Metabolomic changes after coffee consumption: new paths on the block

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Abstract: Several studies suggest that regular coffee consumption may help preventing chronic diseases, but the impact of daily intake and the contribution of coffee metabolites in disease prevention are still unclear. The present study aimed at evaluating whether and how different patterns of coffee intake (one cup of espresso coffee/day, three cups of espresso coffee/day, one cup of espresso coffee/day and two cocoa-based products containing coffee two times per day) might impact endogenous molecular pathways. To reveal this challenge, a three-arm, randomized, cross-over trial was performed in 21 healthy volunteers who consumed each treatment for one month. Urine samples were collected to perform untargeted metabolomics based on UHPLC-IMS-HRMS. A total of 153 discriminant metabolites were identified. Several molecular features were associated with coffee consumption, while others were linked with different metabolic pathways, such as phenylalanine, tyrosine, energy metabolism, steroid hormone biosynthesis and arginine biosynthesis and metabolism. This information has provided new insights into the metabolic routes by which coffee and coffee-related metabolites may exert effects on human health.

Keywords: coffee, cocoa, biomarker, metabolomics, caffeine, xenobiotics.

1. Introduction

Coffee is one of the most appreciated and consumed beverages worldwide. Besides the pleasant aroma and taste, it is considered an important source of bioactive compounds, mainly caffeine, trigonelline, chlorogenic acids, cafestol, and kahweol (Ludwig et al., 2014). In many epidemiological studies, regular coffee consumption has been associated with a reduced risk of several chronic diseases, such as type 2 diabetes, atherosclerotic heart disease, and stroke, as well as of neurodegenerative conditions, like Parkinson's and Alzheimer's diseases (Bidel & Tuomilehto, 2013; Ding et al., 2014; Elbaz et al., 2016; Huxley et al., 2009; Larsson, 2014; Malerba et al., 2013; Wu et al., 2017). Most meta-analyses have shown an apparent dose-response effect, with the lowest disease risk achieved with the consumption of about 3–5 cups/day (Grosso et al., 2017; Poole et al., 2017). However, the dose – a cup of coffee – is not a standardized measurement, and compound content of a dose also varies with the brewing method (Ludwig et al., 2014). Of note, no association has already been found between circulating coffee-related metabolites and physiological responses, making the mechanisms through which coffee exerts its potential preventive effects still widely undisclosed.

Among the plant matrixes with high content in bioactive phytochemicals, cocoa is also gaining increasing attention in nutrition research (EFSA, 2012; Kim et al., 2014; Sansone et al., 2015). Cocoa and its derived products mainly contain flavan-3-ols and theobromine, a closely related analogous of caffeine (Kim et al., 2014). Cocoa products may enhance the preventive effects of regular coffee consumption, and, in turn, cocoa-based products containing coffee, combining the phytochemical content of both coffee and cocoa, may be regarded as a potential candidate to increment the levels of putatively protective metabolites in the context of a balanced diet (Mena et al., 2017).

It is worth noting that, except for trigonelline, coffee and cocoa-related phytochemicals are extensively transformed by human metabolism, and the gut microbial catabolism. These derived compounds, rather than the parent molecules, are circulating molecules that might exert a beneficial action in human health. To date, the complete pool of circulating metabolites resulting from coffee and cocoa consumption still needs to be disclosed. In this frame, metabolomics allows a comprehensive description of the metabolites in a biological sample, providing information on exposure to exogenous metabolites and on levels of endogenous metabolites from metabolic pathways, thus allowing the study of biochemical processes modulation (Scalbert et al., 2014). In most recent years, the number of metabolomic studies applied to answer nutritional questions has raised. In particular, metabolomic profiling has been widely used to map biomarkers of intake, which are metabolites generated from compounds present in a specific food (Madrid-Gambin et al., 2016; Michielsen et al., 2018; Münger et al., 2017; Rothwell et al., 2019; Vázquez-Manjarrez et al., 2019). On the other hand, untargeted metabolomics approaches aim at identifying not only biomarkers specifically associated with a given food like coffee, but also metabolites that may reflect the biological effects of specific dietary components. This strategy may help in elucidating the contribution of coffee metabolites in disease prevention and in shedding light on the underlying mechanisms (Gibbons et al., 2015; Wishart, 2008). For instance, a comprehensive metabolomic analysis of serum samples following coffee

intake (up to 8 cups of coffee/day) revealed that metabolites from the endocannabinoid and fatty acid acylcholine pathway decreased in response to coffee consumption whilst those of the steroid pathway generally increased (Cornelis et al., 2018). Moreover, induction of fatty acid metabolism, mainly related to carnitine derivatives, was observed in urine after 30 days following green coffee bean extract consumption (Peron et al., 2018). Shi and colleagues have also identified several plasma metabolites specifically associated with filtered and boiled coffee consumption and used them to estimate filtered or boiled coffee intake and to find associations with type 2 diabetes risk (Shi et al., 2020).

Based on those previous studies, there is still room for discovering novel pathways of coffee metabolic effects using metabolomics. This untargeted metabolomics study has revealed new molecular pathways affected by coffee and cocoa intake, linking metabolic pathways to different levels of coffee and cocoa intake.

2. Materials and methods

2.1 Chemicals

HPLC-grade methanol, acetonitrile, and acetic acid were purchased from Sigma-Aldrich (Taufkirchen, Germany); bidistilled water was obtained using Milli-Q System (Millipore, Bedford, MA, USA). MS-grade formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA) and ammonium acetate (Fluka, Chemika-Biochemika, Basil, Switzerland) were also used. Leucine-enkephalin, used as lock mass standard and Major Mix for collisional cross-sectional (CCS) calibration were purchased from Waters (Milford, USA.).

2.2 Subjects

Twenty-one volunteers were recruited in Parma (Northern Italy) to participate in the study. Inclusion and exclusion criteria and main subject clinical characteristics have already been published (Mena et al., 2017). Briefly, 21 subjects, 10 males (2 smokers) and 11 females (6 smokers), aged 25.9 ± 0.5, BMI 22.3 ± 0.6 kg/m^2, were enrolled. The study was conducted according to the guidelines of Good Clinical Practice and the Declaration of Helsinki. All subjects provided written informed consent before study entry, and they all completed the intervention study.

2.3 Study design

A three-arm, randomized cross-over trial was performed in 21 healthy volunteers, as previously reported (Mena et al., 2017). The study was approved by the Ethics Committee for Parma Hospital and University (AZOSPR/0015693/6.2.2.) and registered on ClinicalTrials.gov on May 21, 2017 (NCT03166540). Briefly, participants had to consume three different treatments in a random order for one month: (1) one cup of espresso coffee/day (at 9.00 AM, namely 1C group), (2) three cups of espresso coffee/day (at 9.00 AM, 12.00 noon, and 3.00 PM, namely 3C group) and (3) one cup of espresso coffee/day and two cocoa-based products containing coffee (CBPCC) twice per day (coffee at 9.00 AM and two CBPCC at 12.00 PM and 3.00 PM,

namely PC group). The randomization list was generated using Random Number Generator Pro (Segobit Software). Volunteers were supplied with a single-serve coffee machine (Essenza EN 97.W, De' Longhi Appliances S.r.l., Treviso, Italy) and coffee capsules (Capriccio, Nespresso Italia S.p.a., Assago, Italy) to standardize raw material, brewing method and cup volume, and also with the CBPCC (Pocket Coffee, Ferrero Commerciale Italia S.r.l., Alba, Italy). Minimal dietary restrictions were given to volunteers two days before and on each sampling day to exclude other sources of coffee/cocoa-related phytochemicals apart from those provided by the assigned treatment. The sampling day corresponded to the last day of each intervention period. On the sampling day, urine from each volunteer was collected at baseline (t0) and different collection periods within 0–3 h, 3–6 h, 6–9 h, and 9–24 h. Samples used for this study corresponded to the period 9-24 h. The volume of urine collected during each period was measured, and two 2 mL samples were stored at −80 °C until analysis.

2.4 Urine sample preparation

Urine samples were prepared as described elsewhere (Want et al., 2010). Briefly, urine samples were thawed on ice before analysis and centrifuged for 10 min at 10,000g to remove particulates. 50 µL of supernatant were diluted with 100 µL of Milli-Q water. Quality control samples consisting of all urine samples to form a pool were analyzed for the study and injected every 9 samples to allow for the performance of the analytical system in terms of retention times, mass accuracy and signal intensities to be evaluated. Three technical replicates of each sample were injected. All samples were acquired in a randomized order.

2.5 UHPLC-TWIMS-QTOF analysis

ACQUITY I-Class UPLC separation system coupled to a VION IMS QTOF mass spectrometer (Waters, Wilmslow, UK) equipped with electrospray ionization (ESI) interface was employed.

Samples were injected (5 µL) and chromatographically separated using a reversed-phase C18 HSS T3 ACQUITY column 2.1 × 100 mm, 1.7 µm particle size (Waters, Milford, MA, USA). A gradient profile, as previously described (Want et al., 2010) was applied. In short, water (eluent A) and acetonitrile (eluent B), both acidified with 0.1% formic acid, were used as mobile phases. Initial conditions were set at 1% B followed by a linear change to 15% B in 3 min and 50% B in 3 min. Finally, 95% B was achieved at 9 min prior to holding at 95% for 1 min to allow for column washing before returning to initial conditions. Column recondition was completed over 3 min, providing a total run time of 14 min. The column was maintained at 40 °C and a flow rate of 0.5 mL/min used.

Mass spectrometry data were collected in both positive and negative electrospray mode over the mass range of *m/z* 70−1000. Source settings were maintained using a capillary voltage, 2.5 kV; cone voltage, 40 V; source temperature, 120 °C; desolvation temperature, 500 °C and desolvation gas flow, 800 L/h. The TOF analyzer was operated in "sensitivity mode" and data acquired using HDMSE (Rodriguez-Suarez et al., 2013), which is a data-independent approach (DIA) coupled with ion mobility. The ion mobility device within the Vion was calibrated using the Major Mix IMS calibration kit (Waters, Wilmslow, UK) to allow for CCS values to be

determined in nitrogen. The calibration covered the CCS range from 130-306 $Å^2$. The TOF was also calibrated prior to data acquisition using sodium formate (Waters, Wilmslow, UK) and covered the mass range from 151 Da to 1013 Da. TOF and CCS calibrations were performed for both positive and negative ion mode. Data acquisition was conducted using UNIFI 1.8 (Waters, Wilmslow, UK).

2.6 Data processing and multivariate modelling

Data processing and compound identification were conducted using Progenesis QI Informatics (Nonlinear Dynamics, Newcastle, UK). Each UPLC-MS run was imported as an ion-intensity map, including *m/z* (*m/z* range 70-1000) and retention time, that were then aligned in the retention-time direction (0-8.5 min). From the aligned runs, an aggregate run representing the compounds in all samples was used for peak picking. This aggregate was then compared with all runs, so that the same ions are detected in every run. Isotope and adduct deconvolution were applied, to reduce the number of features detected. Data were normalized according to creatinine intensity in each sample.

Unsupervised principal components analysis (PCA) with pareto scaling was performed to check the quality of the raw data. Afterward, the variables were filtered, retaining entities with coefficients of variation (CV) lower than 30% across the QCs. From the analysis of the variance (ANOVA) significant features were selected, retaining those presenting, simultaneously, fold change >2, and Benjamini-Hochberg FDR adjusted *p*-value (q value) < 0.01. In parallel, multivariate supervised models, including least-squares discriminant analysis (PLS-DA) were built and validated using SIMCA software (v. 16.0.2, Sartorius Stedim Data Analytics, Sweden). Cross-validation of the PLS-DA model using one-third leaving out approach and permutation testing were applied to validate and to exclude overfitting by inspecting model parameters (goodness-of-fit R^2Y and goodness-of-prediction Q^2Y). The variable influence in projection analysis (VIP) was further used to identify the compounds that have the highest discrimination potential (VIP value threshold >1.2). The resulting significant features to both ANOVA p-Values < 0.01 and VIP >1.2 were subjected to the identification.

Metabolites were identified by publicly available database searches including Lipid Metabolites and Pathways Strategy (LIPID MAPS) (Fahy et al., 2009), Human Metabolome database (HMDB) (Wishart et al., 2013), and METLIN (Smith et al., 2005), as well as by fragmentation patterns, retention times and collision cross-sections. CCS values were searched against "MetCCS Predictor" database (Zhou et al., 2016) containing *m/z* and CCS values by selecting a ΔCCS of 5% for metabolite matching. Based on the Metabolomics Standards Initiative (Sumner et al., 2007), metabolites were annotated as level III (putatively characterized), level II (putatively identified compounds) and level I (identified compound), as reported in **Table 1**. Level I identification was performed by comparison of rt and fragmentation pattern with the standard collect in our UNIFI library, created by running a mix of standards with the same analytical method.

2.7 Metabolic pathway analysis

Identified metabolites were submitted to the Pathway and Network Analysis modules in MetaboAnalyst 4.0 (Chong et al., 2018) using HMDB identifiers. For the former analysis, Fishers' exact test and relative-

betweenness centrality were the algorithms respectively selected to perform pathway enrichment analysis and pathway topology analysis, using the current KEGG version of "homo sapiens" library. For the network analysis, the Metabolite-Metabolite Interaction Network mode was chosen.

3. Results and Discussion

3.1 Multivariate modelling and metabolite identification

An untargeted metabolomics approach was used to explore metabolome changes in urine in response to different patterns of coffee consumption. UHPLC-TWIMS-QTOF data sets, obtained in positive and negative ionization modes, were separately submitted for data analysis. A total of 15714 and 19591 features were initially peak picked for positive and negative modes, respectively. Most likely, the high number of detected features was due to the use of ion mobility between LC and MS detector. Indeed, Rainville and co-authors have quantified that the features detected in urine increased up to 41% when adding a further dimension of separation as provided by ion mobility between the LC system and the Q-TOF, most likely due to a combination of separation of co-eluting compounds and noise reduction (Rainville et al., 2017).

At first, the PCA of non-averaged samples was employed to explore the data obtained. Score scatter plots for both positive and negative ionization data are depicted in **Figure 1S**. Both PCA plots demonstrated a grouping of samples associated with coffee consumption. Afterward, technical replicates were merged, and both unsupervised and supervised models were constructed. PLS-DA (**Figure 1**) applied on positive and negative datasets showed a clear separation between 1C and the other two treatments (PC and 3C), displaying excellent goodness-of-fit (R^2Y) and good prediction ability (Q^2). Cross-validation of both PLS-DA models indicates that 100% of urine samples analyzed in positive ionization mode were correctly classified, while in ESI(-) the percentage of total correct classification was 98.4% since one sample was not correctly predicted (1C sample predicted as PC). Permutation plots are depicted in **Figure 2S**.

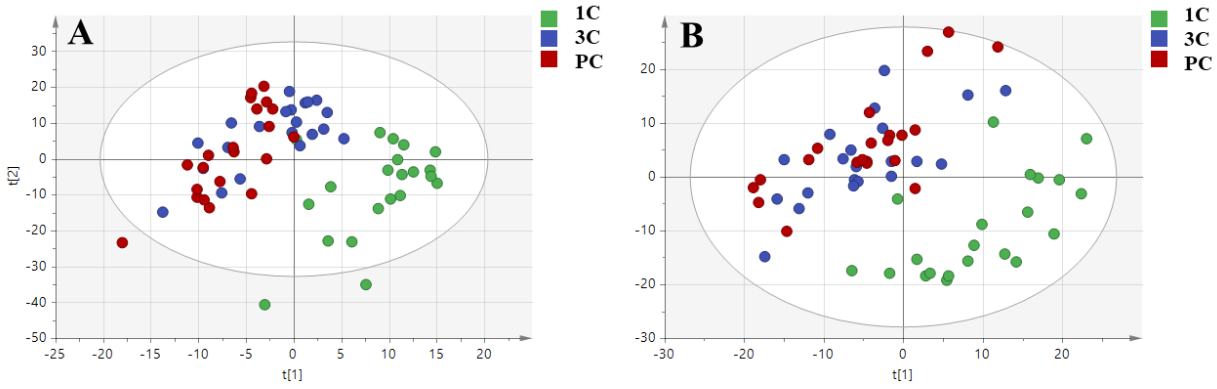

Figure 1. (**A**) PLS-DA model built with positive ionization data (R^2Y = 0.95, Q^2 = 0.651) and (**B**) negative ionization data (R^2Y = 0.906, Q^2 = 0.769). The intervention treatment groups (1C, 3C, PC) are color coded accordingly.

Subsequently, significant features were selected, retaining those presenting, simultaneously, fold change >2, and FDR adjusted *p*-value (q value) < 0.01 and merged with those showing VIP >1.2. This filtering step

returned a dataset with 3590 significant features for both polarities, which were subjected to the identification. This last step is considered the bottleneck of the whole metabolomics workflow, which remains a major analytical challenge. With mass fragmentation and CCS matching, 153 identifications were assigned out of 3590, meaning that less than 5% of the significant features were translated into knowledge. All significant features are reported, for completeness, in **Table 1S**. We assume that the vast majority of the unidentified features may correspond to coffee or cocoa chemicals and their metabolites. These beverages have a tremendous chemical complexity, and their derived metabolites are largely undocumented and thus absent from reference databases. Endogenous metabolites involved in human metabolic pathways are currently better covered in databases. The annotation of discriminant metabolites, information regarding their biochemical class, and statistical parameters (ANOVA P-Value and fold change) of each metabolite are reported in **Table 1**. Further analytical details on accurate mass, detected adduct, formula, error ppm, CCS value and retention time are summarized in **Table 2S**. In parallel to 3-groups modelling, 2-groups comparison was performed between 3C vs 1C, PC vs 1C and 3C vs PC. PLS-DA models were built, and their plots showing excellent clustering are summarized in **Figure 3S**. Significant metabolites from the binary comparisons with their fold changes are reported in **Table 1**.

Table 1. Statistically significant urine metabolites annotated using UHPLC-TWIMS-QTOF.

3.2 Metabolism of coffee and cocoa phytochemicals (biomarkers of intake)

More than one hundred out of the 153 annotated metabolites were generated from phytochemicals present in coffee and the CBPCC. These metabolites belonged to various chemical classes, including cinnamic acids, imidazopyrimidines, naphthofurans, pyridine derivatives, phenols, and benzene derivatives, among others (**Table 1**).

As expected, we observed an increase in urinary levels of caffeine and its related metabolites (dimethylxanthines, monomethylxanthines, and methyluric acids, with the sole exceptions of theobromine and its metabolites) upon increased coffee consumption, reaching the highest intensities for the 3C treatment (3 cups of coffee per day). After being rapidly absorbed and metabolized in the liver, caffeine and its metabolites are slowly removed from the circulation (Martínez-López, Sarriá, Baeza, et al., 2014).

Trigonelline followed the same trend in urine samples, with the highest mean value for 3C and lowest in 1C group. Conversely to caffeine, trigonelline is excreted unmetabolized (Madrid-Gambin et al., 2016), and has been found to significantly correlate with coffee consumption. Indeed, it has been proposed as a biomarker of coffee intake, alone (Rothwell et al., 2019) or in combination with 1-methylxanthine and cyclo(isoleucylprolyl) (Rothwell et al., 2018). However, neither cyclo(isoleucylprolyl) nor the diterpene atractyligenin glucuronide, recognized as specific biomarkers of coffee consumption, were detected in the present study (Rothwell et al., 2014). This observation reinforces the potential of trigonelline to serve as a candidate biomarker of coffee intake.

Another important class of coffee-derived metabolites is that of cinnamic acids, which originated mainly from the metabolism of chlorogenic acids, the main phenolic compounds found in coffee (Ludwig et al., 2014). This metabolism takes place both at the upper and lower level of the gastrointestinal tract, with the latter involving the gut microbiota. Once absorbed, coffee hydroxycinnamates are then subjected to phase II metabolism at the hepatocyte level and enter into circulation (Ludwig et al., 2014). In line with this prediction, coumaric acid-sulfate was the most significant marker of this class of compounds, with the highest intensity in the 3C treatment and lowest in the 1C group. However, these compounds cannot serve as selective biomarkers of coffee intake, because of their very poor specificity and their colonic origin (Madrid-Gambin et al., 2016; Rothwell et al., 2018), which is inevitably affected by a high inter-individual variation due to intrinsic variability of the human gut microbiota (Bento-Silva et al., 2020).

Investigating the PC intervention, in which coffee and cocoa intake were combined, an increased amount of theobromine derivatives, kahweol oxide glucuronide and nicotinamides was observed in urine (**Table 1**). Theobromine is known to be found in cocoa and to be rapidly absorbed and converted in, among other metabolites, 3,7-methyluric acid, 3-methylxanthine, 7-methylxanthine and 3-methyluric acid (Martínez-López et al., 2014). These four metabolites were found to be greatly excreted after the PC treatment and, in particular, 3,7-methyluric acid and 3-methyluric were good discriminant markers for both positive and negative ionization modes (**Table 1 and Table 2S**). On the other hand, although kahweol oxide glucuronide and nicotinamides mostly derive from coffee consumption, they changed notably as a consequence of the PC treatment. Both metabolites might be considered markers of different roasting processes or coffee brewing styles, different from the espresso (Gross et al., 1997; Lang et al., 2013). Thus, this result might indicate that the cocoa-based products most likely contained a coffee with a phytochemical composition different to that of the espresso coffees consumed by the volunteers.

Moreover, the significant presence of compounds coming exclusively from cocoa constituents rather than coffee, like flavan-3-ol metabolites and phenethylamines, was observed. Eight phenyl-γ-valerolactone glucuronides and sulfates were detected as markers for the PC treatment, these having been reported to originate by colonic microbial catabolism of flavan-3-ols (Mena et al., 2019). In particular, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and its glucuronide and sulfate derivatives were among the metabolites showing the most significant fold changes compared to the espresso coffee treatments (1C and 3C).

3.3 Biological interpretation of significant markers

Untargeted metabolomics allowed the identification of unrelated metabolites to coffee and cacao. The role of these metabolites was evaluated by pathway analysis.

3.3.1 Pathway analysis

The pathway analysis shows the main metabolic routes modulated by the modes of coffee consumption under study (**Figure 2**). All the identified pathways resulted in being upregulated following coffee intake (**Table 3S**).

As previously stated, caffeine is the main bioactive compound in coffee beans and abundantly present in coffee. Once ingested, it is rapidly absorbed and metabolized into more hydrophilic metabolites that can be excreted in the urine. Up to nine metabolites corresponding to this pathway were identified in urine samples following coffee intake, highly impacting on the urinary metabolome. Other metabolic routes influenced by the intake of coffee were the metabolism and biosynthesis of specific amino acids (in particular phenylalanine, tyrosine and arginine), ascorbate and aldarate metabolism, ubiquinone and other terpenoid-quinone biosynthesis, galactose metabolism, purine metabolism, nicotinate and nicotinamide metabolism, steroid hormone biosynthesis and citrate cycle (TCA cycle), as depicted in **Figure 2**.

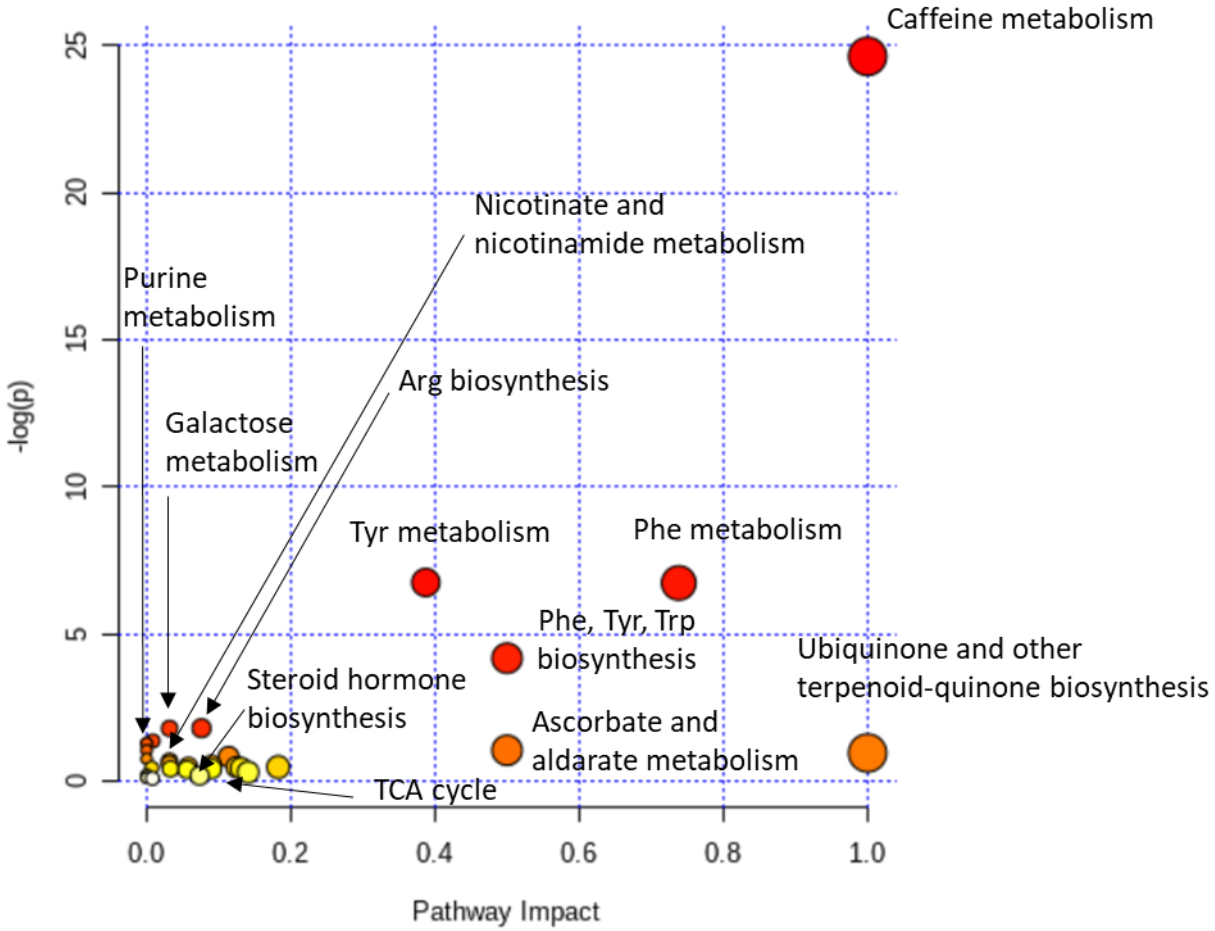

Figure 2. Pathway analysis performed with all the significant metabolites identified. The output displays metabolic pathways arranged by scores from pathway enrichment (y-axis) and topology analysis (x-axis). The color and size of each circle are based on *p*-values and pathway impact values, respectively (from yellow to red, the –log(*p*) increases, the bigger the circle size, the higher the pathway impact value).

Phenylalanine metabolism resulted as the second most perturbed pathway (**Figure 2**). In particular, the route arising from the catabolism of phenylalanine into phenylacetic acid was significantly altered. In this sense, phenylalanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis and tyrosine metabolism were influenced by coffee consumption. Other pathways involving amino acids affected by the mode of coffee consumption were arginine biosynthesis and metabolism. Arginine is a semi-essential amino acid that has many functions, including being involved in the urea cycle, as a precursor of nitric oxide, creatine, glutamate,

and proline, and it can be converted into glucose and glycogen if needed (Wishart et al., 2013). On the other hand, the activation of nicotinate and nicotinamide metabolism is probably a consequence of the presence of trigonelline and other pyridines (*N*-methylpyridinium and niacin) in coffee (Lang et al., 2013), as well as of caffeine for purine metabolism. The impact on the TCA cycle (via citric acid) suggests an influence on energy metabolism. The effects of coffee and its constituents on energy metabolism has been observed and extensively studied (Astrup et al., 1990; Bracco et al., 1995; Dulloo et al., 1989) with several mechanisms of action having been proposed and reviewed (Grosso et al., 2017; Stohs & Badmaev, 2016), but none involving the TCA cycle. A similar effect towards the TCA cycle was observed by Takahashi and colleagues (Takahashi et al., 2014) in mice when, through an integrated multi-omics study, researchers found that TCA cycle-related proteins in mice were upregulated upon coffee consumption. Among these upregulated proteins, NADH dehydrogenase (ubiquinone), which may explain the presence of ubiquinone and other terpenoid-quinone biosynthesis among the main perturbed pathways. The underlying mechanism by which coffee, through one or more of its components, determines this upregulation of TCA cycle enzymes is still unknown.

3.3.2 Functional response metabolites (biomarkers of effect)

A dose-dependent increase following coffee intake (1 and 3 cups of espresso coffee per day) was also observed for unusual pathways that did not involve bioactive coffee compounds directly (**Figure 3**). This was the case of some amino acids, purine nucleosides and steroids.

3.3.2.1 Amino acids

Several amino acids are naturally present in coffee. However, the significant presence of arginine and phenylalanine in the urinary metabolome upon coffee consumption (3C) (**Table 1**) is more likely due to to the activation of metabolic pathways leading to their syntheses, as described above (Kulapichitr et al., 2019; Ludwig, Clifford, et al., 2014). To the best of our knowledge, the link of arginine with coffee seems to be new, since no other study identified changes in arginine levels upon coffee consumption, nor suggested a potential effect of coffee on human health through the modulation of arginine biosynthesis. Cornelis and collaborators did not report arginine but found a slight decrease in homoarginine levels following coffee intake (Cornelis et al., 2018). Homoarginine is an endogenous non-proteogenic amino acid produced from arginine and lysine by the catalytic action of arginine:glycine amidinotransferase (AGAT) (Rodionov et al., 2016; Tsikas & Wu, 2015). Recent epidemiological studies have demonstrated an association between low circulating concentrations of L-homoarginine and an increased risk of cardiovascular and all-cause mortality (Atzler et al., 2015; Pilz et al., 2015). The high levels of free arginine in urine, after the 3C treatment, could have different implications—for example, a decreased conversion into homoarginine or reduced production of dimethylarginines, among others. If homoarginine favors human health by promoting nitric oxide synthesis, endogenous dimethylated derivatives of arginine (asymmetric dimethylarginine and symmetric dimethylarginine) are generally accepted cardiovascular risk factors (Jarzebska et al., 2019; Tsikas & Wu, 2015). Notably, asymmetric dimethylarginine slightly increased (3.6 fold-change) after the PC treatment

compared to a lower coffee dose (1C). These observations should be investigated more in-depth to explore any possible association with disease prevention or development.

Phenylalanine is the essential amino acid precursor of tyrosine. Via tyrosine metabolism, it is also a precursor for catecholamines, like dopamine. Regarding phenylalanine, increased levels of this amino acid were registered as a consequence of high coffee consumption (3C vs 1C and PC vs 1C) (**Table 1**), likely having an impact on the synthesis and metabolism of tyrosine and dopamine. This result is in contrast with the findings of the EPIC-Potsdam Study on the evaluation of various biomarkers as potential mediators of the association between coffee consumption and incident type 2 diabetes (Jacobs et al., 2015). Actually, in that study authors reported an inverse association between coffee consumption and plasma levels of phenylalanine in men. However, they couldn't suggest any plausible biological explanation for this association and a consequent linkage with type 2 diabetes. Dopamine levels also raised after higher coffee intake, highlighting an increased amount of catecholamines as a consequence of coffee consumption. Increased dopamine levels may represent a potential neuroprotective mechanism exerted by coffee (de Lau & Breteler, 2006).

3.3.2.2 Purine nucleosides

A higher excretion of deoxyadenosine was observed for the 3C treatment, mostly as a nucleoside (with a fold change of 17.6 when comparing 3C vs 1C) but also esterified with a phosphate group forming the corresponding nucleotide (with a fold change of 3.2 when comparing 1C vs 3C). This compound is a critical component of DNA, and it is linked with coffee consumption for the first time. Its increased levels at the highest coffee intake might be a result of enhanced purine metabolism, as shown by the pathway analysis. Disorders in purine metabolism have been associated with various diseases, such as gout (Richette & Bardin, 2010), multiple sclerosis (Amorini et al., 2009), and certain cancers (Struck-Lewicka et al., 2014). However, the purine compounds showing altered levels in those studies were different from deoxyadenosine and an important role was played by uric acid, the final product of purine degradation. In our study, uric acid increased only slightly following coffee intake (**Table 1**), but this is likely due to caffeine metabolism (Myers & Wardell, 1928). This enhanced deoxyadenosine production, not accompanied by increased uric acid excretion, may necessitate further investigation.

3.3.2.3 Steroids and steroid derivatives

The link between coffee and steroid metabolism is not new and has been largely studied mainly because of the possible association with female cancer risk (Ferrini & Barrett-Connor, 1996; Kotsopoulos et al., 2009; Larsson et al., 2009; Lucero et al., 2001; Sisti et al., 2015) and adverse effects during pregnancy (Dinçer et al., 2020; Doepker et al., 2018). In particular, in the present study, an impact on steroid hormone biosynthesis and augmented levels of progesterone, estriol, (carboxymethyl)estrone, methoxy-estradiol glucosiduronic acid and estriol glucuronide (**Table 1**) were reported. While most are inactive metabolites usually excreted in the urine, progesterone and estriol are hormones that can affect human health, particularly in female subjects in delicate conditions, such as pregnancy, lactation, or menopause (Ito, 2007; Pasqualini, 2005). Cornelis and colleagues

(Cornelis et al., 2018) also found a significant plasmatic enrichment of steroid metabolites after coffee intake but linked to the androgen pathway. This could be due to differences in the study population (at elevated risk of type 2 diabetes vs. healthy subjects), sampling times, analysed samples matrix (plasma vs urine) or in the types of intervention since subjects in that study had to consume higher doses of coffee (4 to 8 cups per day), and the brewing method was not standardized. Augmented levels of steroid hormones (estriol and progesterone) after 3C treatment compared to 1C are very relevant to consider, but their association with female cancers is controversial, both in pre- and post-menopausal women (Ferrini & Barrett-Connor, 1996; Ganmaa et al., 2008; Kotsopoulos et al., 2009; Li et al., 2011; Sisti et al., 2015). Further research aimed at elucidating increased hormone levels will be of great interest. A recent meta-analysis associated coffee intake with probable decreased risk of breast and endometrial cancers, among other pathologies, with the lowest risk reached with the consumption of about 4-5 cups/day (Grosso et al., 2017), thus reducing any concern about this specific association.

3.3.2.4 Others

The boost to ascorbate and aldarate metabolism to produce high amounts of D-glucuronate is possibly due to the increased need, following coffee consumption, to remove the many xenobiotic substances introduced, considering that glucuronidation of exogenous compounds is the first phase II mechanism involved in the detoxification of reactive electrophiles and the production of polar metabolites that diffuse less across membranes. Regarding the functional effect of the treatment including cocoa (PC treatment) on pathways associated to specific metabolic responses, it should be noted that the only major route, beyond those related to the metabolism of cocoa xenobiotics, regulated by this intervention in comparison to the other treatments was phenylalanine catabolism into phenylacetic acid.

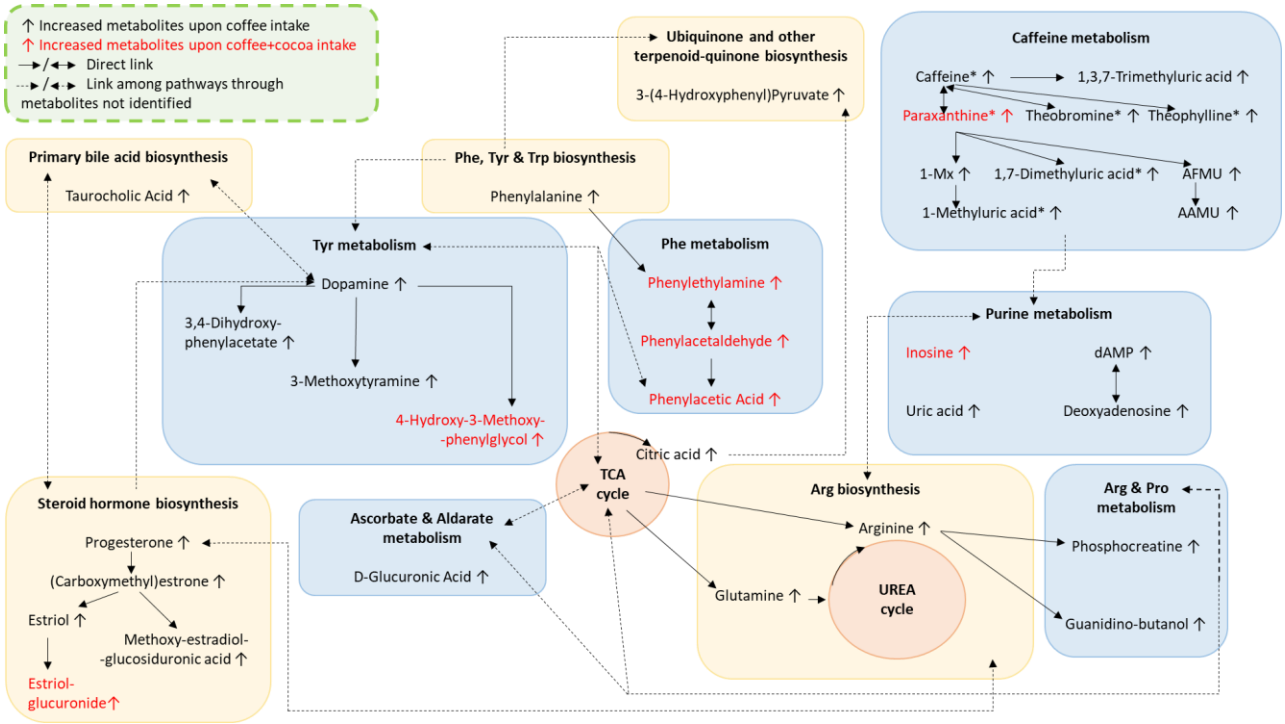

Figure 3. Detailed metabolic pathways involved and the significant metabolites (endogenous and exogenous) identified in urine after coffee or coffee and cocoa intake and their inter-connections. Information on pathways has been drawn from KEGG database.
* indicates level I identified metabolites.
Phe: phenylalanine; Tyr: tyrosine; Trp: tryptophan; Arg: arginine; Pro: proline; 1-Mx: 1-methylxanthine, AFMU: 5-Acetylamino-6-formylamino-3-methyluracil; AAMU: 5-Acetylamino-6-amino-3-methyluracil; TCA cycle: tricarboxylic acid cycle/citrate cycle.

Conclusions

In conclusion, this study showed that – in controlled but realistic conditions – it is possible to detect changes in the metabolome that are associated with different modes of coffee consumption. First, we demonstrated that the use of a holistic approach, such as untargeted metabolomics, may disclose not only the fate of coffee components after ingestion, but also how coffee can modulate endogenous metabolome changes. Indeed, besides the already known coffee and cocoa biomarkers of intake, we detected endogenous metabolites from the phenylalanine, tyrosine and arginine biosynthesis and metabolism, energy metabolism and steroid hormone biosynthesis that were affected by the three modes of coffee consumption and may, in turn, potentially influence human health. Although only 5% of the detected features were identified, our results unveil the complex metabolic pathways that may be modulated by coffee and cocoa consumption.
In the future, the observed changes should be further validated by quantitative measurement in the kinetics of all key metabolites of the modulated pathways, and their biological meaning and potential implications in disease prevention should also be investigated in specifically designed intervention studies.

Supplementary information available: Figure 1S. Unsupervised principal components analysis (PCA) models built from non-averaged samples run in positive and negative ionization modes. **Figure 2S.** Permutation plots obtained for (A) positive and (B) negative ionization 3-groups comparison data. **Figure 3S.** PLS-DA plots for two-group comparisons in positive and negative ionization modes. **Table 1S.** ESI positive and ESI negative non-identified significant features ranked by ANOVA P-Value**. Table 2S.** Chromatographic and spectrometric characteristic of significant annotated metabolites. **Table 3S.** Results from the pathway analysis, performed with all the significant metabolites. In particular, are reported the main metabolite routes perturbed by coffee consumption, the number of compounds matched on the total number of compounds in the pathway and the pathway impact value calculated from pathway topology analysis.

ACKNOWLEDGMENTS

Authors thank the volunteers for participating in the study, and Yevgeniy Topolskyy, Francesca Zicca, Debora Fede, Luciano Notarpietro, Matteo Chini, and Chiara Bandini for their assistance in the conduction of the study.

DECLARATIONS

Sources of funding: This work was partially funded by Soremartec Italia S.r.l. (Alba, Italy). The funder approved the final trial protocol prior to its implementation, but it was not involved in the design of the study, data analysis and interpretation, nor the drafting of this manuscript.

Conflict of Interest: DDR has received research grants from Soremartec. The other authors have no conflict of interest to declare.

Data availability: Data matrices have been deposited in the following dataset: Righetti, Laura; Favari, Claudia; Mena, Pedro (2020), "Metabolomic changes after coffee consumption: new paths on the block ", Mendeley Data, V1, doi: 10.17632/5w936rthzg.1

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Table 1. Statistically significant urine metabolites annotated using UHPLC-TWIMS-QTOF. Metabolite levels that increase in response to the first considered treatment are shaded red and metabolite levels that decrease are colored green. The fold change value is calculated as the ratio between the highest mean concentration reported in a specific treatment (1C, 3C or PC) for a metabolite and the lowest mean concentration reported for the same metabolite in the respective treatment.

Class	Sub Class	Putative identification	q Value	Fold Change		
				3C/1C	3C/PC	PC/1C
Azoles	Imidazoles	3-(Imidazol-5-yl)lactate[c]	6.3E-03	0.5	0.4	1.3
		4-Amino-1H-imidazole-5-carboxylic acid[*, c]	1.0E-16	2.4	1.2	2.0
Benzene and substituted derivatives	Aniline and substituted anilines	4-Aminophenol[*, c]	1.0E-16	2.8	0.8	3.5
		Aniline[*, b]	3.8E-09	1.9	0.6	3.2
	Anilines	4-Methoxyaniline[c]	1.2E-11	3.8	0.9	4.4
	Benzaldehydes	3-Hydroxybenzaldehyde[*, c]	4.5E-12	1.9	n.s.	n.s.
	Benzenesulfonic acids and derivatives	3,4-Dihydroxybenzenesulfonic acid[b]	2.8E-08	1.4	0.9	1.6
	Benzoic acids and derivatives	Hydroxybenzoic acid[*, c]	1.0E-16	2.5	1.1	2.2
		4-Hydroxybenzoic acid-4-O-sulfate[*, c]	3.9E-07	1.1	0.5	2.3
	Benzyl alcohols	Benzyl Alcohol[*, c]	1.0E-07	n.s.	1.6	n.s.
	Phenethylamines	Tyramine[c]	1.3E-13	8.8	0.7	13.4
		Phenethylamine[*,b]	1.0E-16	7.0	0.8	8.5
	Phenols and derivatives	Phloroglucinol[c]	7.7E-03	1.3	1.1	1.2
		Resorcinol[c]	2.7E-08	1.4	0.9	1.6
	Phenylacetaldehydes	Phenylacetaldehyde[b]	4.0E-02	0.8	0.3	3.1
	Phenylmethylamines	4-Hydroxybenzylamine[c]	1.0E-16	5.9	1.0	6.2
		Phenylmethanamine[*, c]	1.0E-16	3.8	0.8	4.5
	Phenylpyruvic acid derivatives	3-(4-Hydroxyphenyl)Pyruvate[*, c]	1.3E-07	n.s.	3.5	n.s.
Benzenoids	Phenols	6-Hydroxydopamine[*, c]	2.4E-11	1.8	n.s.	n.s.
Carboxylic acids and derivatives	Amino acids, peptides, and analogues	Glutamine[*, c]	1.4E-10	2.9	n.s.	n.s.
		3,4-Dihydroxy-L-Phenylalanine[c]	8.6E-10	1.4	1.0	1.3
		6-acetamido-3-aminohexanoic acid[c]	2.1E-09	1.2	0.5	2.5
		Arginine[c]	1.0E-16	7.7	n.s.	n.s.
		Phenylalanine[c]	7.2E-14	3.2	1.2	2.7
		3-Methoxy-L-Tyrosine[c]	1.3E-04	1.7	n.s.	n.s.
		4-Hydroxy-L-Phenylglycine[*, c]	4.4E-10	1.7	n.s.	n.s.

		Dihydrodipicolinic acid[b]	2.2E-02	3.0	1.9	1.6
		N,N-Dimethylarginine (ADMA)[c]	3.5E-02	0.7	0.6	3.6
		N5-(1-Carboxyethyl)-ornithine[c]	3.2E-12	1.7	0.5	3.6
		N-Acetylleucine[c]	9.6E-04	2.0	0.8	1.7
		N-Acetyl-L-lysine[c]	4.3E-05	2.2	1.1	2.1
		O-Acetyl-L-homoserine[b]	9.1E-13	4.8	4.5	1.1
		Phosphocreatine[*, c]	6.4E-13	2.0	1.4	n.s.
		Tilarginine acetate[*, c]	2.9E-04	0.9	0.7	n.s.
		4-Methylene-L-glutamate[c]	2.9E-12	4.4	1.3	3.4
	Dicarboxylic acids and derivatives	Glutarate[c]	2.4E-03	1.5	n.s.	n.s.
	Tricarboxylic acids and derivatives	Citric Acid[c]	6.2E-04	n.s.	n.s.	0.6
Cinnamic acids and derivatives	Cinnamic acids	Trans-Cinnamate[*, c]	2.0E-03	1.7	1.3	1.3
		4-methoxycinnamic acid[b]	1.3E-04	2.0	1.8	1.1
	Hydroxycinnamic acids and derivatives	coumaric acid-sulfate[*,b]	1.0E-16	3.6	1.3	2.8
		Dimethoxycinnamic acid [b]	1.7E-04	1.1	2.1	0.5
		Caffeic acid-4′-sulfate[b]	1.6E-04	1.9	1.7	1.1
		Ferulic acid[b]	2.7E-03	1.6	1.8	0.9
		m-Coumaric acid[c]	3.7E-02	1.6	1.2	1.3
		p-Coumaric acid[c]	1.2E-02	1.7	2.0	0.9
		m-Dihydrocoumaric acid-sulfate[b]	2.8E-02	0.7	1.4	0.5
Coumarins and derivatives	Hydroxycoumarins	7-Hydroxy-3-(4-methoxyphenyl)-4-propylcoumarin[c]	1.2E-04	1.4	1.0	1.3
Diazines	Pyrazines	3,5-diethyl-2-methylpyrazine[b]	1.0E-16	1.2	0.2	5.9
	Pyrimidines and pyrimidine derivatives	5-Acetylamino-6-formylamino-3-methyluracil[b]	1.6E-02	2.4	1.2	2.0
Dihydrofurans	Furanones	L-Ascorbic acid 2-glucoside[b]	2.3E-07	3.1	1.3	2.4
		3-Hydroxy-4,5-dimethyl-2(5H)-furanone[c]	7.1E-07	2.6	2.2	1.2
Fatty Acyls	Fatty acid esters	Sorbate[*, c]	9.1E-10	1.8	n.s.	0.9
	Fatty acids and conjugates	Ethylmalonic Acid[c]	1.4E-02	1.7	n.s.	1.3
		Hydnocarpic acid[b]	6.3E-04	2.6	1.3	2.1
		Hydroxy-methylsuccinic acid[*, c]	1.0E-16	1.7	0.3	5.3
	Lineolic acids and derivatives	Methyl Jasmonate[c]	3.3E-11	n.s.	n.s.	2.3
Glycerophospholipids	Glycerophosphates	Sn-Glycero-3-Phosphocholine[c]	1.0E-16	n.s.	n.s.	7.1

Hydroxy acids and derivatives	Beta hydroxy acids and derivatives	O-Methyl-(epi)catechin-sulfate[*, b]	1.0E-16	2.8	0.4	7.3
Imidazopyrimidines	Purines and purine derivatives	1,3,7-Trimethyluric acid*[b]	1.9E-06	4.5	1.3	3.4
		1,3-Dimethyluric acid*[,a]	1.2E-06	2.8	1.3	2.1
		1,7-Dimethyluric acid*[,a]	1.1E-06	2.5	1.1	2.2
		1-Methyluric acid[*,a]	1.0E-16	2.1	1.1	2.0
		3,7-Dimethyluric acid[*,a]	1.0E-16	1.9	0.2	8.1
		3-Methyluric acid[a]	1.0E-16	2.1	0.4	5.8
		3-Methylxanthine[*, b]	1.0E-16	1.8	0.3	5.4
		7-Methylxanthine[*, b]	1.0E-16	1.7	0.3	4.9
		1-Methylxanthine[b]	1.5E-06	2.5	1.3	1.9
		Caffeine[*, a]	5.3E-09	4.0	1.2	3.3
		Paraxanthine[*, a]	1.0E-16	1.8	0.3	6.7
		Theobromine[*, a]	4.0E-07	2.2	1.1	2.0
		Theophylline[*,a]	1.5E-07	2.9	1.3	2.3
		Uric acid*[,c]	1.2E-02	1.1	1.2	1.1
Indoles and derivatives	Indoles and derivatives	Indole[c]	4.1E-04	n.s.	n.s.	1.6
	Indolyl carboxylic acids and derivatives	3-Methylindolepyruvate*[,b]	4.6E-10	4.7	1.9	2.4
		Indole-3-methylethanoate[c]	2.1E-10	3.3	1.8	1.9
	Tryptamines and derivatives	5-Hydroxy-L-Tryptophan[c]	1.1E-04	0.9	1.4	0.7
Keto acids and derivatives	Gamma-keto acids and derivatives	Hydroxy-oxovaleric acid[c]	7.0E-09	1.9	1.3	1.5
	Medium-chain keto acids and derivatives	2-Oxoadipate[c]	1.3E-02	n.s.	n.s.	1.5
	Short-chain keto acids and derivatives	Oxo-pentenoic acid[c]	1.4E-02	1.4	1.1	1.3
Organonitrogen compounds	N-arylamides	5-Acetylamino-6-amino-3-methyluracil[*,b]	1.0E-16	2.7	1.1	2.3
Organooxygen compounds	Alcohols and polyols	3-Dehydroshikimate[*]	6.2E-12	2.1	n.s.	n.s.
		Pantothenic acid[b]	4.2E-11	4.5	2.4	1.9
	Carbohydrates and carbohydrate conjugates	D-Sorbitol[c]	1.0E-16	7.8	n.s.	n.s.
		Galactitol*[,c]	3.8E-02	1.2	0.6	2.0
		1-Ribosylnicotinamide[b]	2.4E-08	2.7	1.4	2.0
		D-Glucuronic Acid[c]	3.0E-04	2.0	n.s.	n.s.
		Inosine monophosphate[c]	1.0E-16	9.0	n.s.	n.s.
		Mannitol[c]	1.0E-16	10.0	n.s.	n.s.

		Raffinose[c]	1.0E-16	n.s.	n.s.	8.8
	Carbonyl compounds	1-Methyl-2-pyrrolecarboxaldehyde*[,c]	1.1E-03	1.8	n.s.	1.8
Peptidomimetics	Hybrid peptides	Carnosine[c]	9.0E-09	5.5	n.s.	n.s.
Phenyl-γ-valerolactones and phenylvaleric acids	Phenyl-γ-valerolactones	5-(3′-Hydroxyphenyl)-γ-valerolactone[*,b]	1.0E-16	n.s.	n.s.	17.4
		5-(Phenyl)-γ-valerolactone-3′-sulfate[*,b]	1.0E-16	n.s.	0.05	n.s.
		5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone[b]	1.0E-16	n.s.	0.02	n.s.
		5-(4′-Hydroxyphenyl)-γ-valerolactone-sulfate[*,b]	1.0E-16	n.s.	0.04	1.2
		5-(4′-Hydroxyphenyl)-γ-valerolactone-sulfate[b]	1.7E-02	1.1	0.85	1.2
		5-(4′-Hydroxyphenyl)-γ-valerolactone-O-glucuronide[*,b]	1.0E-16	n.s.	0.04	n.s.
		5-(Methoxyphenyl)-γ-valerolactone-sulfate[c]	1.0E-16	n.s.	n.s.	8.3
		5-(Dihydroxyphenyl)-γ-valerolactone-sulfate (3′,4′,5′)*[,c]	1.4E-08	n.s.	n.s.	2.4
	Phenylvaleric acids	5-(Hydroxyphenyl)valeric acid*[,c]	1.7E-02	0.5	1.0	2.1
		5-(Phenyl)valeric acid-sulfate*[,c]	1.9E-03	0.3	0.8	3.1
Phenols	Benzenediols	3,4-Dihydroxyphenylacetate[c]	4.5E-03	1.8	n.s.	n.s.
		3-Hydroxymethyl-phenol*[,c]	6.0E-03	1.7	1.5	1.1
		Dopamine*[,c]	1.8E-08	1.4	1.0	1.4
	Benzenetriols and derivatives	4-Hydroxycatechol[c]	5.7E-09	3.0	2.8	1.1
		Methylcatechol sulfate*[,b]	1.1E-08	1.4	0.8	1.7
		Methylpyrogallol sulfate[*, c]	1.0E-10	1.9	1.3	1.5
	Methoxyphenols	3-Methoxy-4-Hydroxymandelate[c]	2.1E-07	n.s.	n.s.	2.4
		3-Methoxytyramine*[,c]	1.0E-04	1.9	n.s.	n.s.
		4-Hydroxy-3-Methoxyphenylglycol [c]	2.1E-04	n.s.	n.s.	2.3
		DL-Normetanephrine*[,c]	3.6E-03	n.s.	n.s.	1.5
		Vanillin 4-sulfate[*,b]	1.0E-16	2.5	0.7	3.4
Prenol lipids	Sesquiterpenoids	Farnesyl Diphosphate[c]	1.3E-02	n.s.	n.s.	2.7
Purine nucleosides	Purine 2'-deoxyribonucleosides	Deoxyadenosine[c]	1.0E-16	17.6	n.s.	3.9
		Deoxyadenosine monophosphate[c]	1.1E-11	3.2	n.s.	n.s.
Pyridine nucleotides	Nicotinamide nucleotides	Nicotinamide Mononucleotide[c]	1.0E-16	n.s.	0.03	n.s.
Pyridines and derivatives	Pyridinecarboxylic acids and derivatives	5-Hydroxy-6-methylnicotinic acid *[,b]	1.7E-03	3.3	2.2	1.5
		6-amino nicotinamide[*, b]	1.0E-16	2.0	0.3	6.6
		N,N-Diethylnicotinamide[b]	5.7E-03	1.4	0.8	1.6

	Hydropyridines	2-Hydroxypyridine[*, c]	6.7E-12	3.0	1.1	2.7
		2,6-Dihydroxypyridine[c]	2.7E-05	1.6	1.2	3.0
Steroids and steroid derivatives	Steroidal glycosides	estriol 3-glucuronide[*, b]	3.5E-13	3.6	0.9	4.1
	Bile acids, alcohols and derivatives	Taurocholic Acid[c]	2.9E-13	n.s.	14.2	n.s.
	Estrane steroids	Estriol *[,b]	9.6E-05	2.7	1.2	2.3
		2-methoxy-17beta-estradiol 3-glucosiduronic acid[b]	1.5E-02	2.4	1.3	1.9
		3-O-(Carboxymethyl)estrone *[,b]	2.7E-03	2.5	1.5	n.s.
	Pregnane steroids	Progesterone[c]	1.0E-16	3.8	n.s.	n.s.
Benzene and substituted derivatives	Miscellaneous	Mandelic acid *[,c]	1.4E-03	1.8	2.0	0.9
		Phenylacetic Acid *[,b]	1.1E-03	1.7	0.9	2.0
		Phenylethanol[c]	1.8E-07	2.1	n.s.	n.s.
Benzimidazoles		Dimethylbenzimidazole[c]	3.1E-06	5.8	n.s.	n.s.
Miscellaneous		((17-Oxoestra-1,3,5(10)-trien-3-yl)oxy)acetic acid *[,b]	7.5E-09	3.2	0.8	3.8
		2-Deoxyinosose[*, b]	1.0E-16	1.6	0.3	5.4
		4-Oxocyclohexanecarboxylic acid *[,b]	4.4E-09	1.5	0.8	1.8
		Guanidino-butanol[b]	5.1E-02	1.3	2.7	0.5
		Hydroxy-(indol-yl)ethanamine[c]	1.1E-02	1.0	1.2	0.8
		Trigonelline[*, b]	2.9E-10	2.2	1.3	1.7
		Trihydroxy-5alpha-cholan-24-yl sulfate[*, c]	1.0E-16	3.7	1.7	n.s.
		Kahweol oxide glucuronide[*, b]	2.6E-13	3.7	0.8	4.4
Phenol esters		Phenyl Acetate[*,c]	1.0E-16	4.0	n.s.	n.s.
Phenylpropanoic acids		3-(2-Hydroxyphenyl)Propanoate *[,c]	4.8E-05	1.0	1.9	0.5
		3-(3′-Hydroxy-4′-methoxyphenyl)propionic acid[c] (Dihydroisoferulic acid)	1.1E-03	1.8	2.2	n.s.
		3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid[b] (Dihydroferulic acid)	3.9E-03	1.8	2.1	0.5
		3-(4-Hydroxyphenyl)Lactate[b]	9.1E-07	2.2	n.s.	n.s.
Purine nucleosides		2-Aminoadenosine[*, c]	1.4E-03	0.9	0.7	1.3
		Inosine[*, c]	4.4E-03	n.s.	n.s.	1.8
Pyrrolizines		2,3-Dihydro-5-(3-hydroxypropanoyl)-1H-pyrrolizine[b]	8.4E-03	3.0	1.3	2.3

Class and Sub class have been taken from Human Metabolome Database (HMDB); q value is the FDR adjusted p-value. n.s.: not significant according to p-value<0.01 in the 2-groups comparison.
[*] indicates metabolites common to both to both corrected p-Value<0.01 and VIP>1.2.
[a] identified metabolites (level I).
[b] putatively identified metabolites (level II).

687 [c] putatively characterized metabolites (level III)
688 1C, 3C and PC are the three treatments under investigation, respectively characterized by the consume for a month of one cup of espresso coffee/day, three cups of espresso coffee/day and one cup of espresso coffee/day and
689 two cocoa-based products containing coffee (CBPCC) twice per day.

690