influenced by other factors including pigment retention by food matrix and digestive decomposition (Choo et al., 2019). Tesoriere et al. (2008) illustrated various and significant degrees of loss (up to 70%) of purified BET and VUL along the simulated digestion in spite of a minor loss of IND. Thus, the lower availability of BET and VUL is considered a bottleneck for their potential health effectiveness regardless of their promising bioactivities. Data regarding cellular effects of betalains on the immune and endothelial cell models (Cheok et al., 2018; Ahmadi et al., 2020) need to be elaborated in view of low body uptake and therefore, low circulating concentration of betalains. In terms of the intestinal barrier function, some studies revealed an increased expression of transmembrane scaffolding proteins (e.g., zonula occludens) and stimulated differentiation of mucin-secreting cells following polyphenol consumption (Pierre et al., 2013; Del Bo et al., 2021). Likewise, the impacts of betalains on the intestinal epithelial tight junctions and mucin generation merits additional exploration.

Chapter IV

Bioavailability and Excretion profile of Betacyanins – Variability and Correlations between different excretion routes

4.1 Abstract

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 addresses the knowledge gap in betalain bioavailability, transformation and excretion following a 2-week ingestion of beetroot juice, which also included elaborations on inter-individual and inter-compound variabilities. Analysis of renal and faecal excretion profiles in humans revealed very poor bioavailability (f_e % of 0.13%) and fast elimination of pigments (k_{re} of 0.16 h^{-1}), while the dominant proportion underwent severe depletion during GI transit, evidenced by decarboxylation, deglucosidation and dehydrogenation; this was coherently supported by the pigment metabolite profiles in both pig digesta and human faeces. There were 11 and 14 betacyanin compounds being characterised in urinary and intestinal excretion profiles of human volunteers, respectively. 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 pigment variabilities after colonic fermentation compared with systemic metabolism, likely attributed to the increasing complexity and diversity of the intestinal environment with gut microbiota as major driver. To conclude, intestinal uptake and systemic metabolism of betacyanins are intimately associated with their intestinal biotransformation, with gut microbiota serving as a crucial factor. Results of the current study could greatly advance our knowledge in the metabolomic fates of betacyanin pigments regarding the mechanisms underlying their absorption, metabolism and excretion, contributing to future research on their systemic efficacy and gut-related benefits.

4.2 Introduction

There is emerging *in vitro* and *in vivo* research emphasising the health-promoting properties of betalain pigments including anti-lipidaemia (Reddy et al., 2005; Wróblewska et al., 2011), anti-hyperglycaemia (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; Wang et al., 2022b).

There is some evidence from *in vivo* trials reporting 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 the 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, 2016a; 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 variety of intrinsic (e.g., physiological conditions) and extrinsic factors (e.g., dose, food matrix, exposure duration). It is generally evaluated *in vivo* by analysing 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; Wiczowski et al., 2018). According to a limited number of pharmacokinetic studies (Tesoriere et al., 2004a; Frank et al., 2005; Wiczowski 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., 2004a; 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 faeces. 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, 2011a; 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 indeed to analyse 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 faecal excretion of betanin *in vivo* was initially explored by Krantz et al. (1980) who indicated recovery fractions of 3% in rat faeces after a single oral dose; whereas a significantly higher recovery of betanin (41%) was determined *ex vivo* after incubating the pigment with dissected large intestine of rat (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.

4.3 Aims

The present study aimed to –

- Investigate the bioavailability and biotransformation of betacyanins, the main betalain group, based on i) renal and faecal excretion profiles in humans following consumption of beetroot juice and ii) intestinal betacyanin degradation profiles in pig digesta following administration of beetroot supplemented feed.
- Visualise from human trial, the inter-individual variability in responsiveness as well as the correlations between differentially transformed betacyanins excreted through the different routes.

4.4 Method

Methods of this chapter primarily involved the executions of an *in vivo* human study (2 weeks) and an animal trial (2 weeks), with the interventions of red beetroot juice and beetroot-supplemented feed, respectively (Section 2.3.1 – 2.3.4). The collected samples (i.e., urine, stool, digesta) were processed and submitted to (U)HPLC-MS for the purpose of betacyanin characterisation and quantification (Section 2.3.5). The development and optimisation of analytical methods were carried out throughout this project, and were largely dependent on the accessibility and performance of analytical facilities. The statistics concentrated on the pharmacokinetics and metabolomics of betacyanins following oral administration, involving the principal coordinates as well as correlation analysis.

4.5 Results

4.5.1 Human study compliance and profile of red beetroot juice

After health screening, 18 healthy volunteers were enrolled who consumed the diluted red beetroot juice (250 mL) for 14 consecutive days. 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 faecal excretion of betacyanins. The diet patterns of individuals were monitored during the intervention, suggesting a marked inter-subject variability ($p < 0.05$) but minor for intra-subject ($p > 0.05$) regarding the approximate ingestion amount and frequency of digestible carbohydrates, polyphenol-rich fruit and vegetables, red and white meat, polyphenol-rich drinks, and dairy products (distance matrix shown in Appendix 6). The SBP and DBP of participants hardly fluctuated between baseline ($101 \pm 13 / 66 \pm 8$ mmHg) and Day 14 ($103 \pm 13 / 67 \pm 7$ mmHg) ($p > 0.05$).

The betacyanin content in red beetroot juice (208 ± 7 μ mol 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 optimised specifically for detection of betacyanins.

4.5.2 Urinary excretion profile and kinetics of betacyanins

Upon 24 h post-prandial window of beetroot juice consumption, the area under renal excretion rate curves (AURC₀₋₂₄) of individual compounds was determined, from which the recovery fraction (fe %) to administered dose was estimated and is demonstrated in Table 4-1. A representative chromatogram of urinary excreted betacyanins is shown in Fig. 4-1A.

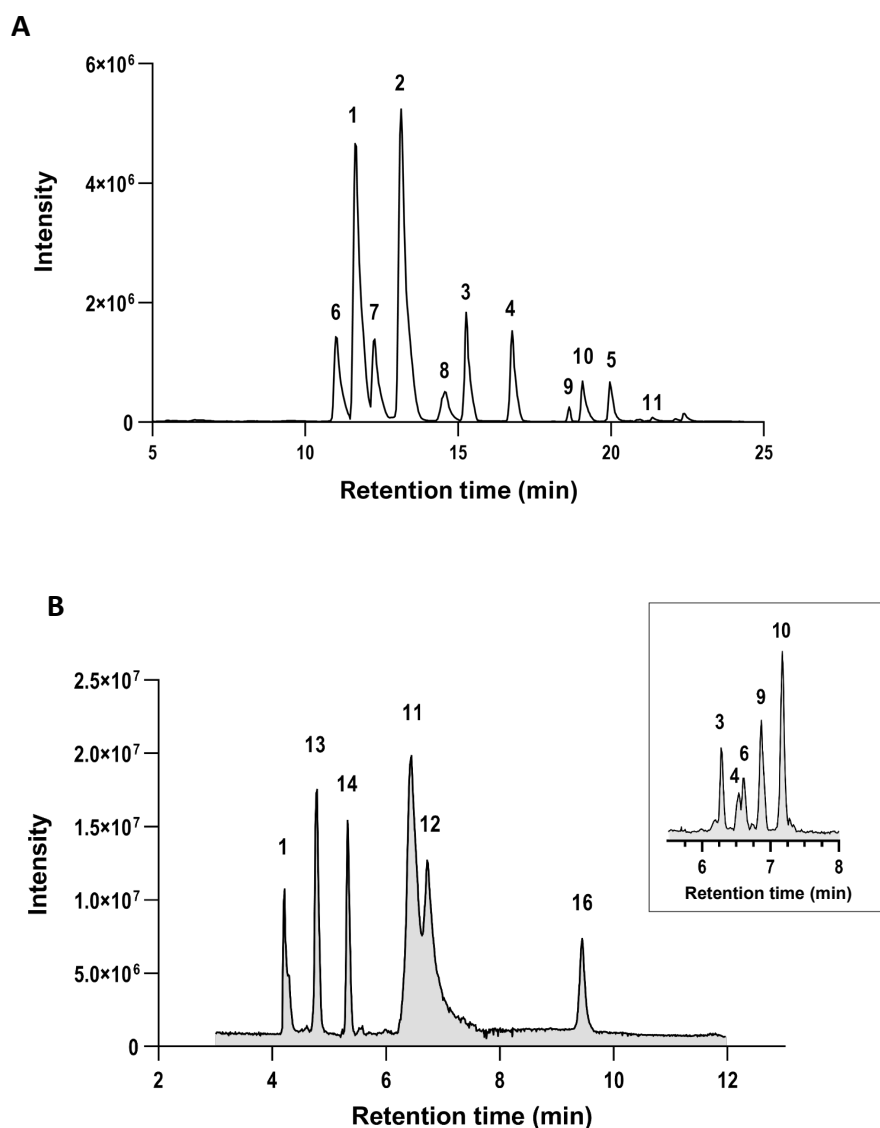


Figure 4-1. MS chromatograms of betacyanins acquired from urine (A) and stool samples (B) in human trial. The inset of (B) shows betacyanins in stool other than compound **11** and **12** at the retention time of 6-7.5 min. Note that (A) was acquired from HPLC-MS (Shimadzu 2020 MS), while (B) was obtained from the UHPLC-MS technique (Thermo-Vanquish with TSQ Quantiva) with modified analytical methods (Section 2.3.5.2 and 2.3.5.3). The numbers of compounds are presented in Table 4-1.

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 BSL urine. 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 AURC₀₋₂₄ 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, 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 AURC₀₋₂₄ 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 rate of different betacyanins are illustrated in Fig. 4-2 with individual excretion displayed in dots. The compound 2,17-bidecarboxy-betanin was not included in the kinetic and compositional profiles (Fig. 4-2 & 4-3) 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 AURC₀₋₂₄, no significant difference was found between excretion rate curves of stereoisomers in this study. According to Fig. 4-2C, (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, proportion of (iso)betanin was gradually outweighed by the

¹ The bold numbers in brackets were linked to the compound numbers in Table 4-1.

others, and eventually the dominance of (iso)betanidin from 8-12 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 the next section.

Table 4-1. Urinary and faecal excretion profiles of betacyanin derivatives in human trial. Data of AURC and k_{re} are presented as mean $\pm$ 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 [#]			Faecal excretion composition (%) [‡]	
				AURC ₀₋₂₄ (nmol) [*]	k_{re} (h ⁻¹)	fe (%)	D3	D14
1	Betanin	551, 389	Juice, urine, stool	86.29 $\pm$ 4.86 (31.3) ^a	0.29 $\pm$ 0.01 ^a	0.10	0.79 ^{ac}	1.81 ^{ac}
2	Isobetanin	551, 389	Juice, urine, stool	90.65 $\pm$ 4.63 (32.9) ^a	0.26 $\pm$ 0.01 ^a	0.09	0.43 ^a	0.55 ^a
3	Betanidin	389, 345	Juice, urine, stool	37.06 $\pm$ 3.15 (13.4) ^b	0.06 $\pm$ 0.02 ^b	9.74	1.59 ^a	3.68 ^a
4	Isobetanidin	389, 345	Juice, urine, stool	24.57 $\pm$ 2.47 (8.9) ^{bd}	0.08 $\pm$ 0.02 ^b	8.39	2.33 ^a	3.80 ^a
5	Neobetanin	549, 387	Juice, urine, stool	3.83 $\pm$ 0.27 (1.4) ^c	0.19 $\pm$ 0.02 ^c	0.03	0.00 ^a	0.08 ^{ac}
6	17-Decarboxy-betanin	507	Juice, urine, stool	2.51 $\pm$ 0.14 (0.9) ^c	0.16 $\pm$ 0.01 ^{cd}	0.09	10.57 ^{ac}	13.20 ^{abc}
7	17-Decarboxy-isobetanin	507	Juice, urine	3.58 $\pm$ 0.26 (1.3) ^c	0.18 $\pm$ 0.02 ^c	1.23	-	-
8	15-Decarboxy-betanin	507	Juice, urine	2.12 $\pm$ 0.12 (0.8) ^c	0.16 $\pm$ 0.02 ^{cd}	0.22	-	-
9	2,17-Bidecarboxy-neobetanin	461	Urine, stool	19.49 $\pm$ 1.57 (7.1) ^d	0.06 $\pm$ 0.01 ^b	-	6.75 ^{bcd}	5.37 ^{abc}
10	2,15,17-Tridecarboxy-neobetanin	417	Urine, stool	5.52 $\pm$ 0.53 (2.0) ^c	0.11 $\pm$ 0.02 ^{bd}	-	22.20 ^{abc}	15.78 ^{abc}
11	2,17-Bidecarboxy-betanin	463	Urine, stool	0.24 $\pm$ 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 $\pm$ 2.83	0.16 $\pm$ 0.01	0.13	-	-
Total concentration of faecal betacyanins (nmol/g stool)				-	-	-	12.18 $\pm$ 4.56	19.92 $\pm$ 8.59

* Proportion of individual compounds (%) respect to total excreted amount in urine was displayed in brackets.

[#] Abbreviation – AURC₀₋₂₄, area under the urinary excretion rate curve (0-24 h); k_{re} , urinary excretion rate constant; fe (%), eliminated fraction in urine respect to dose.

[‡] There was no compositional difference between D3 and D14 groups.

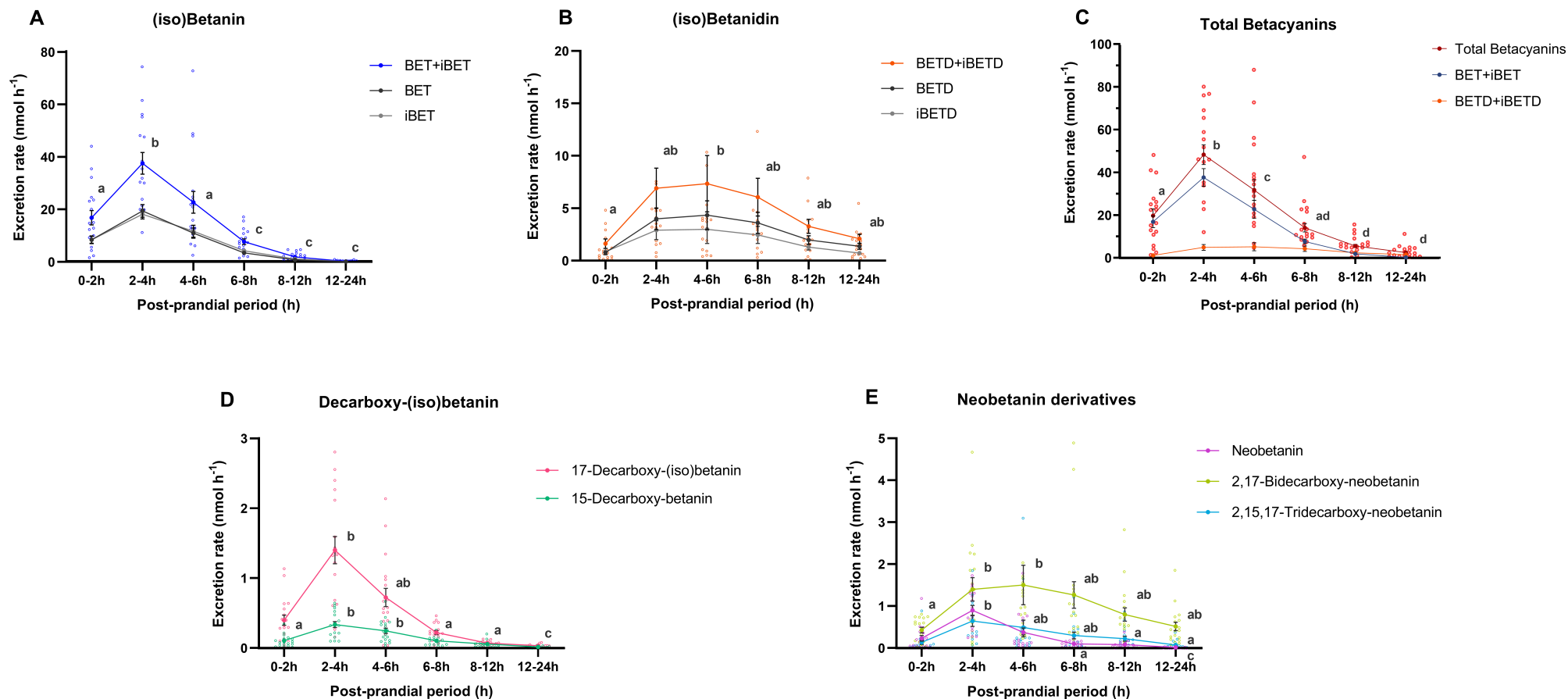
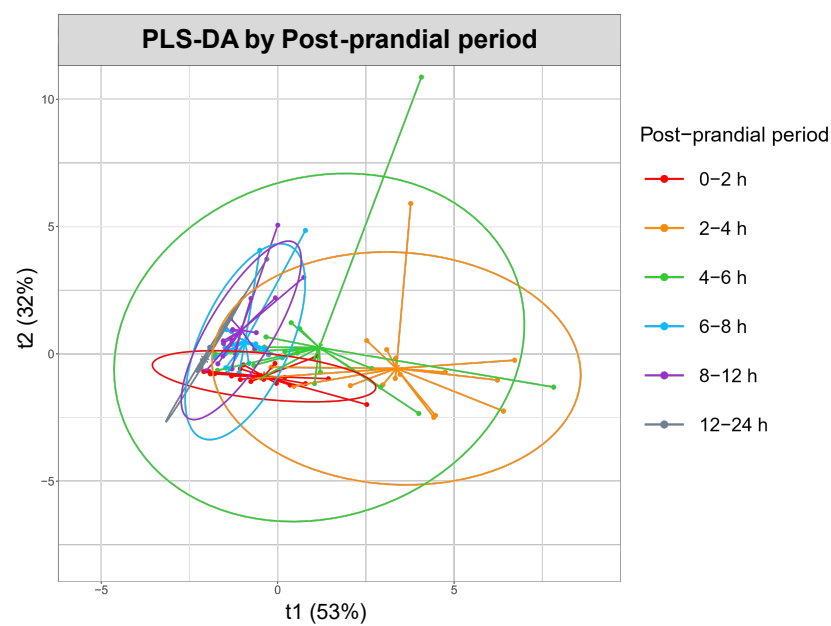
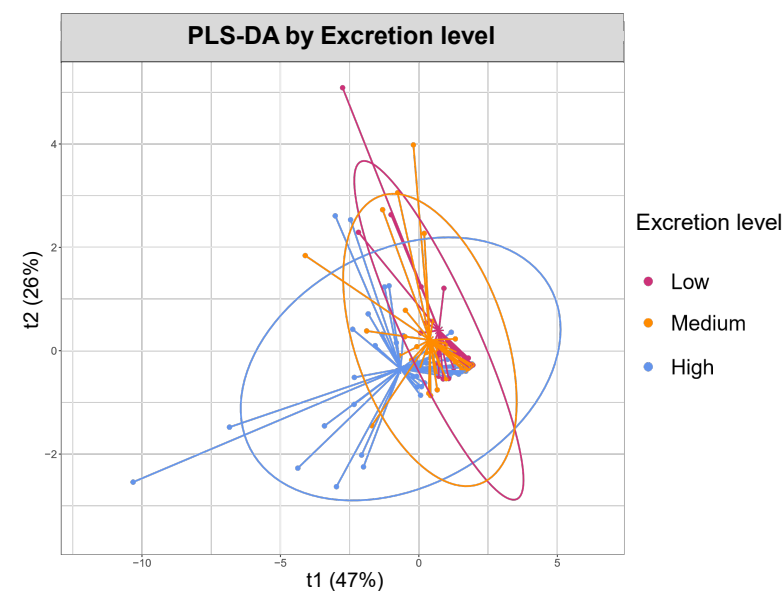
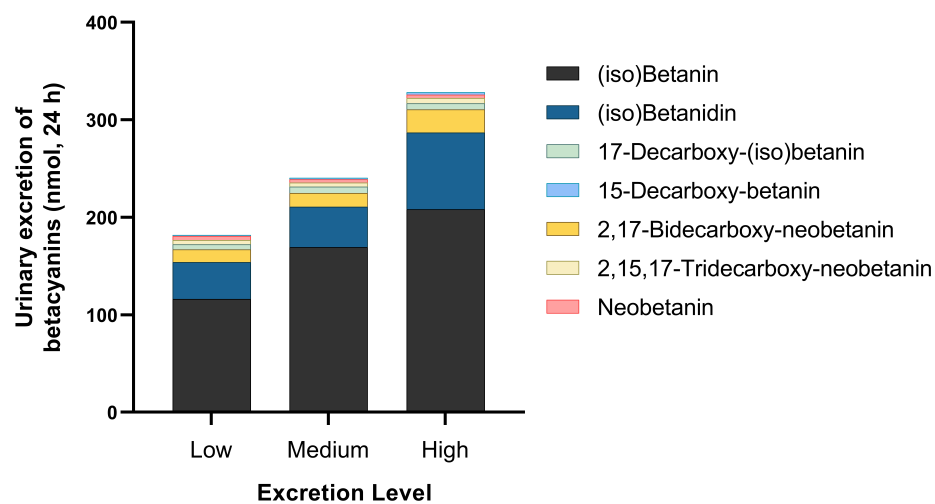


Figure 4-2. Urinary excretion profiles of native betacyanins and their metabolites against time intervals following acute beetroot juice consumption, including (A) betanin and isobetanin (BET, iBET), (B) betanidin and isobetanidin (BETD, iBETD), (C) total betacyanins, and (D), (E) other derivatives. Data are presented as mean $\pm$ 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 ($p < 0.05$). Note that inter-compound difference at each specific time point is not compared.

4.5.3 Inter-individual variation and stratification based on urinary excreted betacyanins

The PLS-DA scores (Fig. 4-3A and B, and Appendix 7A) 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 Q²_Y value of 0.123. Fig. 4-3A 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 maximally amplified at 4-6 h followed by a restoration to nearly BSL condition during 6-24 h. The plot was in line with excretion curves in Fig. 4-2 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 excretors (n=8) based on their total amount of renal excretion with the inter-group difference ($p < 0.05$). The PLS-DA score plot (Fig. 4-3B) showed a notably larger variance in excretion profiles of high excretors compare with low and medium. According to Fig. 4-3C, the augmentation in AURC₀₋₂₄ from low to high excretors was mainly contributed by the increases in (iso)betanin (116.0 vs. 208.1 nmol), (iso)betanidin (37.9 vs. 78.6 nmol), and 2,17-bidecarboxy-neobetanin (13.1 vs. 23.8 nmol), whereas the composition regarding native betacyanins and metabolites remained similar between groups. Inter-subject variation was further elucidated via the subject-based proximity matrix, 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 Appendix 7B.

A**B****C**

D

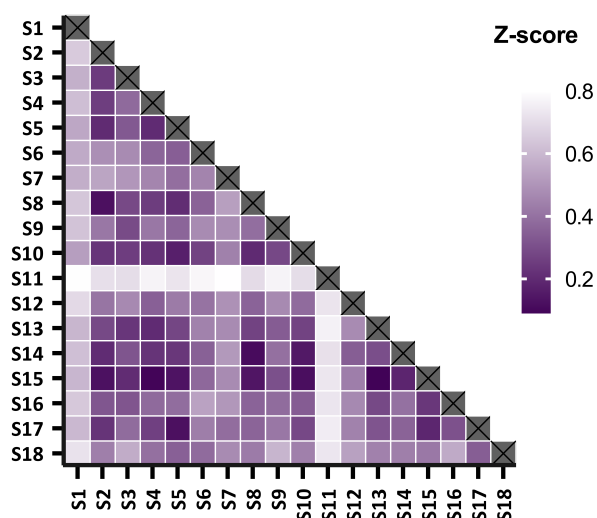


Figure 4-3. Variability in urinary excretion profiles of betacyanins and subject stratification according to betacyanin excretion level, as illustrated by the PLS-DA score plots of urine samples (A) at different time intervals after consumption of red beetroot juice, and (B) at each stratified excretion level. It was followed by (C) the compositional histogram of betacyanin metabolites and (D) the proximity matrix between subjects acquired from the urinary parameters.

4.5.4 Characterisation of betacyanin catabolites in human faeces

By employing the UHPLC-MS techniques, there were in total 14 betacyanin derivatives identified in human faecal samples with corresponding m/z values and composition as listed in Table 4-1. The chromatogram (Fig. 4-1B) has illustrated 11 catabolites out of 14 with individual compounds occupying more than 1% of total betacyanins in stool. 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 characterised 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). The existence of other catabolites cannot be excluded considering the limitations in detection scope and sensitivity of this study.

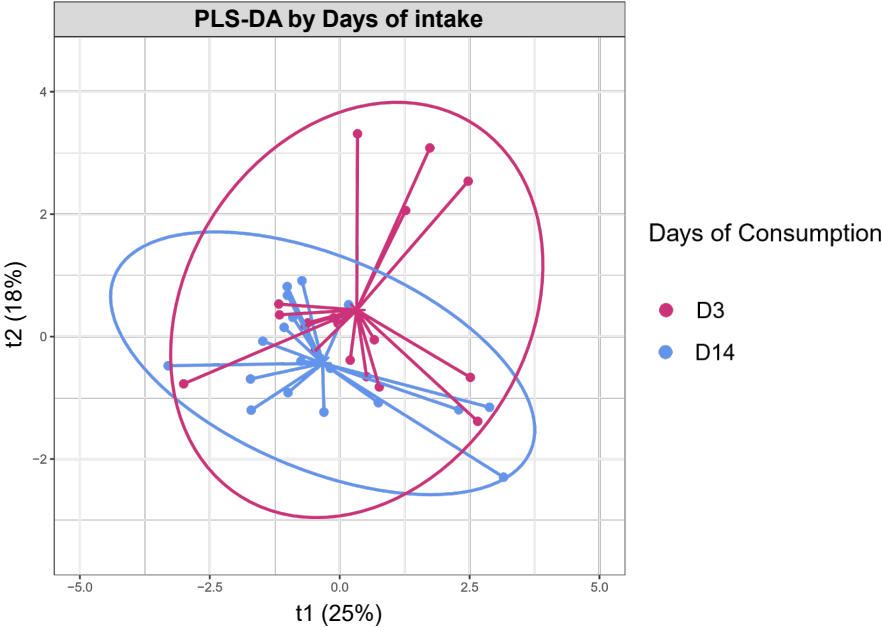
Like urinary excretion, native betacyanins and catabolites were absent from BSL stools. Betacyanin concentrations in D3 and D14 stool samples were 12.2 ± 4.6 and 19.9 ± 8.6 nmol/gram of stool, respectively, from which the proportion of catabolites (77-84%) has apparently outweighed that of native betacyanins (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 inter-subject variability in faecal excretion profiles, the non-parametric hypothesis tests did not provide an explicit interpretation for the divergence among betacyanins in the current study.

4.5.5 Inter-individual variation of faecal excreted betacyanins and stratification

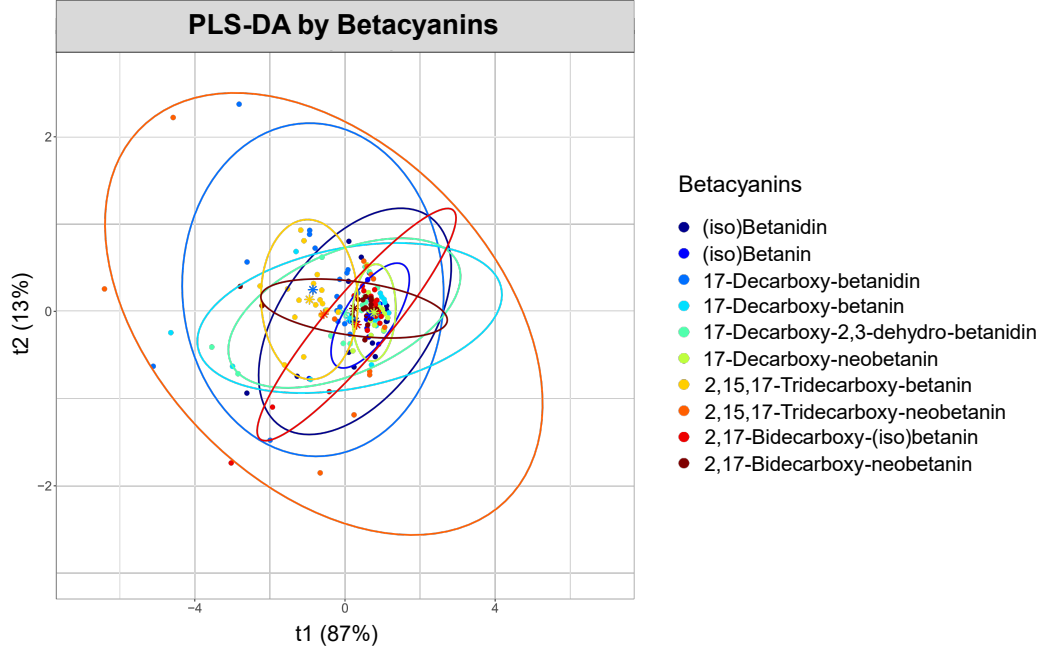
Betacyanin profiles of intestinal excretion were discriminated by PLS-DA models from the perspectives of intervention duration and individual compounds. As illustrated in Fig. 4-4A, the greater dispersion of D3 samples compared to D14 has revealed a larger variation among profiles of the former regardless of the averagely lower pigment level. Yet, the convergence of most samples implied some degree of resemblance in the feature of faecal profiles between two groups. The score plot of individual compounds (Fig. 4-4B) was in accord with the significant difference demonstrated in Table 4-1, 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.

A



B



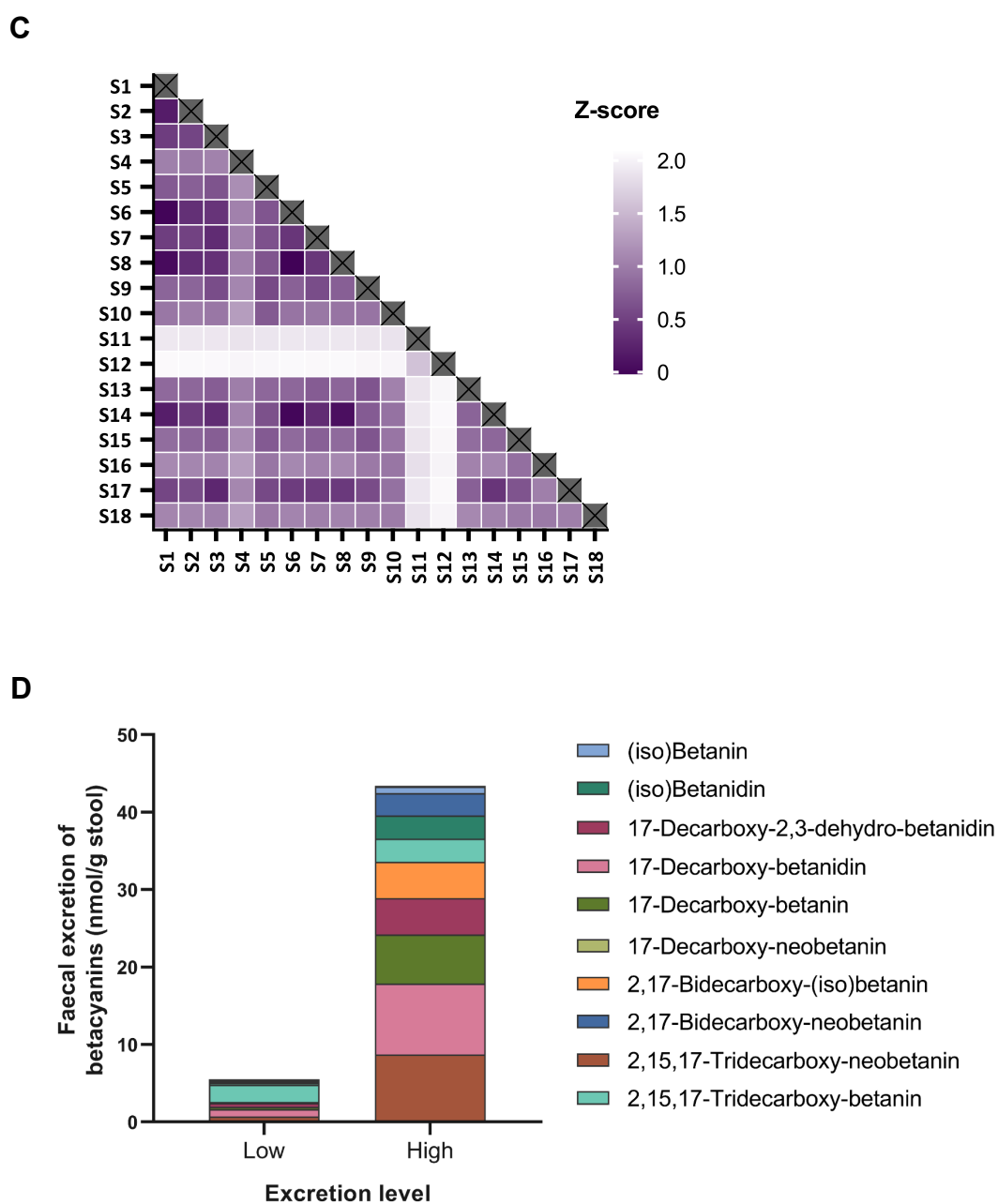
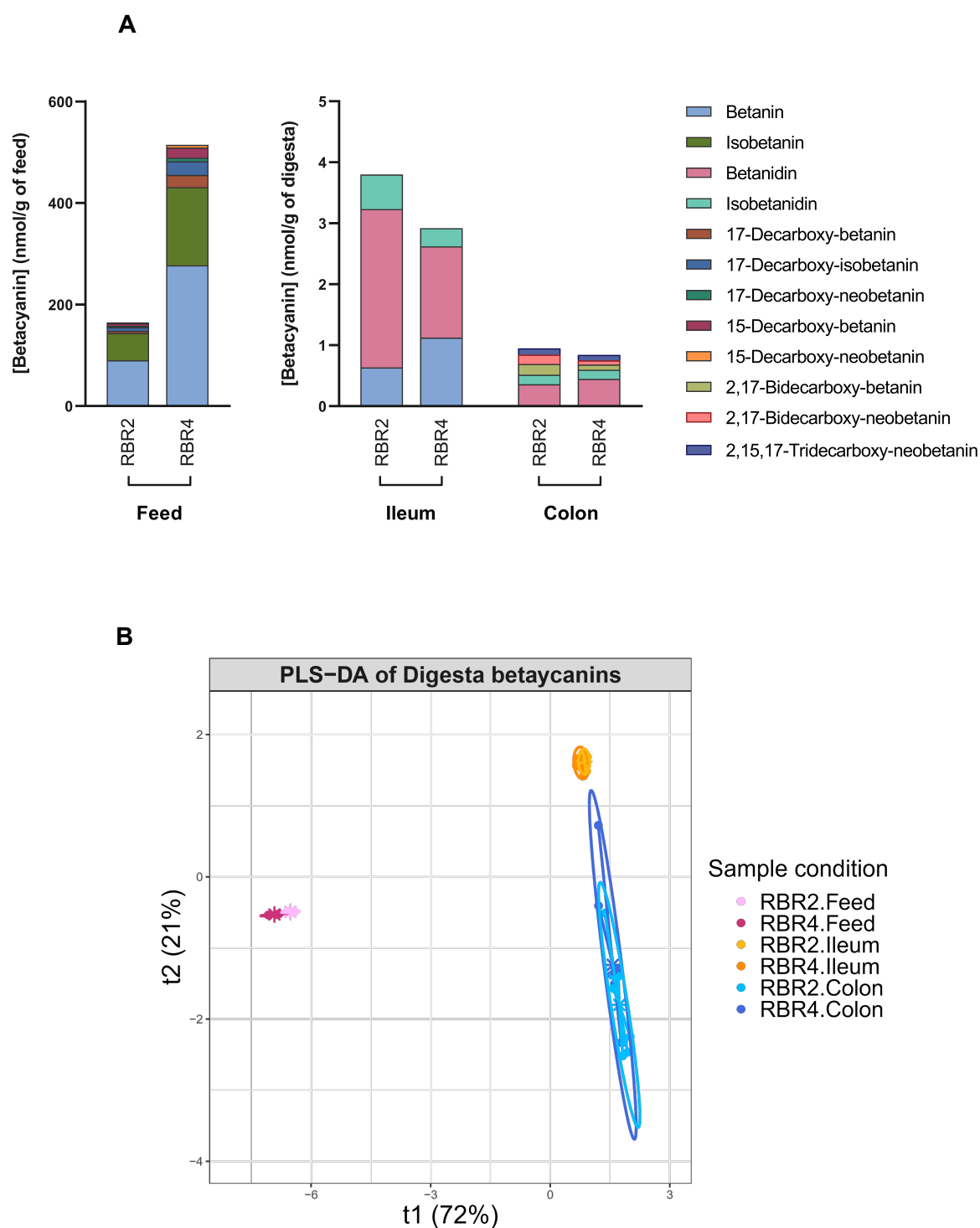


Figure 4-4. Variability of betacyanin excretion profiles in stool and stratification according to betacyanin excretion level, which are demonstrated in the PLS-DA score plots of stool samples grouped by (A) different days after ingesting red beetroot juice and by (B) individual betacyanin derivatives. It is accompanied with the exhibition of (C) subject-based proximity matrix and (D) the compositional histogram of betacyanins in stools from low and high excretors.

Significant inter-individual variation was observed when comparing faecal betacyanin profiles with urinary extraction profiles, which was reflected by the overall greater z-scores in proximity matrix with apparent deviations of subject 11 and 12 (Fig. 4-4C). Stratification was also carried out based on the corresponding betacyanin level in stools, into low (n=13) and high excretors (n=5) with significant difference ($p = 0.01$). It was evidenced from Fig. 4-4D that faecal betacyanin composition of low excretors was predominated by 2,15,17-tridecarboxy-betanin, while that of high excretors 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 faecal excretion profile of individual subjects in the relative composition (%) was presented in Appendix 8B.

4.5.6 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 1.4% and 0.4% of the content in feed, respectively (Fig. 4-5). The inter-group difference was also recognised 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. 4-5B. No betalains were detected in the BSL samples.



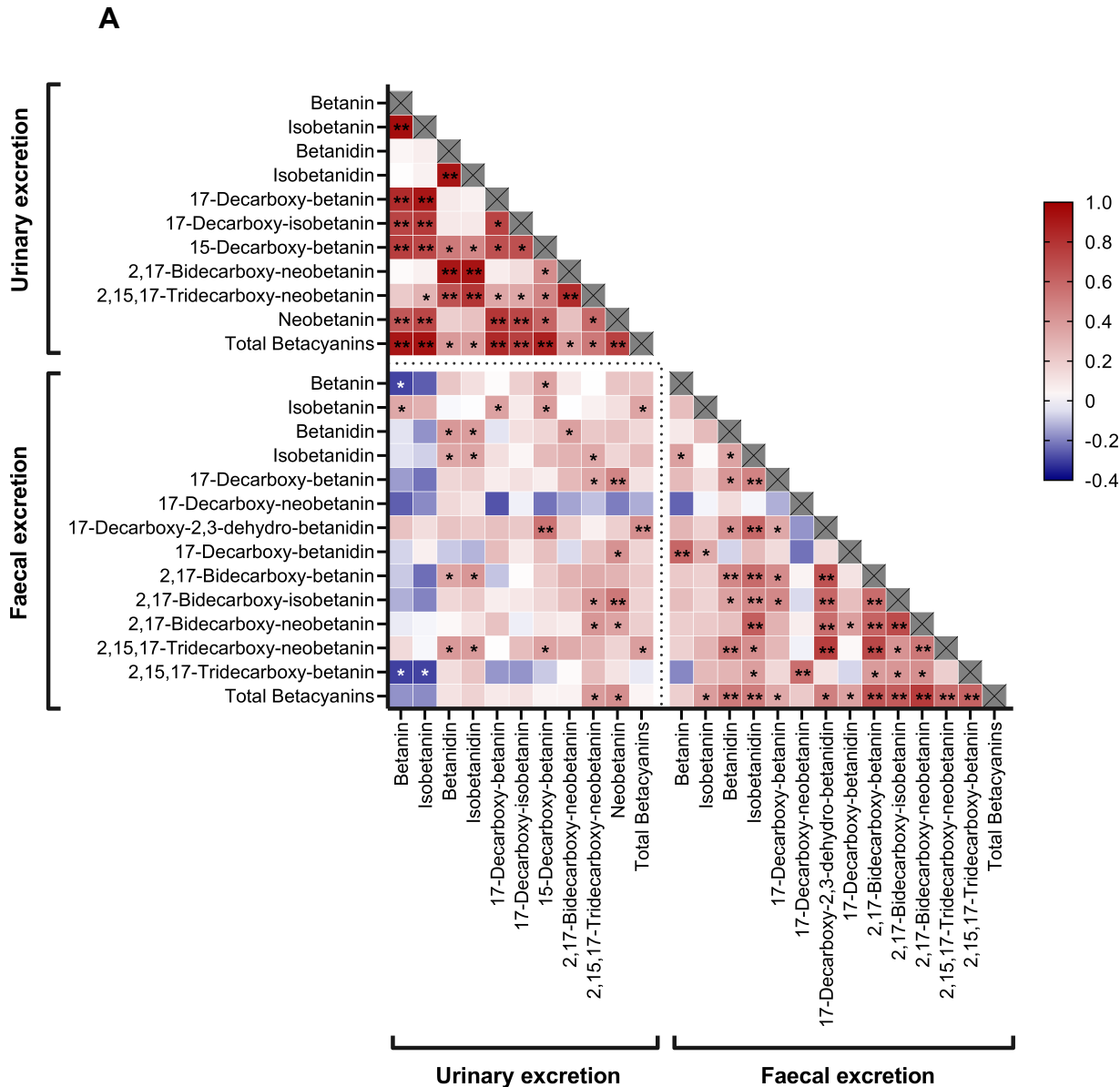
4.5.7 Correlation between urinary and intestinal excreted betacyanins of human and lifestyle factors

The tendency and strength of relationship between urinary and faecal 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. 4-6A has demonstrated an overall positive relationship among betacyanin compounds in renal and faecal 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 regards to faecal excretion, the positive tendency in correlation was evidently attenuated compared with renal excretion, and the significant relationship was mainly observed among the more fragmented 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-faecal 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 faeces. Moreover, the study reported a positive correlation between urinary and faecal (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 faecal excretion regarding the total betacyanin levels.

Associations between betacyanin excretion and the factors of diet, anthropometric, and lifestyle were presented in Fig. 4-6B and C. With regards to diet components, intake of meat and fish was discovered to be positively correlated with urinary and faecal 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 neobetananin derivatives in both urine and stool. With regards to other factors, results suggested exercise and frequent defecation as positive contributors to betacyanin excretion in this study.



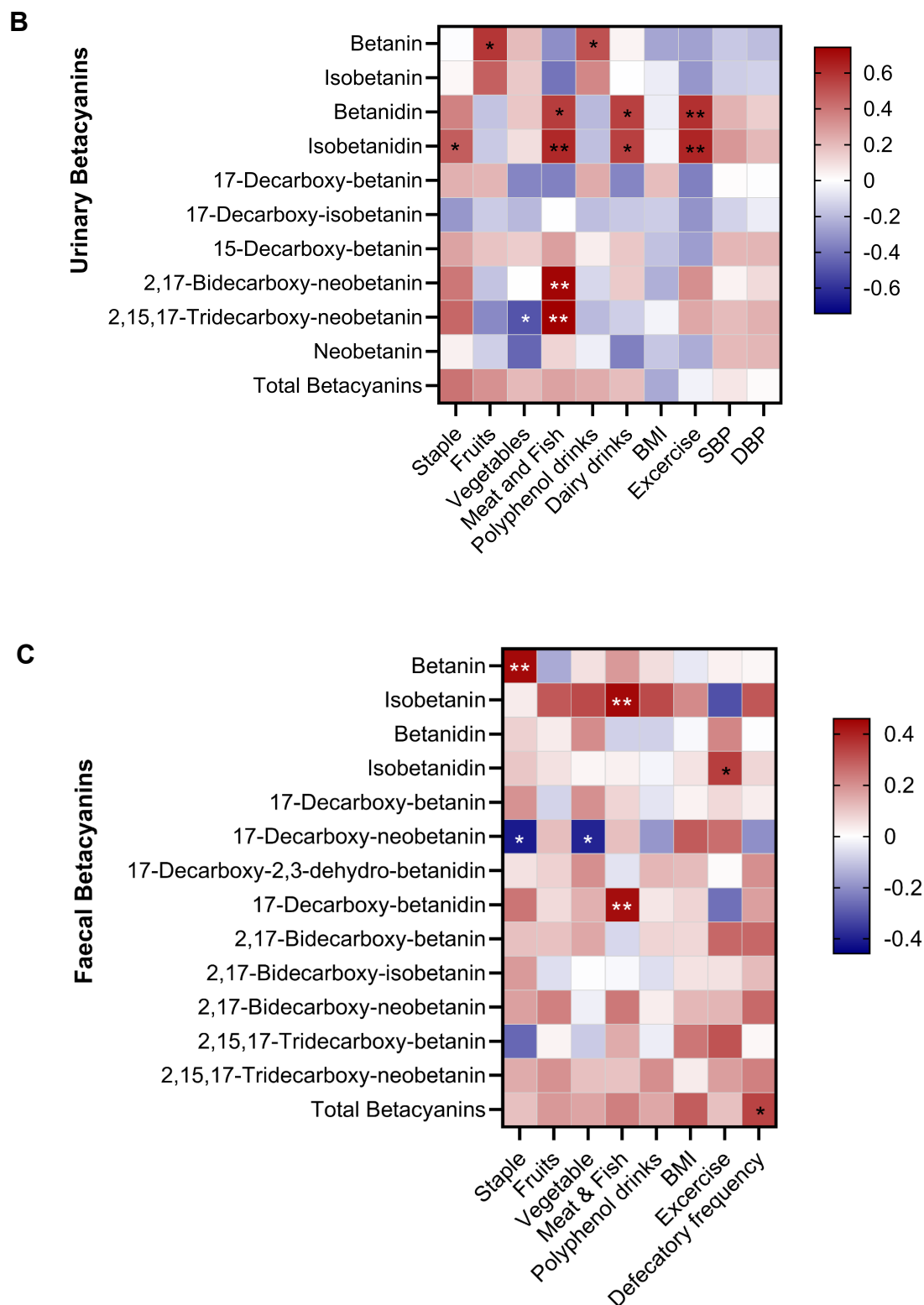


Figure 4-6. Correlation matrices between (A) urinary and faecal excretion of betacyanins, (B) among urinary excreted betacyanins and dietary/anthropometric/lifestyle factors, and (C) among faecal betacyanins and dietary/anthropometric/lifestyle factors. Correlations with strong evidence are marked by stars: * $p < 0.05$, ** $p < 0.01$. Noted the pigment correlations in urine were analysed via Pearson rank correlation test while the rest was of Spearman rank test.

4.6 Discussion

With a growing recognition of the importance of betalain health benefits in humans, the basis of intestinal excretion of betacyanin pigment and corresponding features still remains under-investigated. The study herein, 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 on the interconnections between bioavailability and bioaccessibility of betalain pigments.

The results confirmed the low urinary excretion rates thus bioavailability of betacyanins as observed in Wiczowski 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 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 metabolism following the ingestion. It is presumably caused by a constant dissipation of pigments, and the reducing number as well as diameter of paracellular passages from jejunum to colonic epithelia (Watson et al., 2001). The urinary pharmacokinetics of total betacyanin was previously studied by Frank et al. (2005) and Wiczowski et al. (2018) in healthy volunteers following a single oral administration of red beetroot juice. Higher values of f_e % (0.28% and 0.30%, respectively) and lower k_{re} (both 0.07 h^{-1}) were reported, in comparison to current work. There were also earlier studies, Kanner et al. (2001) and Tesoriere et al. (2004a) addressing the bioavailability of (iso)betanin, indicating the f_e % 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 Wiczowski et al. (2018) that carried out the human intervention with merely beetroot juice, 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 had a potential consequence of 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 the current study with 13 females and 5 males, there were no gender-related differences in the urinary pharmacokinetics, although this requires further assessment with a more balanced sample size.

In partial agreement with Wiczowski et al. (2018), our results indicated the overall dominance of (iso)betanin (64.1%) in urinary betacyanin composition and an overtaking by (iso)betanidin at the later stage of excretion. Three betacyanin metabolites were identified in urine which were lacking presence in beetroot juice and were therefore likely generated during hepatic and/or kidney detoxification. It supported the hypothesis that (iso)betanin are likely 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 the 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 ruled out (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. The (iso)betanidin, 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 behaviour of aglycones and other metabolites (e.g., 2,17-bidecarboxy-neobetanin) from (iso)betanin was likely caused by their generation from the systemic metabolism of native (iso)betanin. It is also proposed that the deglycosidation 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 ($0.4-3.2 \times 10^{-6} \text{ cm s}^{-1}$) compared to dietary phenolic compounds ($2-8 \times 10^{-6} \text{ cm s}^{-1}$) (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 low lipophilicity. As the most abundant betacyanins, (iso)betanin (MW > 500 Da) possess 9 hydrogen bond donors and 14 acceptors (i.e., non-Lipinski compound) that primarily contribute to the molecular polarity and solubility, which yet 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 faecal excretion of betacyanins

were previously explored by Krantz et al. (1980) via an animal study which recovered 3% of betanin from rat faeces 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 faeces and its 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. 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. 4-7. The degree and pattern of biotransformation were proposed to be principally determined by their chemical scaffolds. The betacyanin derivatives recognised 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 sulphate-conjugated pigments were not detected in urine and faecal samples therefore lacking evidence on Phase II metabolism. The detected catabolites 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 fibre-rich whole beetroot thanks to the physicochemical preservation exerted by the outermost compounds as well as the food matrix (Tesoriere et al., 2008; Wiczowski et al., 2018; Sawicki et al., 2020).

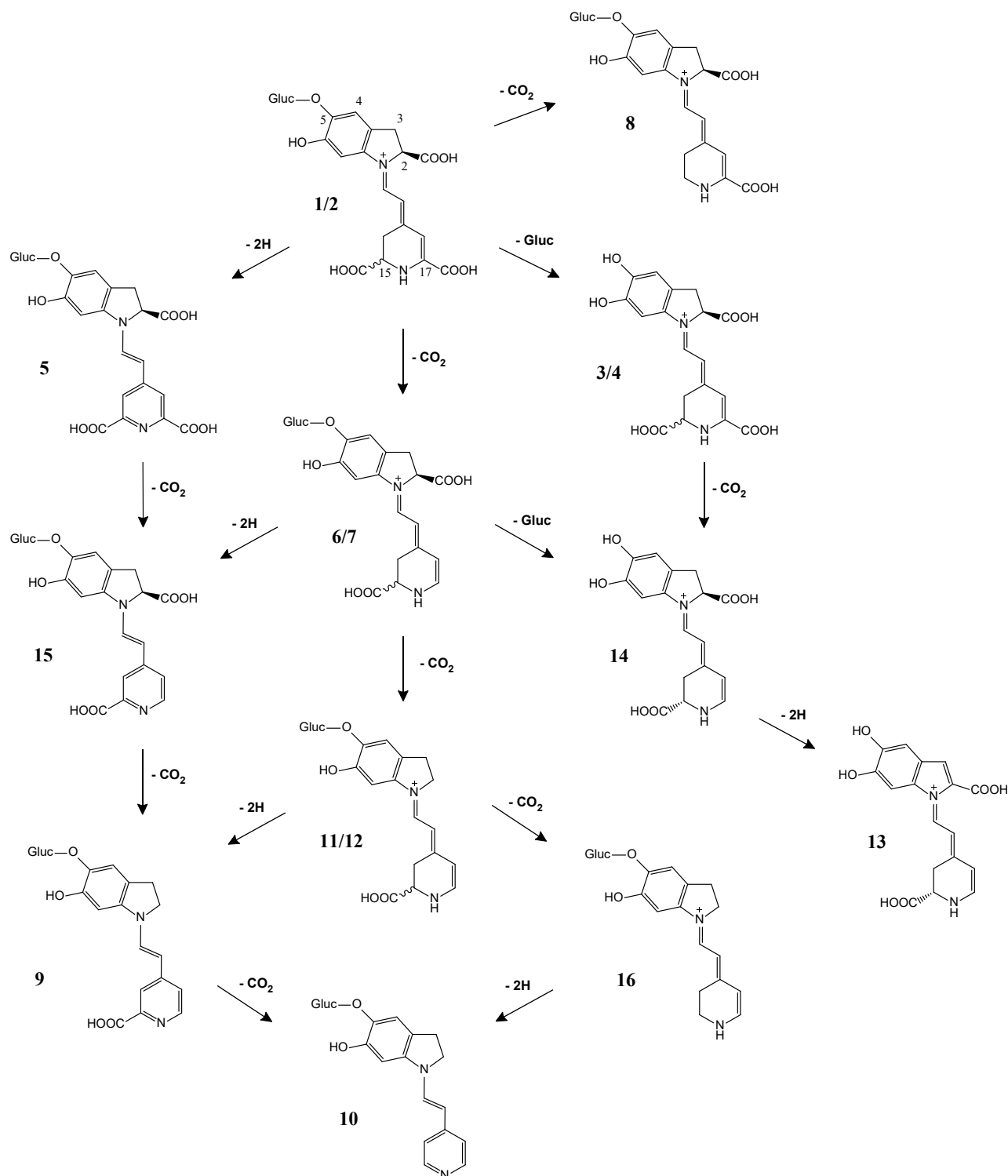


Figure 4-7. Proposed mechanistic pathway of betacyanin transformation and catabolite formation. The numbers of compounds are linked to those in Table 4-1.

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 faecal and faecal, and to urinary and faecal 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. 4-6), 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 faecal (iso)betanidin, it was hypothesised 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 hence renal excretion of (iso)betanin was accompanied with the decreased intestinal excretion of betacyanins. Concurrently an increment in luminal (iso)betanin concentration thus the bioaccessibility could potentially improve the 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; Wiczowski 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 pigment.

According to individual excretion level, the study stratified the participants into low/(medium)/high groups representing their innate abilities to uptake and metabolise 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. 4-6B and 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, 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 was supposed that gut microbiota primarily influences the bioavailability of betacyanins by catalysing their biotransformation, e.g., via deconjugation and reduction, thereby altering their physiochemical features and pharmacokinetics in the human body (Cussotto et al., 2021). Moreover, gut microbiota was found to participate in the 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 was increasingly explored by recent studies (Capper et al., 2020; Wang et al., 2022a). The result in Chapter V 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 faeces was markedly increased after red beetroot juice consumption. Noted that *Bifidobacterium* and *Coprococcus* are well-known

fermentative taxa contributing to the SCFAs production, which are inferred to contribute to the depletion of betacyanins. 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; details will be demonstrated in Chapter V. It is therefore essential to link the intestinal excreted betacyanins to various biological markers (e.g., mucosal microbiome) so as 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 faecal excretion could be determined via a single-dose trial and/or *in vitro* fermentation experiment. Furthermore, the demonstration of systemic metabolomic signature is recommended by analysing 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 faecal excretion and their correlation with bioavailability, future research should continue broadening the scope of metabolite characterisation and enhancing the detectability of fragments, with further emphasis on the effects of dose and food matrix as well as faecal excretion profiles of betaxanthins.

Chapter V

Effect of Two-week Red beetroot juice consumption on Modulation of Gut Microbiota in healthy Human volunteers

5.1 Abstract

Red beetroot has been increasingly highlighted as a source of bioactives, i.e., betalains and polyphenols, some of which exert strong radical scavenging and anti-inflammatory properties. With very little research exploring the intestinal effects of red beetroot consumption, the present pilot study investigated the gut microbial changes following red beetroot consumption, via a 14-day intervention trial in healthy adults. Despite the absence of significant changes in alpha- and beta-diversities before and after intervention, the study demonstrates transient deviations in abundance of specific taxa e.g., *Romboutsia* and *Christensenella*, after different days of intervention compared to baseline ($p < 0.05$). The enrichment of *Akkermansia muciniphila* and decrease of *Bacteroides fragilis* were observed after 3 days of juice consumption ($p < 0.05$), followed by a restoration in abundance after 14 days. *A. muciniphila* has been inversely associated with obesity, diabetes, inflammation, and metabolic disorders, whereas *B. fragilis* is potentially enterotoxigenic causing inflammatory diarrhoea. Consumption of beetroot juice additionally increased the production of total SCFAs especially (iso)butyric acid in stools ($p < 0.05$). With native betacyanins and catabolites detected in stool after juice consumption, betacyanins were found to correlate positively with *Bifidobacterium* and *Coprococcus*, and inversely with *Ruminococcus* ($p < 0.1$), potentiating the significant rise in (iso)butyric acid content ($172.7 \pm 30.9 \mu\text{mol/g}$ stool). Study findings indicate the potential of red beetroot to influence gut microbial populations and catabolites associated with these changes, emphasising the putative health implications of red beetroot on intestinal as well as systemic health. Further research needs to assess the long-term implications of beetroot on the gut microbial changes including mucosal microbiota and the physiological consequences for the gut and the host organism.

5.2 Introduction

The structure of human gut microbiota is composed of bacteria, virus, protozoa, archaea and fungi, with the microbial biomass ascending distally in the intestinal tract from duodenum and jejunum (10^{2-3} g^{-1}), to ileum and cecum (10^{4-7} g^{-1}), and reaching the maximum density in the colon ($10^{11-12} \text{ g}^{-1}$) (David et al., 2014). Despite considerable inter-individual variations, the human gut microbiota is dominated by the phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, accounting for more than 90% of all bacteria. It is well-known that the gut microbiota plays an important role towards human health in diverse aspects including fermentation, metabolism, immunomodulation and neurobehavioral traits (Hakansson and Molin, 2011a).

Dysbiosis of gut microbiota is generally featured by a decrease in bacterial richness and diversity in tandem with the disturbed compositional balance between commensal and putatively pathogenic species. It has been intimately associated with a wide array of enterobacteria-dependent disorders such as inflammatory bowel disease (IBD), obesity, diabetes mellitus, allergic reaction, and depression (Karlsson et al., 2013). For example, the interplay between gut microbiota and IBD pathogenesis, comprising Crohn's disease and ulcerative colitis, has been studied *in vivo* via the gut microbial signatures of IBD-positive models (Gevers et al., 2014; Ni et al., 2017), which were often characterised by the reduced abundance of genus *Faecalibacterium* and rise of *Escherichia* in the stools (Manichanh et al., 2006; Lo Presti et al., 2019). Therefore, it is a wide consensus that the metabolic and immune regulations in the intestinal environment are profoundly influenced by the inherent bacterial community (Wu and Wu, 2012; Khan et al., 2019; Alam et al., 2020).

There are a variety of intrinsic and extrinsic properties shaping microbial features. Compared to hereditary factors, environmental factors (e.g., diets, lifestyle, and health condition) are reckoned as stronger determinants to gut microbial development, with diet being one of the most pivotal confounders (Niederreiter et al., 2018). Major changes of diet components (e.g., carbohydrates and proteins) could induce a rapid perturbation in the genetic

composition and metabolic functions of the gut microbes due to the mutual interactions between enteric bacteria and undigested nutritional substrates; this is followed by a long-term adaptation of intestinal homeostasis (Wu et al., 2011; David et al., 2014). Even though permanent shift of gut microbiota might necessitate prolonged dietary adjustments, the benefits of a therapeutic dietary approach are gradually recognised for the improvement of gut microbial environment hence the host health.

Red beetroot (*Beta vulgaris* L.) is regarded as a rich source of betalains, polyphenols, and nitrates. While numerous studies have emphasised the antiradical and anti-inflammatory properties of phenolic phytochemicals, there is emerging attention towards their ability in alteration of gut microbes. Animal research, for instance, documented a connection of non-absorbed flavan-3-ols and anthocyanins residing in the mice guts with the stimulated growth of *Akkermansia* genus and *Bacteroidetes* phylum, and with the depleted *Firmicutes* phylum (Masumoto et al., 2016; Gu et al., 2019). Colonic fermentation in the presence of polyphenol-rich extract is also associated with elevated generation of short-chain fatty acids (SCFAs) especially the proportion of butyrate (Jakobsdottir et al., 2013; Wang et al., 2018). Both polyphenol and SCFAs are believed to contribute to the regulation of intracellular signal transduction pathways hence the enhancement of metabolic outcome, resulting in delayed onset or prevention of systemic inflammation. The nitrates in beetroot are widely known for their powerful hypotensive effect, which have meanwhile demonstrated potential to influence gastrointestinal motility (Walker et al., 2018; Capper et al., 2020). Additionally, Hu et al. (2020) elucidated the role of nitrates in regulating microbial dysbiosis, maintaining colon consistency, as well as inhibiting the p53 transduction pathway. This suggests the potentiality of nitrates in gut microbiota alteration and anti-colitis, albeit more research evidence will be required to draw the firm conclusion.

Resembling polyphenols, betalains are another group of phytochemicals with positive biological activities have so far been largely overlooked. Betalains, especially the betacyanin subgroup, were discovered to efficiently suppress the formation of reactive oxygen species in cell-free and cellular conditions, which has highlighted their ability to mitigate oxidative

stress and restore cellular redox balance (Wang et al., 2022b). Latest *in vivo* and *in vitro* research have also expounded the potent immunomodulatory properties of betalains, specifically by the molecular interference with pro-inflammatory and/or redox-sensitive signalling cascades. This modulates the expression of downstream target proteins and enzymes that ultimately mediate cellular inflammatory responses and redox status (Krajka-Kuzniak et al., 2013; Tesoriere et al., 2014; Gómez-Maqueo et al., 2019; Fernando et al., 2022). These biological functions have been further associated with the remission of hyperglycaemia, type 2 diabetes mellitus, cardiovascular disease, IBD and other chronic illnesses (Khan, 2016a).

With regards to microbiota modulation, the antibacterial properties of betalains was evidenced by a few *in vitro* studies showing the inhibited proliferation of *Escherichia coli* and *Salmonella typhimurium* following the treatment of betalain-rich extracts (Velićanski et al., 2011; Hayek and Ibrahim, 2012). However, investigation of the influence of beetroot components on gut microbiota is still lacking from *in vivo* aspect. Capper et al. (2020) carried out an 8-week parallel intervention trial with 36 older participants (age 67 ± 6 years) who consumed whole beetroots or a control diet every two days. The evident shifts in gut bacteria in the elderly were reported, specifically phylum *Bacteroidetes* and genus *Alistipes*; it was accompanied with an increase in SCFA concentrations and lowering of blood pressure following whole beetroot ingestion. An earlier study in a high-fat-diet fed rodent model found an enhanced gut microbiome response with decreased ratio of *Firmicutes* to *Bacteroidetes* phyla following a 14-week feeding of high-fat diet supplemented with red pitaya betacyanins compared with control (Song et al., 2016).

5.3 Aims

Given the scarcity of research exploring the *in vivo* impact of red beetroot on human gut bacteria and metabolites, the present pilot study aimed to –

- Explore the effect of red beetroot juice on the modulation of gut microbiome in healthy individuals.
- Investigate for the first time the association between non-absorbed betacyanin catabolites and microbiota changes during intestinal fermentation of beetroot.

5.4 Method

Methods of the present chapter involved again the 2-week human trial with the consumption of red beetroot juice (Section 2.3.1 – 2.3.3). Moving on from the previous chapter on betalain bioavailability, the current analytical focus was shifted to potential alterations in human gut microbiome while linking to the data of betacyanin metabolism. Betacyanins in the juice and human stools were examined via UHPLC-MS technique (Section 2.3.5.1 and 2.3.5.3). With regards to gut microbiome analysis (Section 2.3.6), the 16S rRNA gene from human stools were amplified, sequenced, and compared to the database, followed by the analyses on microbiome diversities and differential abundance via QIIME2 package and R software. At the meantime, the study investigated the relative population of target species as well as SCFAs content via qPCR and chromatography, respectively; the data, together with gut microbiome, were eventually correlated with native betalains and metabolites.

5.5 Results

5.5.1 Subject characteristics and dietary intake

As mentioned in Chapter IV, all eighteen participants, with the mean age of 29.1 ± 6.1 and BMI of $22.5 \pm 2.4 \text{ kg m}^{-2}$ (mean $\pm$ SD), completed the study with high compliance (98%) and no drop-out. According to food diary and FFQs, marked inter-individual diversity existed between the diet patterns of participants in terms of the intake frequencies of staple, polyphenol-rich fruit and vegetables, polyphenol-rich beverage, and meat dishes ($p < 0.05$). However, there was no evident change of their individual diet pattern along the intervention period ($p > 0.05$). The SBP and DBP were not markedly changed before and after the beetroot juice consumption.

5.5.2 Presence of betacyanins and their metabolites in juice and in faecal samples

The composition of betacyanins and polyphenols in the beetroot juice is illustrated in Fig. 5-1A and B. The native pigments consisted dominantly of betacyanin glucosides, i.e., (iso)betanin (86.7%) and neobetanin (9.3%), whilst minor proportions were found in degraded forms such as aglycones (i.e., (iso)betanidin) and decarboxylates, which, together with glucosides, made up to a total content of 114.5 mg betacyanins per portion of juice. By contrast, polyphenols were present in the juice at lower amount (15.6 mg/portion) compared with betalains, with principal components being chlorogenic acid (65.7%) and ferulic acid (17.4%). Of note, vulgaxanthin I, a common betaxanthin in red beetroot, was not detectable in the beetroot juice, therefore further analysis of which was infeasible in the current study.

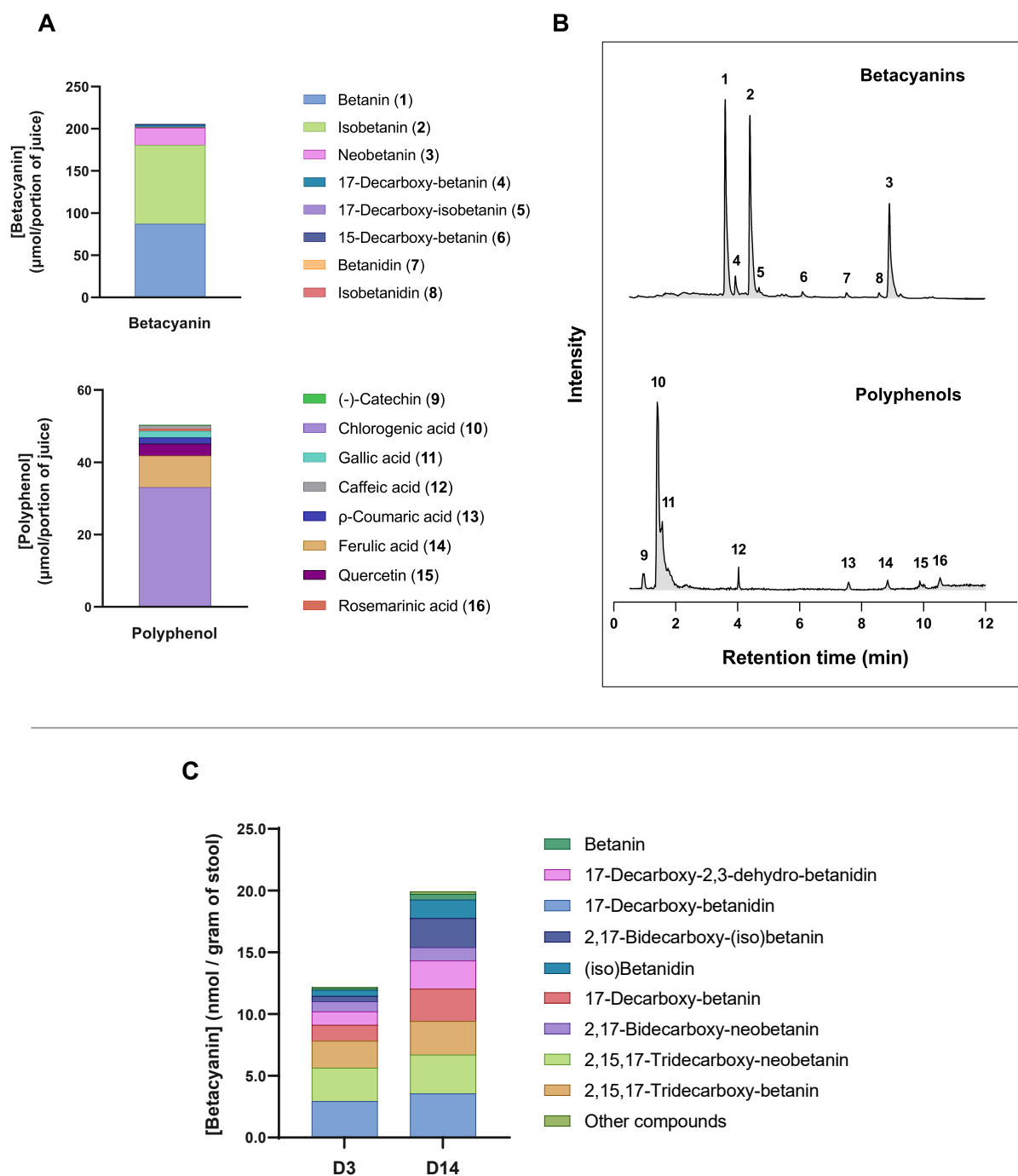


Figure 5-1. Profiles of red beetroot juice and the faecal excreted betacyanins after juice ingestion, illustrated in (A) the compositional histogram of betacyanins and polyphenols of juice with (B) corresponding chromatograms, as well as (C) the composition of dominant betacyanin catabolites in human stool on D3 and D14 acquired via UHPLC-MS. Detailed composition of catabolites was tabulated in Table 4-1.

By employing UHPLC-MS techniques in the current study, there were 11 major catabolites of betacyanin being identified in faecal samples. As demonstrated by the cumulative histogram of faecal excretion profiles (Fig. 5-1C), stools at D14 had higher total amounts of excreted betacyanins compared to D3, which was majorly contributed by the increase in 17-decarboxy-betanin, 2,17-bidecarboxy-(iso)betanin, 17-decarboxy-2,3-dehydro-betanidin, and (iso)betanidin. However, there were no marked differences in the levels of specific metabolites between D3 and D14 when performing the conventional statistical analysis, due to the notably large inter-individual variability of faecal excreted betacyanins. With regards to composition, all the stool samples contained dominantly the catabolised forms of betacyanins (76.9-84.3%), with the principal compounds comprising 17-decarboxy-betanidin, 2,15,17-tridecarboxy-neobetanin, and 2,15,17-tridecarboxy-betanin that together dominated over 50% of characterisable catabolites. Native and catabolised betacyanins were not detected in baseline stool samples. The detailed description could be found in Chapter IV.

5.5.3 Taxonomic diversity and distribution of microbial communities

The richness and diversity within the microbial community were demonstrated using phylogenetic alpha-diversity indices – Shannon, Chao1, Simpson and phylogenetic diversity (Fig. 5-2). Species in faecal samples of BSL, D3 and D14 possessed similar community diversity as indicated by Shannon entropy ($p > 0.05$), whereas post-consumption groups (i.e., D3 and D14) showed higher phylogenetic diversity than BSL, especially at D14 ($p = 0.039$). There was no difference being observed between post-consumption groups. Chao1 and Simpson indices with adjusted p -values did not confirm the significant divergence in the total species abundance and in the uniformity of species distribution between three groups ($p > 0.05$). However, both Chao1 and Simpson indices of post-consumption groups revealed higher convergence in distribution compared with BSL.

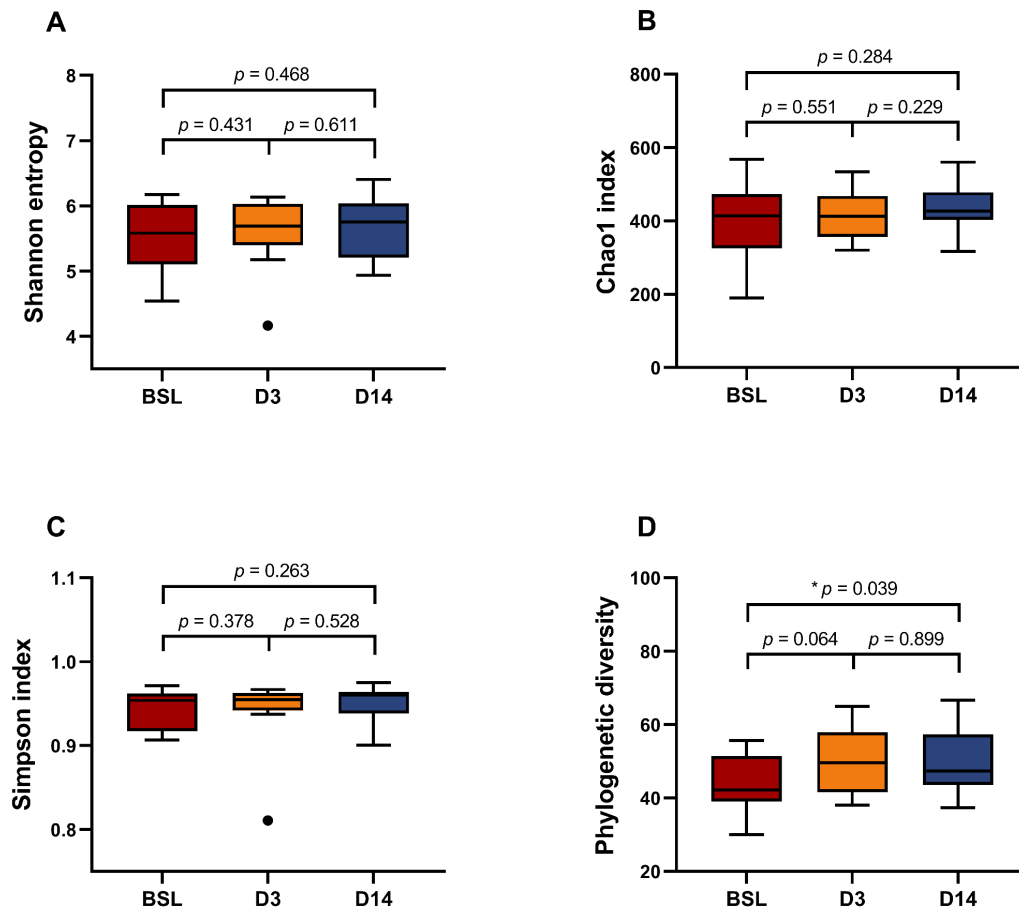


Figure 5-2. Alpha diversity indices of human gut microbiome on different days of beetroot juice consumption illustrated with (A) Shannon, (B) Chao, (C) Simpson and (D) phylogenetic diversity whole tree indices. Significant difference was analysed by Wilcox and Tukey test and was indicated as * ($p < 0.05$).

Analysis of beta-diversity is presented in Fig. 5-3 using unweighted phylogenetic Unifrac distance with significance calculated by PERMANOVA method. The clustering of three groups in the principal coordinates analysis (PCoA) plot overlapped to a large extent ($p = 0.993$), suggesting that the community composition before and after juice consumption was phylogenetically similar. The plot of non-metric multi-dimensional scaling analysis (NMDS), nevertheless, indicated that samples at BSL and D14 groups were more clustered compared with the D3 group. Consistent results were also acquired from the analysis of weighted Unifrac and Bray Curtis distance. Meanwhile, inter-individual variation of microbial community was visualised by the marked intra-group difference in PCoA, as well as by the unweighted Unifrac distance between distinct subjects as displayed in matrix (Fig. 5-3C).

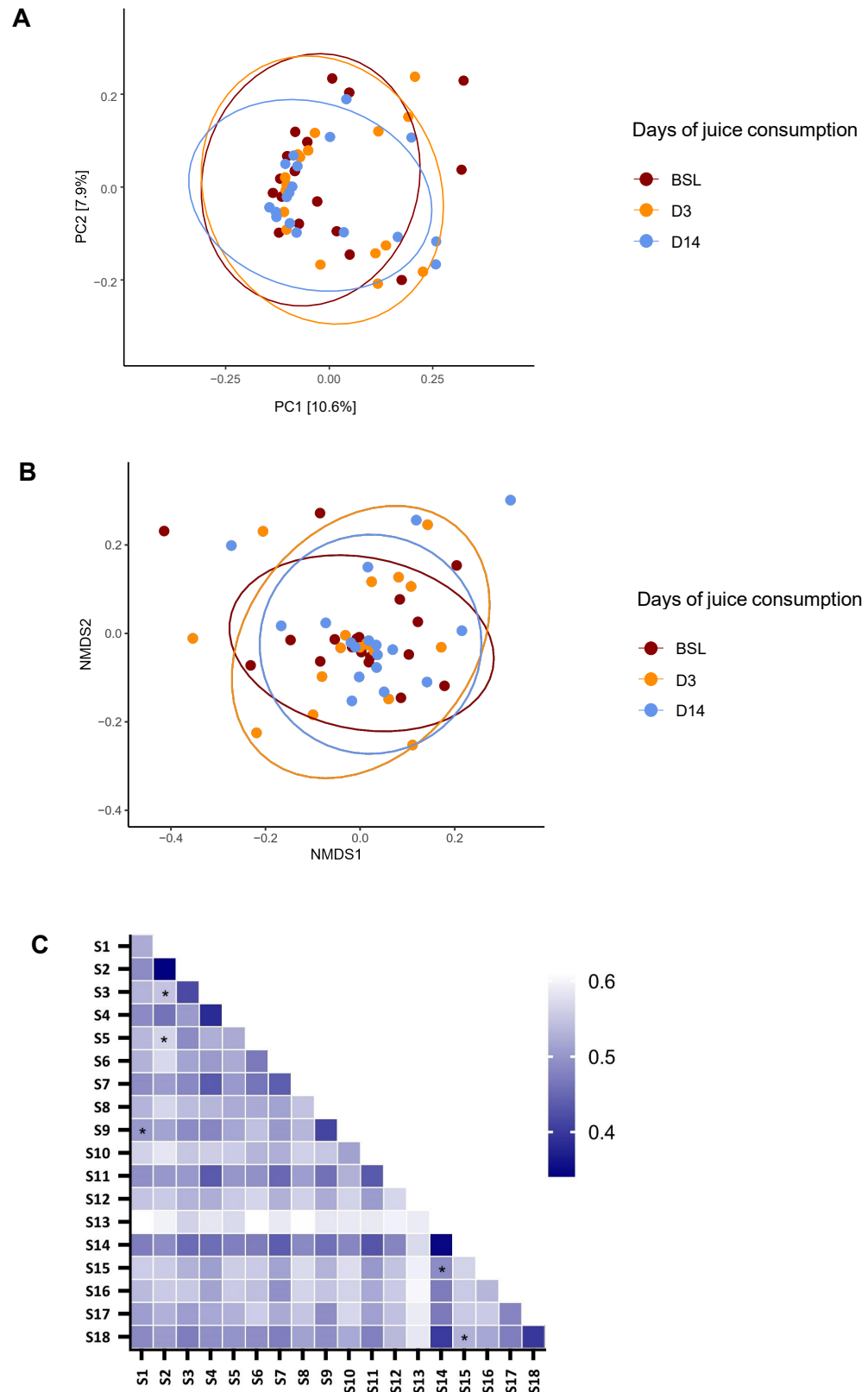


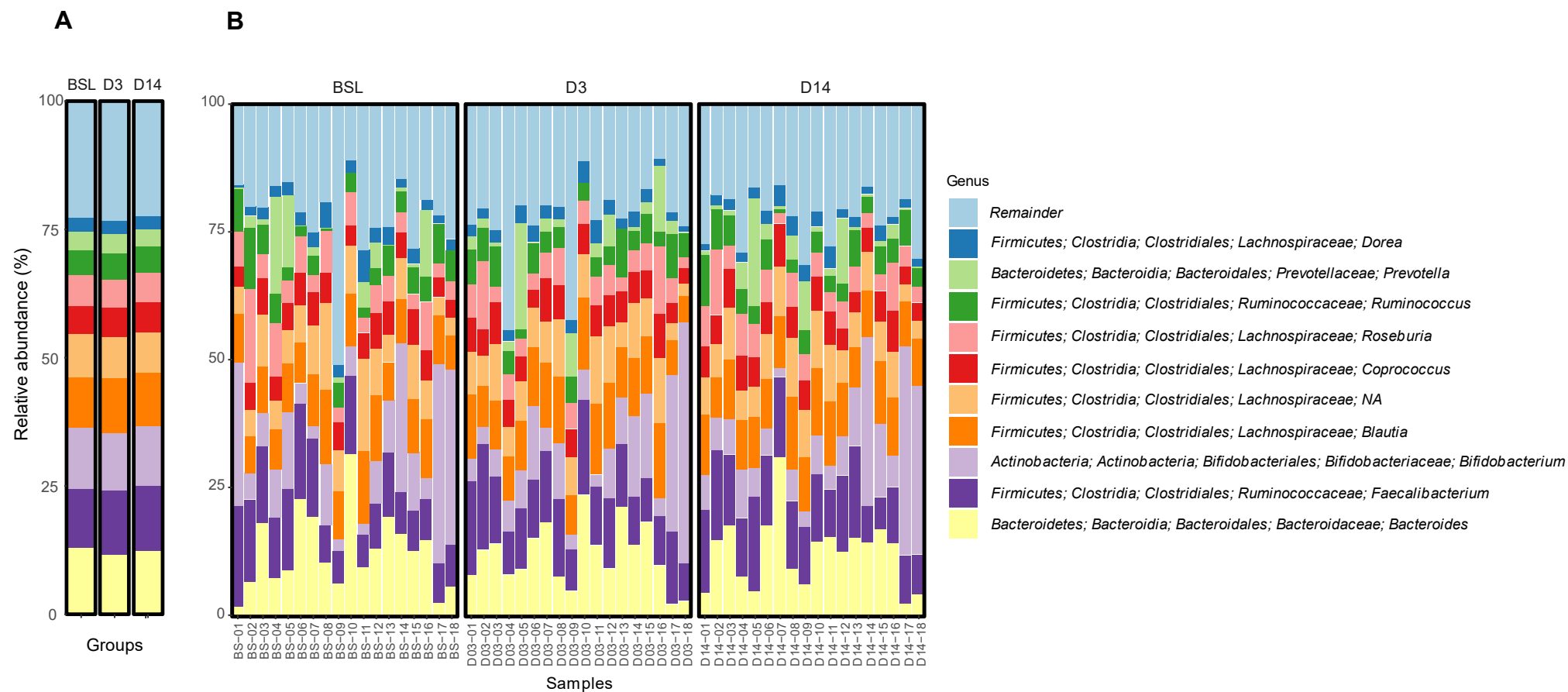
Figure 5-3. Beta diversity of gut microbiome on different days of beetroot juice consumption depicted with (A) the PCoA, (B) NMDS, and (C) subject-based matrix of unweighted Unifrac distance (n=18). Significant difference between subjects in (C) were analysed by Wilcox and Tukey test and was indicated as * ($p < 0.05$).

5.5.4 Comparison of taxonomic composition before and after beetroot juice consumption

The taxonomic composition of each group and sample was demonstrated in the distribution histogram of relative abundance (Fig. 5-4A and B) which displayed the dominant 10 genera from the taxonomic ranks, e.g., *Bacteroides*, *Faecalibacterium*, *Bifidobacterium*, and *Blautia*. They were subsumed by the most representative phyla in relative abundance, comprising *Firmicutes* (45-81%), *Bacteroidetes* (4-35%), *Actinobacteria* (3-52%), *Euryarchaeota* (0-20%), *Proteobacteria* (1-4%), and *Verrucomicrobiota* (0-3%). The change in taxonomic population between different days of beetroot juice consumption was visualised in a group-based heatmap (Fig. 5-4C), from which the difference in taxa composition was observed between D3 and other groups. Compared to BSL, the Log10 abundance of species *Prevotella copri*, *Bacteroides plebeius*, and *Bacteroides ovatus* were lower in D3, which then partially recovered in D14. A mild decrease in the level of *Rominococcus bromii* and an increase in *Bacteroides eggerthii* were also observed along the intervention.

The significance of inter-group variation in taxonomic abundance and distribution was further examined by the differential abundance analysis employing Wald hypothesis test and FDR-adjusted *p* values. By comparing among groups, taxa with statistical significance at 95% confidence interval were summarised and expressed in Log2-fold change relative to specific groups (Fig. 5-5). Concordant with PCoA plot, there were more taxa characterised with significant abundance from comparisons involving D3 than BSL and D14. At the genus level, *Arcobacter*, *Parvibacter*, *Parapusillimonas* and *Lactococcus* were decreasingly abundant in D3 group compared with BSL, whereas *GCA-900066755* abundance was increased. D14 microbiome has demonstrated the enrichment of genera *UC5-1-2E3* and *Solobacterium*, as well as the lowering of *Christensenella* compared with BSL group. Regarding taxonomic difference between D3 and D14 groups, the latter possessed significantly higher populations of genera *Phocaeicola* and *Haemophilus*, and less abundant *Anaerofilum*, meanwhile families of *Hungateiclostridiaceae*, *Leuconostocaceae*, and *Peptococcaceae* were markedly enriched in D14 group. Furthermore, abundances of several taxa were discovered to shift significantly

across the juice consumption period. Results indicated a decline in abundance of genus *Romboutsia* after 3 days of juice consumption which was then recovered after day 14. Conversely, there was an increment in population of genus *DTU014* around day 3, followed by an overall recession afterwards to the approximate BSL level. The decreasing tendency in abundance of *Bacteroidales* order along the intervention period was also noteworthy, which was aligned with the reduction of its lower-ranked species as shown in composition heatmap (Fig. 5-4C). In this study, the 14-day beetroot juice consumption did not exert significant impact at the phylum and class levels of gut microbiota.



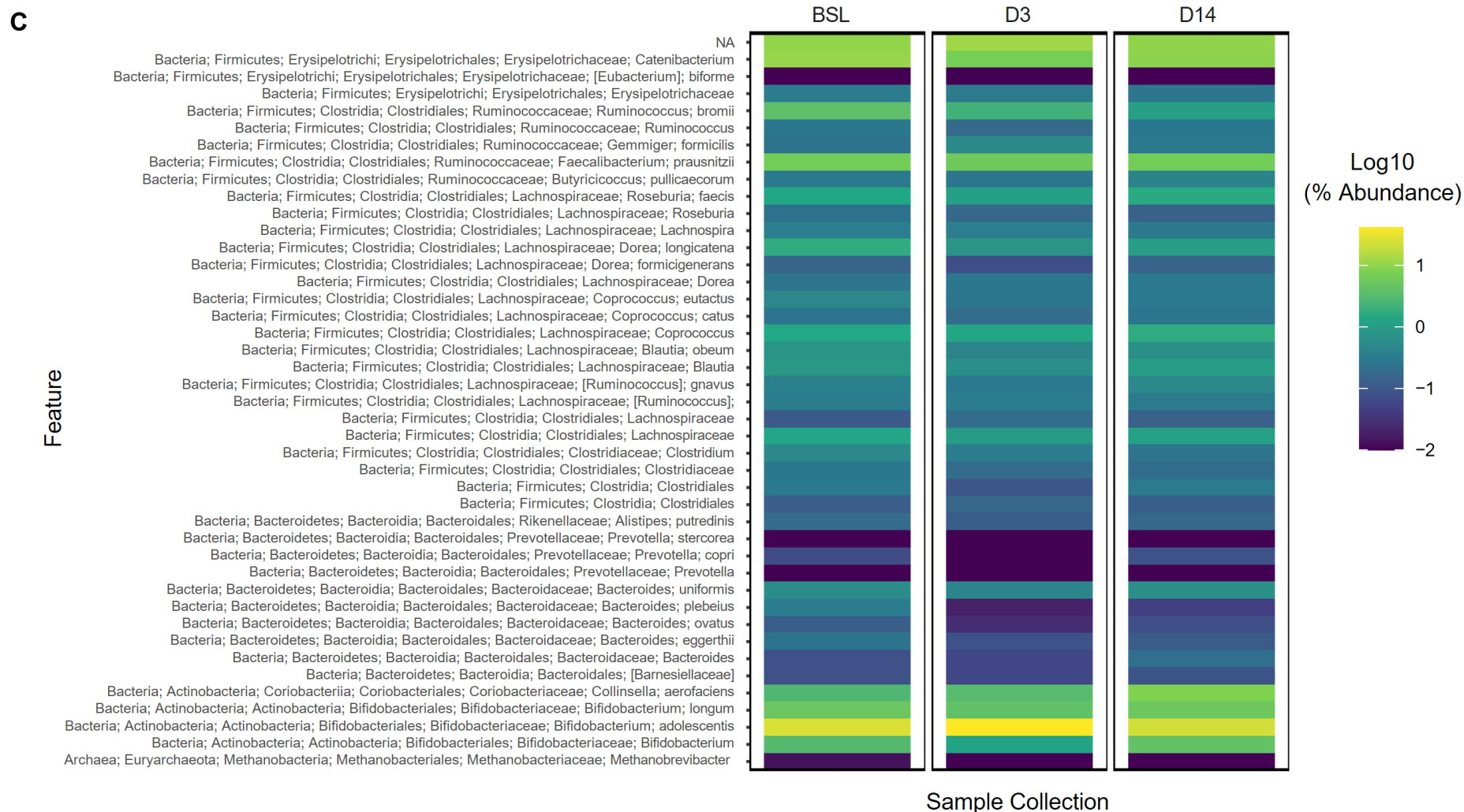


Figure 5-4. The taxonomic composition and abundance alterations in the BSL, D3 and D14 groups, demonstrated in (A) group-based and (B) sample-based taxonomic composition histogram of relative abundance of top genera, and (B) group-based heatmap with observable enrichment and depletion of specific taxa.

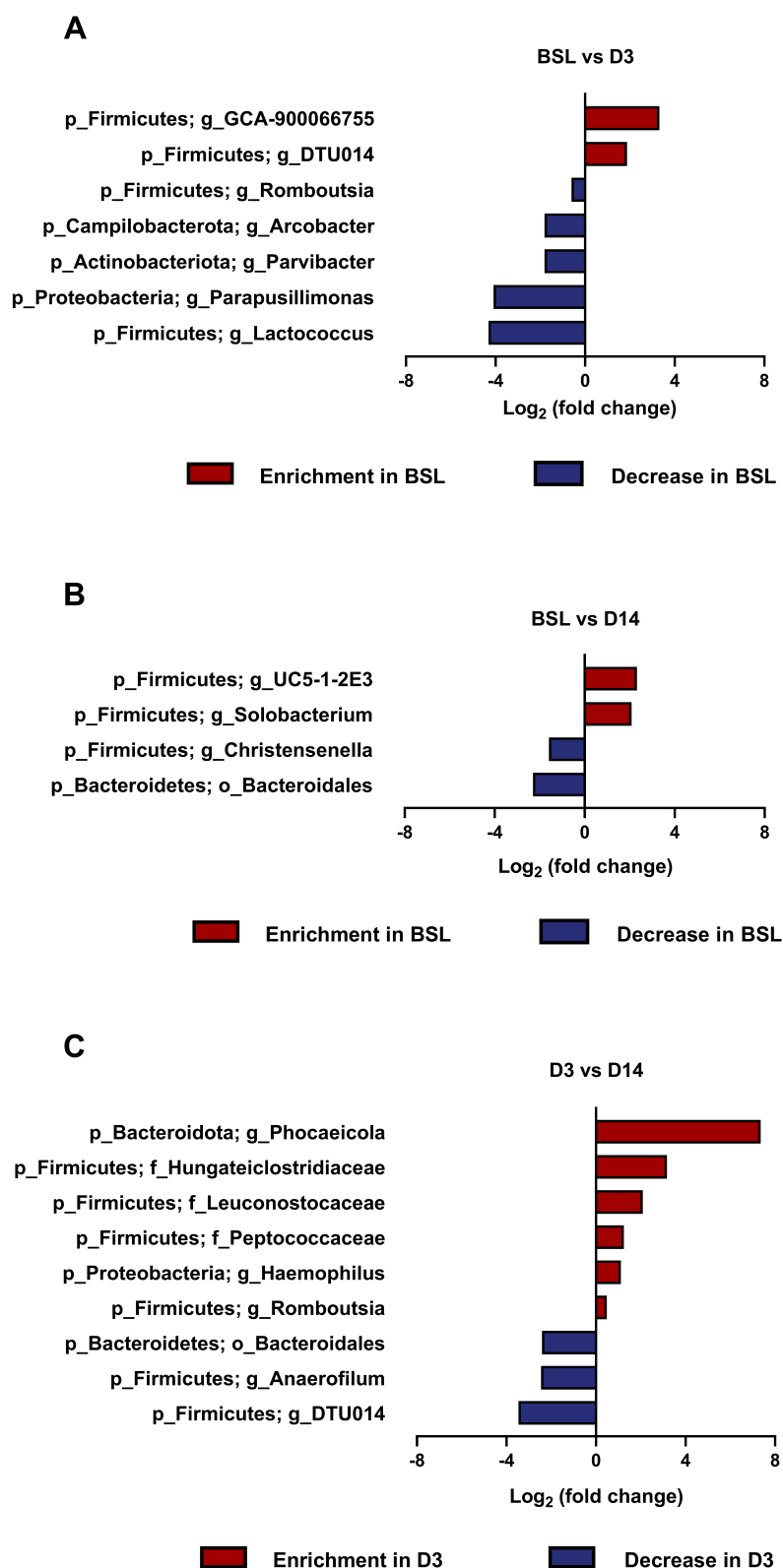


Figure 5-5. Inter-group comparisons expressed in the Log₂-fold change of taxa with significantly different abundance between (A) BSL and D3, (B) BSL and D14, and (C) D3 and D14. Note the fold changes in (A) and (B) were respect to BSL, and changes in (C) were respect to D3, using FDR-adjusted Wald hypothesis test ($p < 0.05$).

5.5.5 Relative abundance of target microbial species

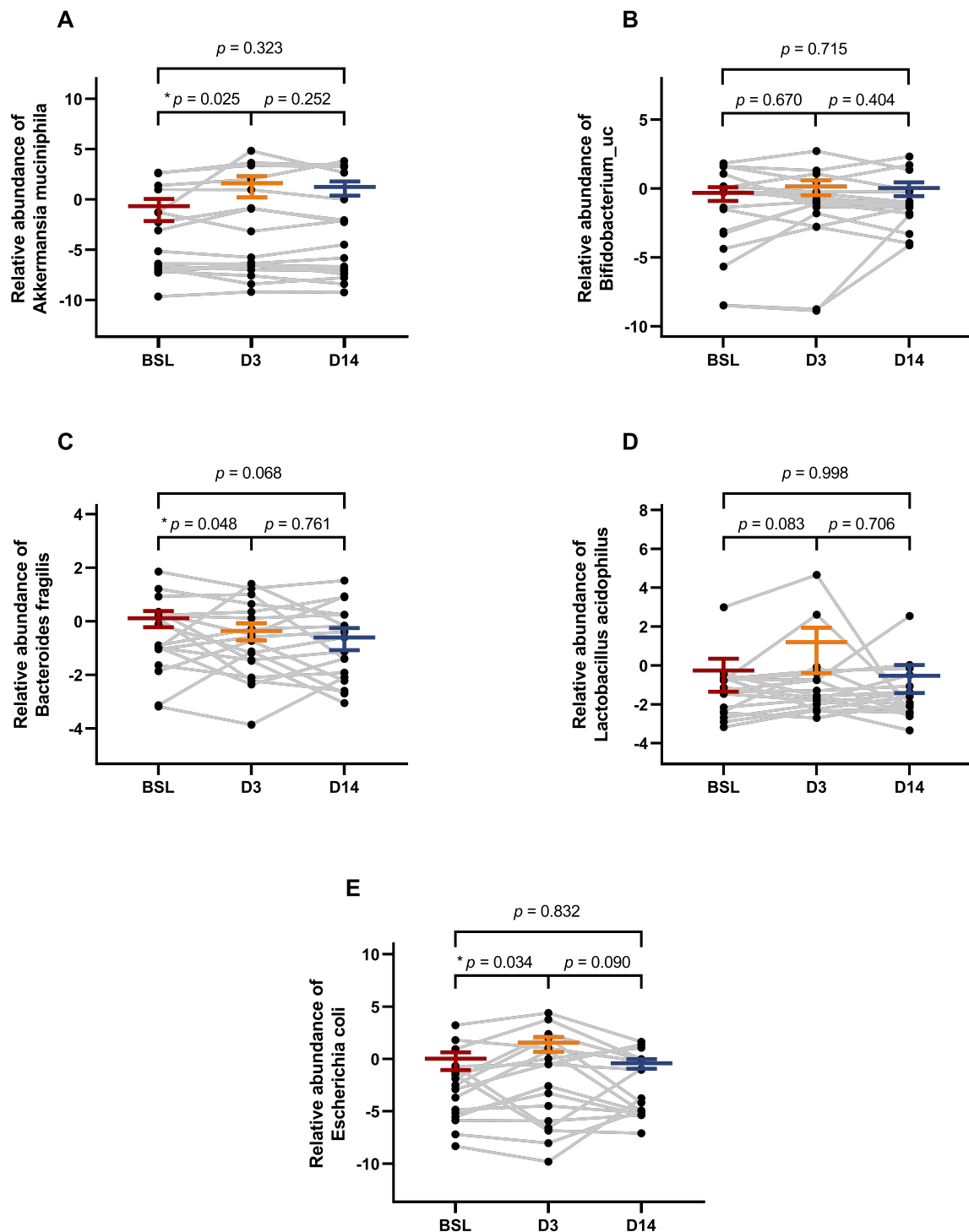


Figure 5-6. Quantitative changes of the relative species abundance (in Log 2 scale) of (A) *Akkermansia muciniphila*, (B) *Bifidobacterium_uc*, (C) *Bacteroides fragilis*, (D) *Lactobacillus acidophilus*, and (E) *Escherichia coli* after day 3 and 14 of juice consumption. Data are mean with SEM, and significance was analysed by Wilcoxon test ($p < 0.05$).

Relative abundances of target species in samples at different days were examined using real-time qPCR. According to Fig. 5-6, most differences were observed when comparing between BSL and D3 species, represented by enriched populations of *Akkermansia muciniphila* and *Escherichia coli*, as well as the reductions in *Bacteroides fragilis* ($p < 0.05$). Consistent with beta-diversity and composition heatmap, the study observed a restoration of D14 abundance from D3 towards the approximate BSL level in all tested species, except *B. fragilis* which showed overall decreasing trend along the intervention period ($p < 0.1$). The modest enrichment of *Lactobacillus acidophilus* in D3 compared to BSL, and modest decrease of *E. coli* from D3 to D14 were also displayed ($p < 0.1$). No difference was observed in the abundances of *Bifidobacterium_uc*, *Lachnospiraceae_uc* and *Roseburia_uc* between three groups (Appendix 12).

5.5.6 SCFA content

Table 5-1. SCFA concentrations ($\mu\text{mol/g}$ stool), and the ratio between specific acids in stools at BSL, D3 and D14 following beetroot juice consumption. Data are mean with SEM ($n=18$), and different letters indicate inter-group significance respect to each SCFA ($p < 0.05$).

SCFAs	BSL	D3	D14
Acetic acid	110.4 $\pm$ 13.5	175.6 $\pm$ 44.1	124.9 $\pm$ 8.4
Propionic acid	29.4 $\pm$ 5.7	36.3 $\pm$ 14.0	18.7 $\pm$ 2.2
Butyric acid	50.6 $\pm$ 10.7 ^a	130.7 $\pm$ 24.9 ^b	124.6 $\pm$ 22.9 ^b
Isobutyric acid	16.4 $\pm$ 6.9 ^a	42.0 $\pm$ 8.8 ^b	48.4 $\pm$ 10.3 ^b
Valeric acid	19.6 $\pm$ 6.6	22.4 $\pm$ 9.1	72.7 $\pm$ 28.0
Isovaleric acid	6.7 $\pm$ 2.8	2.4 $\pm$ 1.3	5.0 $\pm$ 2.6
Butyric/Propionic acid ratio	2.4 $\pm$ 0.5 ^a	6.5 $\pm$ 1.6 ^b	7.1 $\pm$ 1.2 ^b
Linear/Branched acid ratio	18.4 $\pm$ 5.4	9.7 $\pm$ 4.0	10.1 $\pm$ 4.2
Total	233.0 $\pm$ 24.8 ^a	409.3 $\pm$ 80.5 ^b	394.3 $\pm$ 50.7 ^b

There were six SCFA compounds detected and quantified in this study (Table 5-1). The composition was dominated by acetic acid (32-47%) and (iso)butyric acid (29-44%), whereas

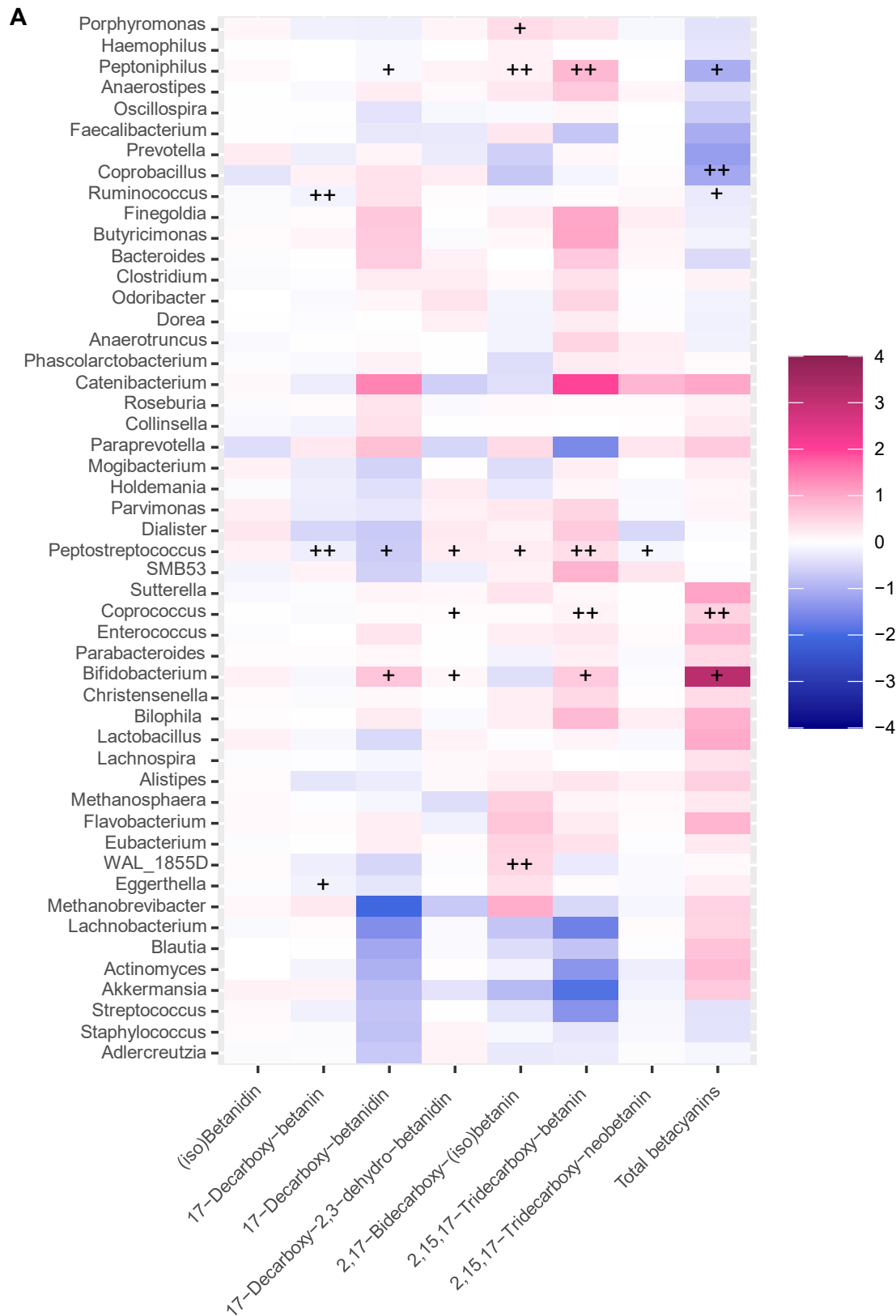
propionic acid (5-13%) and (iso)valeric acid (11-20%) were relatively minor. During the intervention, the total amount of SCFAs in D3 and D14 stools was remarkably higher compared to BSL stool. It was mainly attributed to an evident enrichment of butyric and isobutyric acids, which was also accompanied with a marked rise in butyric:propionic acid ratio in the stools of post-intervention groups ($p < 0.05$). Other than these, there were no significant changes being observed elsewhere.

5.5.7 Association between betacyanin catabolites, SCFAs, and microbial profile

The correlation matrix in Fig. 5-7A demonstrates the Spearman relationship between taxa in genus and concentrations of distinct betacyanin catabolites, revealing some genera, such as *Peptostreptococcus* and *Peptoniphilus*, being heavily and differentially influenced by the intervention. The lower-matrix genera were found positively correlated with the total excreted amount of betacyanins e.g., *Bifidobacterium* ($p < 0.1$) and *Coprococcus* ($p < 0.05$), whereas those at upper matrix were inversely affected by total pigments e.g., *Peptoniphilus* and *Ruminococcus* ($p < 0.1$). Among the catabolites identified, 2,15,17-tridecarboxy-betanin and 2,17-bidecarboxy-(iso)betanin have displayed different extends of promotional effect to distinct genera (e.g., *Peptostreptococcus*, *Coprococcus*, *WAL_1855D*), meanwhile the significant effects of 17-decarboxy-betanin appeared to be suppressive (e.g., *Ruminococcus*, *Peptostreptococcus*) ($p < 0.05$). The compound 17-decarboxy-betanidin showed a mix of positive and negative correlations with taxonomic data.

Associations between betacyanin catabolites, species of interest and SCFAs were further explored by correlation matrices. According to Fig. 5-7B, a negative correlation between 2,15,17-tridecarboxy-betanin and relative abundance of *Roseburia_uc* was the sole strong relationship identified ($p < 0.05$). The total excreted amount of betacyanins was positively associated with contents of (iso)butyric acid, (iso)valeric acid, and total SCFAs ($p < 0.05$). Specifically, 2,17-bidecarboxy-(iso)betanin, 2,15,17-tridecarboxy-betanin, and 17-decarboxy-betanidin demonstrated a strong relationship to (iso)butyric acid, meanwhile 2,17-bidecarboxy-(iso)betanin was also significantly related to (iso)valeric acid. By correlating

SCFAs with gut microbiota taxa (Appendix 13), (iso)butyric acid and total SCFA contents showed a positive relationship with genera *Blautia*, *Collinsella*, *Lachnobacterium*, and *Lactobacillus*, as well as an inverse association with *Ruminococcus* ($p < 0.1$).



B

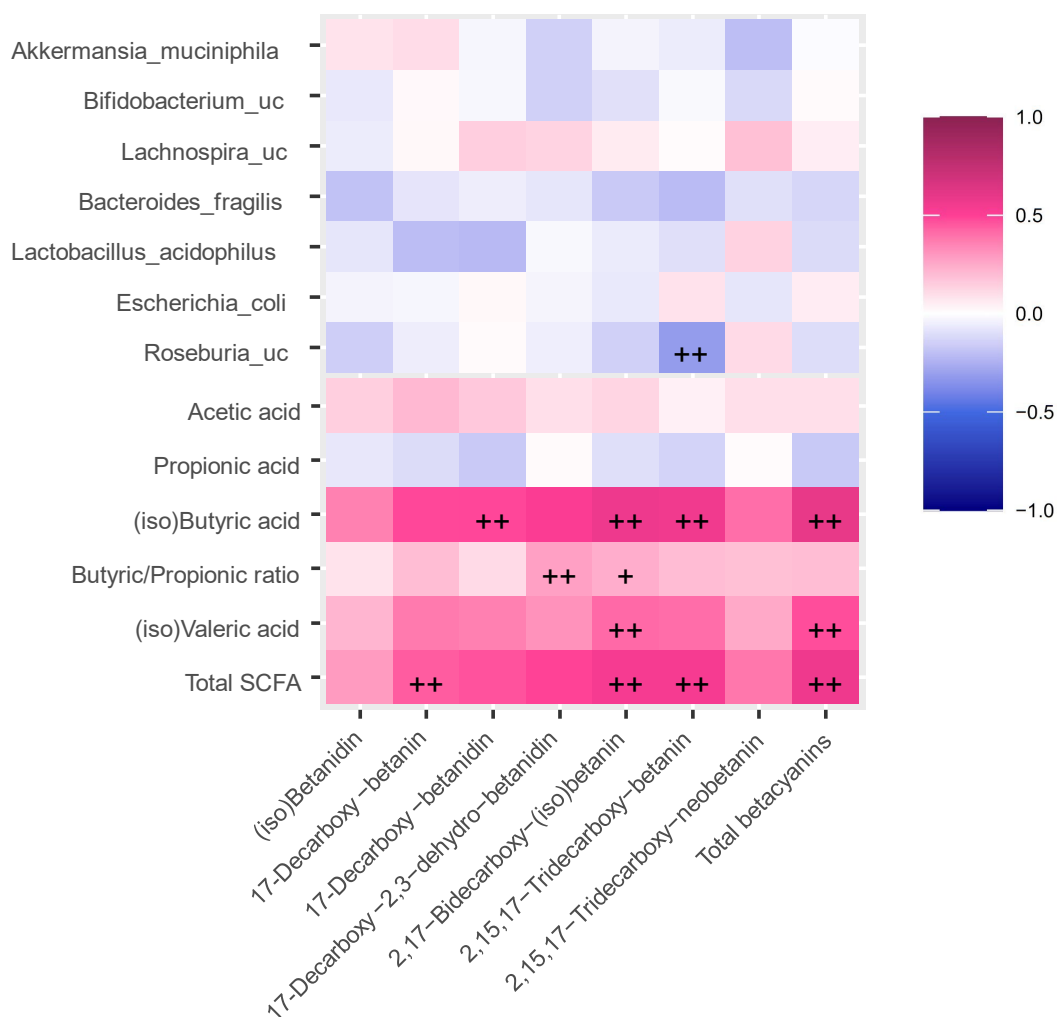


Figure 5-7. Spearman's rank correlation matrix demonstrating the relationship of faecal excreted betacyanins with (A) gut microbiota abundance at genus level, and with (B) relative abundance of target species plus SCFA contents. Correlations with strong evidence were labelled as: + $p < 0.1$, ++ $p < 0.05$.

5.6 Discussion

Beetroot and its pigments are increasingly explored in terms of their bioavailability and post-absorptive bioactivity, however, there is little research to date addressing the effect of beetroot phytochemicals on the gut microbiota. This pilot study, involving a 14-day period of beetroot juice consumption, presented with alterations of specific taxa in the human gut microbiome after 3 days, followed by a long-term adaptation after 14 days. This study also exhibited the profiles of catabolised betacyanins at the two time intervals, revealing a strong relationship between several taxa and betacyanins. These changes in microbiota and catabolites are anticipated to pose further influence on the regulation of metabolism and intestinal immune system.

There is a clear consensus regarding the poor availability of bioactive compounds that usually becomes a bottleneck to explain subsequent physiological effects. As one of the most enriched bioactive groups in red beetroot, betalains, especially betacyanins, possesses notoriously low absorption intensity reflected by their urinary excretion rate in the previous chapter (Clifford et al., 2017; Wiczowski et al., 2018), while the principal amount of pigment is anticipated to pass through the GI tract undergoing sequential biotransformation with potential involvement of enterohepatic circulation. The present study has detected trace amount of native betacyanins (e.g., (iso)betanin and (iso)betanidin) in stool samples (5.2–9.9% of total faecal betacyanins), which proved their capability of reaching the lower GI tract where potential interactions with gut bacteria may occur. Results also suggested dehydrogenation, decarboxylation and deglucosidation as the main mechanisms of betacyanin transformation, whereas glucuronic acid or sulphate conjugated betacyanins were not detected in stool samples.

The current study results indicated no statistically significant difference in alpha diversity and inter-group phylogenetic distance following beetroot juice consumption. This might be interpreted by the large inter-individual variability of gut microbiota profiles considering that the habitual diet of each participant was maintained during the intervention

period. More significant changes on taxonomic composition were observed at D3 compared with BSL and D14, in relation to the relative abundances at genus and family levels. It revealed a notable acute influence of beetroot juice consumption to gut bacterial community followed by a general repercussion after 14 days of intervention. By associating microbiota with betacyanin profile, the changes in bacterial composition were shown to be greatly influenced by the individual and total concentrations of excreted pigments.

This study discovered an enrichment in populations of the genus *GCA-900066755* and *UC5-I-2E3* (f_ *Lachnospiraceae*²) following beetroot juice consumption, compared to baseline. Higher population of *Lachnospiraceae* is often associated with rising production of butyrate as an important anti-inflammatory mediator, with concomitant beneficial effects to alleviate inflammatory bowel conditions (Parada Venegas et al., 2019). Indeed, Frank et al. (2011) and Gevers et al. (2014) reported a decline in *Lachnospiraceae* in the faeces of IBD patients. Other marked changes in abundance involved the decrease in genus *Romboutsia* and rise in *DTU014*. According to Bojović et al. (2020), there was an increased presence of *Romboutsia ilealis* tested in gut samples from patients with neurodevelopmental disorders, while its family, *Peptostreptococcaceae*, showed potential linkage to risk of colorectal cancer (Ahn et al., 2013). On the other hand, members of *DTU014* were characterised as acetate-oxidising bacteria that contribute to carbohydrate fermentation and methane production (Hassa et al., 2021). As a less abundant taxa, its physiological and metabolic effects in the human gut remain to be explored.

Moreover, this study indicated an overall decreasing trend of the *Bacteroidales* order following beetroot juice consumption, which was partly in alignment with Capper et al. (2020). The Gram-negative members of o_ *Bacteroidales* are abundantly present in the colon, including relatively well-studied families such as *Bacteroidaceae*, *Prevotellaceae* and *Rikenellaceae*. Among them, several commensal species were reported to be colitogenic such as *Bacteroides fragilis* and *Prevotella copri*. Based on this finding, we further examined the

² Note that g_ represents genus level, f_ for family level, and o_ for order level taxonomy.

microbial abundance of *B. fragilis* by qPCR. As a common anaerobic taxon in human gut, the involvement of *B. fragilis* in the inflammatory diarrhoea and related bowel diseases was confirmed by multiple studies (Swidsinski et al., 2005; Sears et al., 2014). The virulence of enterotoxigenic *B. fragilis*, sourced from the production of metalloprotease toxin, serves a role in colitis potentially by triggering NF- κ B signalling impacting on synthesis of pro-inflammatory interleukins in intestinal epithelial cells. With other characterised mechanisms, e.g., Stat3 transcription factor, it may contribute to colitis-colorectal carcinoma (CRC) (Chung et al., 2018; Niederreiter et al., 2018). Boleij et al. (2015), for example, revealed a higher rate of positive test of *B. fragilis* toxin from colon tumorigenic lesions compared with controls (89% vs 67%). A subset of the commensal species, *E. coli*, has also been reported to participate in CRC development, especially the adherent-invasive *E. coli* that were found to be prevalent coloniser in the mucosa of Crohn's disease individuals (Eaves-Pyles et al., 2008; Bonnet et al., 2014). The genotoxic subspecies *E. coli* with expression of colibactin demonstrated remarkable promotion of the tumorigenesis in colitis-susceptible mouse model (Arthur et al., 2012). Bonnet et al. (2014) further discovered the positive relationship between the abundance of pathogenic *E. coli* and the progression of colon cancer development. In this study, changes in taxonomic abundance showed a clear decrease of *B. fragilis* and increase of *E. coli*, which may have resulted from an integrated impact of the components in red beetroot containing betalains, polyphenols, nitrates, and sugars.

In addition, the current study has observed an enrichment of *Akkermansia muciniphila* ($p < 0.05$) and *Lactobacillus acidophilus* species ($p < 0.1$) after short-term intake of beetroot juice (D3). There is increasing evidence emerging on the positive association between the intake of healthy diets (e.g., polyphenol-rich), and the presence of *A. muciniphila* with associated beneficial metabolic outcomes (Jayachandran et al., 2020). As a mucin-degrading bacterium, *A. muciniphila* abundance was correlated inversely with pro-inflammatory markers (e.g., tumour necrosis factor- α (TNF- α)) and positively with lipid metabolic markers (e.g., acetyl-CoA carboxylase- β) in mice fed with high-fat diet (Schneeberger et al., 2015). Furthermore, Li et al. (2016) illustrated *A. muciniphila*-mediated amelioration of a disrupted intestinal epithelial barrier, systemic inflammation, and atherosclerosis lesions, with further

implications to mitigate IBD, obesity and cardiometabolic diseases. Meanwhile, probiotic *L. acidophilus* and *Bifidobacterium* appear to be promising candidates for the treatment of clinical conditions with abnormal indigenous microbiota and altered gut mucosal barrier functions, associating with immunity, metabolic health, and psychiatric wellness. Significant reductions of *Lactobacillus* populations in the gut were also discovered in IBD subjects compared with healthy individuals (Zhuang et al., 2018). Overall, the PCR data were in line with the beta-diversity and differential taxonomy results in terms of the strong shift of microbiome from BSL to D3 followed by repercussion at D14, as well as the remarkable decrease of *Bacteroides* abundance along the intervention.

Emerging studies found the abundance of these taxa highly susceptible to alteration by dietary components such as polyphenols and fibre (Hervet-Hernández et al., 2009; Park et al., 2020). Several phenolic compounds that are predominant in red beetroot (e.g., chlorogenic acid, ferulic acid, and quercetin) have been reported to mitigate bowel inflammation, firstly, through the stimulation of probiotics (e.g., *Bifidobacterium*) and associated SCFA generation; and secondly via immunoregulatory mechanisms (Lin et al., 2019; Liu et al., 2019). In particular, chlorogenic acid, as the most abundant polyphenol in beetroot juice, was discovered to raise *A. muciniphila* and the genus *Lactobacillus*, and to inhibit generation of intestinal inflammatory markers, i.e., TNF- α , in dextran sulphate sodium-induced colitis in mice (Zhang et al., 2019). These polyphenols were presumed to act in concert with betalains to impact the microbial profile of subjects in this study. Further measurements need to consider more defined intervention products to investigate the interactions and synergistic effect of individual/mixed components, and to target specifically the proportion of toxigenic subspecies among interested taxa.

During intestinal fermentation, energy is produced in the form of SCFAs, primarily acetate, propionate, and butyrate, leading to a cascade of anti-inflammatory and anti-cholesterolemic functions that overall benefit the host (Parada Venegas et al., 2019). Chemically, the organic acids acidify the colon environment which to some extent increases the mineral release and absorption in bowel. A randomised mice trial also reported the lipid-

lowering and hormone-regulatory effects with increased SCFA generation that alleviated obesity and improved insulin sensitivity in mice (Lin et al., 2012). As a predominant SCFA in faeces, acetic, propionic and butyric acids are derived from carbohydrate glycolysis. Acetate is involved in lipid and cholesterol biosynthesis, whereas propionate and butyrate serve roles in the signalling and activation of intestinal gluconeogenesis that potentially benefit the control of body weight and glycaemia (De Vadder et al., 2014). In addition, butyrate is essential for the maintenance of energy homeostasis and integrity of colonic mucosal layers that facilitate the stabilisation of cell phenotypes (Cui et al., 2019). However, the branched SCFAs (i.e., isobutyric and isovaleric acids) are primarily produced from the fermentation of undigested amino acids (e.g., leucine, valine), which is often accompanied by the accumulation of undesirable metabolites such as ammonia and indole. The isobutyric acid further showed potential association with the hyperlipidaemic profile in plasma and corresponding gut bacterial composition. The beetroot juice consumption in the present study was leading to an increase in total SCFA content especially butyric acid which implies the prebiotic character of beetroot components in reinforcing epithelial development and intestinal immune function. The slightly increased proportion of branched SCFAs, nonetheless, deserves additional research despite the insignificant difference of the current result. Future work may also consider investigating the SCFA content in the physiological fluids of subjects for a holistic analysis (Cardona et al., 2005; Granado-Serrano et al., 2019).

The relationship between betacyanin catabolites and microbiome variables was further expounded by correlation matrix in this study, indicating the strong bidirectional influences between betacyanins and genera *Bifidobacterium*, *Coprococcus*, *Peptostreptococcus*, *Peptoniphilus*, and *Ruminococcus*. The metabolic profile of betacyanin pigments was presumably determined by a vast array of intrinsic (e.g., physiological conditions and genetic varieties) and extrinsic factors (e.g., habitual diet matrix, dose). During the transit in GI tract, pigments are subject to increasing fragmentation from proximal to distal intestinal regions, evidenced by their strikingly low recovery from defecated materials. The gut microbial action, especially in the colon, serves as a powerful driver of the phytochemical biotransformation, mechanisms of which are potentially associated with the richness and composition of specific

taxa (Sorrenti et al., 2020). Previous studies investigating the gut microbial metabolism of polyphenols reported a strong hydrolytic potential of diverse genera (e.g., *Bifidobacterium*, *Lactobacillus*, *Bacteroides*) towards anthocyanins and other polyphenolic glucosides resulting from their intrinsic β -glucosidase activity (Bokkenheuser et al., 1987; Ávila et al., 2009). Indeed, several species of *Lactobacillus* and *Enterococcus* were shown to encode decarboxylase genes responsible for the transformations of tyrosine and *L*-DOPA compounds which are precursors of betacyanins (van Kessel et al., 2019). There were also studies revealing the capability of heterocyclic ring cleavage of genera *Bacteroides*, *Clostridium*, and *Eubacteria* towards core structure of polyphenols, which, in the case of betalain pigments warrants future investigation (Winter et al., 1991; Schneider et al., 1999). Furthermore, the structure-activity relationship between betacyanins and other natural compounds should be clarified under fermentation conditions.

Meanwhile, gut bacteria appeared to be mutually influenced by the unabsorbed betacyanins which selectively augmented and suppressed proliferation of specific taxa. As probiotics, *Bifidobacterium* and its species ferment bifidogenic compounds that help maintain the host immune homeostasis and alleviate the pathogen-associated diarrhoea (Picard et al., 2005). *Peptoniphilus* and *Coprococcus* are also known as promoters for butyrate generation via carbohydrate glycolysis, with *Coprococcus* being further involved in the mechanism of vitamin B metabolism (Nogal et al., 2021), which might explain the significant rise in (iso)butyric acid concentration after intervention. The decrement of fermentative taxa on the intestinal mucosa is often accompanied with reduced SCFA production and intestinal barrier dysfunction (Kowalska-Duplaga et al., 2019; Lo Presti et al., 2019). On the other hand, *Ruminococcus* is commonly present in the gut of healthy individuals. Yet Granado-Serrano et al. (2019) revealed a positive relationship between the population of *Ruminococcus* and isobutyric acid in faeces, linking to hypercholesterolemia signature. In addition, evidence from several studies indicated the robust association between *Ruminococcus gnavus* species and Crohn's disease, as the degree of *R. gnavus* enrichment was positively correlated with disease progression (Hall et al., 2017; Nishino et al., 2018). The pathogenic mechanism of *R. gnavus* was proposed to involve the biosynthesis of glucorhamnan polysaccharide with strong

TNF- α -inducing activity hence corresponding inflammatory response (Henke Matthew et al., 2019). Therefore, an assessment of relative abundance of *R. gnavus* in faecal samples will be valuable in future studies. As demonstrated by *in vitro* experiments (Chapter III), the anti-inflammatory properties of betacyanins were mainly reflected by immunoregulation, specifically by downregulating the expression of pro-inflammatory markers (e.g., COX-2 and iNOS) as well as augmenting expression of antioxidant enzymes (e.g., HO-1 and NQO-1) (Krajka-Kuzniak et al., 2013; Gómez-Maqueo et al., 2019; Wang et al., 2022b). Results from this study further revealed the microbiota-modulatory role of native and catabolised betacyanins in the intestinal environment which are speculated to play a part in the anti-inflammatory activity of this pigment.

It is noteworthy that results reported in this study should be interpreted in view of some limitations. For example, betalain-rich food also comprises a number of other potentially relevant compounds, such as polyphenols and nitrates; and further study should therefore consider dissecting individual components and their contribution on the microbiota. Secondly, there is controversy about the similarity and therefore the relevance of faecal microbiome to mucosal microbiome since the latter relates more closely to the bowel conditions (Lo Presti et al., 2019). With sufficient accessibility to biopsy, further investigation should ascertain the value of faecal microbiome data and the influence of red beetroot components on intestinal inflammation. At last, future work could address the impact of a longer-term exposure to beetroot which takes the adaptation of diet into consideration.

Chapter VI

General Discussion and Future Perspectives

6.1 Betalains and their promising evidence from *in vitro* and *in vivo* research

As a group of phytochemicals with intensive dyeing characteristics, betalains possess a series of biochemical properties that have been associated with desirable health benefits and potential for functional food development. Recent studies of common betalains have demonstrated strong radical scavenging and anti-inflammatory properties relating to the prevention and amelioration of chronic diseases such as type 2 diabetes mellitus, cardiometabolic disease and cancer. Nonetheless, the low bioavailability is regarded as a bottleneck for the beneficial bioactivities of this pigment group *in vivo*. The thesis started from an overview of the currently available evidence on absorption and metabolism of betalains (Chapter I) which serves as a foundation for this project. It is comprised of *in vivo* and *in vitro* investigations on the bioaccessibility, intestinal uptake, biotransformation profile, and excretion of two prevalent betalains, betanin and indicaxanthin. Meanwhile the corresponding influential factors have been discussed, such as pigment type, dosage, and food matrix. By identifying the scarcity of research in the intestinal metabolism of betalains with individual heterogeneity, and the gap of knowledge regarding pigment impact on gut microbiota, investigations of this project attempted to address the relevant topics by performing *in vitro* cell culture experiments and an *in vivo* human intervention study, which are followed by the recommendation of work in prospect.

The present project has partially explored the human bioavailability and enteric bioactivity of common betalains found in beetroot and cactus pear using the methodology delineated in Chapter II. Results in Chapter III demonstrated the poor uptake and transport efficiencies of betanin, vulgaxanthin I and indicaxanthin across intestinal epithelia of human (Caco-2 cell line), which was supplemented by the immunomodulatory results showing positive impacts of tested betalains on the critical cell signalling and redox balance of

enterocytes, especially betanin (Wang et al., 2022b). The investigations of dextrin-coated betanin and beetroot extracts were also incorporated, suggesting the effects of divergent matrices to the absorption and molecular behaviour of pigments.

Next, the path of project leads to the *in vivo* absorption and metabolic fate of betacyanins which was assessed via a human study with intervention of 14-day consumption of red beetroot juice. Chapter IV has analysed and contrasted the composition of betacyanins during renal and intestinal excretions, with evidence provided on the inter-compound correlation and variability in multiple facets. Regardless of the notable inter-subject variation, the poor bioavailability and rapid elimination of betacyanins were confirmed by urinary pharmacokinetics. The excretion profile supported the hypothesis that passive diffusion was the major trans-epithelial mechanism of (iso)betanin, which is consistent with the findings in cell culture experiments. With the significant impact of gut microbiota, betacyanins were subjected to a progressive metabolism and fragmentation along the GI tract predominantly via decarboxylation and dehydrogenation, indicating a larger inter-individual variability of intestinal metabolism of pigments compared with systemic. Furthermore, pigments and metabolites remained in the GI tract were demonstrated to modulate specific microbial taxa in the human gut (e.g., *Romboutsia* and *Bacteroidales*) in Chapter V, featured by an enriched abundance of *A. muciniphila* and suppressed *B. fragilis*. The alteration in gut microbiota as well as incremented generation of SCFAs following the intervention have further suggested the implication of red beetroot components to promote human gut health likely via the alleviation of intestinal inflammation (Wang et al., 2022a). To summarise, the aforementioned results elucidated the metabolic kinetics and gut-associated biofunctions of betalain pigments from *in vitro* and *in vivo* perspectives that have overall fulfilled the project aims regarding the absorption, metabolism and health beneficial activities of betalains in the gut.

6.2 Absorption, metabolism, and excretion of betalains

The study findings of intestinal absorption and renal pharmacokinetics of betalains along with literature from Chapter I have established a relatively clear comprehension of betalains' fate following oral administration (Fig. 6-1). Deduced from bioaccessibility literature, the decay of betalain compounds begins in the oral cavity and is intensified during gastric digestion (Tesoriere et al., 2008; Montiel-Sánchez et al., 2021). Meanwhile a proportion of betanin was found to be promptly absorbed through the gastric wall in the form of glucosides (Takahashi et al., 2017; Sawicki et al., 2020). Reaching the intestinal phase, native betalains come into close contact with mucus layer and enterocytes, where they are proposed to be readily absorbed via paracellular passive diffusion (Tesoriere et al., 2013; Wang et al., 2022b). The absence of betalain metabolites in the cellular uptake experiments suggests the deglucosidation of betacyanins and Phase II conjugation (e.g., glucuronidation, sulfation) to be non-essential during their trans-epithelial transport, contrary to most flavonoids. Pigment molecules absorbed in the stomach and intestine are subsequently carried to the liver via portal bloodstream where they possibly experience a series of Phase I and II metabolisms. Enterohepatic recirculation may also play a part by shuttling the conjugated betalains back to intestine via biliary excretion, which could be deconjugated and reabsorbed at the lower intestine (Gropper and Smith, 2012). Following the liver detoxification, pigment compounds are distributed and metabolised in the systemic circulation then organs and tissues where they potentially exert various biological actions such as anti-inflammation and radical quenching until excretion through kidneys. Results indicated (iso)betanin as the predominant betacyanins discharged in urine instead of (iso)betanidins or other derivatives, implying a minor degree of pigment modification during systemic metabolism, with deglucosidation as the dominant mechanism in metabolic pathway.

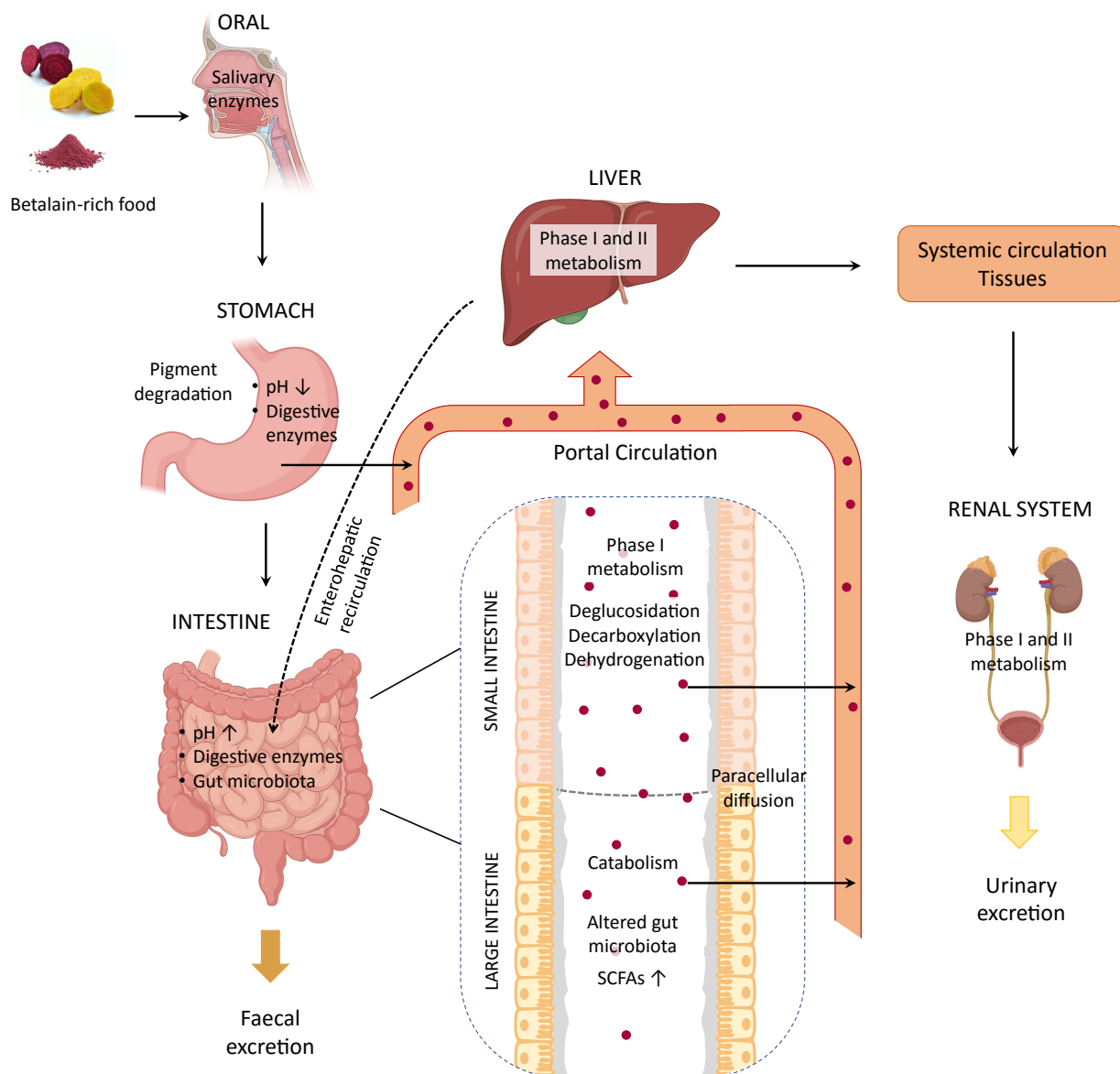


Figure 6-1. Proposed absorption, metabolism, and excretion of betalain pigments following oral administration of betalain-rich matrix. The figure was adapted from Frayn (2010) and Catalkaya et al. (2020) and modified according to the betalain results.

By contrast, the major fraction of ingested betalains is believed to be unabsorbed experiencing more extensive decomposition when transiting from proximal to distal intestine. It is supposedly driven by the physiological conditions (e.g., temperature, pH) and multi-enzymatic reactions involving the digestive, brush boarder and gut microbial enzymes. In the phase of intestinal digestion, the hydrolysis of sugar moiety from betacyanin structure is likely catalysed by β -glucosidase excreted by enterocytes and several fermentative bacteria

such as *Bifidobacterium*. Multiple decarboxylation is presumed to be facilitated by the decarboxylase-expressing taxa such as *Lactobacillus*; whilst the core scaffold of pigment might be further fragmented into simpler molecules via the Schiff base hydrolysis and cleavage of heterocyclic and aromatic rings with the assistance of gut microbiota. These deductions were drawn according to previous investigations on the anthocyanin and amino acid metabolism (Ávila et al., 2009; van Kessel et al., 2019), nonetheless their structure-activity relationship to betacyanins and betaxanthins regarding biotransformation still remains to be explored. With diminishing fraction of native compounds and metabolites (e.g., aglycones) being absorbed at distal intestinal region, pigments remaining in the lumen are eventually eliminated via faeces.

The metabolic fate of betalains in GI tract is significantly diverged between individuals due to a vast array of influential factors (Marín et al., 2015; Sorrenti et al., 2020). Exogenously the digestive resistance and absorption efficiency of betalain compounds are primarily determined by their physiochemical characters and ingested matrix. For example, the present project and previous literature have demonstrated the lower degradation and greater uptake intensity of tested betaxanthins compared to betanin. Alterations of dose and food matrix are associated with different synergistic and inhibitory interactions between pigments and nutrient network. Meanwhile endogenously, the level of betalain biotransformation is directly influenced by the digestive and metabolic powers of the host which are intimately linked to the genetic variation and health status; these also contribute to shaping the gut microbiota of hosts regarding the taxonomic richness and diversity which undoubtedly confer the metabolic and catabolic specificities of the pigments as aforementioned hence associated bioactivities. All the factors above have served indispensable roles in the bioaccessibility, absorption, metabolism and elimination of betalains that raise a great uncertainty to their biological effectiveness in real life.

6.3 Modulatory functions of betalains in the intestine

There is increasing recognition towards the systemic bioactivities of dietary betalains, e.g., anti-atherogenesis and anti-diabetes, based on *in vitro* and *in vivo* evidence (Tesoriere et al., 2015; Dhananjayan et al., 2017). However, given the weak absorbability and severe metabolism of pigments in digestive tract, it is speculated that the health-promoting cellular effects of betalains are considerably compromised at a specific body site after their intestinal absorption and systemic distribution. The major proportion of ingested pigments, however, are anticipated to transit through the entire digestive tract unabsorbed, undergoing bioconversion into multiple secondary metabolites that exert bidirectional interactions with human physiological pathways. It is therefore sensible to consider in an integrative manner targeting the symbiotic interactions between betalain metabolism and microbiota communities, and their relationship to the network of immunomodulation in human intestine.

By performing a pilot human study, this project has explored the modulatory effect of red beetroot phytochemicals, especially betacyanins, on human gut microbiota which is considered an underdeveloped research area compared to polyphenols and carotenoids due to their low prevalence. The consumption of red beetroot juice for 14 days led to a boost in SCFAs production and an acute alteration in specific species of human gut microbiota (e.g., *A. muciniphila*) followed by a restoration in a longer term. By associating with the metabolic pattern of betacyanins, mutual influences between pigments and gut microbiota were partially revealed, showing a positive relationship between specific metabolites and SCFA-promoting genera, *Bifidobacterium* and *Coprococcus*, meanwhile an inverse correlation of pigment with IBD-related *Coprobacillus*. Linking to other physiologies stated in Chapter V, red beetroot components have demonstrated the prebiotic characteristics with potential health benefits of preventing/mitigating cardiometabolic diseases, diabetes, and local inflammation of the host.

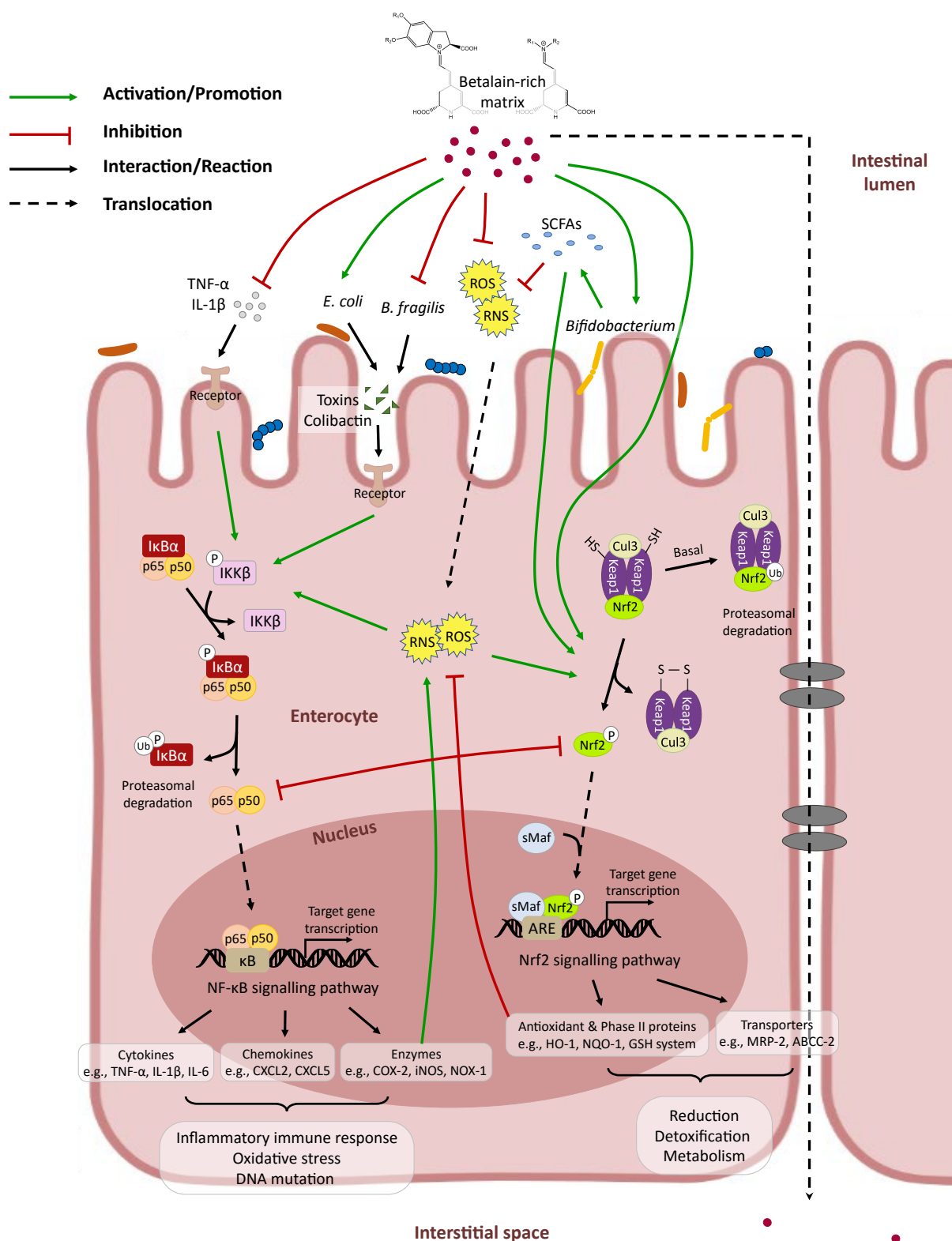


Figure 6-2. Proposed interplay of betalain compounds with molecular signalling pathways in the enterocytes, and with relevant gut bacteria in the intestinal lumen based on the evidence from current study and reference (Wardyn et al., 2015; Parada Venegas et al., 2019). The figure was partly adapted from Peng et al. (2020) and Niwano et al. (2022).

There is emerging concept of the crosstalk between gut microbiota and mucosal immune homeostasis categorised into the innate (i.e., epithelium and antigen-presenting cells) and adaptive immunities (i.e., lymphocytes). Compiling *in vivo* evidence revealed the crucial role served by the intestinal immunity in regulating gut bacterial composition and functions; and reciprocally the microbial activities and metabolic by-products (e.g., SCFAs) are tightly related to the homeostasis in localised and even systemic immune response (Cebra et al., 1998; Bouskra et al., 2008). The dysbiosis of gut microorganisms with compromised immune functions are often responsible for the chronic inflammatory disorders in the digestive system linking further to carcinoma, cardiovascular and neurodevelopmental diseases (Catalkaya et al., 2020). This project has underlined the immunomodulatory effects of three prevalent betalains at the intestinal epithelium which conferred the non-specific mucosal immune defence at the inflammatory condition. During the exposure to dietary betalains at certain concentrations, these compounds appeared to powerfully suppress the generation of pro-inflammatory mediators (e.g., IL-6, iNOS) whilst promoting the release of antioxidant enzymes (e.g., NQO-1, GPX-1) by interfering with NF- κ B and Nrf2 signalling pathways of enterocytes, respectively, which ultimately contribute to alleviating the inflammation symptoms. The anti-inflammatory activities of pigments were similarly observed on the immune cells (e.g., macrophage) and in other organs (Krajka-Kuzniak et al., 2013; Allegra et al., 2014). Aside from immunomodulation, the intrinsically reducing betalains are capable of quenching the oxidative stress from overproduced ROS in the intestine. Noted the increasing colonic ROS level is related to a reduced taxonomic diversity, for example, lower populations of *Firmicutes* and *Clostridiales*, and the ROS accumulated in the enterocytes often leads to a higher risk of DNA damage and tumorigenesis (Hakansson and Molin, 2011b). The putative modulatory mechanisms by betalains in the context of crosstalk between Caco-2 cell signalling and gut bacteria were illustrated in Fig. 6-2.

The cellular signalling pathways are essential in orchestrating the innate and adaptive immune systems that participate in the regulation of host response to gut microbial perturbation. The NF- κ B signalling cascade, despite its association with colitogenesis, is critical for the activation of protective response of the innate immune system during microbial

infection and tissue lesions. Nonetheless, its engagement with adaptive immunity often causes extensive and sustained inflammation with destabilised DNA, which could ultimately result in the autoimmune dysfunction and tumour development. Among the CRC-related taxa (e.g., *Peptostreptococcus*, *Fusobacterium*), some pathogenic bacteria are well-known in exacerbating the intestinal inflammation and carcinogenesis partially via NF- κ B modulation, such as *E. coli* and *B. fragilis* (Nakatsu et al., 2015; Khan et al., 2019). As aforementioned, enteropathogenic *E. coli* and *B. fragilis* with expressed virulence factors (e.g., colibactin and metalloprotease toxin) and other synergistic pathways (e.g., Stat3, Wnt/b-catenin) could markedly promote the NF- κ B signalling of host cells hence the release of downstream pro-inflammatory mediators (e.g., IL-8, CXCL5) which eventually cause genomic damage and contribute to the CRC signatures (Sahu et al., 2017; Peng et al., 2020). On the other hand, supplementation of *A. muciniphila* species was associated with the inhibition of LPS-induced protein binding and downstream NF- κ B pathway in the liver (Zhao et al., 2017). The SCFAs produced in abundance by the gut bacteria such as *Bifidobacterium* and *Roseburia* could also serve to mitigate local inflammation and improve epithelial integrity via the suppression of pro-inflammatory enzymes and radical by-products (Parada Venegas et al., 2019). This is working in concert to the actions of Nrf2 pathway with its downstream antioxidant and detoxifying enzymes that efficiently scavenge the intracellular ROS and reactive nitrogen species (RNS), and with the mutual constraints between Nrf2 and NF- κ B signalling in the cells (Liu et al., 2012; Oliveira Corrêa et al., 2016).

6.4 Future perspectives and social implications

Overall, the *in vitro* and *in vivo* evidence of this project has contributed to the understanding of the beneficial impacts of betalain compounds in the human intestine through the regulation of luminal microbiota and epithelial immunity. Findings of the project were deemed to be valid, consistent, and free of significant contradictions. The *in vitro* Caco-2 cell model with rigorous control of experimental conditions is advantageous in the relatively high throughput and reproducible outcomes, while the *in vivo* human trial provides a holistic physiology to the context of betalain bioavailability despite the inextricably high environmental variation. However, this project could still be improved in terms of the research scope and methodology. For example, given that the intestinal absorption kinetics and immunoregulation of two prominent betaxanthins were covered *in vitro* (Chapter III), an *in vivo* trial is recommended to further examine their metabolism and excretion as well as potentials in gut microbiota alteration. In addition, a commercial red beetroot juice with rich betacyanins was employed as intervention of the current human trial, hence potential biological effects of other ingredients could not be precluded, e.g., nitrates and phenolic compounds. Future study is recommended to utilise the purified betacyanins or betaxanthins with the preconditions of food safety and sufficient yield from purification. Notwithstanding, it is imperative to assess the divergence in biological impacts between pure betalains and plant extracts in order for establishing the beneficial effectiveness of dietary pigments, which supports the innovation of betalain-based functional foods and/or supplements. Thirdly, the investigation in prospect could employ *in silico* techniques, e.g., molecular docking, to provide a novel perspective to the interplay between pigments and vast arrays of biomolecules related to microbial and intestinal barrier functions. At last, the health impacts of betalains and betalain-containing matrix should be evaluated at a longer time scale taking physiological adaptation into account.

The current findings contribute to deciphering the relationship between the bioavailability and gut-related bioactivities of betalains, allowing us to tailor the pigment type and oral dose for it to become an alternative candidate in the prevention and/or counteraction of gut inflammatory disorders. Besides, betalains, especially betacyanins, are further reported

to exert chemopreventative, anti-hypertensive, anti-hyperglycaemic, and anti-cholesterolemic properties by interfering with different biological mechanisms therefore pose influence on the systemic health of hosts. With betalain-rich fruits and vegetables serving as possible sources of functional food, the industrial food processing and innovation are advised to concentrate on enhancing the digestive resistance of pigments and consequently their levels of exposure in the lower GI tract. For instance, the instability of betalains can be efficiently mitigated by co-pigmentation and encapsulation techniques which are frequently utilised in the phytochemical studies and drug development (Janiszewska, 2014; Khan, 2016b; Mueller et al., 2018). The micro-encapsulation technique of betalain has only become a popular choice of preservation recently in food industry, where the coating polysaccharides (e.g., pectin) could effectively decelerate the decomposition of hygroscopic and oxygen-sensitive materials such as betalains and polyphenols, and lengthen their storage period (over 6-month at 25 °C) (Azeredo et al., 2007; Obón et al., 2009). This may also shed light on facilitating the administration and improving bioavailability of betalain pigments as well as other phytochemicals although with the concern of matrix effect.

In addition, future industries could consider the application of potential synergistic effect of polyphenols to betalains preservation and absorption, as well as the prebiotic benefits of betalain-containing fibre for gut health. Fibres of the red beetroot, resembling those of sugar beets, mainly comprise of cellulose, hemicellulose and pectin. They are suggested of high value in regulating nutrient metabolisms and gut microflora which have been associated with the risk lowering of obesity, diabetes mellitus and coronary heart disease (Thorsdottir et al., 1998; Thibault et al., 2001). Apart from general benefits of fibre to bowel health such as relieving constipation, an animal trial showed an increase in activities of detoxifying enzymes (e.g., superoxide dismutase and catalase) in colon after oral administration of red beetroot fibre, implying its potential to ameliorate chronic inflammation and colonic lesions (Bobek et al., 2000). In addition, sugar beet pectin, i.e., arabino-oligosaccharides (Fig. 6-3), was indicated to selectively provide the substrates and accessibility to the beneficial *Lactobacillus* and *Bifidobacterium* species hence the butyrate generation. It is regarded as a viable candidate for mitigating clinical conditions of abnormal indigenous microflora and aberrant gut

mucosal barrier functions (Holck et al., 2011; Tian et al., 2017). Research of the relevant fields should be extended to red beetroot fibre to further understand the physiological effects and nutritional value of the red beetroot matrix.

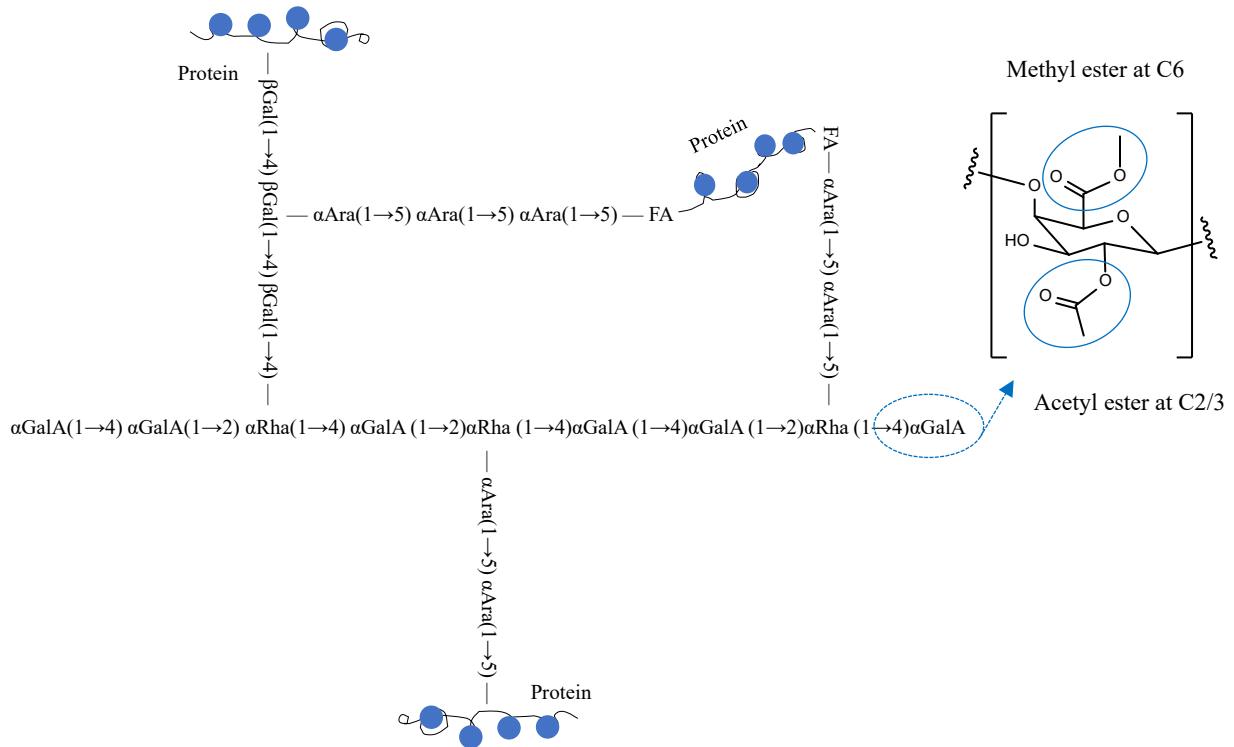


Figure 6-3. Simplified chemical structure of beetroot pectin with plausible transformations (e.g., methylation and acetylation), properties of which could greatly impact the alteration trajectory of gut microbiota. GalA – Galacturonic acid; Gal – Galactose; Rha – Rhamnose; Ara – Arabinose; FA – Ferulic acid (Rejaini and Salehi, 2016).

Conclusion

Betalains have sparked more interests in the phytochemical studies for their potential health benefits denoting their advanced implication in food industry and pharmaceuticals. Despite the bioactivities, investigations on the uptake and metabolic fate of betalains have lagged behind, meanwhile little is known about their interactions with gut microbiota of human hence relevant physiological effects. The current project ultimately aimed to examine the bioavailability and metabolic course of food-derived betalains in the human body, bolstered by the immune- and gut microbial manipulations of the pigments which may unravel the impacts of regular consumption of betalain-rich foods, particularly betacyanins.

The aims were addressed by the methodologies categorised into cell culture model, analytical chemistry, and human trial. Albeit the relatively higher uptake of vulgaxanthin I and indicaxanthin, both *in vitro* and *in vivo* results have concurred with the low bioavailability of (iso)betanin which is regarded as the main barrier of its systemic biological function. Results, in addition, suggested the minor transformation of betalains during stages of intestinal absorption and hepatic metabolism. The fate of pigments, especially betacyanins, remained in the intestinal lumen was further explored, revealing their progressive decomposition along the tract and positive modulatory effects to the intestinal immunity and gut microbiota. The evidence proved the radical scavenging, anti-inflammatory, and prebiotic characters of betalain as well as betalain-rich matrix which would contribute ultimately to the alleviation or prevention of inflammatory gut disorders. Nonetheless, significant individual heterogeneity was recognised in the context of betalain metabolism and bioactivity *in vivo* aside from their dose dependency and betalain specificities; it is likely dependent upon the genetic variation and health status of the hosts.

Results of this project could significantly advance our understanding of betalains' bioavailability and their nutritive value to the intestinal wellness of human, allowing the maximisation of their health advantages via functionalised foods. Meanwhile, the findings further emphasised the urge in the functional food industry to improve the digestive resistance and subsequent bioavailability of betalains via novel processing techniques and research into diverse betalainic species.

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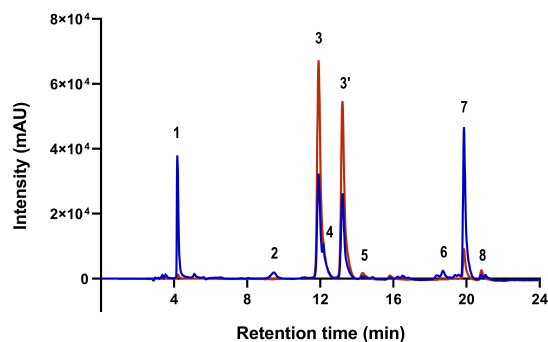
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Appendix

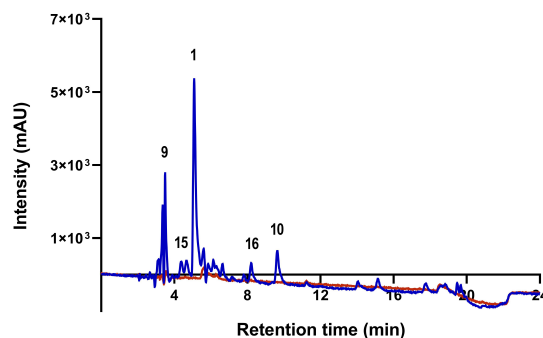
Appendix 1. Content of major betalain compounds detected in different types of plant sources. Replicates (n=3) were calculated from calibration curves of purified betanin, vulgaxanthin I and indicaxanthin. Values are mean $\pm$ STD expressed in the equivalent of standards above. The numbers after each compound were linked to those in Appendix 2.

Plant source	Individual betalains	Betalain content ($\mu\text{g/g}$ of food)
Red beetroot	Betanin (3)	1311.1 $\pm$ 23.5
	Isobetanin (3')	416.4 $\pm$ 7.3
	17-Decarboxy-betanin (2)	33.2 $\pm$ 1.9
	15-Decarboxy-betanin (5)	127.9 $\pm$ 2.1
	Vulgaxanthin I (1)	8699.6 $\pm$ 187.2
	Neobetanin (7)	43.1 $\pm$ 0.7
Yellow beetroot	Vulgaxanthin I (1)	1486.4 $\pm$ 65.9
	Indicaxanthin (10)	148.1 $\pm$ 23.5
	Portulacaxanthin I (9)	420.2 $\pm$ 38.5
Red prickly pear	Betanin (3)	236.6 $\pm$ 2.1
	Indicaxanthin (10)	2428.9 $\pm$ 18.6
	Portulacaxanthin I (9)	322.6 $\pm$ 13.2
Yellow prickly pear	Betanin (3)	5.6 $\pm$ 0.5
	Vulgaxanthin I (1)	106.4 $\pm$ 22.7
	Indicaxanthin (10)	3128.0 $\pm$ 43.5
	Portulacaxanthin I (9)	436.8 $\pm$ 21.9
Red pitaya	Betanin (3)	717.8 $\pm$ 7.0
	Isobetanin (3')	124.8 $\pm$ 1.4
	Phyllocactin (11)	1049.3 $\pm$ 36.3
	Indicaxanthin (10)	504.2 $\pm$ 30.7
Red amaranthin tricolor	Amaranthin (13)	411.0 $\pm$ 5.3
	Isoamaranthin (13')	45.4 $\pm$ 0.7

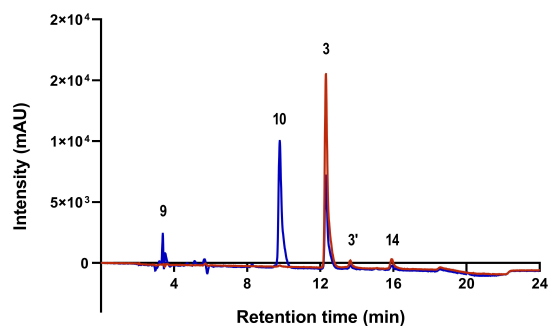
(A). Red Beetroot



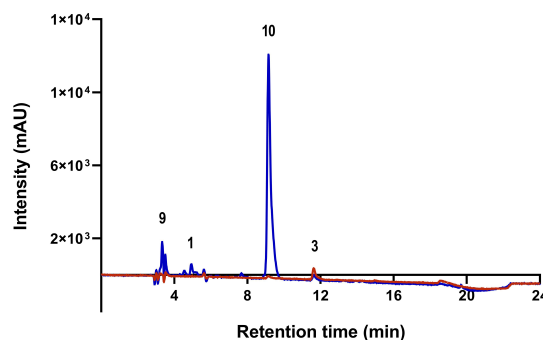
(B). Yellow Beetroot



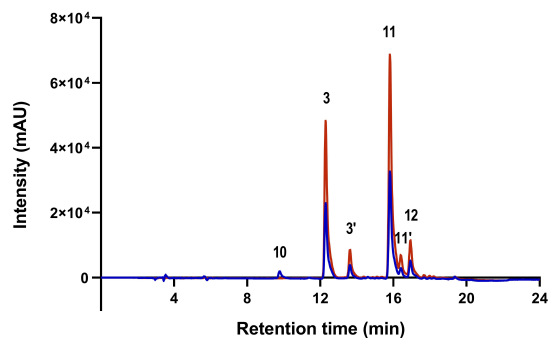
(C). Red Prickly Pear



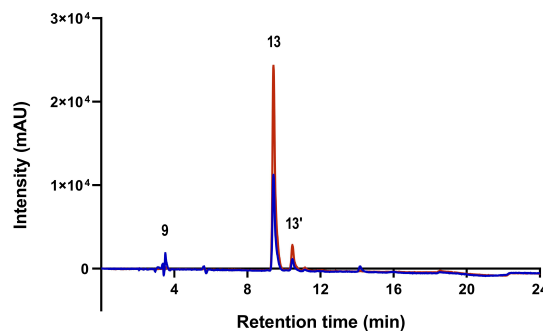
(D). Yellow Prickly Pear



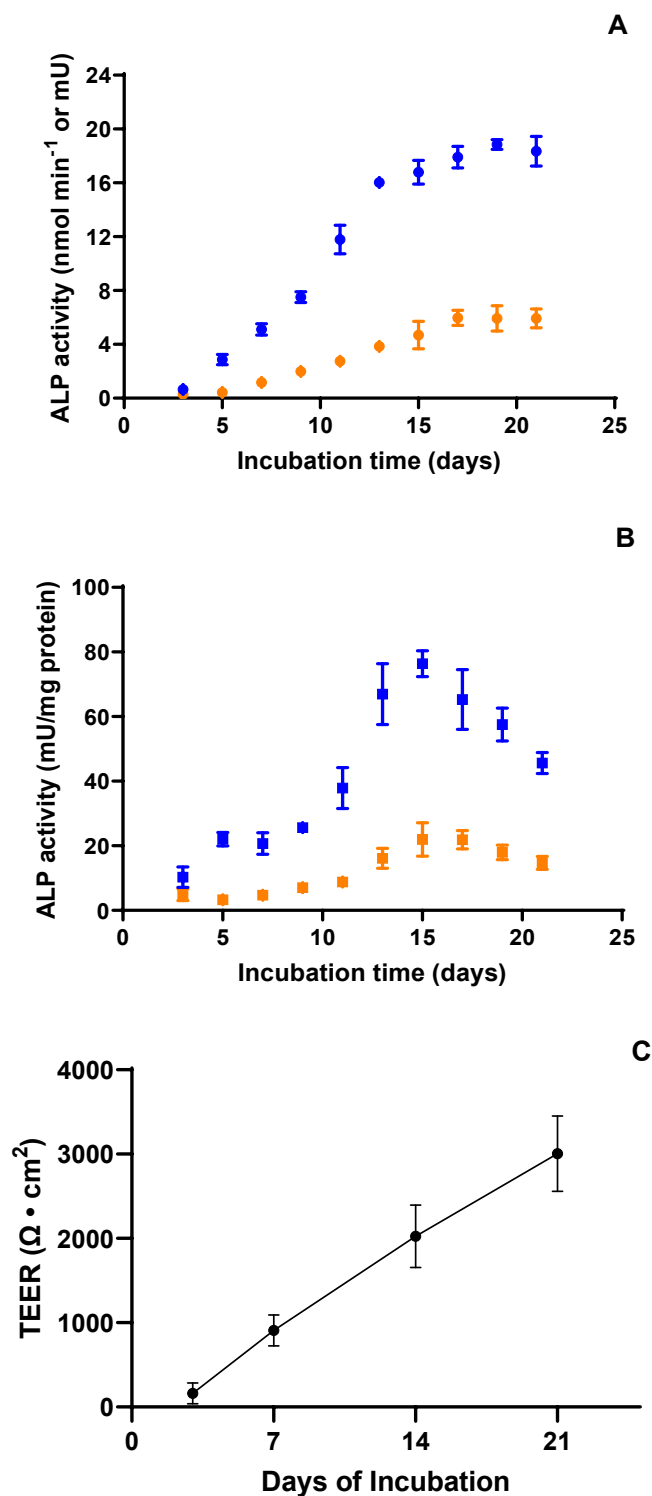
(E). Red Pitaya



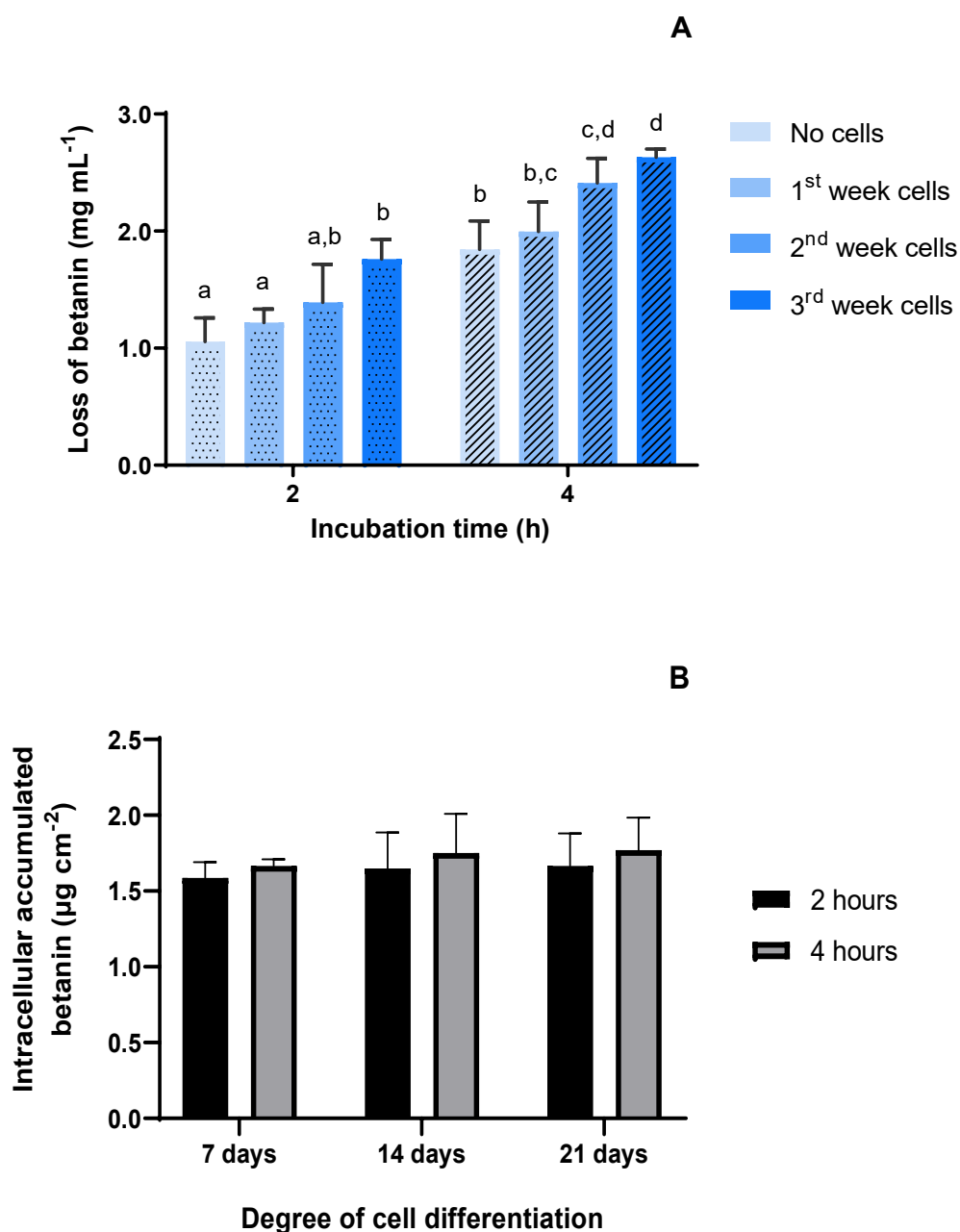
(F). Red Amaranth Tricolor



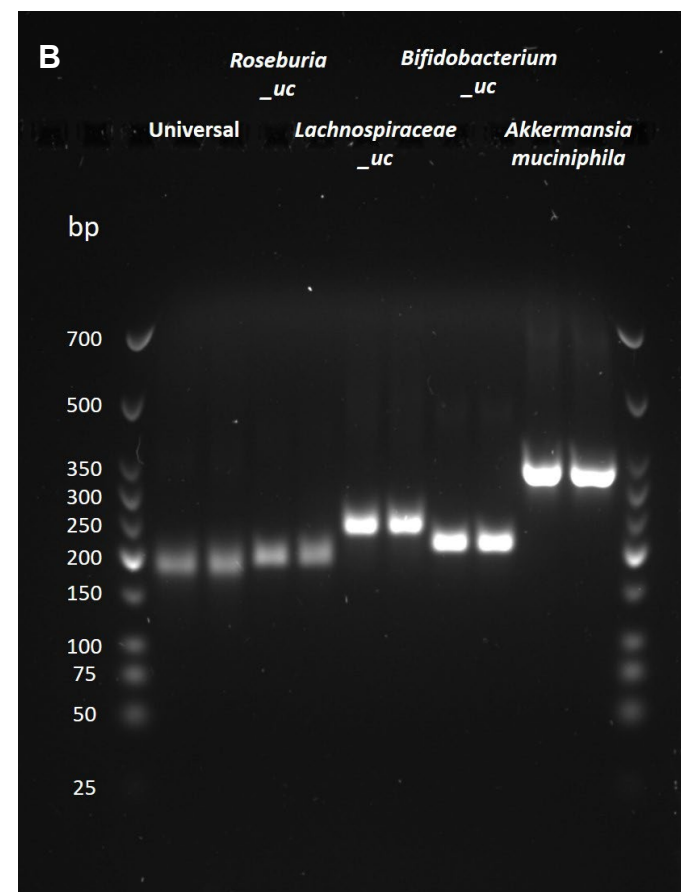
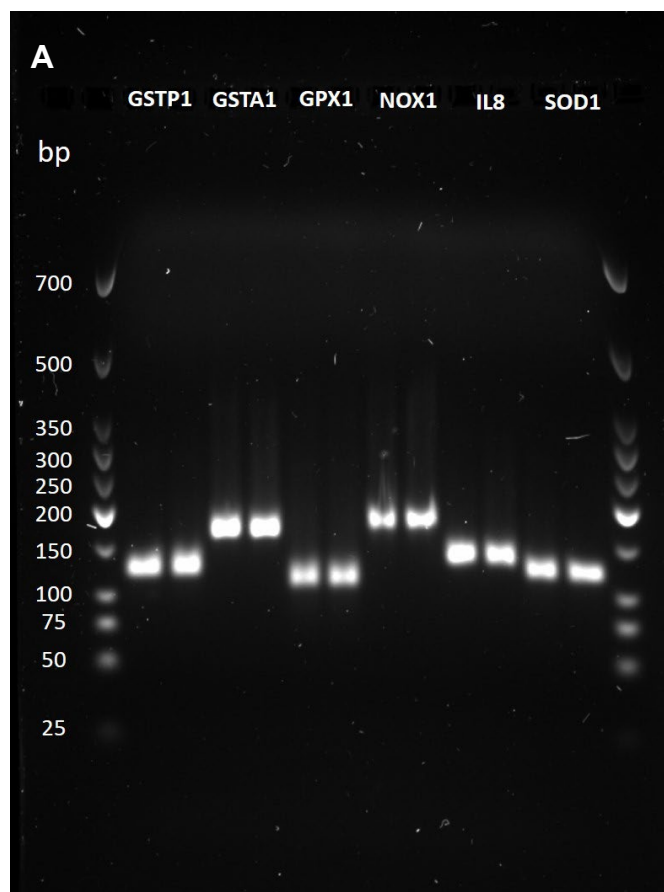
Appendix 2. Typical chromatographs of (A) red beetroot, (B) yellow beetroot, (C) red prickly pear, (D) yellow prickly pear, (E) red pitaya, and (F) red amaranth tricolor, at the detection wavelength of 536 nm (—) and 486 nm (—). Components identified: 1 – Vulgaxanthin I; 2 – 17-Decarboxy-betainin; 3/3' – Betainin/Isobetainin; 4 – Miraxanthin V; 5 – 15-Decarboxy-betainin; 6 – 2,17-Bidecarboxy-neobetainin; 7 – Neobetainin; 8 – 17-Decarboxy-neobetainin; 9 – Portulacaxanthin I; 10 – Indicaxanthin; 11/11' – Phyllocactin/Isophyllocactin; 12 – 4-*O*-Malonyl-isobetainin; 13/13' – Amaranthin/isoamaranthin; 14 – Betanidin; 15 – Miraxanthin III; 16 – γ -Aminobutyric acid-betaxanthin.



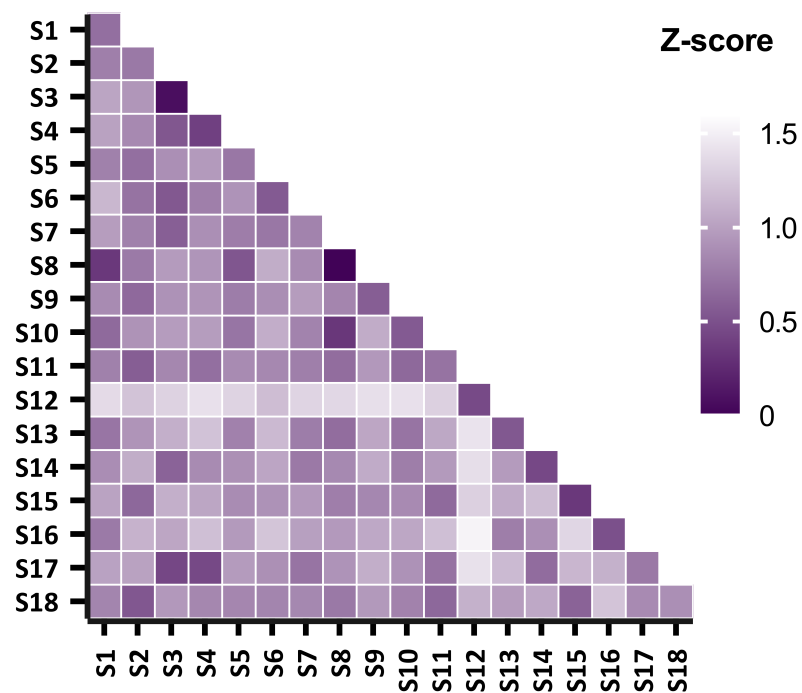
Appendix 3. ALP activity and TEER value of Caco-2 cell membrane after specific days of differentiation. (A) Non-normalised activity of adhered ALP (●) and suspended ALP (○). (B) Normalised activity of adhered ALP (■) and suspended ALP (□) with total protein content of Caco-2 cells (n=6). (C) Non-normalised TEER values along the incubation of Caco-2 cells in 6-well plate transwell (n=4). Data are mean ± STD from at least 3 independent cell passages.



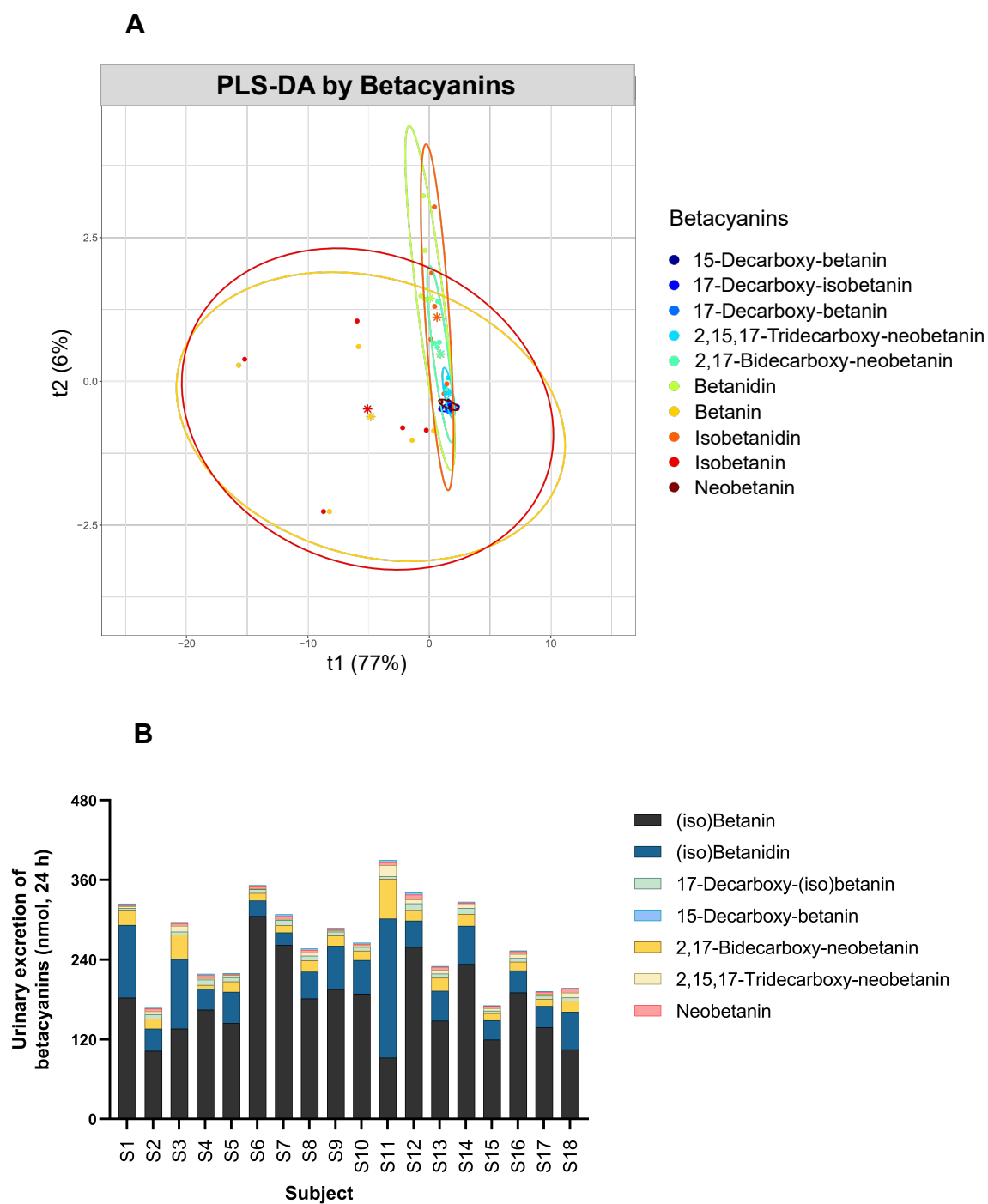
Appendix 4. Preliminary test of the intracellular accumulation of dextrin-coated betanin by Caco-2 cells at various degrees of differentiation. (A) Reduction of betanin concentration (dextrin-coated) in medium (Δ [betanin]) after incubation without Caco-2 cells, and with first-week, second-week, and third-week Caco-2 cells, meanwhile under incubation duration of 2 h (dotted) and 4 h (slashed). (B) The intracellular accumulated betanin per unit of area, with specific incubation times and degrees of cell differentiation. Data are means with STD of 3 replicates from different passages of Caco-2 cells. Different letters indicated the presence of significant difference ($p < 0.05$).



Appendix 5. Agarose gel electrophoresis of PCR products of designed human primers (A) and previously published bacterial primers (B). Only part of the target genes is displayed as examples.

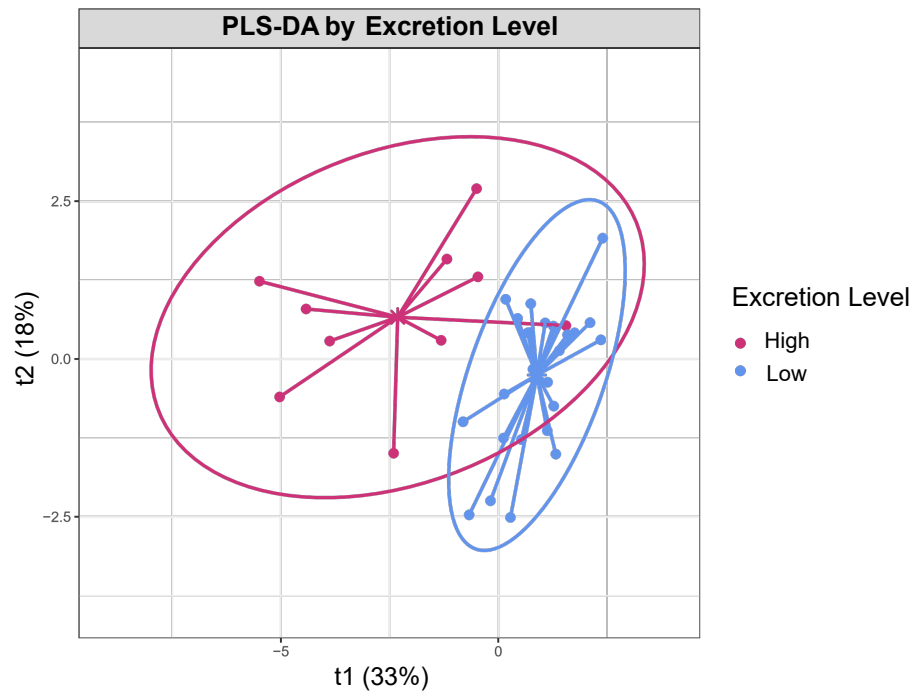


Appendix 6. The proximity matrix of diet patterns between subjects acquired from their intake frequencies of staple, fruits and vegetables, fish and meat, and polyphenol-rich drink during the intervention.

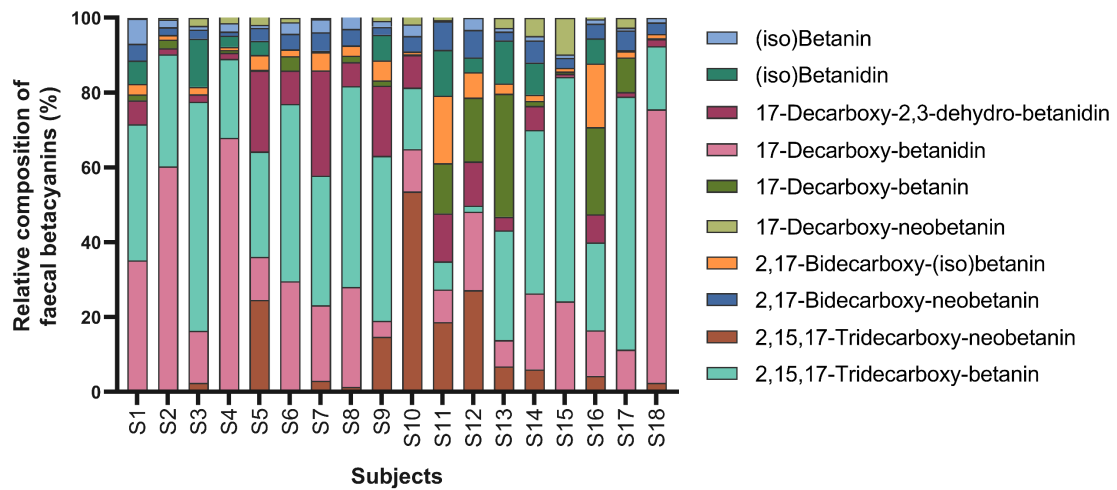


Appendix 7. PLS-DA plots according to the content and composition of betacyanins in urine (A) and the subject-based compositional histogram of betacyanins following urinary excretion (B).

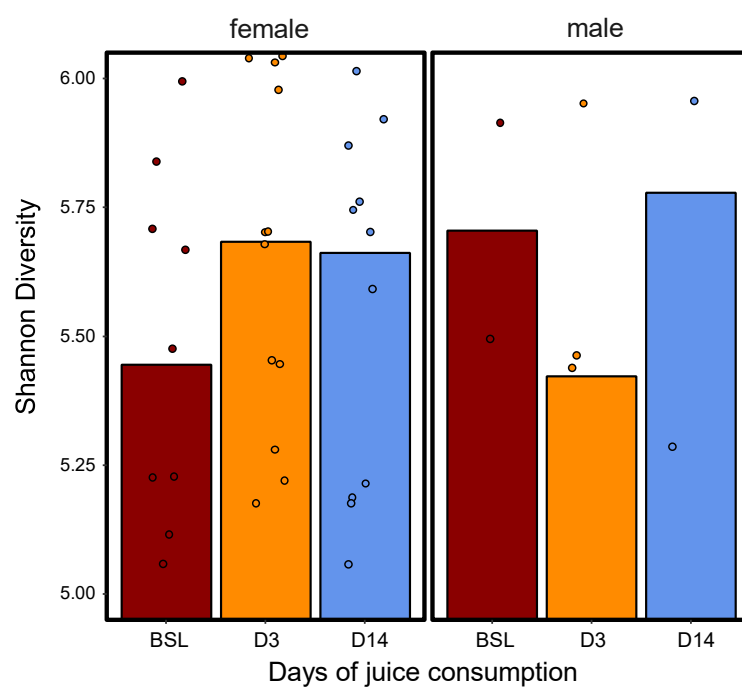
A



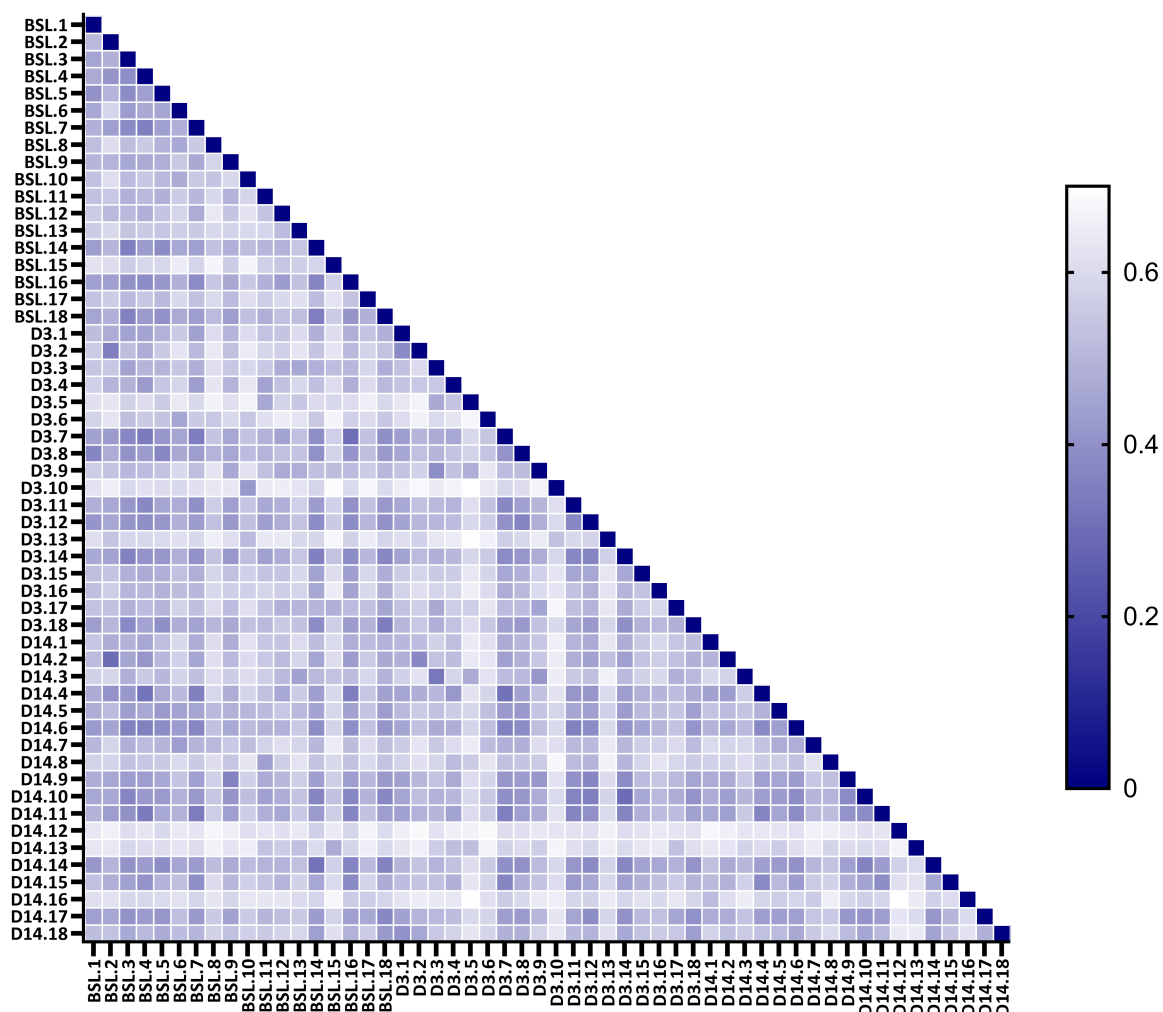
B



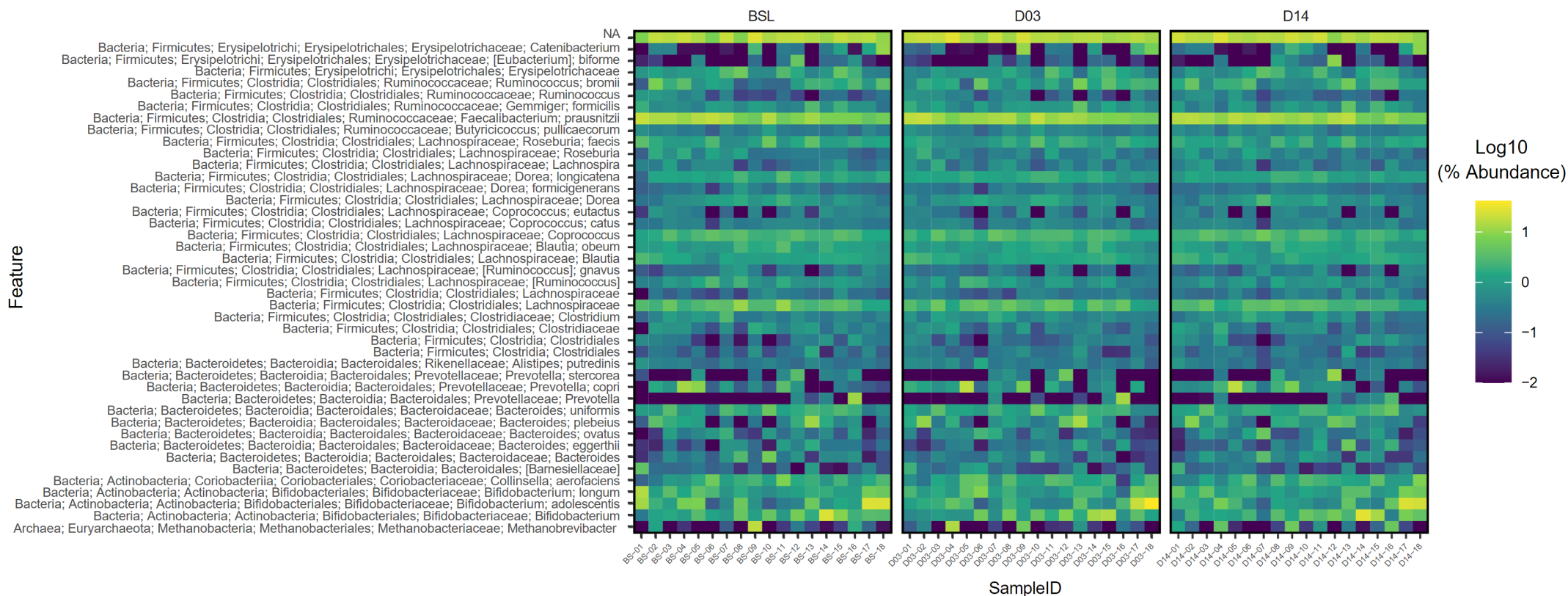
Appendix 8. PLS-DA plots categorised by the low and high faecal excretion level of betacyanins (A) and the subject-based compositional histogram of betacyanins in stool with relative concentrations (B).



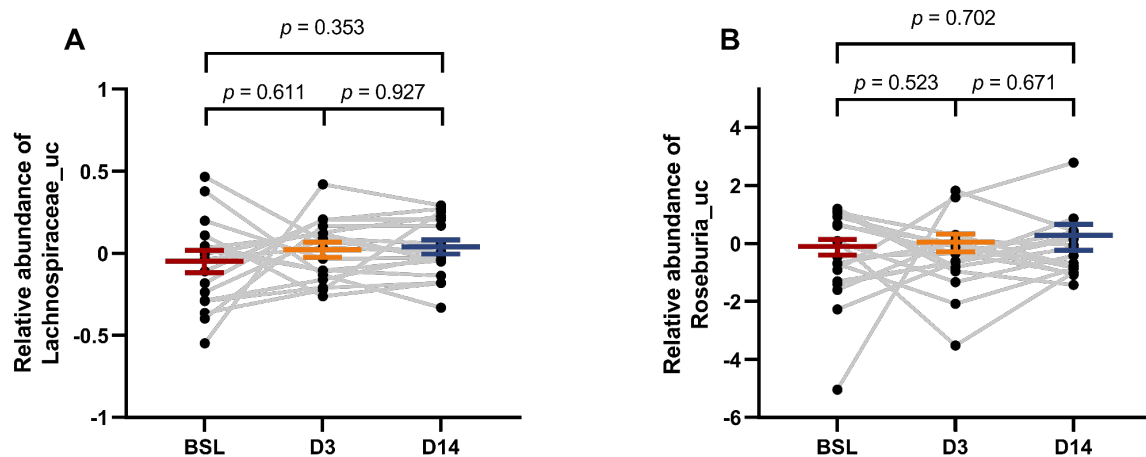
Appendix 9. Boxplots of alpha diversity indices from samples at BSL, D3 and D14, based on Shannon index categorised by gender.



Appendix 10. Beta diversity of gut microbiome reflected by the inter-individual variation displayed in the sample-based distance matrix (unweighted Unifrac).

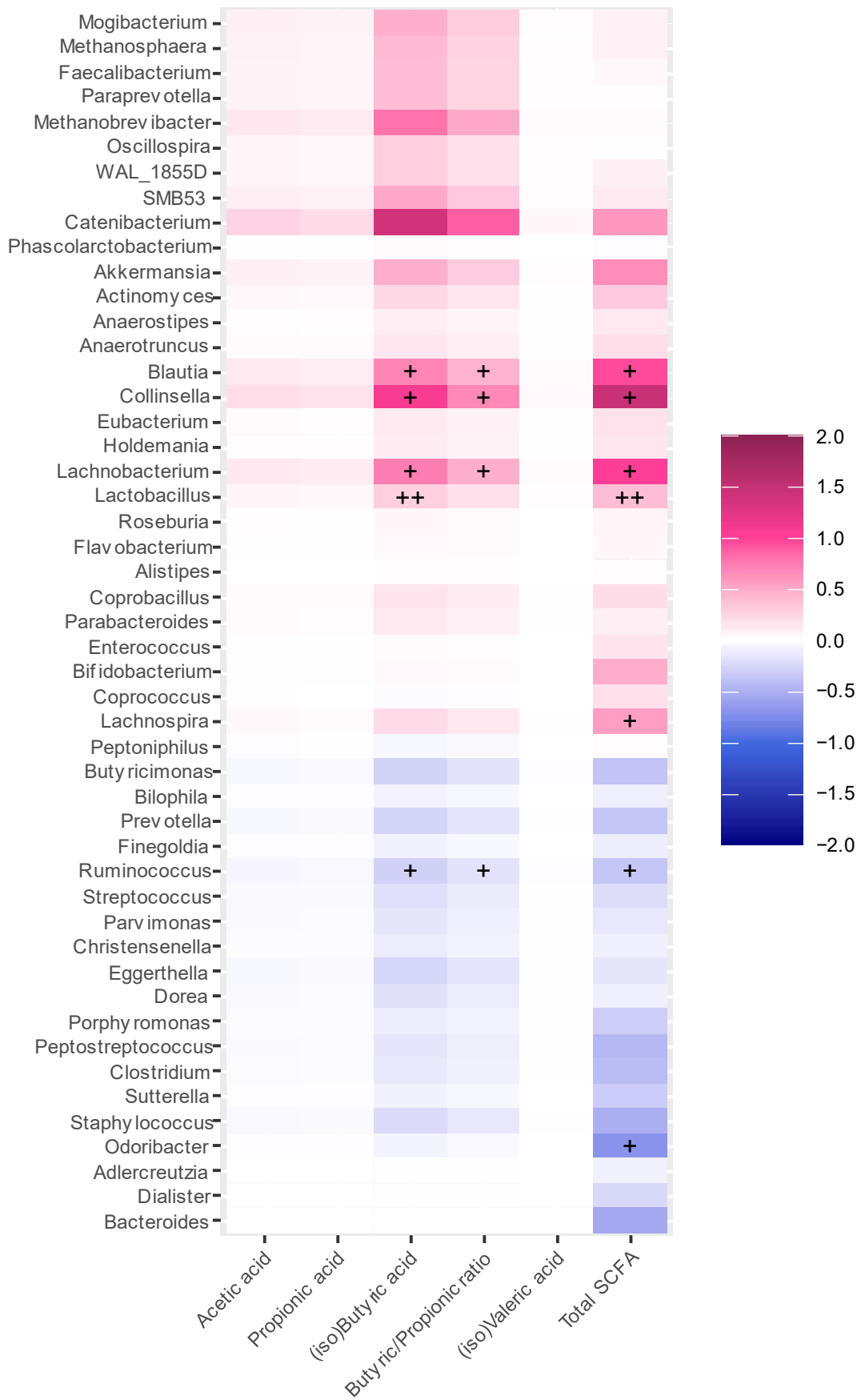


Appendix 11. The sample-based heatmap of taxonomic composition and proportion of gut microbiome.

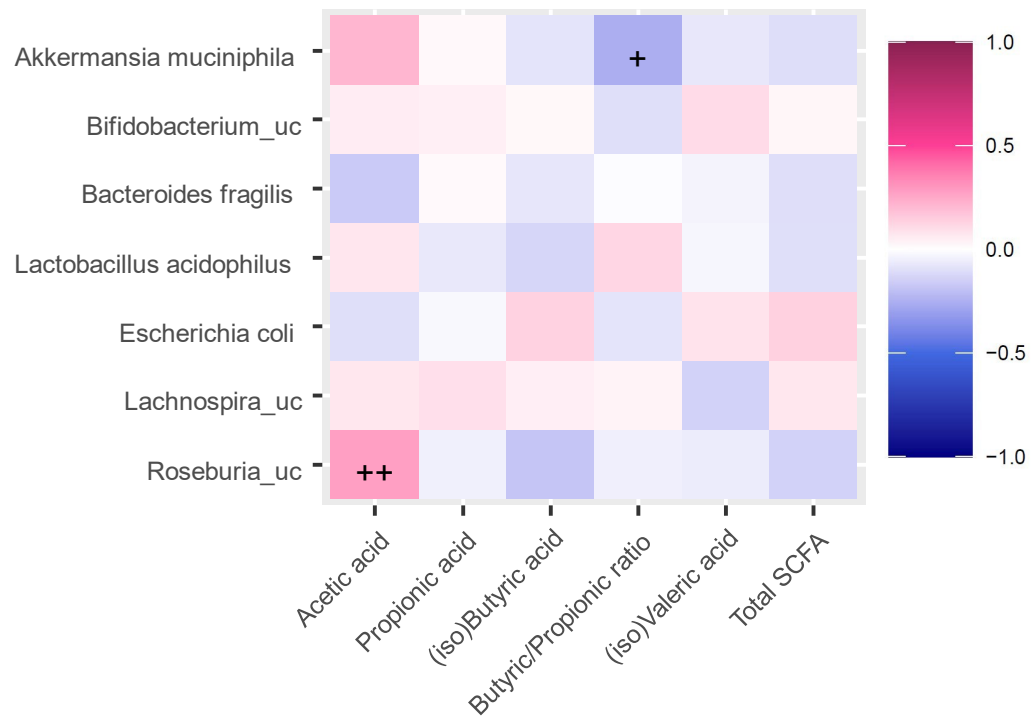


Appendix 12. Changes of the relative species abundance of (A) *Lachnospiraceae_uc* and (B) *Roseburia_uc* on different days of juice consumption. Data were displayed as mean with SEM whose difference was analysed by Wilcoxon test.

A



B



Appendix 13. Spearman's rank correlation matrix between (A) SCFAs and gut microbiota abundance at genus level, and (B) SCFAs and species of interest. Correlations with evidence were marked as: ⁺ $p < 0.1$, ⁺⁺ $p < 0.05$.

Appendix 14. Participant information sheet of the human study of beetroot juice (AREA 20-085).

31/03/2021



Participant Information Sheet

Project title: Pilot study on the effect of beet intake on betalain bioavailability and modulation of gut microbiota in human volunteers

You are invited to take part in this study. Before you decide whether or not to take part, please carefully read the information sheet and make sure you understand why the study is conducted and what will be involved. If you have any questions, please use the contact details below. Thank you for reading this.

What is the purpose of this study?

More attention has been paid to betalains, a group of phytochemicals, whose radical scavenging and anti-inflammatory properties suggest their potential health benefits in mitigation of chronic diseases. The knowledge on their uptake and effects in the gut is however very limited. This human trial aims to investigate the absorption and excretion of beet-sourced betalain compounds in the human body, and to observe the gut microbiome composition before and after betalain consumption. After consumption of beetroot juice, samples collected from participants will be analysed for presence of betalain compounds and microbiome composition.

Am I suitable to participate?

We are looking for healthy volunteers aged ≥ 18 years old with BMI of 19-25 kg m⁻², who are able and willing to drink beetroot juice daily over 2 weeks, who also agree to provide urine and stool specimens during the study.

You should NOT participate in this study if you are:

- Intolerant or allergic to beetroot and derivative foods
- Heavy smoking (≥ 25 cigarettes/day) and alcohol drinking (≥ 7 drinks/week)
- Feeling unwell, or have communicable health condition(s)
- Diagnosed with hypertension or hypotension
- Diagnosed with chronic diseases e.g. diabetes, digestive system diseases, liver and kidney diseases
- Pregnant or lactating
- Diagnosed with cancer
- Taking any dietary supplements or prescribed medication
- Participating in other clinical trials that may have impact on outcomes

If I participate in this project, what will I have to do?

If you decide to take part, you will be asked to fill in a health screening and Covid-19 screening questionnaires that enables the researcher to assess if you can participate in the study. Before study starts, you and the researcher will arrange a short session (< 30 min) at the University of Leeds campus, during which this study can be explained in detail. Questions related to methodology, ethical consideration, confidentiality, withdrawal, etc., will be answered. The study will only be started after receiving your completed consent form, and future visits can be arranged.

You will be asked to attend visit(s) to University of Leeds campus to collect items for study and/or dropping samples. During the trial and 7 days before the trial, you will be asked to reduce your intake of food and drink that are rich in betalains and polyphenols as suggested in Food Guide, except for beet juice provided. Food diaries will be completed for 7 days pre-trial and during the trial for monitoring purpose. On the day before the study starts, you will be asked to fast overnight (i.e. no food intake after 10 pm), and to refrain from alcohol intake. Please contact the researcher if you have any questions.

Due to the current pandemic situation, all the urine and stool samples will need to be collected by yourself at home with provided materials. Procedures will be guided in detail by the instructions for collecting, storing, and transporting the urine and stool specimens (SOP documents). Before starting any beet juice consumption, urine and stool samples will need to be collected at the beginning of study. On Day 1 of study, you will be asked to consume a

1

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specific amount of red beetroot juice and sandwich provided as breakfast. Please follow the instruction for the daily consumption of red beet juice. Then urine samples are collected at post-prandial 0-2h, 2-4h, 4-6h, 6-8h, 8-12h, and 12-24h. From Day 2, you will be asked to consume a specific portion of red beet juice during lunch and dinner (see Instruction) for next 13 days while maintaining your habitual diet. Again, a stool sample needs to be collected after 3 and 14 days of beetroot juice consumption. Due to Covid-19, it is recommended that sampling could be done safely at home. Samples need to be stored in a biohazard-safe container in a household freezer. Samples need to be dropped off at the University (School of Food Science) within 3 days of sample collection. Exact location and time are to be arranged with researcher before each visit. In total, 3 times of drop-off will be arranged with you. After completing all the stages, you will receive vouchers valued £20 as a compensation for your time.

What are the benefits and risks of the participation?

Slight hunger may be felt on Day 1 due to restricted food intake during the first 3 hours of the study. During the intervention, your urine and stool are likely to appear red in colour. This situation is expected and completely normal, but if you still have concern you may contact the GP. There is also a low risk of mild hypotension, with possible symptoms of light dizziness, sick or blurred vision after 10 min of beet juice consumption. To ease hypotension, please change postures more slowly and drink more water. Please record your adverse effect in the Record of Consumption and Events during Intervention. If you suffer from any symptoms of vomit and/or diarrhea during period of juice consumption, please report the situation to researcher and cease the progress of juice drinking and sample collection. If you do not wish to continue, please see the study withdrawal section. By participating in this study, you will make an important contribution to increase the knowledge on the availability of beet-sourced betalains to human body, as well as their impact on gut health.

Can I withdraw from this study and how?

Yes. You can withdraw from the study at any time without giving any reasons. Please contact the researcher at your earliest convenience about the withdrawal decision. Correspondingly your data and collected samples will be destroyed once withdrawn. However, they will not be destroyed after all visits have been completed.

Will my information and data be kept confidential?

Yes. Personal information and all your data will be kept confidential and be destroyed after the research is completed. Digit codes will be allocated to all samples and data for the purpose of anonymity. Raw information in both electronic and paper forms will be anonymised and locked in a University of Leeds repository, whose accessibility is restricted to the research team members. Processed results will be presented in a grouped manner to ensure the data of individuals is non-identifiable. For more information, please visit <https://dataprotection.leeds.ac.uk/research-participant-privacy-notice/>

Who has reviewed this study?

This research study has been reviewed by University of Leeds Research Ethics Committee, with ethical approval granted (AREA 20-058).

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If you have no questions and you agree to participate, please complete the participant consent form.

If you want to make a complaint, please contact the Ethics Secretariat at University of Leeds:

ResearchEthics@leeds.ac.uk Tel. no.: +44 (0)1133434873