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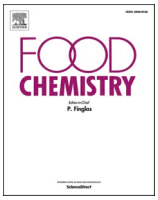

Effect of two-week red beetroot juice consumption on modulation of gut microbiota in healthy human volunteers – A pilot study

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ABSTRACT

With very little research exploring intestinal effects of red beetroot consumption, the present pilot study investigated gut microbial changes following red beetroot consumption, via a 14-day intervention trial in healthy adults. Compared to baseline, the study demonstrates transient changes in abundance of some taxa e.g., *Romboutsia* and *Christensenella*, after different days of intervention ($p < 0.05$). Enrichment of *Akkermansia muciniphila* and decrease of *Bacteroides fragilis* ($p < 0.05$) were observed after 3 days of juice consumption, followed by restoration in abundance after 14 days. 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 a significant rise in (iso)butyric acid content (172.7 ± 30.9 μmol/g stool). Study findings indicate the potential of red beetroot to influence gut microbial populations and catabolites associated with these changes, emphasizing the potential benefit of red beetroot on intestinal as well as systemic health.

1. Introduction

It is well-acknowledged that the gut microbiota plays an imperative role towards human health in multiple aspects involving fermentation, metabolism, immunomodulation and neurobehavioral traits (Hakansson & Molin, 2011). The human gut microbiota is comprised of virus, bacteria, protozoa, archaea and fungi, whose density ascends distally in the intestinal tract from $10^{2\text{-}3}$ g^{-1} in duodenum and jejunum, to $10^{4\text{-}7}$ g^{-1} in ileum and cecum, and to the maximum in colon ($10^{11\text{-}12}$ g^{-1}) (David et al., 2014). Despite large inter-individual variations, the gut bacterial community is dominated by the phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* accounting for > 90 % of bacteria.

A dysbiosis of gut microbiota is often featured by decreased bacterial richness and diversity in tandem with the disturbed compositional balance between commensal and putatively pathogenic species, such as a reduced abundance of genus *Faecalibacterium* and rise of *Escherichia* in stools of Crohn's disease patients (Lo Presti et al., 2019; Manichanh et al., 2006). It has been intimately linked to a wide array of enterobacteria-dependent disorders including inflammatory bowel disease (IBD), obesity, diabetes mellitus, allergic reaction, and depression (Karlsson, Tremaroli, Nielsen, & Bäckhed, 2013). Therefore, in a general consensus, gut microbiota serves a strong influential role in the metabolic and immune regulation in the intestinal environment.

There are a variety of intrinsic and extrinsic properties shaping the quantitative and qualitative microbial features. Compared to the heredity, environmental factors (e.g., diets, lifestyle, health condition) are reckoned as stronger determinants to shape gut microbiota, especially the diet as one of the most pivotal confounders (Niederreiter, Adolph, & Tilg, 2018). With mutual interactions between gut microbes and undigested nutritional substrates, major changes of diet components (e.g., carbohydrates and proteins) could result in a rapid modulation of genetic composition as well as the metabolic functions of intestinal bacteria, followed by a long-term adaptation of intestinal homeostasis (David et al., 2014; Wu et al., 2011). Though permanent shift of gut microbiota might necessitate prolonged dietary changes, the advantages of a therapeutic dietary approach are gradually recognized toward improvement of gut microbial environment hence the host health.

The antiradical and anti-inflammatory activities of phytochemicals (e.g., polyphenols) have been emphasized by a number of studies, with emerging attention being paid to their ability for alteration of gut microbes. For example, animal studies documented the linkage of non-absorbed flavan-3-ols and anthocyanins residing in the mice guts to

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the promoted growth of *Akkermansia* genus and *Bacteroidetes* phylum, and to the depleted *Firmicutes* phylum (Gu et al., 2019; Masumoto et al., 2016). Colonic fermentation in the presence of polyphenol-rich extract is also associated with elevated production of short-chain fatty acids (SCFAs) especially the proportion of butyrate (Jakobsdottir et al., 2013; Wang et al., 2018). Both polyphenol and SCFAs contribute to the delay or prevention of systemic inflammation via modulating intracellular signal transduction pathways while enhancing the metabolic outcome.

Red beetroot (*Beta vulgaris* L.) is regarded as a rich source of betalains, polyphenols, and nitrates. The nitrate content in beetroot is well known for the potent hypotensive effects, but nitrates have also demonstrated potential to modulate gastrointestinal motility (Capper et al., 2020; Walker et al., 2018). Besides, Hu et al. (2020) elucidated the role of nitrates in regulating microbial dysbiosis, maintaining colon consistency, as well as suppressing the p53 transduction pathway, suggesting the potential of nitrates in gut microbiota alteration and anti-colitogenic effects, although conclusive evidence will be required from further research.

Resembling polyphenols, betalains are a further group of phytochemicals with biological properties yet have so far been largely overlooked. Betalains, especially the subgroup of red betacyanins, were discovered to efficiently suppress the generation of reactive oxygen species in cell-free and cellular environments, hence highlight the ability to mitigate oxidative stress and cellular redox balance (Wang et al., 2022). Latest *in vivo* and *in vitro* research has also expounded the potent immunomodulatory properties of betalains, demonstrated by the molecular interference with pro-inflammatory and/or cyto-protective signaling pathways leading to modulation of downstream target proteins and enzymes which drive inflammatory processes and cellular antioxidant status (Fernando, Sergeeva, Frutos, Marshall, & Boesch, 2022; Gómez-Maqueo, García-Cayuela, Fernández-López, Welti-Chanes, & Cano, 2019; Krajka-Kuzniak, Paluszczak, Szaefer, & Baer-Dubowska, 2013; Tesoriere, Attanzio, Allegra, Gentile, & Livrea, 2014). These biological properties have been further associated with the amelioration of hyperglycemia and type 2 diabetes mellitus, cardiovascular disease, IBD and other chronic conditions (Khan, 2016).

There is evidence for antimicrobial properties of betalains, with a few *in vitro* studies documenting strong potency of betalain-rich extracts to inhibit growth of *Escherichia coli*, as well as *Salmonella typhimurium* (Hayek & Ibrahim, 2012; Velićanski, Cvetković, Sinisa, Vulić, & Djilas, 2011). However, *in vivo* studies are so far lacking to explore the effect of beetroot on gut microbiota. Capper et al. (2020) carried out an 8-week parallel intervention study in 36 older participants (age 67 ± 6 years) who consumed whole beetroot or a control diet every-two days. They reported evident shifts in gut bacteria in the elderly, specifically phylum *Bacteroidetes* and genus *Alistipes*, accompanied by an increase in SCFA concentrations and lowered blood pressure following whole beetroot consumption. An earlier study in rodents reported an improved gut microbiome response with reduced ratio of *Firmicutes* to *Bacteroidetes* phyla following a 14-week feeding period with red pitaya betacyanin-supplemented feed (Song et al., 2016).

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, and to investigate for the first time the association between non-absorbed betacyanin catabolites and microbiota changes during intestinal fermentation of beetroot.

2. Materials and methods

2.1. Materials and reagents

Red beetroot concentrate (in 30 mL pouches) for the human study was kindly provided by Active Edge commercials (Hartley Wintney, UK). QIAamp PowerFecal Pro DNA extraction kit and Gel Extraction Kit were obtained from Qiagen (Manchester, UK). The SCFA standards (i.e., acetic, propionic, (iso)butyric, (iso)valeric, and hexanoic acid) were purchased from Sigma-Aldrich (Dorset, UK). Organic solvents (e.g., methyl-*tert*-butyl-ether, LCMS-grade methanol) and other reagents were sourced from Fisher Scientific (Loughborough, UK) unless specified.

2.2. Human study design and participant recruitment

This study 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 University guidance was put in place during the pandemic. All participants were fully informed of the study details and their consent was obtained prior to trial initiation. Eighteen healthy participants (13 females, 5 males) were recruited from the University of Leeds. The number of participants for this study was in line with other microbiome-based studies (Kaczmarek et al., 2019; Wiczkowski, Romaszko, Szawara-Nowak, & Piskula, 2018). Screening of potential participants was performed with the following inclusion criteria: generally healthy, age between 20 and 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.

2.3. Intervention study

Participants were invited onto the University campus to attend an initial visit and receive detailed trial explanations and undergo anthropometric measurements. They were provided with instructions and instruments for conducting the entire trial off site, which included the procedures of beetroot drink preparation, stool sampling, and blood pressure monitoring. 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 for this study, the manufacturer's suggestions for daily consumption of concentrated juice were followed, and with reference to the literature (Wiczkowski et al., 2018). In addition, the acceptable daily intake (ADI) for nitrate (3.7 mg/kg body weight) was taken into consideration, as stated by the EU Food Safety Authority, to lower the risk of hypotension.

Two weeks before the study initiation, participants were asked to abstain from consumption of betalain-containing food according to the food guide. For a period of 14 consecutive days, participants were asked to dilute 30 mL of beetroot concentrate into 250 mL with drinkable water on each day and consume with their habitual lunch and dinner (2 × 125 mL). Participants were asked to sample stool at baseline (BSL) before juice consumption, and after 3 days (D3) and 14 days (D14) of beetroot juice consumption for the purpose of microbiome classification and betalain characterization. The participants were asked to refrain from excessive intake of polyphenols and their habitual diets were monitored via food diary and weekly food frequency questionnaires (FFQ). The systolic and diastolic blood pressures of participants were measured each week by the participants to check on potential hypotensive effects of beetroot juice.

2.4. DNA extraction, qPCR amplification and sequencing

Fecal specimens were collected by participants at their respective residences at indicated time points (BSL, D3, D14) during intervention using stool collection bags (Longniddry, UK) and DNase/RNase-free tubes. Specimens were stored at −20 °C immediately after sampling and then transported within two days in freezer bags onto campus for storage at −80 °C. The microbial genomic DNA of fecal samples (*ca.* 200

mg) was extracted using QIAamp PowerFecal Pro DNA Kit which included the stages of DNA isolation and purification following the manufacturer's instruction. The purity and quantity of total genomic DNA were monitored via NanoQuant plate (Tecan Spark 10 M plate reader) and fragment analyzer system (5400 Agilent). Depending on individual concentration (300–560 ng μL^{-1}), the DNA was diluted to 1 ng μL^{-1} for further analytical procedures.

The 16S rRNA gene (V3 and V4 regions) was amplified via polymerase chain reaction (PCR) 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 Qubit 2.0 Fluorometer (Thermo Scientific, UK) and Agilent Bioanalyzer 2100 system. According to manufacturer's instruction, equimolar PCR products were pooled, end-repaired, tagged and ligated with Illumina adapters. The library sequencing process was performed on the Illumina NovaSeq PE250 platform by Novogene Co., ltd (Cambridge, UK), and the paired-end reads were assigned to corresponding samples and cleaned for the purpose of statistical analysis.

2.5. Real-time quantitative PCR of target bacterial strains

Real-time qPCR was conducted on genomic DNA of fecal samples to determine presence of specific bacterial strains whose primers are listed in Supplementary Table S1. Primers of target strains were based on relevant literature, with primer specificity verified by BLAST with NCBI database tool and PCR product size confirmed through agarose gel electrophoresis. Diluted genomic DNA samples were mixed with SybrGreen based reagent (SensiFast SYBR Green, SLS ltd, UK) following the manufacturer's instruction, and real-time qPCR was performed via StepOne Plus Real-Time PCR System (Applied Biosystems, UK) with 40 thermal cycles of 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 normalized with corresponding universal gene levels followed by calculation based on the Livak ($2^{-\Delta\Delta CT}$) method.

2.6. SCFA measurement

The analytical method was modified based on De Baere et al. (2013) and Diotallevi et al. (2022). The fecal specimens (100 mg) were suspended in water (12 %, w/v) and acidified with phosphoric acid (1.5 % v/v). The SCFA in the fecal suspension (100 μL) were obtained by liquid–liquid extraction, twice performed, using methyl *tert*-butyl ether (2 × 100 μL) through horizontal vortex (5 min) and centrifugation (20,000*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 spectrophotometric detector (Tokyo, Japan). Compounds were separated using Kinetex XB-C18 column (100 mm × 2.1 mm, 2.6 μm, Phenomenex, UK) with mobile phases of (A) NaH_2PO_4/H_3PO_4 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 using standard calibration curves generated from the chromatograms at 210 nm.

2.7. Analysis of betalains and polyphenols

Fecal mass (100 mg) was extracted twice in 0.5 % formic acid through horizontal vortex, sonication and centrifugation (20,000*g*, 8 min, 4 °C), from which the aqueous suspensions (15 %, w/v) were obtained. Then, native betacyanins and catabolites were separated from fecal suspension via solid-phase extraction (SPE) method using Strata-X polymeric reversed-phase cartridges (33 μm, 200 mg, 6 mL), with a method was based on Sawicki, Topolska, Romaszko, and Wiczkowski (2018). The suspension-loaded cartridges were washed with 0.1 % formic acid prior to elution of retained pigments using methanol. The eluents were dried via Genevac centrifugal concentrator EZ-2 (low bp mode, 1 h) followed by Labconco freeze dryer (−55 °C), resuspended in water (100 μL) and centrifuged (22,000*g*, 10 min, 4 °C).

Betacyanin pigments from processed fecal samples were quantified by Thermo-Vanquish UHPLC following identification with TSQ Quantiva electrospray ionization sources (ESI-MS) (Thermo Scientific, UK). The reverse-phase LC was performed within Kinetex XB-C18 column (100 mm × 2.1 mm, 2.6 μm) at 40 °C. The binary mobile phases were established by (A) 2 % formic acid (v/v) and (B) 100 % methanol. With discrete sample injection volume (5 μL) and flow rate (0.2 mL min^{-1}), the proportion of solvent B was increased from 5 to 25 % (0–2 min); to 95 % (2–5 min), and was held for 5 min before returning to initial 5 % (10–15 min). Chromatograms were generated by ESI-MS source in positive ion mode, with spray voltage of 3500 V, ion transfer temperature of 325 °C and vaporizer temperature of 275 °C. Characterization of compounds was achieved based on corresponding parent ion (m/z value) and retention times in comparison with reference (Nemzer et al., 2011; Sawicki et al., 2018). Note that all the compounds were quantified in equivalents of purified betanin standard (Fernando et al., 2022).

Similarly, betalains and polyphenols in the beetroot concentrate were analyzed via UHPLC-MS after 10-fold dilution, centrifugation (20,000*g*, 10 min, 4 °C) and filtration (0.2 μm membranes). The reverse-phase liquid chromatography utilized the same column, injection volume and temperature as aforementioned, except the binary mobile phases which were (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 increased 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). Quantification and identification of individual compounds were achieved by MS in the negative ion mode with spray voltage of 2500 V, and calculation performed using calibration curves with corresponding standards.

2.8. Data analysis and statistics

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) (Bokulich et al., 2018; McDonald et al., 2012). With yielded information of corresponding abundance, alpha and beta-diversities were processed in QIIME2 software and visualized via R-4.1.2 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 analyze 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 (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 analyzed and presented as mean with SEM using R, SPSS, and/or 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 test, with significance level of $p < 0.05$.

3. Results

3.1. Subject characteristics and dietary intake

All eighteen participants, with a mean age of 29.1 ± 6.1 and BMI of 22.5 ± 2.4 kg m^{-2} (mean ± SD), completed the study with high compliance (98 %) and no drop-out. According to food diary and FFQs, marked inter-individual diversity exerted 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 systolic and diastolic blood pressures (SBP/DBP) of participants hardly fluctuated between baseline (101 ± 13/66 ± 8 mmHg) and Day 14 (103 ± 13/67 ± 7 mmHg) ($p > 0.05$).

3.2. Presence of betacyanins and their metabolites in juice and in fecal samples

The composition of betacyanins and polyphenols in the beetroot juice is illustrated in Fig. 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 the 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, and was therefore precluded from further analysis in the current study.

By employing UHPLC-MS technique in the current study, 11 major catabolites of betacyanin could be identified in the fecal samples. As demonstrated by the cumulative histogram of fecal excretion profiles

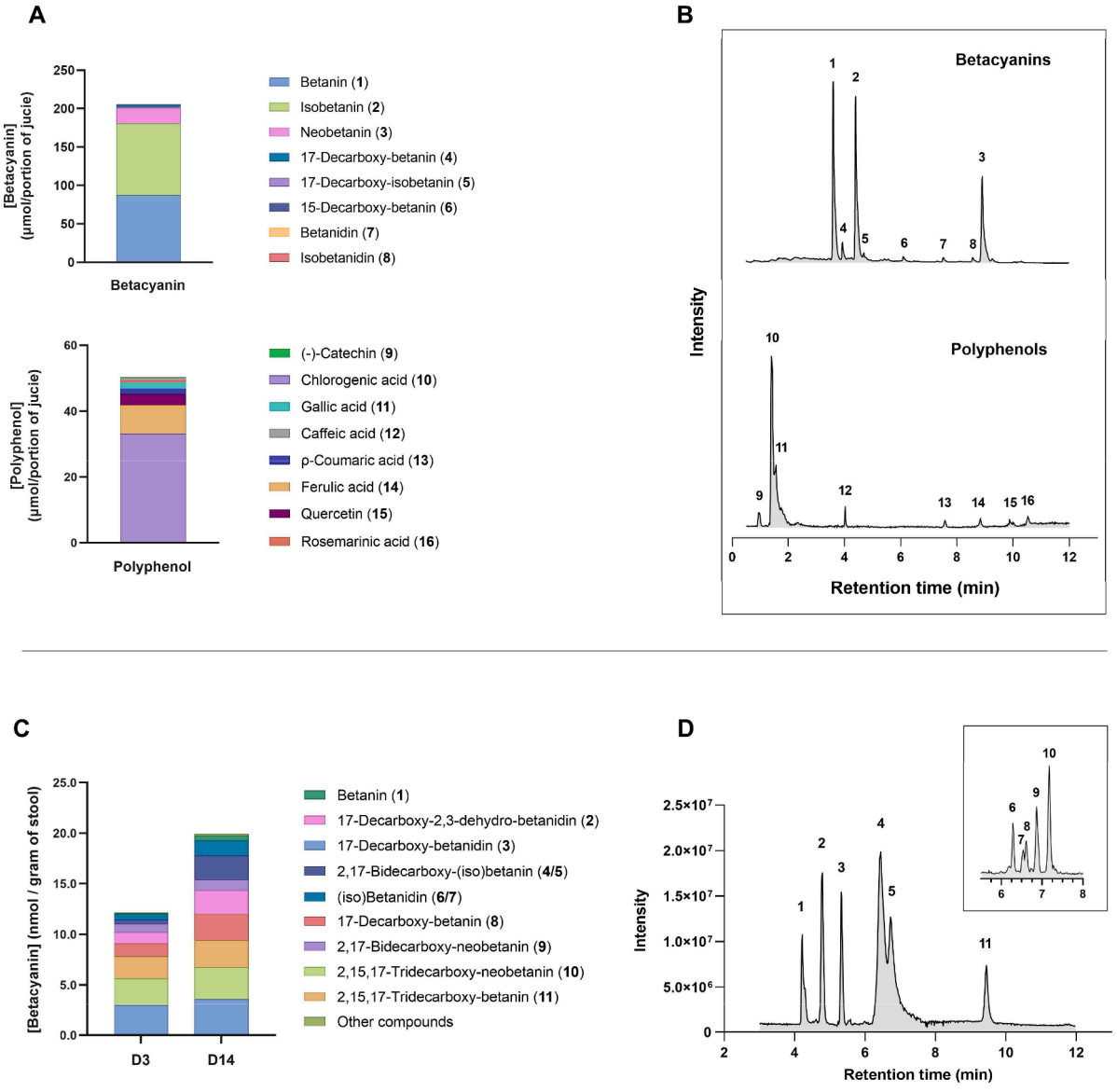

Fig. 1. Profiles of red beetroot juice and the fecal excreted betacyanins after juice ingestion, illustrated in (A) the compositional histogram of betacyanins and polyphenols of juice with (B) corresponding chromatograms, as well as (C) the composition of dominant betacyanin catabolites in human stool with (D) the chromatogram acquired via UHPLC-MS. Note the separate compound numbers in (A) and (B) from (C) and (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 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 large inter-individual variability of betacyanins present in fecal samples. With regards to composition, all the stool samples contained dominantly the catabolized 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 characterizable catabolites. Native and catabolized betacyanins were not detected in baseline stool samples.

3.3. Taxonomic diversity and distribution of microbial communities

The richness and diversity within the microbial community were demonstrated using Shannon and phylogenetic alpha-diversity indices (Fig. 2A-B). Species in fecal samples at 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 (Fig. S1) 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.

Analysis of beta-diversity is presented in Fig. 2C 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 ($p = 0.993$), indicating that the community composition before and after juice consumption was phylogenetically similar. The plot of non-metric multidimensional scaling analysis (NMDS), nevertheless, indicated that samples at BSL and D14 groups were more clustered compared with the D3 group (Fig. S2). Consistent results were also acquired from the analysis of weighted Unifrac and Bray Curtis distance. Meanwhile, inter-individual variation of microbial community was visualized by the marked intra-group difference in PCoA, as well as by the unweighted Unifrac distance between distinct subjects as displayed in the matrix (Fig. 2D).

3.4. Comparison of taxonomic composition before and after beetroot juice consumption

The taxonomic composition of each sample was demonstrated in the distribution histogram of relative abundance (Fig. 2E) 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 visualized in a group-based heatmap (Fig. 3A), 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 summarized and expressed in Log2-fold change relative to specific groups (Fig. 3B-D). Concordant with PCoA plot, there were more taxa characterized 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. 3A). In the current study, the 14-day beetroot juice consumption did not exert a significant impact at the phylum and class levels of gut microbiota.

3.5. Relative abundance of target microbial species after juice consumption

Relative abundances of target species in samples at different days were examined using real-time qPCR. According to Fig. 4, 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 (Fig. 4S).

3.6. SCFA content

There were six SCFA compounds detected and quantified in this study (Table 1). The composition was dominated by acetic acid (19–33 %) and (iso)butyric acid (20–28 %) whereas propionic acid (3–9 %) and (iso)valeric acid (4–12 %) were relatively minor. During the intervention, the total amount of SCFAs in D3 and D14 stools was markably higher compared to BSL stool. It was mainly attributed to an evident enrichment of butyric and isobutyric acids, which was also accompanied by 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.

3.7. Association between betacyanin catabolites, SCFAs, and microbial profile

The correlation matrix in Fig. 5A 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

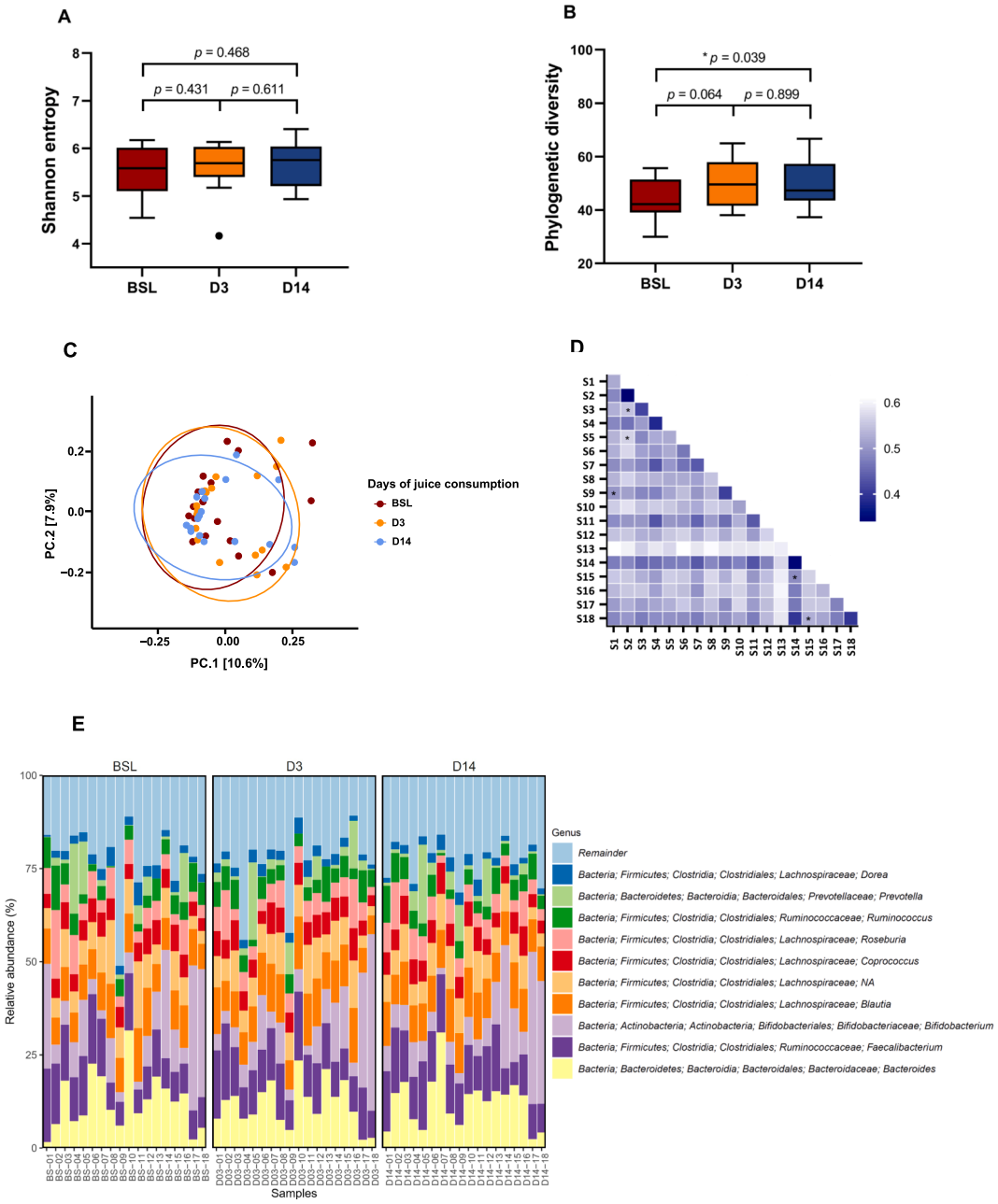

Fig. 2. Alpha and beta diversities of gut microbiome on different days of beetroot juice consumption depicted with (A) Shannon index, (B) phylogenic diversity whole tree index, (C) PCoA of unweighted Unifrac distance, (D) subject-based matrix of unweighted Unifrac distance, and (E) taxonomic composition histogram of relative abundance of top genera. Significant differences in (A), (B) and (D) were analyzed by Wilcox and Tukey test and was indicated as * ($p < 0.05$).

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, strains of interest and SCFAs were further explored by correlation matrices. According to Fig. 5B, 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 (Fig. S5), (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* ($p < 0.1$).

4. 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

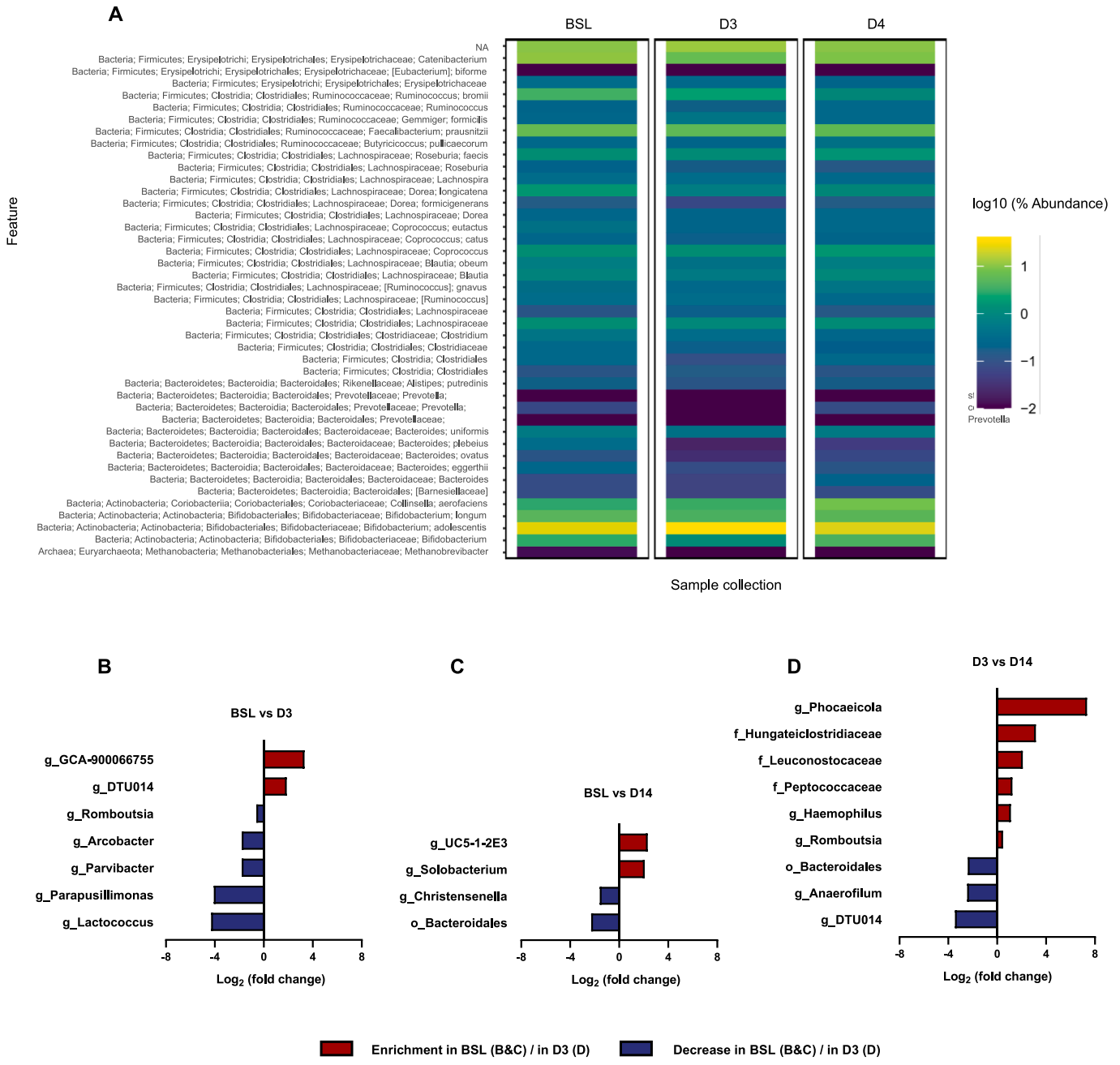

Fig. 3. Changes in taxonomic composition and abundance in the BSL, D3 and D14 sample groups. (A). Enrichment and depletion of specific taxa. The comparisons were carried out as Log2-fold change between BSL and D3 (B), BSL and D14 (C), and D3 and D14 (D) ($p < 0.05$). Note the fold changes in (B) and (C) were respect to BSL, and changes in (D) were respect to D3.

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 catabolized 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 (Clifford et al., 2017; Wiczkowski 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 fecal 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

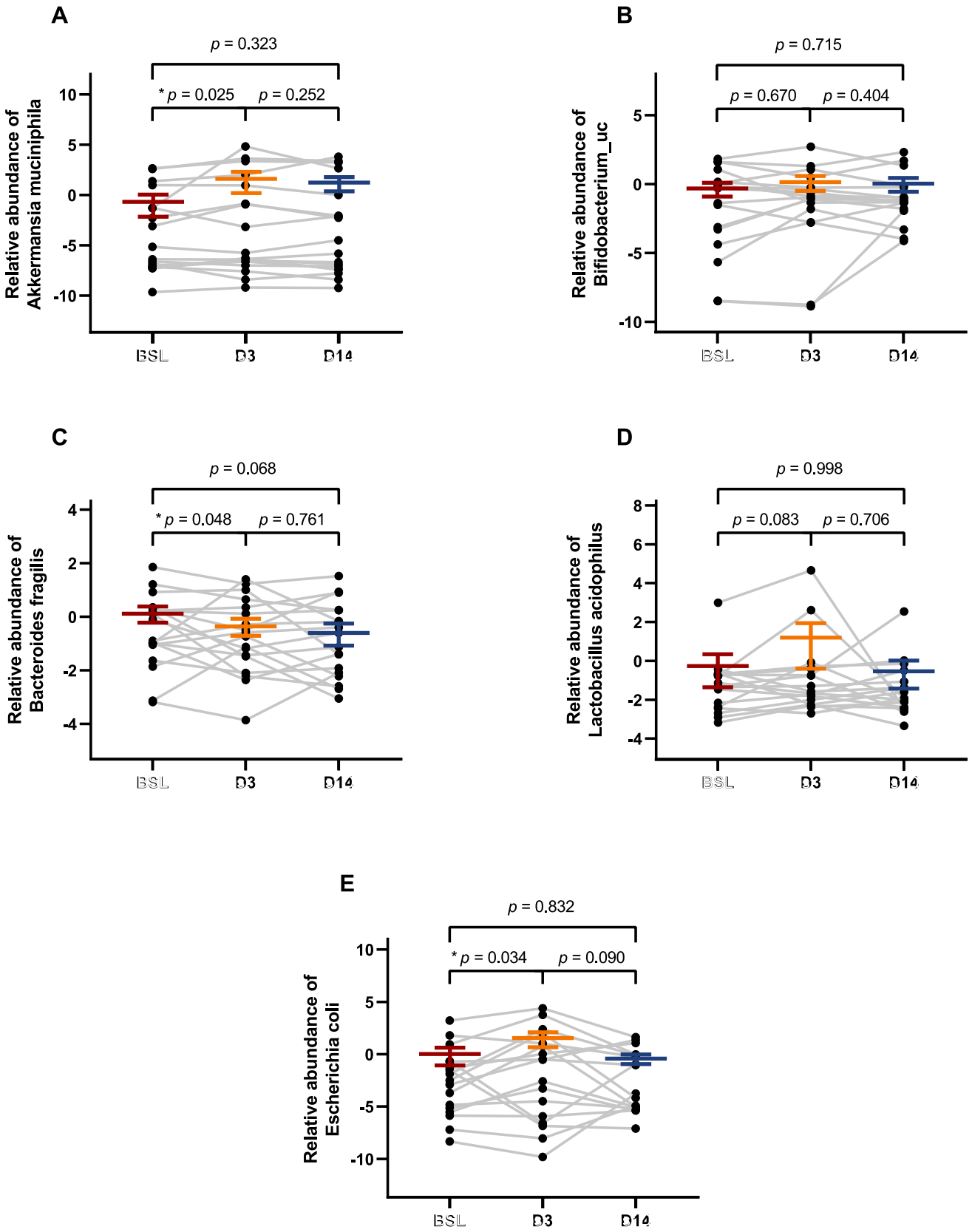

Fig. 4. Quantitative changes of the relative species abundance (in Log 2 scale) of (A) *Akkermansia muciniphila*, (B) *Bifidobacterium_uc*, (C) *Bacteroides fragilis*, (D) *Lactobacillus acidophilus*, and (E) *Escherichia coli* after day 3 and 14 of juice consumption. Data are mean with SEM, and significance was analyzed by Wilcoxon test ($p < 0.05$).

Table 1
SCFA concentrations (μmol/g stool) and butyric/propionic acid ratio in stools at BSL, D3 and D14 following beetroot juice consumption. Data are mean with SEM (n = 18). Differing letters indicate inter-group significance respect to each SCFA ($p < 0.05$).

SCFAs	BSL	D3	D14
Acetic acid	110.4 ± 13.5	175.6 ± 44.1	124.9 ± 8.4
Propionic acid	29.4 ± 5.7	36.3 ± 14.0	18.7 ± 2.2
Butyric acid	50.6 ± 10.7 [a]	130.7 ± 24.9[b]	124.6 ± 22.9[b]
Isobutyric acid	16.4 ± 6.9 [a]	42.0 ± 8.8[b]	48.4 ± 10.3[b]
Butyric/Propionic acid ratio	3.9 ± 1.1 [a]	8.5 ± 2.0[b]	10.2 ± 1.8[b]
Valeric acid	19.6 ± 6.6	22.4 ± 9.1	72.7 ± 28.0
Isovaleric acid	6.7 ± 2.8	2.4 ± 1.3	5.0 ± 2.6
Total	330.1 ± 41.2 [a]	615.3 ± 105.6[b]	655.2 ± 97.3[b]

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 feces 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 characterized as acetate-oxidizing 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

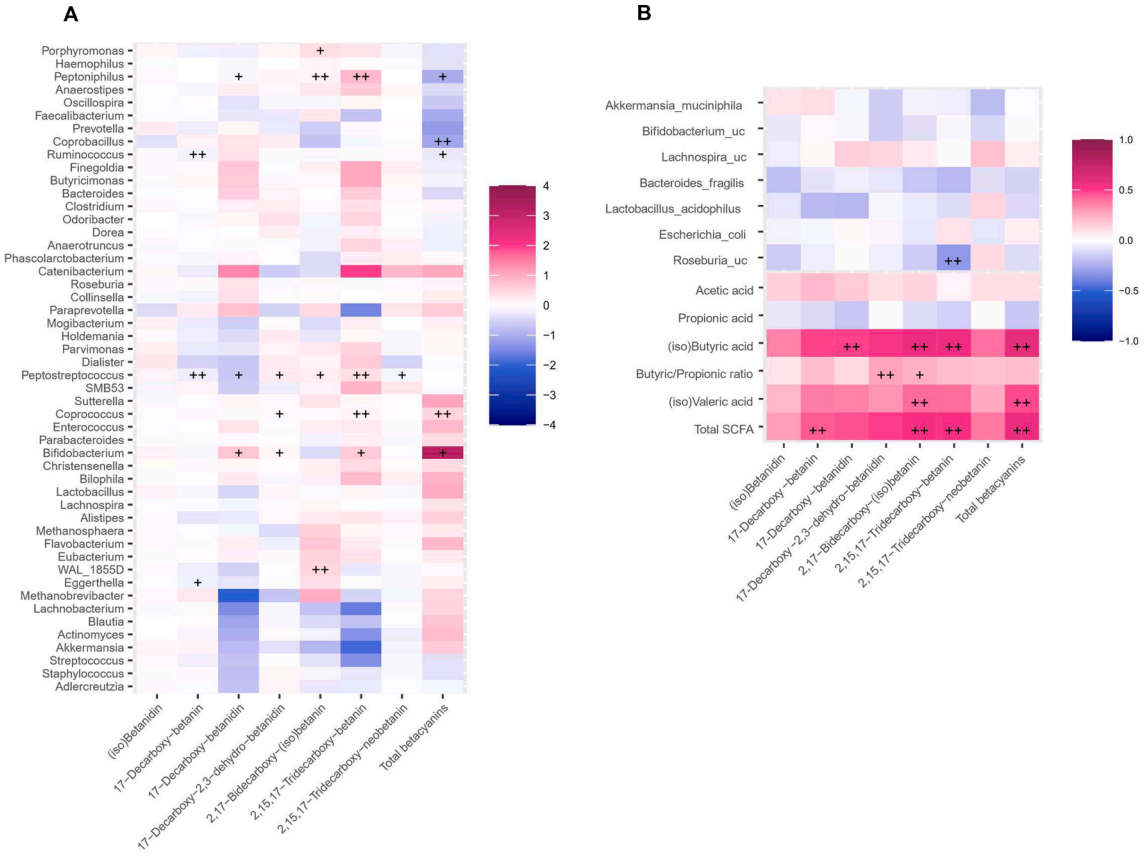

Fig. 5. Spearman's rank correlation matrix demonstrating the relationship of fecal excreted betacyanins with (A) gut microbiota abundance at genus level, and with (B) relative abundance of target species plus SCFA contents. Correlations with strong evidence were labelled as: $^{+}p < 0.1$, $^{++}p <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

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 *UC5-1-2E3* (*f_Lachnospiraceae*[1]) 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 feces 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 characterized as acetate-oxidizing 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 microbial abundance of *B. fragilis* by qPCR. As a common anaerobic strain in human gut, the involvement of *B. fragilis* in inflammatory diarrhea and related bowel diseases was confirmed by multiple studies (Sears, Geis, & Housseau, 2014; Swidsinski, Weber, Loening-Baucke, Hale, & Lochs, 2005). The virulence of enterotoxigenic *B. fragilis*, sourced from the production of metalloprotease toxin, serves a role in colitis potentially by triggering NF-κB signaling impacting on synthesis of pro-inflammatory

[1] Note that g_ represents genus level, f_ for family level, and o_ for order level taxonomy.

interleukins in intestinal epithelial cells. With other characterized 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 strain, *E. coli*, has also been reported to participate in CRC development, especially the adherent-invasive *E. coli* that were found to be prevalent colonizers in the mucosa of Crohn's disease patients (Bonnet et al., 2014; Eaves-Pyles et al., 2008). The genotoxic strains of *E. coli* with expression of colibactin demonstrated remarkable promotion of tumorigenesis in a 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 intake of polyphenols and healthy diets and the presence of *A. muciniphila* and associated beneficial metabolic outcomes (Jayachandran, Chung, & Xu, 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, Lin, Vanhoutte, Woo, and Xu (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 disease. 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 discovered in IBD subjects compared with healthy individuals (Zhuang et al., 2018). Overall, the current PCR data are 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 strains highly susceptible to alteration by dietary components such as polyphenols and fiber (Hervert-Hernández, Pintado, Rotger, & Goñi, 2009; Park, Choi, & Lee, 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, Piao, & Song, 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 sulfate sodium-induced colitis in mice (Zhang, Jiao, Wang, Lin, & You, 2019). It can be assumed that these polyphenols may have acted in concert with betalains to impact the microbial profile of subjects in this study. Further studies should consider more defined intervention products to investigate the effect of individual components, as well as their interactions; and to target specifically the proportion of toxigenic subspecies among interested strains.

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 effects 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 the bowel. A recent study conducted in mice reported the lipid-lowering and hormone-regulatory effects with increased SCFA production that alleviated obesity and improved insulin sensitivity (Lin et al., 2012). As a predominant SCFA in feces, acetate is derived from carbohydrate glycolysis and is involved in lipid and cholesterol biosynthesis whereas propionate and butyrate serve roles in signaling 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 stabilization of cell phenotypes (Cui et al., 2019). The consumption of beetroot juice in the current study was leading to an increase in total SCFA content especially (iso)butyric acid which implies a prebiotic character of beetroot components to reinforce epithelial development and intestinal immune function.

The relationship between betacyanin catabolites and microbiome variables was further demonstrated 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 phytochemical biotransformation, mechanisms 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 several genera (e.g., *Bifidobacterium*, *Lactobacillus*, *Bacteroides*) towards anthocyanins and other polyphenolic glucosides resulting from their intrinsic β-glucosidase activity (Ávila et al., 2009; Bokkenheuser, Shackleton, & Winter, 1987). 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 (Schneider, Schwiertz, Collins, & Blaut, 1999; Winter, Popoff, Grimont, & Bokkenheuser, 1991). 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 diarrhea (Picard et al., 2005). In addition, *Bifidobacterium*, *Peptoniphilus* and *Coprococcus* are 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 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 fecal samples will be valuable in future studies. As demonstrated *in vitro*, the anti-inflammatory properties of betacyanins were mainly reflected by immunoregulation, specifically by downregulating pro-inflammatory markers (e.g., cyclooxygenase-2 and inducible NO-synthase) as well as augmenting expression of antioxidant enzymes (e.g., heme oxygenase-1 and NAD(P)H quinone dehydrogenase 1) (Gómez-Maqueo et al., 2019; Krajka-Kuzniak et al., 2013). Results from this study suggest a microbiota-modulatory role of native and catabolized 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 relevance of the fecal compared to the mucosal microbiome since the latter relates more closely to the bowel conditions (Lo Presti et al., 2019). Further studies *in vivo* e.g. involving biopsies from patients could ascertain the value of fecal microbiome data.

5. Conclusion

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. The current study aimed to investigate the relationship between red beetroot juice consumption and human gut microbiota in the context of the microbiota-modulatory capacity of betacyanins in healthy adults. The present study indicated no significant changes in alpha- and beta-diversities between BSL, D3, and D14 samples. However, marked changes in abundance of specific taxa (e.g., *Romboutsia* and *Bacteroidales*) were observed, as well as enrichment of *A. muciniphila* and decrease in *B. fragilis* populations. *A. muciniphila* has been inversely associated with obesity, diabetes, inflammation, and metabolic disorders, whereas *B. fragilis* is potentially enterotoxigenic causing inflammatory diarrhea. Consumption of beetroot juice additionally increased the production of total SCFAs especially (iso)butyric acid in stools. Correlations between betacyanin variables and microbial taxa suggest a role of red beetroot pigments in modulating gut bacteria with putative health implications. 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.

6. Data availability

The 16S rRNA raw sequences in fastq as well as sample metadata of this study have been submitted to NCBI BioSample database (BioProject ID PRJNA866103, TBC).

CRediT authorship contribution statement

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw 16S rRNA sequences (fastq files) generated from the study were deposited to NCBI Sequence Read Archive (with BioProject)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134989.

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