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Unravelling phenolic metabotypes in the frame of the COMBAT study, a randomized, controlled trial with cranberry supplementation

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ABSTRACT

Cranberry (poly)phenols may have potential health benefits. Circulating (poly)phenol metabolites can act as mediators of these effects, but they are subjected to an extensive inter-individual variability. This study aimed to quantify both plasma and urine (poly)phenol metabolites following a 12-week intake of a cranberry powder in healthy older adults, and to investigate inter-individual differences by considering the existence of urinary metabotypes related to dietary (poly)phenols. Up to 13 and 67 metabolites were quantified in plasma and urine respectively. Cranberry consumption led to changes in plasma metabolites, mainly hydroxycinnamates and hippuric acid. Individual variability in urinary metabolites was assessed using different data sets and a combination of statistical models. Three phenolic metabotypes were identified, colonic metabolism being the main driver for subject clustering. Metabotypes were characterized by quali-quantitative differences in the excretion of some metabolites such as phenyl-γ-valerolactones, hydroxycinnamic acids, and phenylpropanoic acids. Metabotypes were further confirmed when applying a model only focused on flavan-3-ol colonic metabolites. 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone derivatives were the most relevant metabolites for metabotyping. Metabotype allocation was well preserved after 12-week intervention. This metabotyping approach for cranberry metabolites represents an innovative step to handle the complexity of (poly)phenol metabolism in free-living conditions, deciphering the existence of metabotypes derived from the simultaneous consumption of different classes of (poly)phenols. These results will help contribute to studying the health effects of cranberries and other (poly)phenol-rich foods, mainly considering gut microbiota-driven individual differences.

1. Introduction

Increasing evidence from cohort studies and intervention trials suggests that modest long-term intake of (poly)phenols can reduce the risk of chronic diseases, including cardiometabolic diseases, cognitive decline, and neurodegenerative diseases (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). In particular, American cranberry (*Vaccinium macrocarpon*) (poly)phenols have shown beneficial effects in the prevention of urinary tract infections, vascular diseases and cognitive disfunctions (Ângelo et al., 2017; Flanagan et al., 2022; Rodriguez-Mateos et al., 2014,2016). Cranberry is a rich source of different classes of (poly)phenols, in particular type-A oligomeric proanthocyanidins and flavan-3-ol monomers, flavonols, anthocyanidins, and phenolic acids. Following ingestion, (poly)phenols are poorly absorbed in the upper intestinal tract and reach the colon, where they undergo modifications by the gut microbiota, being converted to smaller catabolites that are easily absorbed and then conjugated by human phase II enzymes (Del Rio et al., 2013). Indeed, many circulating (poly)phenol metabolites, including benzaldehydes, benzene derivatives (catechols and pyrogallols), and phenolic acids (benzoic, phenylacetic, phenylpropanoic, cinnamic and hippuric acids), derive from the metabolism of these (poly)phenols classes (Bento-Silva et al., 2020; Mena et al., 2018; Rodriguez-

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Mateos et al., 2014). Interestingly, the two main classes of cranberry (poly)phenols (hydroxycinnamic acids and flavan-3-ols) share common phenolic metabolites in their degradation pathways, as well as high inter-individual variability in their production (Del Rio et al., 2010; Stalmach et al., 2014; Tomas-Barberan et al., 2014). Flavan-3-ol catabolism also generates specific metabolites such as phenylvaleric acids, 4-hydroxyphenylvaleric acids, and phenyl-γ-valerolactones (PVLs), which have shown protective activities in *in vitro* and *in vivo* studies (Mena et al., 2019).

To date, few studies have evaluated the metabolism of cranberry (poly)phenols in humans, mainly focusing on the analysis of structurally-related anthocyanin metabolites (Milbury et al., 2010) or PVLs, which are considered biomarkers of flavan-3-ol intake (Favari et al., 2020). Only a few comprehensive investigations have been conducted on several classes of cranberry (poly)phenols (Feliciano et al., 2016, 2017; McKay et al., 2015; Wang et al., 2012) or on the impact of chronic cranberry consumption on the quali-quantitative profile of (poly)phenolic metabolites (Heiss et al., 2022). In addition, the inter-individual variation in the plasma and urinary profile has been scarcely addressed despite its potential contribution to cranberry health effects. In this sense, a recent work has investigated the existence of metabolic phenotypes (aka metabotypes) for cranberry flavan-3-ols, defining three clusters of individuals based on differences in excreted amount and profile of dihydroxyPVLs, monohydroxyPVLs and 3-(hydroxyphenyl)propanoic acids (HPPAs) (Mena et al., 2022). A methodological workflow for the definition of urinary phenolic metabotypes has also been proposed, defining a strategy to specifically search for metabolic phenotypes not characterized by the dichotomic excretion/non-excretion of specific phenolic metabolites (Mena et al., 2022). This metabotyping approach may be a strategy to handle the inter-individual variability in (poly)phenol bioavailability and to investigate its influence on the monitored health effects (Narduzzi et al., 2022). Metabotyping could be, therefore, a key success factor in personalized approaches, supporting tailored dietary advice to decrease disease risks and promote health at a population level (Palmnas et al., 2019). However, rather than focusing on individual classes of (poly)phenols, comprehensive translatable approaches are needed.

The present study aimed to identify and quantify (poly)phenol metabolites in human biological samples (plasma and urine) after chronic intake of cranberry in healthy older adults, investigating the inter-individual differences in the profile of phenolic metabolites in fasted spot urine samples. The pipeline proposed by Mena et al (Mena et al., 2022) for cranberry flavan-3-ols was used here to investigate the existence of (poly)phenol metabotypes for the first time, in the context of a randomized, controlled clinical trial with no major dietary restrictions.

2. Materials and methods

2.1. Materials

All chemicals and solvents used in this study were of analytical grade, and are indicated in Supplementary Information S1.1.

2.2. Intervention study

Plasma and urine samples were obtained from the COMBAT study (The impact of Cranberries On the Microbiome and Brain in healthy Ageing sTudy), which was fully reported in (Flanagan et al., 2022). The study was registered at clinicaltrials.gov as NCT03679533 and at ISRCTN as ISRCTN76069316, and was conducted according to the guidelines laid down in the current revision of the Declaration of Helsinki. Informed consent was obtained for all participants, and all procedures involving human subjects were approved by the University of East Anglia's Faculty of Medicine and Health Sciences Ethical Review Committee (Reference: 201,819 – 039) and the Health Research Authority (IRAS number: 237251; HRA number: 18/HRA/1339). The COMBAT study was a single-centre, 12-week randomized, double-blind, placebo-controlled parallel intervention trial designed to investigate the impact of cranberry consumption on the gut microbiome, cognition, and brain physiology. It involved the recruitment of 60 healthy older adults, both male and female, aged between 50 and 80 years old. 31 subjects were assigned to the control group, and the remaining 29 were assigned to the treatment group. The intervention consisted of the intake of freeze-dried cranberry powder (Cranberry Institute, USA) in a dose providing 375 mg proanthocyanidins, 85 mg flavonols, 59 mg anthocyanins, and 70 mg of phenolic acids per day. Details are reported in Table S1 in Supplementary Information and in the study by Flanagan and colleagues (Flanagan et al., 2022). The intervention was provided in sachets (4.5 g each) of cranberry powder designed to be incorporated into food and beverages. Participants took two sachets daily, one in the morning and one in the evening, to maximize the physiological impact according to the current knowledge of bioavailability (Rodriguez-Mateos et al., 2014). The control group was supplemented with a placebo powder. Apart from adding the powder, participants were asked not to change their dietary or caloric intake. However, they were asked to reduce their consumption of certain vitamin supplements and to avoid the intake of supplements that could significantly influence the outcome measures, or supplements having a high flavonoid intake, defined as more than 15 portions of flavonoid-rich foods per day (Irvine et al., 2018). In particular, participants were screened and were eligible if they consumed < 15 portions of high polyphenol foods per day. After the participants were randomized into the study, they were asked to keep the same diet as usual except for limiting certain high-polyphenol foods. Spot plasma and urine samples were collected before the treatment (baseline visit V1, 0 weeks) and after the intervention (follow-up visit V2, 12 weeks). Plasma samples were not collected from 15 subjects (8 in the cranberry group and 7 in the control group) after the intervention because of the outbreak of the COVID-19 pandemic. For the study purpose, plasma and urine samples were analyzed to obtain data on metabolite concentration for a total of 210 observations (90 for plasma and 120 for urine). These observations were used for statistical analyses.

2.3. Sample analysis

Urinary extraction of phenolic metabolites was performed using microelution solid phase extraction (μSPE) according to validated protocols, with small modifications (Feliciano et al., 2017). Briefly, plasma (350 μL) and urine (250 μL) were diluted (1:1) with phosphoric acid 4% to limit phenolic-protein interactions. Each sample (600 μL for plasma and μL 400 for urine) was loaded on a 96 well μSPE plate, washed with water (200 μL) and 0.2% acetic acid (200 μL), and finally eluted with methanol (60 μL for plasma and 200 μL for urine). Samples were analyzed through high-performance liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS/MS) as described by Castello et al (Castello et al., 2018). More details are indicated in Supplementary Information S1.2. Up to 74 compounds were simultaneously monitored in selective reaction monitoring (SRM) mode, and 67 metabolites were detected and quantified (Table S2 in Supplementary Information). Chromatograms, mass spectral data and data processing were performed using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.). The nomenclature used follows the current recommendations for (poly)phenol catabolites (Kay et al., 2020). Plasma metabolite data were expressed as nmol/L, while urinary concentrations were normalized using creatinine concentration and expressed as μmol/mol creatinine. Urinary creatinine analysis was performed by uHPLC-MS/MS after dilution of samples with water 0.1% formic acid (1:1000) (details are described in Supplementary Information S1.3). Sums of urinary metabolites belonging to the same aglycone moiety were calculated, defining 20 different phenolic classes. Hippuric acid derivatives (namely, hippuric acid and 4-hydroxyhippuric acid) were not taken into consideration because they have a heterogeneous origin (both exogenous and endogenous) and their inclusion did not modify the models

presented herein. This way, two different data sets were considered, one (DS1) consisting of 120 observations (samples) and 65 variables corresponding to individual metabolites, and one (DS2) consisting of 120 observations (samples) and 19 variables corresponding to phenolic classes. Moreover, one additional data set (DS3) considering individual metabolites belonging to PVLs and HPPAs was considered according to previous evidence about flavan-3-ol metabolites (Mena et al., 2018,2022). DS3 consisted of 120 observations (samples) and 17 variables, including metabolites belonging to HPPAs (isomers 3′ and 4′), monohydroxyPVLs (isomers 3′ and 4′), and dihydroxyPVLs (3′,4′).

2.4. Statistical analysis

All data were expressed as mean values ± standard error of the mean (SEM). Tests for assessing data normality and homoscedasticity were performed using the Kolmogorov–Smirnov and Levene's tests, respectively. Comparisons between the control and the treatment groups were made by independent sample Student *t* test for normally distributed variables or Mann-Whitney *U* test for non-normally distributed variables. Comparisons between concentrations at the baseline and follow-up visits were made by dependent sample Student *t* test for normally distributed variables and Wilcoxon test for non-normally distributed variables. Differences were considered significant at p value ≤ 0.05. These statistical analyses were performed using IBM SPSS Statistics version 26 (IBM, Chicago, IL, USA).

Principal component analysis (PCA) was carried out to investigate inter-individual differences in the urinary excretion of (poly)phenol metabolites. PCA was performed using SIMCA 16.0.1 software (Sartorius Stedim Data Analytics, Umea, Sweden). Data sets were not subjected to any data transformation, since Mena et al (Mena et al., 2022) suggested that this strategy results in better models compared to logarithmic and power transformation. Data sets were subjected to three different mean centering plus scaling methods: only mean centering, unit variance (UV) scaling or autoscaling, and Pareto scaling. Centering converted the data to variations around zero instead of around the mean; UV scaling used standard deviation as scaling factor, while Pareto scaling used the square root of standard deviation (van den Berg et al., 2006). All models were set by default with two principal components (PCs). The parameters used to evaluate the quality of each model and data interpretability were R^2X and Q^2, which measure the model fit (or explained variation) and the predictive ability, respectively. The models obtained pre-treating data with mean centering plus UV scaling were selected, since the results were in accordance with those obtained by Mena et al (Mena et al., 2022). Results obtained pre-treating data with the other two methods (mean centering, and mean centering plus Pareto scaling) are not shown.

To investigate the causes behind the observed inter-individual variation in the excretion of (poly)phenol metabolites, partial least squares discriminant analysis (PLS-DA) was applied using SIMCA 16.0.1 software. Different explorative PLS-DA models were carried out to define the optimal number of clusters and the observations in each cluster. The model that maximized the validation parameters was selected; this final model considered 3 clusters. Observations were assigned to clusters based on their PC scores. Variables were autoscaled applying centering plus unit variance scaling. Model validity was assessed by R^2X (variation in the data), R^2Y (variation in the cluster), Q^2 (goodness of fit of the validation), the random permutation test, and CV-ANOVA within the SIMCA package. The most relevant metabolites from the whole set of metabolites (selection of variables) were identified using the Variable Importance in Projection (VIP) scores, which allows to measure the importance of each variable in the projection used in a PLS model (Acharjee et al., 2013). Variables with VIP scores greater than 1 were considered relevant for the model. The ROC curve was built to assess the classification performance of the PLS-DA models.

The normality of phenolic classes distribution within the clusters identified with PCA using DS1 was checked through the Kolmogorov–Smirnov test. For normally distributed variables, one-way repeated measures analysis of variance (ANOVA) was performed to compare the mean concentrations among the clusters. Post-hoc analysis was conducted using Dunnett's T3 test with Bonferroni correction, since variances in clusters were non equal (heteroscedastic variables). For non-normally distributed variables, non-parametric Kruskal–Wallis test with *post hoc* pairwise multiple comparison was used. Differences were considered significant at p value ≤ 0.05. All these univariate statistical analyses were performed using IBM SPSS Statistics version 26.

3. Results

3.1. Plasma phenolic metabolites

A comprehensive panel of (poly)phenol metabolites was targeted in both plasma and urine samples. A total of 13 metabolites were identified and quantified in plasma (Table 1). These metabolites belong to several phenolic classes, including flavonols, PVLs, cinnamic acids, phenylpropanoic acids, phenylacetic acids, benzoic acids, benzaldehydes, hippuric acids, and catechols. Concentrations in fasting conditions ranged from 10 to 90 nmol/L for all the metabolites without hippuric acid.

When comparing the treatment and placebo groups at baseline (Table 1), there were no statistically significant differences for any metabolite in plasma except for kaempferol-3-glucuronide, which was lower in the treatment group ($p = 0.026$). At the follow-up visit, plasma concentrations of 4 metabolites were statistically higher in the treatment group: 3′,4′-dihydroxycinnamic acid (increased around 68%, $p = 0.001$), 3′-methoxycinnamic acid-4′-glucuronide (108%, $p = 0.005$), hippuric

Table 1
Plasma (poly)phenol metabolite concentration at baseline and at follow-up for placebo and cranberry groups. Data are indicated as mean ± SEM and are expressed as nmol/L. Compounds presenting different uppercase letters indicate statistically significant differences between placebo and cranberry groups at baseline or follow-up. Compounds changing from baseline to follow-up are marked with an asterisk (*). Nomenclature of metabolites is reported as proposed by Kay et al (Kay et al., 2020).

Metabolite	Placebo group, baseline (nmol/L)	Cranberry group, baseline (nmol/L)	Placebo group, follow-up (nmol/L)	Cranberry group, follow-up (nmol/L)
Kaempferol-3-glucuronide	33 ± 7 ^{A}	17 ± 4^{B}	28 ± 5	19 ± 4
5-(Phenyl)-γ-valerolactone-methoxy-glucuronide (3′,4′) isomer 1	17 ± 2	18 ± 2	13 ± 2	18 ± 2
3′,4′-Dihydroxycinnamic acid	20 ± 2	21 ± 3	19 ± 3	32 ± 3 *^{A}
3′-Methoxycinnamic acid-4′-glucuronide	16 ± 3	18 ± 4	12 ± 2^{B}	25 ± 3 ^{A}
3-(3′-Hydroxyphenyl) propanoic acid	77 ± 11	51 ± 9	41 ± 10 *	39 ± 8
3′-Hydroxyphenylacetic acid	68 ± 7	78 ± 9	74 ± 6	87 ± 9
4-Hydroxybenzoic acid	43 ± 16	24 ± 5	38 ± 11	27 ± 3 *
Benzoic acid-4-sulfate	15 ± 2	23 ± 5	14 ± 2	28 ± 5
Benzoic acid-3-sulfate	15 ± 3	26 ± 8	12 ± 2	20 ± 5
4-Hydroxyhippuric acid	43 ± 5	39 ± 4	41 ± 5	43 ± 5
Hippuric acid	2756 ± 234	2824 ± 336	2532 ± 371^{B}	4287 ± 413 *^{A}
4-Hydroxybenzaldehyde	13 ± 1	11 ± 1	18 ± 2 *	19 ± 3 *
2-Hydroxy-4/5-methylbenzene-1-sulfate	32 ± 3	33 ± 3	24 ± 2^{B}	42 ± 3 ^{A}
Total metabolites	3150 ± 250	3184 ± 358	2869 ± 384^{B}	4686 ± 430 *^{A}
Total metabolites without hippuric acid derivatives	351 ± 23	321 ± 28	296 ± 22^{B}	356 ± 23 ^{A}

acid (69%, $p = 0.001$), and 2-hydroxy-4/5-methylbenzene-1-sulfate (75%, $p < 0.001$). The plasma concentration for total metabolites was also significantly higher in the treatment group than in the placebo (increased by 63%, $p = 0.001$) and remained higher even when hippuric acid derivatives were not considered (increased by 20%, $p = 0.033$).

When comparing the baseline and follow-up plasma concentrations within the placebo group, the level of 3-(3′-hydroxyphenyl)propanoic acid significantly decreased its level ($p = 0.026$) while that of 4-hydroxybenzaldehyde increased it ($p = 0.021$). Total metabolites without hippuric acid derivatives showed a small (17%), but significant decrease ($p = 0.045$).Comparisons within the cranberry group accounted for significant increases in 3 metabolites: 3′,4′-dihydroxycinnamic acid ($p = 0.002$), 4-hydroxybenzaldehyde ($p = 0.003$), and hippuric acid ($p = 0.002$). Total metabolites also increased by 1502 ± 84 nM (about 47%) for the treatment group in a statistically significant way when considering hippuric acid derivatives ($p = 0.002$).

3.2. Urinary phenolic profile

In spot urine samples, up to 67 metabolites were quantified (Table S3 in Supplementary Information). They belonged to the following classes: flavonols, flavan-3-ols, PVLs, phenylvaleric acids, cinnamic acids, phenylpropanoic acids, phenylacetic acids, benzoic acids, benzaldehydes, hippuric acids, and benzene derivatives (catechols). Without considering hippuric acid derivatives (hippuric acid and 4-hydroxyhippuric acid), which were the most abundant metabolites (74.3% of total metabolites), PVLs and phenylacetic acids were the most represented classes in all samples (on average, 36.4% and 33.6%, respectively), followed by benzoic acids (9.2%), phenylpropanoic acids (8.8%), and cinnamic acids (8.1%). The less represented classes were flavonols (1.2%), flavan-3-ols (0.8%), catechols (0.4%) and benzaldehydes (0.1%) (Fig. 1).

No statistically significant differences in urinary metabolite concentrations were seen between placebo and treatment groups at baseline (Table S3). At follow-up, 5 metabolites were excreted at significantly higher concentrations in the placebo than in the treatment group: 5-phenyl-γ-valerolactone-methoxy-glucuronide isomer 2 ($p = 0.034$), 5-phenyl-γ-valerolactone-sulfate-glucuronide ($p = 0.046$), 4-methoxyphenylacetic acid-3-sulfate ($p = 0.007$), benzoic acid-4-sulfate ($p = 0.036$), and 4-hydroxyhippuric acid ($p = 0.014$). However, no statistically significant differences were seen in total metabolites. Comparing samples at baseline and at follow-up, 28 and 44 metabolites increased significantly in the treatment and control groups, respectively. Also, total metabolites were higher at follow-up in both groups, although no significance was reached due to the high data variance (Table S3).

3.3. Unsupervised multivariate analysis shows inter-individual variability in the urinary excretion of phenolic metabolites

Unsupervised PCA was carried out to explore the inter-individual and inter-group variability in the urinary excretion of (poly)phenol metabolites. First, all metabolites were considered as variables (data set DS1) except for hippuric acid derivatives (hippuric acid and 4-hydroxyhippuric acid), which were excluded because they derive from different dietary sources -not only phenolics- and endogenous metabolites. Two principal components (PCs) explained 43.1% of the total variability. PC1 and PC2 explained 33.4% and 9.7% of the observed variation, respectively. It was possible to identify a pattern of metabolite (variable) distribution in the loading plots (Fig. 2A), reflecting differences in colonic metabolism according to the aglycone moieties: PVLs grouped together and separated from low molecular weight phenolic acids such as cinnamic, phenylpropanoic, phenylacetic, and benzoic acids. This colonic metabolism-based pattern accounted for the potential existence of phenolic metabotypes and was not associated with the treatment type (placebo or cranberry) or time (baseline or follow-up). This likely indicated that inter-individual differences were greater than study-related differences regarding the spot urinary phenolic profile. Clustering was carried out based on the scores of each observation in the PCA model. Three clusters were defined: observations with a positive score for both PC1 and PC2 were assigned to cluster 1, observations with a positive score for PC1 and a negative score for PC2 were assigned to cluster 2, and observations with a negative score for PC1 and a positive or negative score for PC2 were allocated in cluster 3. Samples in the top right quadrant (cluster 1) were characterized by a higher excretion of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, 5-(hydroxyphenyl)-γ-valerolactone-sulfate (3',4' isomers), 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide, 5-(3'-hydroxyphenyl)-γ-valerolactone-4'-glucuronide, 5-phenyl-γ-valerolactone-sulfate-glucuronide, 5-phenyl-γ-valerolactone-methoxy-sulfate (isomer 1), 5-phenyl-γ-valerolactone-methoxy-glucuronide (isomer 1), 5-phenyl-γ-valerolactone-3'-sulfate, 5-phenyl-γ-valerolactone-3'-glucuronide, 4-hydroxy-5-(hydroxyphenyl)valeric acid-sulfate, and epicatechin-glucuronide (isomer 1). Samples in the

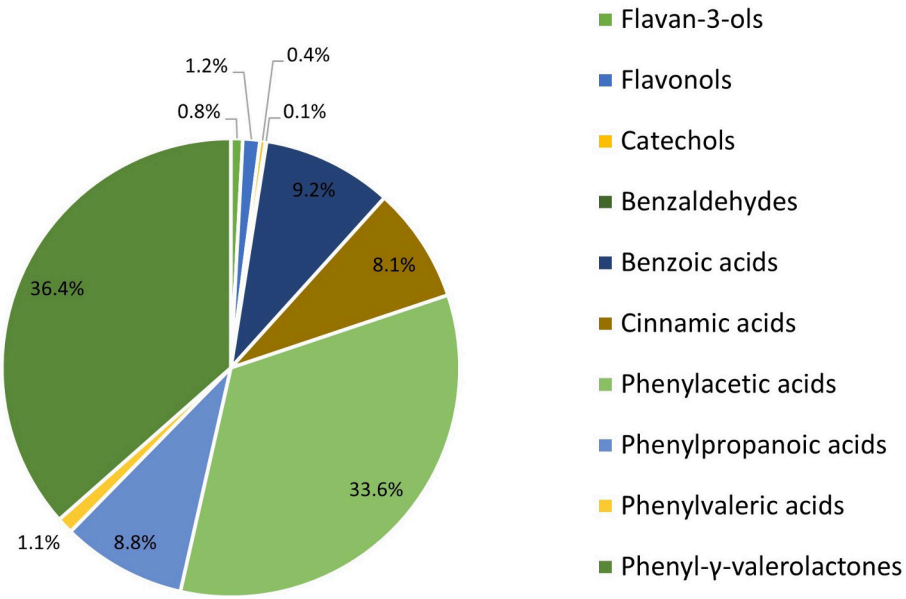

Fig. 1. Phenolic metabolite profile of urine spot samples. The distribution of (poly)phenol metabolite classes does not consider hippuric acid derivatives.

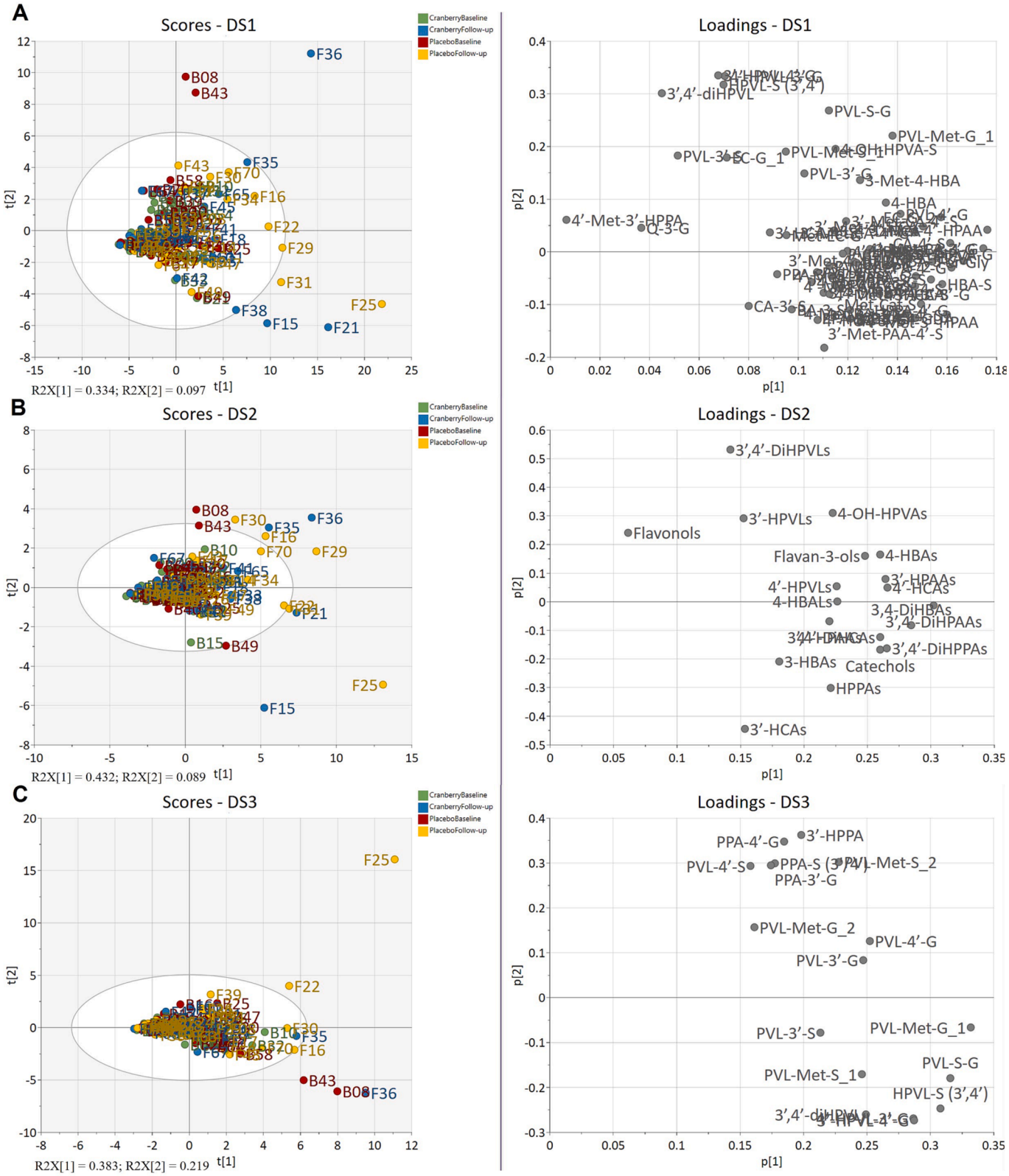

Fig. 2. Score and loading plots resulting after PCA on autoscaled data for individual metabolites (DS1, **A**), phenolic classes (DS2, **B**), and metabolites belonging to HPPAs (isomers 3′ and 4′), monohydroxyPVLs (isomers 3′ and 4′), and dihydroxyPVLs (3′,4′) (DS3, **C**). Metabolite abbreviations in the loading plot are indicated in Table S2 in Supplementary Information. Letters B and F for the observations in the score plot indicate subjects at baseline and follow-up visits, respectively.

bottom right quadrant (cluster 2) were characterized by a more abundant excretion of several low molecular weight phenolic metabolites belonging to cinnamic, phenylpropanoic, phenylacetic, and benzoic acids, benzaldehydes and catechols. Samples in the left quadrants (cluster 3) were characterized by a low excretion of all these metabolites.

Then, phenolic classes instead of individual phenolics were considered variables (data set DS2). Classes were defined grouping metabolites according to their aglycon moiety, obtaining 19 groups: flavonols, flavan-3-ols, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactones (3′,4′-diHPVLs), 5-(3′-hydroxyphenyl)-γ-valerolactones (3′-HPVLs), 5-(4′-hydroxyphenyl)-γ-valerolactones (4′-HPVLs), 4-hydroxy-5-(hydroxyphenyl) valeric acids (4-OH-HPVAs), 3′,4′-dihydroxycinnamic acids (3′,4′-diHCAs), 3′-hydroxycinnamic acids (3′-HCAs), 4′-hydroxycinnamic acids (4′-HCAs), (3′,4′-dihydroxyphenyl)propanoic acids (3′,4′-diHPPAs), 3-(hydroxyphenyl)propanoic acids (HPPAs), 3′,4′-dihydroxyphenylacetic acids (3′,4′-diHPAAs), 3′-hydroxyphenylacetic acids (3′-HPAAs), 4′-hydroxyphenylacetic acids (4′-HPAAs), 3,4-dihydroxybenzoic acids (3,4-diHBAs), 3-hydroxybenzoic acids (3-HBAs), 4-hydroxybenzoic acids (4-HBAs), 4-hydroxybenzaldehydes (4-HBALs), and catechols. This strategy allowed considering only differences arising from the colonic microbial catabolism of (poly)phenols, excluding the inter-individual variation in phase II metabolism. Two PCs accounted for 52.1% of the total variability, PC1 and PC2 explaining 43.2% and 8.9% of the observed variation, respectively. The distribution observed was similar to that already described when considering all the metabolites (DS1), confirming the inter-individual differences due to colonic metabolism (Fig. 2B). As explained above, three clusters were identified, and observations were assigned to each class according to their PC scores. Samples in the top right quadrant (cluster 1) were characterized by a higher excretion of 3′,4′-diHPVLs, 3′-HPVLs, and 4-OH-HPVAs, while samples in the bottom right quadrant (cluster 2) were characterized by a more abundant excretion of 3′-HCAs, HPPAs, and 3-HBAs. Samples in the left quadrants (cluster 3) were characterized by a low excretion of all metabolite classes.

The third data set (DS3) considered was composed only of flavan-3-ol metabolites belonging to diHPVLs, HPVLs, and HPPAs. Two PCs explained 62.5% of the total variability (38.3% and 21.9% for PC1 and PC2, respectively). As observed for all metabolites (Fig. 2C), the treatment type (placebo or cranberry) or time (baseline or follow-up) did not influence the distribution of the observations. Three clusters were set by allocating the observations as previously indicated for DS1: samples in the bottom right quadrant (cluster 2) were mainly characterized by a higher excretion of diHPVLs like 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, 5-(hydroxyphenyl)-γ-valerolactone-sulfate (3',4' isomers), 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide, its isomer 5-(3'-hydroxyphenyl)-γ-valerolactone-4'-glucuronide, 5-phenyl-γ-valerolactone-sulfate-glucuronide, 5-phenyl-γ-valerolactone-methoxy-sulfate (isomer 1), and 5-phenyl-γ-valerolactone-methoxy-glucuronide (isomer 1), as well as by 5-phenyl-γ-valerolactone-3'-sulfate; samples in the top right quadrant (cluster 1) were characterized by a more abundant excretion of 5-phenyl-γ-valerolactone-methoxy-sulfate (isomer 2), 5-phenyl-γ-valerolactone-methoxy-glucuronide (isomer 2), HPVLs like 5-phenyl-γ-valerolactone-3'-glucuronide, 5-phenyl-γ-valerolactone-4'-sulfate, and 5-phenyl-γ-valerolactone-4'-glucuronide, and HPPAs like 3-(hydroxyphenyl)propanoic acid, 3-(phenyl)propanoic acid-sulfate (3'/4' isomers), 3-(phenyl)propanoic acid-3'-glucuronide and 3-(phenyl) propanoic acid-4'-glucuronide. Samples in the left quadrants (cluster 3) were characterized by a low excretion of all the metabolites considered.

For all data sets, PC1 and PC2 resulted in discriminating samples on a quantitative and qualitative basis, respectively. Although PC1 discriminated more than PC2, it was less useful in characterizing specific metabolite patterns. On the contrary, PC2 was very helpful in characterizing metabolic profiles.

3.4. Supervised multivariate analysis: metabotypes in the excretion of phenolic metabolites

Supervised PLS-DA was carried out to predict which metabolite(s) could explain the inter-individual variability obtained in the PCA outcomes, and to stratify subjects into groups sharing a similar excretion profile (Fig. 3 A, B, C). Clustering according to PC scores was used to classify individuals into 3 groups characterized by different metabolite profiles or a low excretion of all metabolites. All the PLS-DA models passed cross-validation by CV-ANOVA ($p = 1.1e^{-31}$ for DS1, $p = 4.3e^{-32}$ for DS2, $p = 1.3e^{-33}$ for DS3) and by random permutation. The model with the highest explained variance was obtained considering DS3 ($R^2X = 0.62$), followed by DS2 ($R^2X = 0.52$). The DS1 model had the lowest explained variance ($R^2X = 0.43$), but it was acceptable and presented the highest predictive ability ($Q^2 = 0.50$). A similar predictive ability was obtained using DS2 ($Q^2 = 0.49$), while this value is lower for DS3 ($Q^2 = 0.43$). R^2Y values were greater than 0.50 for all models (0.57, 0.55 and 0.52 for DS1, DS2, DS3, respectively). These results confirmed model validation and excluded data overfitting. In addition, the ROC curves confirmed the good classification performance of each model (Fig. S1 in Supplementary Information).

For DS1, the most relevant metabolites (VIP score > 1.25) were mainly diHPVL derivatives, epicatechin-glucuronide (isomer 1), 3′-methoxyphenylacetic acid-4′-sulfate, and 4-hydroxy-5-(hydroxyphenyl) valeric acid-sulfate (Fig. S2A in Supplementary Information). This was also observed in the DS2 model, where the highest VIP was recorded for diHPVLs. Other relevant classes were 4-OH-HPVAs, catechols, HPPAs, 3′-HCAs, 3′,4′-diHPAAs, 3-HBAs and 3,4-diHBAs (Fig. S2B in Supplementary Information). For DS3, the highest VIP was for 5-(hydroxyphenyl)-γ-valerolactone-sulfate (3′,4′), followed by other diHPVLs, phenylpropanoic acid-3′-glucuronide, phenylpropanoic acid-4′-glucuronide, and 5-(phenyl)-γ-valerolactone-4′-glucuronide (Fig. S2C in Supplementary Information).

In general, the PLS-DA models were representative of metabotypes reflecting a different microbial metabolism of (poly)phenols. Observations belonging to cluster 1 for DS1/2 and cluster 2 for DS3, which were characterized by a higher excretion of 3′,4′-diHPVLs and 3′-HPVLs, were assigned to metabotype 1. On the contrary, observations belonging to cluster 2 for DS1/2 and cluster 1 for DS3, characterized by a more abundant excretion of 3′-HCAs, HPPAs, and 3-HBAs, were assigned to metabotype 2. The remaining observations belonging to cluster 3 for all models were assigned to metabotype 3, characterized by a low metabolite excretion. Agreement among models, calculated as a percentage of observations with the same metabotype for the models considered, accounted for well-preserved metabotypes regardless of the data set used. A high accordance between the models was found: 87% between models using DS1 and DS2, 73% between models using DS3 and DS1/DS2, and 68% using all models.

Considering the individual metabotype shifts from baseline to follow-up for models DS1 and DS3 (Table 2), half of the subjects (55%) maintained the same metabotype. Most of the metabotype shifts involved metabotype 3 (35% for DS1 and 22% for DS3), which changed into metabotype 2 (18% for DS1 and 8% for DS3) or into metabotype 1 (17% for DS1 and 13% for DS2). The other metabotype shifts involved metabotype 1 (8% for DS1 and 17% for DS3), which changed mainly into metabotype 3 (7% for DS1 and 12% for DS3), and metabotype 2, which was the most stable one, with only 1 and 4 subjects undergoing a change for DS1 and DS3, respectively. Qualitative changes of metabotype, namely shifts from metabotype 2 to 1 or vice versa, were very limited (2% for DS1 and 8% for DS3) and, for DS1, involved only metabotype 1, confirming the stability of metabotype 2. As expected, metabotype shifts obtained with DS2 were similar to the ones obtained with DS1, with the exception of the shift from metabotype 3 to metabotype 2, which was less frequent for DS2. No differences in metabotype shifts were observed between control and cranberry groups.

Fig. 3. Score and loading plots resulting after PLS-DA with clusters obtained using PC score-based models for individual metabolites (DS1, **A**), phenolic classes (DS2, **B**), and metabolites belonging to HPPAs (isomers 3′ and 4′), monohydroxyPVLs (isomers 3′ and 4′), and dihydroxyPVLs (3′,4′) (DS3, **C**). Metabolite abbreviations in the loading plot are indicated in Table S2 in Supplementary Information. Letters B and F for the observations in the score plot indicate subjects at baseline and follow-up visits, respectively.

Table 2
Number and frequency of observations involved in the metabotype shifts observed using models built with data set 1 (DS1), data set 2 (DS2) and data set 3 (DS3).

Shifts	Number of observations			Percentage of observations		
	DS1	DS2	DS3	DS1	DS2	DS3
1–2	1	1	3	2%	2%	5%
1–3	4	4	7	7%	7%	12%
2–1	0	0	2	0%	0%	3%
2–3	1	0	2	2%	0%	3%
3–1	10	11	8	17%	18%	13%
3–2	11	6	5	18%	10%	8%
No shifts	33	38	33	55%	63%	55%

3.5. Univariate statistics account for differences in metabolite excretion among metabotypes

Results from PLS-DA outcomes and discriminating classes were confirmed by univariate statistics. Considering phenolic classes and PC score-based clustering using DS1 (Table 3), the small groups of high excretors (metabotypes 1 and 2) showed statistically significant differences in the urinary excretion of most metabolite classes with respect to metabotype 3. Metabotypes 1 and 2 differed statistically only in the excretion of 3′-HPVLs ($p = 0.001$) and 3′,4′-DiHPVLs ($p = 0.0005$), which were higher for metabotype 1. Metabotype 2 presented higher levels of many phenolic acids like 3′-HCAs, HPPAs, and 3-HBAs, although these differences did not reach statistical significance. Metabotype 1 was also characterized by the highest excretion of total metabolites (without considering hippuric acid derivatives) ($p = 0.010$), while metabotype 3 presented the lowest (Table 3).

Table 3
Urinary excretion of phenolic classes, total metabolites (without considering hippuric acid derivatives) and metabolites belonging to 3′-(hydroxyphenyl)-γ-valerolactones and 3′,4′-(dihydroxyphenyl)-γ-valerolactones per each metabotype defined after applying PCA-based clustering to DS1. Data are indicated as mean ± SEM and are expressed as μmol/mol creatinine. Different lowercase letters indicate significant mean differences between clusters ($p < 0.05$).

Phenolic metabolite class	Metabotype 1	Metabotype 2	Metabotype 3
Flavonols	96.5 ± 41.2 [a]	77.3 ± 33.4 [a]	29.8 ± 7.2[b]
Flavan-3-ols	62.0 ± 6.7 [a]	43.7 ± 4.8 [a]	25.5 ± 1.4[b]
3′,4′-DiHPVLs	3476.6 ± 417.1 [a]	1009.7 ± 133.6[b]	871.8 ± 110.5[b]
3′-HPVLs	159.0 ± 26.2 [a]	61.4 ± 12.1[b]	48.3 ± 5.2[b]
4′-HPVLs	78.3 ± 10.4 [a]	73.0 ± 16.0 [a]	32.6 ± 3.4[b]
4-OH-HPVAs	114.2 ± 17.3 [a]	72.3 ± 14.8 [a]	23.0 ± 2.9[b]
3′,4′-DiHCAs	452.3 ± 57.0 [a]	527.6 ± 69.7 [a]	179.2 ± 15.6[b]
3′-HCAs	31.6 ± 3.4 [a]	70.5 ± 18.4 [a]	20.1 ± 2.0[b]
4′-HCAs	13.2 ± 2.1 [a]	15.3 ± 3.2 [a]	6.0 ± 0.5[b]
3′,4′-DiHPPAs	347.2 ± 36.2 [a]	474.7 ± 78.5 [a]	130.4 ± 11.0[b]
HPPAs	221.5 ± 57.2 [a]	317.6 ± 105.6 [a]	61.9 ± 6.7[b]
3′,4′-DiHPAAs	287.5 ± 29.8 [a]	344.9 ± 37.1 [a]	120.8 ± 7.5[b]
3′-HPAAs	784.3 ± 97.9 [a]	807.7 ± 115.3 [a]	355.1 ± 25.3[b]
4′-HPAAs	1140.3 ± 133.0 [a]	1095.3 ± 115.1 [a]	479.7 ± 39.7[b]
3,4-DiHBAs	77.2 ± 8.0 [a]	100.7 ± 13.0 [a]	28.3 ± 1.9[b]
3-HBAs	91.1 ± 24.5 [a]	152.2 ± 35.1 [a]	40.8 ± 5.3[b]
4-HBAs	432.0 ± 43.4 [a]	426.8 ± 65.7 [a]	176.6 ± 12.0[b]
4-HBALs	6.0 ± 1.0 [a]	7.1 ± 1.1 [a]	2.7 ± 0.2[b]
Catechols	21.1 ± 3.9 [a]	36.2 ± 5.1 [a]	8.3 ± 0.9[b]
Total	**7892.0 ± 523.1 [a]**	**5713.9 ± 467.7[b]**	**2640.9 ± 164.0[c]**
3′,4′-DiHPVL	55.0 ± 9.4 [a]	8.9 ± 1.9[b]	14.0 ± 2.4[b]
HPVL-S (3′,4′)	1944.8 ± 197.2 [a]	583.5 ± 105.9[b]	531.5 ± 72.6[b]
3′-HPVL-4′-G	206.6 ± 40.3 [a]	33.8 ± 4.6[b]	47.2 ± 8.3[b]
4′-HPVL-3′-G	740.2 ± 149.1 [a]	98.7 ± 13.7[b]	136.2 ± 22.5[b]
PVL-Met-S 1	8.1 ± 0.9 [a]	4.4 ± 0.6[b]	3.1 ± 0.3[b]
PVL-Met-S 2	18.8 ± 2.8 [a]	25.5 ± 14.7 [a]	4.6 ± 0.6[b]
PVL-Met-G 1	167.6 ± 15.0 [a]	98.2 ± 10.3[b]	53.9 ± 4.0[b]
PVL-Met-G 2	39.0 ± 7.8 [a]	50.3 ± 6.4 [a]	18.4 ± 2.7[b]
PVL-S-G	296.6 ± 37.1 [a]	106.4 ± 13.0	62.9 ± 8.1[c]
PVL-3′-S	59.5 ± 14.3 [a]	14.6 ± 2.8[b]	15.6 ± 2.6[b]
PVL-3′-G	99.6 ± 13.6 [a]	46.8 ± 10.6[b]	32.7 ± 3.8[b]

Exploring the differences in the excretion of single metabolites belonging to 3′-HPVL and 3′,4′-DiHPVL classes among metabotypes revealed that all the individual metabolites were significantly higher in metabotype 1, except for the isomers 2 of 5-phenyl-γ-valerolactone-methoxy-sulfate (3′,4′) and 5-phenyl-γ-valerolactone-methoxy-glucuronide (3′,4′) (Table 3). These two metabolites were lower in metabotype 3 and did not contribute to differentiating metabotype 1 and 2. So, most 3′-HPVLs and 3′,4′-DiHPVLs may help discriminate cranberry phenolic metabotypes 1, 2, and 3 on the basis of quali-quantitative differences.

4. Discussion

Cranberry supplementation in older adults for 12 weeks has been related to improved episodic memory, through improved regional brain perfusion (Flanagan et al., 2022). A recent study also accounted for the role of cranberry consumption in the vascular system via increased flow-mediated dilation, this positive effect on the endothelial function being associated with (poly)phenol metabolites (Heiss et al., 2022). Our work addressed the (poly)phenolic profile of two biofluids, plasma and urine, after chronic intake of cranberry in healthy older adults, also considering the differential individual excretion of (poly)phenolic metabolites. A total of 13 metabolites were quantified in plasma samples after overnight fasting, in line with other studies reporting acute or chronic consumption of cranberry (Feliciano et al., 2017; Heiss et al., 2022; Rodriguez-Mateos et al., 2016). Some (poly)phenolic metabolites increased upon chronic intake of cranberry, which was used as a measure of compliance with the cranberry treatment (Flanagan et al., 2022). Main increases in plasma phenolic metabolites derived from hydroxycinnamic acid derivatives, such as 3′,4′-dihydroxycinnamic acid and 3′-methoxycinnamic acid-4′-glucuronide, in good agreement with previous studies (Feliciano et al., 2017; Heiss et al., 2022).

A total of 67 metabolites were quantified in spot urine samples, PVLs and phenylacetic acids being the most represented classes. PVLs are specific flavan-3-ol metabolites, while phenylacetic acids can derive from hydroxycinnamic acids and other flavonoids, including flavan-3-ols (Del Rio et al., 2013; Mena et al., 2019; Rodriguez-Mateos et al., 2014). Interestingly, the cranberry treatment did not influence the urinary phenolic profile. This could be due to the sample type and collection time since spot samples were collected, and there was no control on the time of consumption of the 2 cranberry doses during the day. Moreover, the participants' diet was not controlled, and their (poly) phenol intake was not estimated during the intervention, adding a possible confounding factor due to the realistic nature of the intervention. On the other hand, the increase in many phenolic metabolites after the placebo treatment may indicate a spontaneous adjustment of the participants' diet toward healthier dietary patterns during the study, or at least a change in their diet a few days before the follow-up sampling. Indeed, these results were in line with the intervention carried out by Heiss and colleagues (Heiss et al., 2022), where shifts for only some metabolites were recorded in 24-h urines upon 1 month of cranberry powder consumption, and with the conclusions of Feliciano and colleagues (Feliciano et al., 2016), who suggested that only 6.2% of the total ingested (poly)phenols were recovered in urine after acute consumption of cranberry juice.

Applying PCA to urinary data reflected considerable differences in the metabolism of (poly)phenols, mainly attributed to microbial catabolism, and was not associated with the treatment type or time. This indicated that inter-individual differences were somehow greater than study-related differences according to the samples analyzed. Similar results were obtained by Mena et al (Mena et al., 2022) for flavan-3-ol catabolites upon cranberry consumption, where no pattern of metabolite distribution was associated with the intervention study or visit type. Indeed, despite the significant results found in intervention trials with cranberries (Flanagan et al., 2022; Heiss et al., 2022), the beneficial role

of (poly)phenols has not been demonstrated consistently yet because of the heterogeneity in the individual response to their consumption (Manach et al., 2017; Milenkovic et al., 2017). Subjects' heterogeneity is likely related to differences in the microbial metabolism of (poly)phenols, but it could also be influenced by the variability in the consumption of flavan-3-ols and other (poly)phenol classes during the experiment. This second source of variability could not be excluded due to the lack of control of the (poly)phenol intake along the study.

The inter-individual variability in the metabolism and physiological response to (poly)phenols is influenced by several factors, including gut microbiota, genetic background, sex, ethnicity, age, lifestyle (smoking, diet, physical activity), (patho)physiological status and medication (Cassidy & Minihane, 2017; Gibney et al., 2019; Landberg et al., 2019). Inter-individual differences in the colonic metabolism of (poly)phenols have been reported for different classes of these compounds and result in an extensive quali-quantitative variation in the circulating phenolic metabolites (Narduzzi et al., 2022). Handling these individual differences through metabotyping approaches may contribute to understanding the drivers of the existing variability and investigating its impact on the health effects of (poly)phenol-containing foods. A quali-quantitative approach for metabotyping phenolic metabolites has been proposed by our research group, using cranberry flavan-3-ols as an example (Mena et al., 2022). This strategy is based on clustering subgroups of individuals sharing similar metabolic profiles when all subjects produce most of the (poly)phenol metabolites of a specific catabolic pathway but in different proportions, as occurring for flavan-3-ols and other main dietary (poly)phenols. This approach has never been applied before to complex food matrices presenting a wide set of (poly)phenols and for metabolites belonging to different metabolic pathways, as is the case of cranberry. Here, the lack of major differences between control and cranberry treatments may lead to hypothesize that consumption of (poly)phenols from the daily diet mask the cranberry treatment effect, at least regarding the urinary phenolic profile. Therefore, using this metabotyping approach for (poly)phenol metabolites, as done here, represents a new step in handling the complexity of (poly)phenol metabolism in free-living conditions, deciphering the existence of metabotypes derived from the simultaneous consumption of different classes of (poly)phenols. To analyze the inter-individual variability registered in the data and provide methodological insights for future research, PCA was applied considering three different data sets: all individual metabolites (DS1), which may account for differences in phase II conjugation and colonic metabolism; phenolic classes obtained grouping metabolites according to their aglycone moiety (DS2), accounting only for differences in the colonic metabolism; and metabolites belonging to HPPAs, HPVLs and diHPVLs (DS3), related to the metabolism of flavan-3-ols, in line with previous works (Mena et al., 2018,2022). Since no major increases in urinary metabolites were found following treatment with cranberry compared to placebo, all subjects were included as observations, both at baseline and at follow-up. This approach was also used because flavan-3-ols, along with other cranberry (poly)phenols such as hydroxycinnamic acids, are the most consumed (poly)phenols in Western diets (Zamora-Ros et al., 2013; Ziauddeen et al., 2018). All models highlighted a colonic metabolism-based pattern in the metabolite excretion, likely indicating that gut microbiota was the main determinant of the inter-individual variability observed and the main driver of subject clustering into putative metabotypes. However, it is necessary to highlight that clustering could also be influenced by quali-quantitative differences in the consumption of (poly)phenols during the experiment. Subjects clustering in DS1 and DS2 was driven by the excretion of 5-carbon side chain ring fission metabolites, in particular 3′,4′-diHPVLs, 3′-HPVLs and 4-OH-HPVAs, and 3-carbon side chain ring fission metabolites, namely 3′-HPPAs and 3′-HCAs. The former metabolites characterized metabotype 1 and the latter metabotype 2. Metabotype 3 was the most prevalent one and was defined by a low excretion of all metabolites, differing from the other two on a quantitative basis. Similar results were obtained by Mena et al (Mena et al., 2022) investigating the existence of colonic metabotypes in the excretion of cranberry flavan-3-ol metabolites: one group was characterized by a high excretion of 5-(4'-hydroxyphenyl)-γ-valerolactone and 3-(hydroxyphenyl)propanoic acid derivatives, a second group was associated to a relevant excretion of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactones and 5-(3'-hydroxyphenyl)-γ-valerolactones, and a third larger group showed a low metabolite excretion profile.

The DS3 model focused on flavan-3-ols fitted well DS1 insights and previous data (Cortés-Martín et al., 2019; Mena et al., 2018,2022). First, this outcome further confirms the existence of urinary metabolites in the excretion of flavan-3-ol catabolites proposed for tea and in another cranberry study (Mena et al., 2018,2022). Second, flavan-3-ol metabolites may be key in determining (poly)phenol metabotypes, and flavan-3-ol metabotypes may be highly predictive of (poly)phenol metabotypes. This was confirmed by the relevance of 3′,4′-diHPVLs (or its sulfate derivative) as a discriminating class (or metabolite) and the good classification performance of all the models. PVLs, in particular the sulfate and glucuronide derivatives of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, are the main circulating metabolites of flavan-3-ols (Di Pede et al., 2023; Mena et al., 2019), which are in turn the most important dietary class of (poly)phenols together with hydroxycinnamic acids (Zamora-Ros et al., 2016). The role of some PVLs as key phenolic metabolites and their intrinsic microbiota-driven individual variability might be of help to classify individuals and conduct further metabotyping or personalized nutrition studies. Nevertheless, more efforts are needed to understand better the relationships between PVLs, the gut microbiota consortium in charge of their metabolism, and the rest of main dietary classes of (poly)phenols. In addition, results suggested that cranberry (poly)phenols, in particular the A-type oligomeric proanthocyanidins characterizing this fruit, are likely stable in the gut and poorly catabolized, not contributing significantly to the pool of circulating flavan-3-ols or their downstream gut microbiota metabolites. These considerations are in line with the existing evidence from in vitro fermentation studies and human trials, showing that proanthocyanidins are less converted to microbial catabolites than monomers, with type-A oligomers being more resistant than B-type (Di Pede et al., 2022; Hollands et al., 2020; Ou et al., 2014; Wiese et al., 2015).

Last, metabotypes 1 and 2, but not metabotype 3, remained stable over time. This is interesting as it may confirm the stability in the production profile of phenolic metabolites for 12 weeks. The fact that metabotype 3 changed after the intervention period towards metabotypes characterized by higher production of phenolic catabolites should be a positive result if these metabolites are bioactive. This change in the metabotype may account for a shift of the microbiota profile due to increases in specific genera, as already seen for urolithins with non-producers (González-Sarrías et al., 2017) or in the alpha-diversity, since the microbial species behind the catabolic pathways of many (poly)phenols classes are poorly understood. Shifts involving metabotype 3 could also be influenced by changes in the amount of flavan-3-ols ingested with the daily diet, which could be a confounding factor. However, these hypotheses require further research, something warranted.

5. Conclusions

Chronic treatment with cranberry powder for 12 weeks led to changes in the plasma concentrations of circulating phenolic metabolites, in particular hydroxycinnamic acids and hippuric acid. Inter-individual differences in the excretion of phenolic metabolites were assessed using a comprehensive pipeline to unravel phenolic metabotypes, and three metabotypes were identified. They were characterized mainly by quali-quantitative differences in the production of phenolic metabolites of colonic origin, belonging to the catabolic pathway of different classes of dietary (poly)phenols. The most relevant class of metabolites for subjects' metabotyping was 3′,4′-diHPVLs. The identification of metabotypes in the excretion of (poly)phenol metabolites

could be helpful in unveiling the health-promoting activity of phenolic metabolites associated with cranberry consumption. Investigating these inter-individual differences considering gut microbiota profiles and functional measures deserves further efforts. Future intervention trials investigating the beneficial effects of (poly)phenol intake while considering inter-individual differences should (1) control volunteers' diet using dietary records/recalls during the sampling period and the day(s) before, and (2) optimize the time and type of sampling, for example collecting 24 h urine or providing harmonized recommendations on food consumption timing when spot urine samples are collected.

Authors' Contributions

AN, MH, and DV contributed to the conception and design of the study; EF contributed to the day-to-day management of the clinical study and sample acquisition; NT, CF, DDR, and PM contributed to the methodology; AN, MH, DV, DDR, and PM acquired funding; NT performed sample and data analysis and visualized the data; CF, LB, and PM supervised analysis; NT and PM wrote the manuscript. All the authors performed reviewing and editing of the article and consented to the final article.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: DV, MH, and AN received funding from the Cranberry Institute. DDR and PM received a research grant from the Cranberry Institute in the past. The rest of the authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

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