Supporting information

Materials and methods

Metabolomic analysis

Sample preparation

On the day of analysis, plasma samples were placed in the refrigerator for gradual thawing. A pool of all study plasmas was prepared. Samples were vortexed for 30 s and a 50 μL aliquot was subjected to an in-plate hybrid extraction method consisting of deproteinization by acidic solvent precipitation (acetonitrile, 1% formic acid) followed by phospholipid solid phase extraction-mediated removal (OstroTM, Waters). Internal (IS) and external (ES) standards were added to the samples before and after the extraction procedure, respectively, to check for the extraction reproducibility and analytical stability during LC-MS data acquisition, and to prove the successful removal of phospholipids from all the samples (1-O-stearoyl-sn-glycero-3-phosphocholine was used as negative control). The resulting solution was vortexed and placed into a 96-well plate for HPLC-ESI-QToF-MS analysis. No preservatives or stabilizers were used at any stage of the collection and manipulation of the samples. Samples were kept at low temperature using a CoolRack (Biocision, USA).

HPLC-ESI-QToF-MS analysis and data acquisition

The HPLC-ESI-QToF-MS analysis was performed using an Agilent 1200 Series Rapid Resolution HPLC system coupled to a hybrid quadrupole ToF QSTAR Elite (AB Sciex). Prepared samples (5 μL) were injected using a thermostatic auto-injector at 4 °C into a reversed phase Luna®C18 column (5 μm, 50 × 2.0 mm; Phenomenex, USA). The MS acquisition was performed in positive and negative modes using a TurboIonSpray source in full scan mode, within the m/z range 70–850. The parameters of chromatography and mass spectrometer used in the present study were developed previously by our group and have

been reported elsewhere [1,2]. To avoid possible bias, all extracts were analyzed in a unique batch-designed and randomized run sequence order.

Throughout the whole data analysis process, five types of quality control (QC) were analyzed in order to monitor the system stability and functionality, besides the evaluation of the quality and the reproducibility of the acquired data [3–5]. The quality control types analyzed were: QC1: LC-MS water samples; QC2: standard mixture solution; QC3: reinjections of plasma samples in opposite position; QC4: pooled plasma samples prepared by mixing equal volumes from each of the samples; and QC5: reference human plasma.

Data conversion and processing

Data processing parameters are detailed in **Supporting Table S2**. These parameters were defined after taking the maximum deviation in retention time (RT) and mass accuracy in all types of QC samples into consideration [4].

In order to focus on features with the lowest proportion of analytical variation, those features that presented a higher variation (according to the coefficient of variation) within QC4 (pooled samples) than within-study samples ($CV_{QC} > CV_S$) were excluded since it was considered that they contained more analytical variation than biological variation. Moreover, the data filtering strategy "50% rule" was used to reduce the effect of zero values. Therefore, a variable was kept if it was present (i.e, intensity values >0) in at least 50% of all samples for at least one group. After this pretreatment step, a total of 3000 and 894 features were kept in the ESI(+) and ESI(–) data sets, respectively.

The analytical variability across the runs was firstly evaluated by monitoring the standard compounds of QC2 samples; the compounds of IS and ES in study samples; as well as IS, ES and a subset of endogenous metabolites present in QC3 and QC5 samples. This enabled us to determine whether the RT, mass and peak intensities changed over time [4,5].

Then, using an unsupervised multivariate analysis such as principal component analysis (PCA), possible alterations that occurred during data acquisition were evaluated. This method was used for the evaluation of the analytical variability of QCs across the data acquisition [6].

Identification of metabolites

First, clustering analysis with Pearson distance and Ward's method to aggregate the observations (PermutMatrix 1.9.3.0 software) was applied in order to identify the mass features corresponding to the same metabolite: (de)protonated molecules, ^{13}C isotopes, adducts, and in-source fragments mainly derived from the loss of the corresponding glucuronide moiety (−176 Da) or sulphate moiety (−80 Da). Then, metabolites were tentatively identified on the basis of their exact mass using an in-house database mainly focused on the metabolites expected from the intake of dietary phytochemicals using an automated identification algorithm implemented in an R package [7]. Additionally, the databases HMDB, METLIN and MassBank were also queried. Each metabolite was associated with a specific level of identification according to the information used for its annotation. Level I was assigned to those metabolites identified by matching its accurate mass, fragmentation and retention time with authentic standards. Level II corresponded to those annotations based on a comparison of accurate mass and mass fragmentation between the discriminant metabolite and the information provided in the mentioned databases or in scientific bibliography. Level III was reserved for those annotations for which the accurate mass was the only information available [8].

Statistical analysis

Untargeted metabolomics data were analyzed by multivariate statistical methods using SIMCA-P+ 13.0 software (Umetrics, Umea, Sweden). To achieve better results, prior to

multivariate analyses, data was log-transformed and Pareto-scaled to make features more comparable. Initially, principal component analyses (PCA) were used for exploratory purposes: (i) to evaluate possible alterations that occurred throughout the data acquisition phase (if samples are placed in the "score plot" taking into account the order in which they were injected); (ii) to get an overview of the samples; and (iii) to detect any potential outlier sample. Successively, partial least squares discriminant analysis with orthogonal signal correction (OSC-PLS-DA) was used to examine between-groups differences using a multistep approach: firstly, all samples were included in one analysis to examine the overall pattern of the datasets; and then, further models were performed to analyze the separation of each group in pairwise analyses. For each model, the number of selected components was set K – 1, K being the number of included classes. The value of p(corr) was used to select the discriminant features. Only those features that presented a high stability in the p(corr) values within the leave-one-out procedure were kept in the list of discriminating variables. During this procedure, one sample at a time was left out from the original data set, an OSC-PLS-DA model was constructed with the rest of the samples, and the list of p(corr) values for that model was retrieved. This was repeated until all samples had been removed once. Therefore, only those features that had p(corr) values >0.75 in all the models were selected as the discriminant features. New models with raw data (non-OSC filtered; log-transformed and Pareto-scaled) were developed by including the selected features.

Results

Data acquisition quality

The analytical variability across the runs was monitored using data from the standard compounds of QC2 samples; the compounds of IS and ES in study samples, as well as IS, ES and a subset of endogenous metabolites present in QC3 and QC5 samples were injected throughout all the data sets. These analyses covered the RT range from 0.35 min (the earlier-eluting standard: L-carnitine) to 6.95 min (later-eluting standard: glycochenodeoxycholic acid).

Supporting Table S3 shows that the maximum deviation in RT was 0.13 min, and the maximum mass accuracy deviation ranged from 0.3 mDa to 14.0 mDa. With regard to the peak intensities, the coefficient of variation ranged from 0% to 18%.

Additionally, the unsupervised multivariate analyses performed by PCA indicated a clear spatial separation among the different sample classes (plasmas, QC1, and QC2) and replicates of each QC type were positioned strongly clustered among themselves. In addition, **Supporting Figure S1** shows that among plasma samples there was a clear spatial separation between samples from the present study (obtained from rats) and commercially purchased reference human plasma samples, confirming the presence of relevant differences among the composition of human and murine plasma samples.

Thus, data quality results from both univariate and multivariate data analyses of standard mixtures of compounds and plasma samples indicate that no major instrument failures related to a decline in signal intensity, RT shifts or changes in mass accuracy were observed. The data gave confidence both about the robustness of the HPLC-ESI-QToF-MS system operating conditions and the reliability of the data for further statistical analysis of the results to detect biomarkers according to the initial hypothesis [1].

Metabolomic analysis

The OSC-PLS-DA analysis, carried out only in samples from 7 days of diet, showed differences in the plasma metabolomics profiles between the RF, CC and TB groups after 7 days of diet. To better select discriminating metabolites between groups, one-component OSC-PLS-DA models were further performed in a pairwise manner. As a result, all multivariate models resulted in one latent variable model characterized by good robustness and predictability to explain the differences between groups. Only features showing high correlation coefficients in all models developed during the leave-one-out procedure were considered as discriminating metabolites between groups. They were then submitted to the metabolite identification procedure.

References

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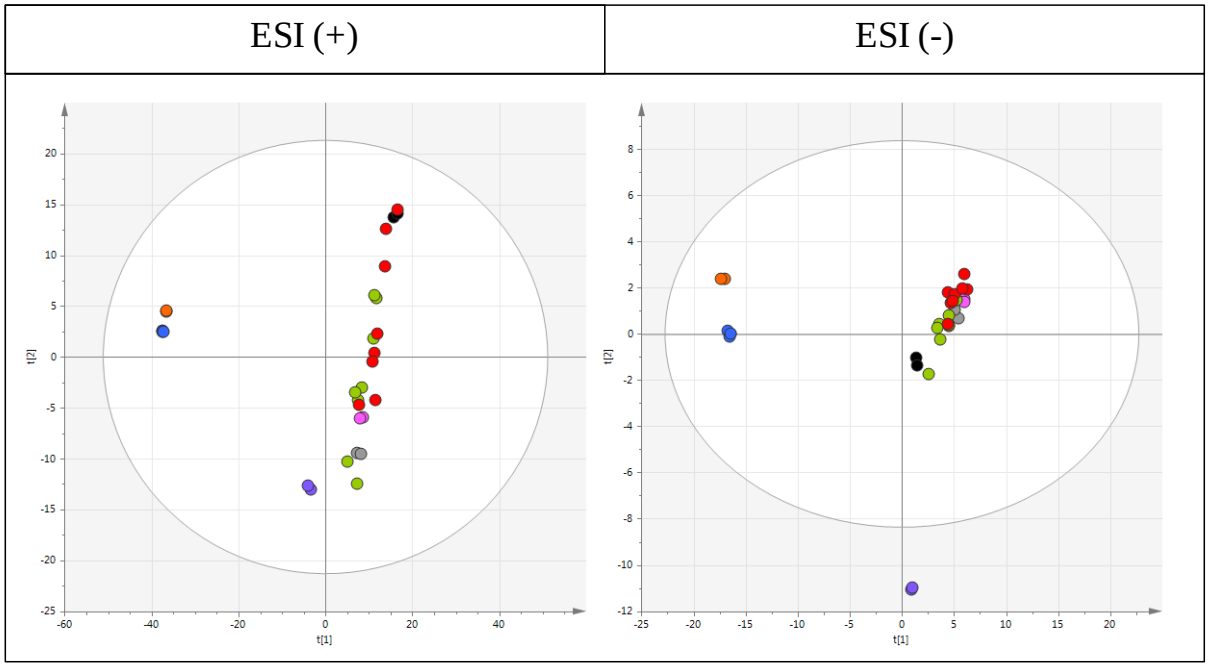
Supporting Figure 1. PCA score plots (PC1 versus PC2) of global data set (biological and QC samples). Plasma samples from the first sub-batch are indicated in green; plasma samples from the second sub-batch in red; QC1 samples in blue; QC2 samples in orange; QC3 samples in pink; and QC5 samples in purple. Reinjected samples (QC4) are linked by the same black/grey color.



Supporting Table S1. Composition of the diets

Components (%)	RF diet	CC diet	TB diet
Proteins	14.1	14.1	14.0
Lipids	3.9	3.9	3.9
Carbohydrates	72.1	71.0	72.0
Insoluble fiber	5.0	5.1	5.0
Soluble fiber	-	0.9	-
Micronutrients	4.9	4.4	4.9
Theobromine	-	0.25	0.25
Phenolic compounds	-	0.4	-

RF, reference diet; CC, diet containing 10% cocoa; TB, diet containing 0.25% theobromine.

Supporting Table S2. Data pre-processing parameters*.

Parameter	Description	ESI(+)	ESI(–)
Peak finding	Subtraction offset (scans)	5	5
	Subtraction multiplication factor	1.5	1.5
	Minimum spectral peak width (ppm)	1	1
	Minimum retention time peak width (scans)	3	3
	Noise threshold	5	5
Peak alignment	Retention time tolerances (min)	0.14	0.10
	Mass tolerance (Da)	0.05	0.05
	Intensity threshold	5	5
	Number of peaks	10,260	4,387

*MarkerViewTM 1.2.1 software (AB Sciex, Toronto, Ontario, Canada).

Supporting Table S3. Variation in retention time, detected mass and intensity of IS, ES and a subset of endogenous metabolites present in QC5 samples (commercially purchased reference human plasma).

Compound	RT (min)		Detected mass (m/z)				Intensity	
	Mean	Shift*	Mean	error (mDa)	Shift* (Da)	max error (mDa)	Mean	CV (%)
ESI(+)								
Carnitine	0.37	0.00	162.1123	0.2	0.00	0.9	122	1
Valine	0.44	0.02	207.1415	0.3	0.00	0.7	744	8
Acetyl-d_3-L-carnitine**	0.44	0.02	118.0841	2.1	0.00	3.4	6	4
Tryptophan	4.64	0.08	205.0968	0.3	0.00	1.8	1	1
Indole-3-acetic-2,2-d_2 acid**	5.71	0.01	178.0828	0.3	0.00	0.6	229	1
Glycocholic acid-(glycyl-1-^{13}C)**	6.71	0.01	467.3179	1.7	0.00	2.2	194	1
ESI(–)								
Tryptophan	4.61	0.01	203.0848	2.2	0.01	5.1	7	7
Indole-3-acetic-2,2-d_2 acid**	5.70	0.00	176.0707	2.1	0.00	3.9	168	3
Dodecanedioic acid	6.42	0.02	229.1482	3.7	0.01	6.9	11	7
Glycocholic acid-(glycyl-1-^{13}C)**	6.71	0.01	465.3103	5.2	0.01	10.4	551	1

*(max – min); **IS, ES. Abbreviations: CV, coefficient of variation; IS, internal standards; ES, external standards; RT, retention time.

Supporting Table S4. Summary of parameters for assessing the OSC-PLS-DA models. RF, reference group; CC, cocoa group; TB, theobromine group; N, number of components in OSC and PLS-DA models, respectively; Â displays the angle between the component and the Y variable; SS, sum of squares, indicating the % of the original variance in the X-block that remains in the corrected X-matrix; R^2X(cum) and R^2Y(cum) are the cumulative modeled variation in the X and Y matrix, respectively; Q^2(cum) is the cumulative predicted variation in the Y matrix; the p-value from CV-ANOVA, based on the cross-validated predictive residuals, is listed for each model (significant result indicates a valid model); R^2 and Q^2 displays the values of the regression lines obtained between the values of the original models and the values obtained in the permutation tests with 999 iterations.

Model		**OSC Filter**				**OSC-PLS-DA Model**					**Permutation test (n=999)**	
		N	**Â**	**remaining SS (%)**	**eigen-value**	**N**	**R^2X (cum)**	**R^2Y (cum)**	**Q^2 (cum)**	**p-value**	**R^2**	**Q^2**
ESI(+)	RF vs CC	3	90.00	50.28	1.06563	1	0.196	0.996	0.723	0.007	0.620	-0.132
	RF vs TB	3	90.00	48.35	1.07446	1	0.230	0.998	0.798	0.003	0.568	-0.170
	CC vs TB	3	90.00	43.42	1.04157	1	0.230	0.996	0.824	0.001	0.523	-0.179
ESI(–)	RF vs CC	2	90.00	65.60	1.13818	1	0.184	0.996	0.675	0.013	0.679	-0.110
	RF vs TB	2	90.00	70.80	1.28674	1	0.205	0.998	0.761	0.004	0.689	-0.142
	CC vs TB	4	90.00	51.18	1.09176	1	0.212	0.994	0.761	0.006	0.511	-0.154

Supporting Table S5. Tentatively identified metabolites in plasma. RF, reference group; CC, cocoa group; TB, theobromine group.

Group	cluster	ion mode	RT	m/z	error	assignation	metabolite	p(corr) a	p(corr) b	p(corr)c
CC & TB	1	+	0.38	176.1017	1.3	[M+H]+	Citrulline	0.849	0.865	-
(CC & TB) > 0	2	–	4.55	218.1059	2.5	[M-H]-	Pantothenic acid	0.952	0.978	-
	3	+	4.71	181.0716	0.4	[M+H]+	Theobromine	0.941	0.962	-
TB	4	+	5.91	228.0523	2.0	[M+H]+	5-(2'-Carboxyethyl)-4,6-Dihydroxypicolinate	-	0.914	0.862
TB > (RF & CC)										
CC	5	+	6.70	466.3137	2.6	[M+H]+	Glycocholic acid	0.794	-	-
CC > RF										
RF	6	–	5.36	190.0533	2.3	[M-H]-	Hydroxyindoleacetic acid	-0.990	-0.984	-
RF > (CC & TB)				146.0626	1.5	[M-H-COO]-		-0.975	-0.970	-
				144.0477	2.2	[M-H-COO-H_2]-		-0.980	-0.963	-

[a]Mean of p(corr) values obtained in all OSC-PLS-DA models of RF group vs CC group developed during the leave-one-out procedure.
[b]Mean of p(corr) values obtained in all OSC-PLS-DA models of RF group vs TB group developed during the leave-one-out procedure.
[c]Mean of p(corr) values obtained in all OSC-PLS-DA models of CC group vs TB group developed during the leave-one-out procedure.