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Untargeted UPLC-MS metabolomics reveals multiple changes of urine composition in healthy adult volunteers after consumption of *curcuma longa* L. extract

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ABSTRACT

Curcuma longa L. is used as food supplement to prevent diseases, although limited studies have been performed on healthy subjects up to now. In the present work, an untargeted UPLC-MS metabolomics approach was applied to study the changes of 24-hours urinary composition on healthy volunteers due to a 28-days daily consumption of a dried *C. longa* extract containing a standardized amount of curcuminoids. Changes in the excretion of different metabolites were observed after supplementation. Curcumin and two metabolic derivatives (hexahydrocurcumin and dihydrocurcumin) were detected in urine, indicating the absorption of the main curcuminoid from the extract and its further metabolism by liver and gut microbiota. For the first time *ar*-turmerone, the main apolar constituent of curcuma, was detected in urine in intact form, and its presence was confirmed by a targeted GC-MS analysis. The increase of tetranor-PGJM and tetranor-PGDM, two prostaglandin-D2 metabolites, was observed, being related to the anti-inflammatory effect exerted by curcuma. The variation of the amounts of HPAG, PAG, proline-betaine and hydroxyphenyllactic acid indicate that the supplementation induced changes to the activity of gut microbiota. Finally, the reduced excretion of niacin metabolites (nicotinuric acid, trigonelline and 2PY) and medium- and short-chain acylcarnitines suggests that curcuma could induce the mitochondrial β -oxidation of fatty acids for energy production in healthy subjects.

Overall, the results indicate that a prolonged daily consumption of a dried curcuma extract exerts multiple effects on healthy subjects, furthermore they show the opportunity offered by untargeted metabolomics for the study of the bioactivity of natural extracts in healthy human volunteers.

1. Introduction

Curcuma longa and curcuma extracts are largely used in phytotherapy and as food supplements due to their supposed health-promoting effects. *C. longa* is well-known for its content in curcuminoids, and among all curcumin, a polyphenol that has been largely investigated for its multiple preventive effects against several diseases, ranging from inflammation to cancer and metabolic disorders (Xu et al., 2018). Many research papers and reviews point out the potential of curcuma and curcuminoids as medicinal species and health-promoting products, mainly due to their anti-inflammatory and antioxidant

activities. On the other hand, the same studies report that their use as drugs and food supplements is hampered by their poor bioavailability and extensive metabolism (Bavarsad et al., 2019; Grover, Shah, Khullar, Gupta, & Behl, 2019; Kwiecien et al., 2019; Li et al., 2019; Salehi et al., 2019; Sanidad, Sukamtoh, Xiao, McClements, & Zhang, 2019; Sun et al., 2018; Sut, Baldan, Faggian, Peron, & Dall'Acqua, 2016; Willenbacher et al., 2019), and results from meta-analyses are discordant (Tabrizi et al., 2019; White, Pasupuleti, Roman, Li, & Hernandez, 2019). Many reasons can be ascribed to the inconsistent results of the clinical trials, but, among the others, the different quality of used products and the experimental design mainly involving pathological models are the most

Abbreviations: GC-MS, Gas Chromatography coupled to Mass Spectrometry; HPLC-DAD, High Performance Liquid Chromatography coupled to Diode Array Detector; NMR, Nuclear Magnetic Resonance; OPLS-DA, Orthogonal Partial Least Squares Discriminant Analysis; PCA, Principal Component Analysis; PLS-DA, Partial Least Squares Discriminant Analysis; RT, Retention Time; UPLC-MS, Ultra Performance Liquid Chromatography coupled to Mass Spectrometry; UPLC-QTOF, Ultra Performance Liquid Chromatography coupled to Quadrupole-Time of Flight Detector

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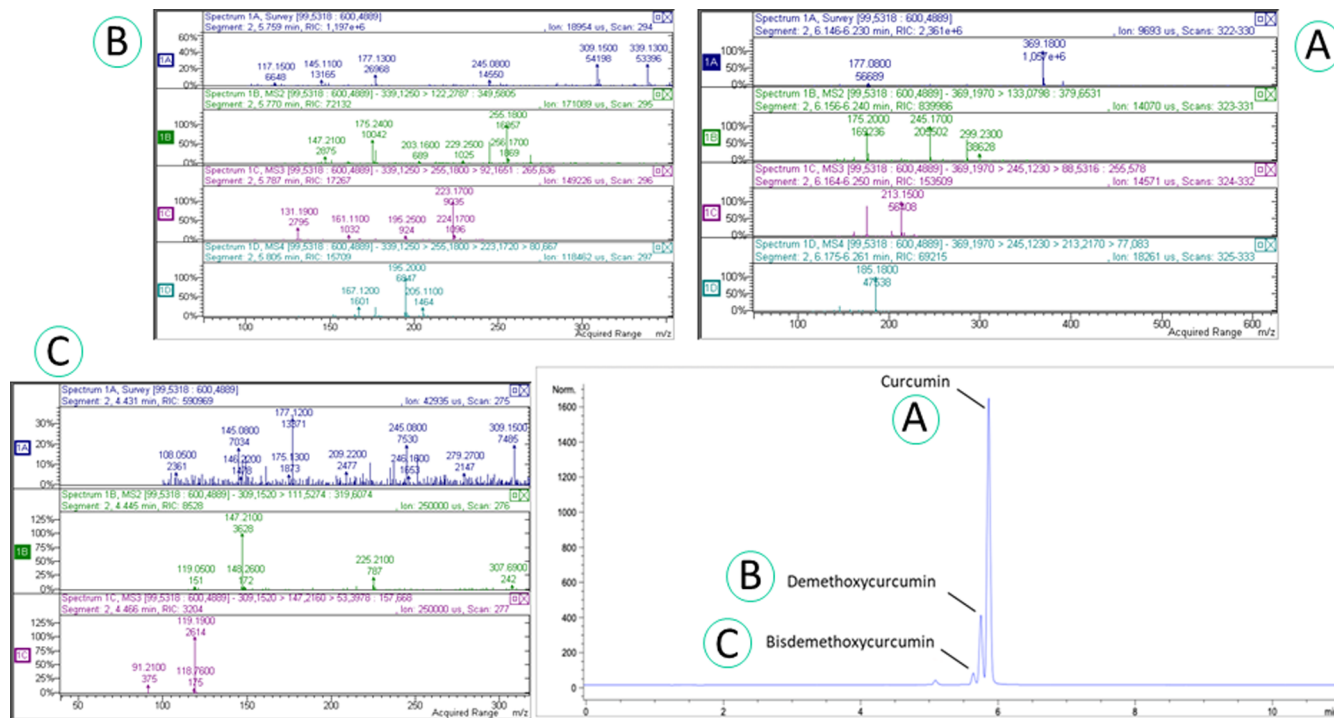


Fig. 1. Chromatogram and fragmentation spectra obtained from the HPLC-DAD-MSⁿ analysis of curcuminoids in *C. longa* extract. The spectra correspond to: A) curcumin; B) demethoxycurcumin; C) bisdemethoxycurcumin.

important. For a better evaluation of the efficacy of curcuma as food supplement and to obtain more reliable data from clinical trials, different research approaches should be adopted, and more specific experimental trials dedicated to nutraceuticals should be performed.

Metabolomics is an approach that is gaining popularity in the study of the complex bioactivity of natural products and nutraceuticals *in vivo*. The use of sensitive and robust instrumentations (UPLC coupled to High Resolution MS, NMR) together with multivariate statistical analysis allow to monitor the complex effects of an intervention on the metabolic composition of biofluids, and finally to correlate these variations to the perturbation of specific metabolic pathways induced by treatment (Wang et al., 2017). Among the biofluids, urine is the most attractive, being the richest sources of metabolic end-products and being its collection scarcely invasive (Zhang & Watson, 2015). Furthermore, it is easily available and sample treatment prior to analysis is relatively simple compared to other biofluids (Law, Han, Mao, & Zhang, 2017).

In two previous works, we used an integrated UPLC-MS and NMR approach to assess the effects of a prolonged supplementation with a dry curcuma extract on urine composition in healthy rats (Dall'Acqua et al., 2014; Dall'Acqua et al., 2016). As a continuation of these works, in this paper we report the study of the effects of a supplementation with a standardized *C. longa* extract on the composition of 24-h urine in healthy adult volunteers. In the pilot study here presented, eight volunteers consumed daily, for 28 days, 500 mg of standardized *C. longa* extract (containing 20% w/w of curcuminoids and 10 mg/g of *ar-turmerone*), and the effects of supplementation were studied monitoring the composition of 24-h urine by a UPLC-QTOF untargeted approach. The aim here was to describe the possible multiple effects of curcuma supplementation in healthy subjects with attention to urinary markers related to inflammation, oxidative status and exogenous metabolites derived from curcuma intake, using a non-invasive approach.

2. Materials and methods

2.1. Materials

The standardized *C. longa* dry extract was obtained from a local supplier. The product was formulated in tablets, each one containing 500 mg of extract (equivalent to 100 mg of curcuminoids) and excipients: cellulose, calcium phosphate, magnesium salts of fatty acids, silicon dioxide, sodic carboxymethylcellulose, hydroxypropylmethylcellulose, and glycerol. Standard curcumin and chloroxylenol were purchased from Sigma Aldrich (Milan, Italy). LC-MS-grade acetonitrile (HiPerSolv CHROMAN-ORM®), formic acid and all the solvents used were purchased from VWR (Milan, Italy). Deionized water used in UPLC analyses was filtered through a Milli-Q system equipped with a 0.22 µm cut-off filter (Millipore).

2.2. Characterization of curcuminoids in dry *C. Longa* extract

10 mg of dried material were weighted and sonicated with 20 mL of methanol for 10 min. Solvent was decanted and separated, then further 15 mL of solvent were used. Liquids were collected, and the volume was adjusted to 50 mL. For the quantification of curcuminoids, an Agilent 1260 HPLC system was used, coupled to an Agilent 1260 diode array detector (DAD) operating at 425 nm and to a Varian 500 MS Ion-Trap mass spectrometer, operating with Electrospray (ESI) source in positive mode. An Agilent Eclipse XDB C-8 column (3.0 mm × 150 mm, 3.5 µm) was used as stationary phase. The mobile phase was composed of solvent A (acetonitrile) and solvent B (water with 1% formic acid). Linear gradients of A and B were used, as follows: 0 min, 30% A; 6 min, 90% A; 9 min, 90% A; 11 min, 30% A. The flow rate was 300 µL/min and the injection volume was 5 µL. Standard solutions of curcumin were prepared in the range 5–50 µg/mL and were used for calibration. The curve was as follows: $y = 48.3x + 8.3$ ($R^2 = 0.9996$). Mass spectra were acquired in the range 100–1000 Da, and the MS parameters were as follows: nebulizing gas, nitrogen; nebulizing pressure, 25.0 psi; needle voltage, 5000 V; capillary voltage, 70.0 V; drying gas temperature, 280 °C; drying gas pressure, 25.0 psi. Three curcuminoids were detected

in the *C. longa* extract, namely curcumin (m/z 369; $19.66 \pm 0.23\%$ w/w), demethoxycurcumin (m/z 339; $1.71 \pm 0.02\%$ w/w) and bisdemethoxycurcumin (m/z 309; $0.88 \pm 0.01\%$ w/w). Compounds were identified by comparison with standard curcumin and comparing the UV-Vis spectra and the MS fragments with literature (Jiang, Somogyi, Jacobsen, Timmermann, & Gang, 2006). A representative chromatogram and the MSⁿ spectra corresponding to the three identified compounds are reported in Fig. 1.

2.3. Extraction and characterization of the apolar fraction of dry *C. Longa* extract

The dried extract (2.5 g) was added of internal standard (chloroxilenol, 200 µg/mL solution) and extracted in a Clevenger apparatus for 3 h, using water (250 mL). The distillate was added of diethyl ether (2 mL) and extracted in separating funnel. The organic layer was finally used for GC-MS analysis. An Agilent 5790 system with single quadrupole MS was used. An HP-5 MS column was employed as stationary phase. For the separation of the apolar constituents of *C. longa* extract, a temperature gradient was programmed, as follows: start from 55 °C, isothermal for 5 min; increase to 260 °C at 5 °C/min; finally, increase to 295 °C at 20 °C/min. Compounds were identified on the basis of their MS spectrum (NIST, 2018) and by calculation of their Kovats index, as reported in NIST database.

Six apolar constituents were characterized in *C. longa* extract, as indicated in Table 1. To estimate their quantitative amounts, we used chloroxilenol as reference compound, assuming the same response factor of the curcuma constituents. Thus, a relative quantification was performed. The most abundant was identified as *ar*-turmerone, with an average relative amount of 10.3 ± 0.02 mg/g. This terpenoid has been already reported as one of the main apolar constituents of *C. longa* by other authors (Dosoky, Satyal, & Setzer, 2019).

2.4. Human pilot trial methodology

Eight healthy adult volunteers (four females and four males, 40 ± 12 y. o., body mass index (BMI) of $18.4\text{--}23.8$ kg m⁻²) were enrolled for the study and informed consent was obtained. The protocol was conducted in compliance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines. The inclusion criteria were as follows: healthy adult male and female subjects aged 18–55 years, not pregnant, without chronic inflammatory (e.g., arthritis, psoriasis and inflammatory bowel disease), cardiovascular (e.g., hypertension) and metabolic diseases (e.g., diabetes, dyslipidemia), having a BMI of $18\text{--}30$ kg m⁻². All subjects were non-smoking and normally active. No medication was taken during the study (wash out period: 7 days). From 7 days before the onset of the study and during the trial, the participants were asked to maintain their usual diet and to avoid the consumption of *Curcuma* species, ginger and dietary supplements, except for the one provided for the study. 24-h control urine was collected at the last day of weeks 1st, 3rd and 4th and, after every harvesting, urine was divided in aliquots of 50 mL in Falcon tubes,

centrifuged at 5,000 rpm for 10 min (centrifuge model: Eppendorf 5702) and finally stored at -80 °C until analysis. After the first 4 weeks, the volunteers started to consume 1 tablet of supplement per day, equivalent to a daily *C. longa* dose of 500 mg (100 mg of curcuminoids), and they collected 24-h urine at the last day of the 1st, 3rd and 4th weeks of treatment. As described before, urine was divided in aliquots of 50 mL in Falcon tubes, centrifuged (5,000 rpm for 10 min) and finally stored at -80 °C until analysis.

2.5. UPLC-QTOF analysis of 24-h urine

To obtain a metabolite profiling of urine samples, a Waters H-Class UPLC system equipped with a Waters Xevo G2 Q-TOF MS was used. The detector was equipped with an ESI ionization source and was operating in positive mode. The sampling cone voltage was adjusted to 40 V, the source offset at 80 V. The capillary voltage was adjusted to 3 kV. The nebulizer gas used was N₂ at a flow rate of 800 L/h. The desolvation temperature was 400 °C. The mass accuracy and reproducibility were maintained by infusing lock mass (leucine-enkephalin, $[M + H]^+ = 556.2771$ m/z) thorough lockspray at a flow rate of 20 µL/min. Centroided data were collected for each sample in the mass range 50–1200 Da, and the m/z value of all acquired spectra was automatically corrected during acquisition based on lock mass. MS^c experiment was simultaneously performed to collect structural information, setting the collision energy to 30 V. An Agilent Zorbax Eclipse XDB C8 (2.1×150 mm, 3.5 µm) column was used as stationary phase. The mobile phase was composed of solvent A (0.1% formic acid in acetonitrile) and solvent B (0.1% formic acid in water). Linear gradients of solvents A and B were used, as follows: 0–2 min, 8% A; 15 min, 48% A; 18 min, 100% A; 20 min, 100% A; 20.5 min, 8% A; 24 min, 8% A. The flow rate was 250 µL/min and the injection volume was 1 µL. Collected urine samples were centrifuged at 13,000 rpm for 10 min (centrifuge model: Eppendorf 5427 R) and then they were randomized and directly injected in the UPLC. A pooled quality control sample (QC) was used to monitor the instrument performance. It was prepared mixing 200 µL of each test urine sample and centrifuging the mixture at 13,000 rpm for 10 min. QC sample was divided in ten aliquots, that were randomly injected throughout the sequence list.

2.6. Statistical data analysis

Centroided and integrated UPLC-MS data were processed using MarkerLynx Application Manager version 4.1 (Waters) to generate a multivariate data matrix. The parameters used for deconvolution, alignment and peak detection were retention-time (RT) range 1–15 min, mass range 50–1200 Da, mass tolerance 0.01 Da. Noise elimination level was set to 6.00, minimum intensity was set to 15% of base peak intensity, maximum masses per RT was set to 6 and, finally, RT tolerance was set to 0.01 min. Isotopic peaks were excluded from analysis. A list of the ion intensities of each peak detected was generated, using RT and the m/z data pairs as the identifier for each ion. The resulting three-dimensional matrix, containing arbitrarily assigned peak index (RT- m/z pairs), sample names (observations), and ion intensity information (variables), was used to perform multivariate analysis in MetaboAnalyst 4.0 platform (<http://www.metaboanalyst.ca/>) (Chong et al., 2018). Variables having > 50% of missing values were excluded and data were then normalized by sum, log-transformed and Pareto scaled. Principal Component Analysis (PCA) was used as explorative model and to detect outliers (samples out from the 95% confident regions), while Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used for group classification and biomarkers discovery. To exclude overfitting, the OPLS-DA model was validated performing permutation test on the responses (100 random permutations), and its quality was evaluated on the basis of the obtained R²_Y and Q² values.

Table 1
Characterization of the apolar fraction of the dry curcuma extract used in the trial.

Name	CAS	RT (min)	K.I.	Relative amount (mg/g)
<i>ar</i> -Turmerol	38142-57-3	30.580	1585	0.49 ± 0.02
<i>ar</i> -Turmerone	532-65-0	32.300	1668	10.3 ± 0.02
(E)-gamma-Atlantone	108549-47-9	32.396	1699	0.23 ± 0.01
Curlone	87440-60-6	33.272	1703	0.52 ± 0.01
(6R,7R)-Bisabolone	72441-71-5	34.488	1760	0.25 ± 0.02
(E)-Atlantone	108645-54-1	35.200	1785	0.22 ± 0.01

2.7. Biomarkers identification

Following the rules of the COSMOS Metabolomics Initiative (<http://cosmos-fp7.eu/msi.html>), we performed a level 2 identification (tentative annotation) of the biomarkers. Tentative identification was performed by calculating the molecular formula by high-resolution m/z values of protonated pseudomolecular ions ($[M + H]^+$) and ion adducts, and searching for both the calculated molecular formula and accurate m/z in freely available web databases (i.e. Human Metabolome Database (HMDB), <http://www.hmdb.ca/>; Metlin, https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage; MassBank, <https://massbank.eu/MassBank/>). The maximum Δ_{ppm} tolerance between experimental and theoretical m/z values was set at 10 ppm. Possible candidates were then screened comparing the MS^e fragmentation spectra with MS/MS data available from HMDB and Metlin.

2.8. Identification of *ar-turmerone* in 24-h urine by GC-MS

To confirm the assignment of *ar-turmerone*, 24-h urine samples were analyzed by GC-MS. Before analysis, the samples were extracted with ethyl acetate and, after anhydrication, the organic layer was concentrated up to 1 mL. The GC-MS method used was the same already described in section 2.3. Also in this case, compounds were identified on the basis of their MS spectrum (NIST, 2018) and by calculation of the Kovats index, as reported in NIST database.

3. Results and discussion

3.1. Untargeted analysis of 24-h urine

A data matrix containing 1523 “RT- m/z ” features was obtained after raw data pre-processing, and it was subsequently analyzed by unsupervised PCA for a preliminary data exploration. The unsupervised analysis showed the robustness of the analytical procedure, as indicated by the QCs tightly grouped at the origin of the score plot (Figure S1), and did not show the presence of any outlier (Figure S2). However, at the same time it did not allow to highlight any difference between urine samples collected during supplementation with *C. longa* (“treated”) and controls (Figures S1 and S2). Urine samples collected at different times during supplementation (1st, 2nd and 4th week) were compared to control samples using PLS-DA, and the models showed a differentiation

between the “treated” and “control” groups at each harvesting time (Fig. 2). However, to avoid any effect of time on the data output, the entire dataset was further analyzed using OPLS-DA, and from this model the variables correlated to the effects of supplementation were selected. The obtained model was characterized by 1 parallel and 5 orthogonal components (Fig. 3). External R2Y and Q2 values were respectively 0.94 (p-value < 0.01) and 0.501 (p-value < 0.01), indicating the robustness of the model. Variables positively correlated to group separation showing + p(corr)[1] > 0.4 (Table 2) and ones negatively correlated showing − p(corr)[1] < −0.3 (Table 3) were selected as significantly descriptive of the model. The p-value of each variable was calculated by *t*-test, comparing the “control” and “treated” groups, and only variables with p-value < 0.05 were considered for the description of the effects of treatment. Among these features, 36 were tentatively identified using the accurate m/z values and the MS^e spectra. Other unidentified features are reported in the [Supplementary Material](#). A representative scheme summarizing the biological effects of curcuma observed in the study and the corresponding metabolites is reported in Fig. 4.

3.1.1. Metabolites derived from *C. Longa* intake

Ten variables were annotated as compounds present in *C. longa* extract, or their derivatives formed by gut and liver metabolism. The main constituent of *C. longa*, curcumin, was tentatively identified among these, but two of its metabolic derivatives, namely hexahydrocurcumin and dihydrocurcumin (Tan et al., 2014; Zhao et al., 2015), were detected among the variables with the highest correlation value (+ p(corr)[1] > 0.46, Table 2). Hexahydrocurcumin can be produced by both reductive metabolism of curcumin predominantly in the liver (Ireson et al., 2001; Marczylo, Steward, & Gescher, 2009) and by reduction of the same parent compound by microbial transformation in the gut, as observed *in vitro* (Younis, Ibrahim, Ibrahim, Abouloud, & Kabbash, 2016) and by detection in feces and urine (Zhao et al., 2015). Dihydrocurcumin is a constituent of *C. longa* but it could also be formed as an intermediate in the reduction of curcumin to tetrahydrocurcumin and hexahydrocurcumin (Ireson et al., 2001), or from the degradation of the same parent compound by different colonic bacteria (Tan et al., 2014). The identification of these metabolites among the most important in describing the effects of supplementation on urine composition confirms that curcumin undergoes an extensive metabolism by gut microbiota and phase I enzymes in the liver.

Other compounds correlated to the metabolism of curcumin were

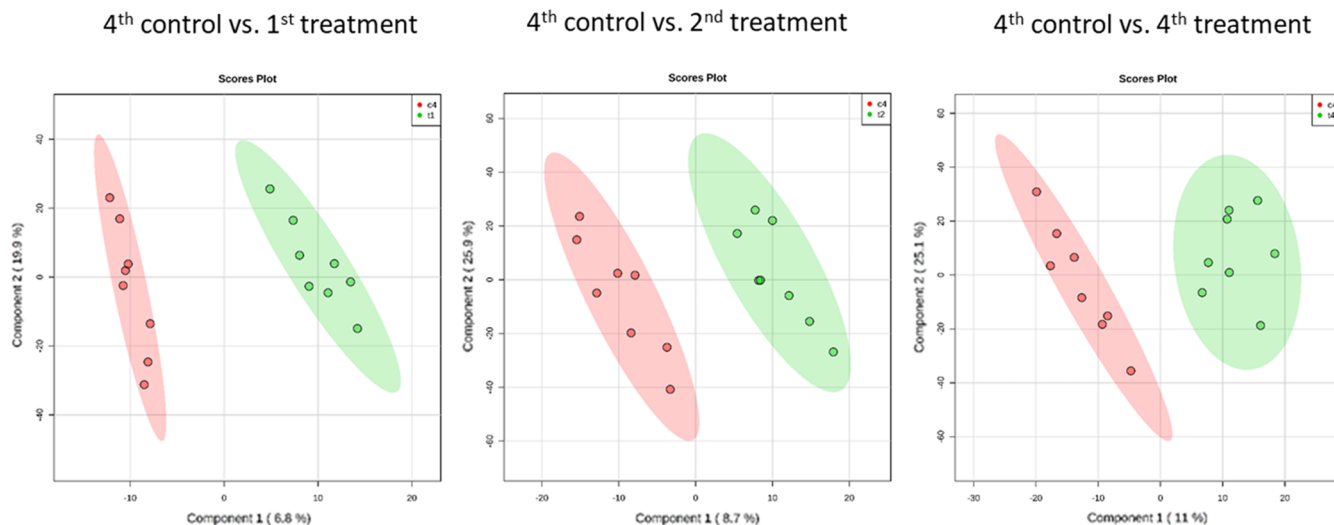


Fig. 2. PLS-DA plots showing comparisons between urine samples collected at the 1st, 2nd and 4th weeks of supplementation (treatment) with *C. longa* extract (green dots) and control urine samples collected at the 4th week (red dots). The circles around observation groups in the score plot display the 95% confidence regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

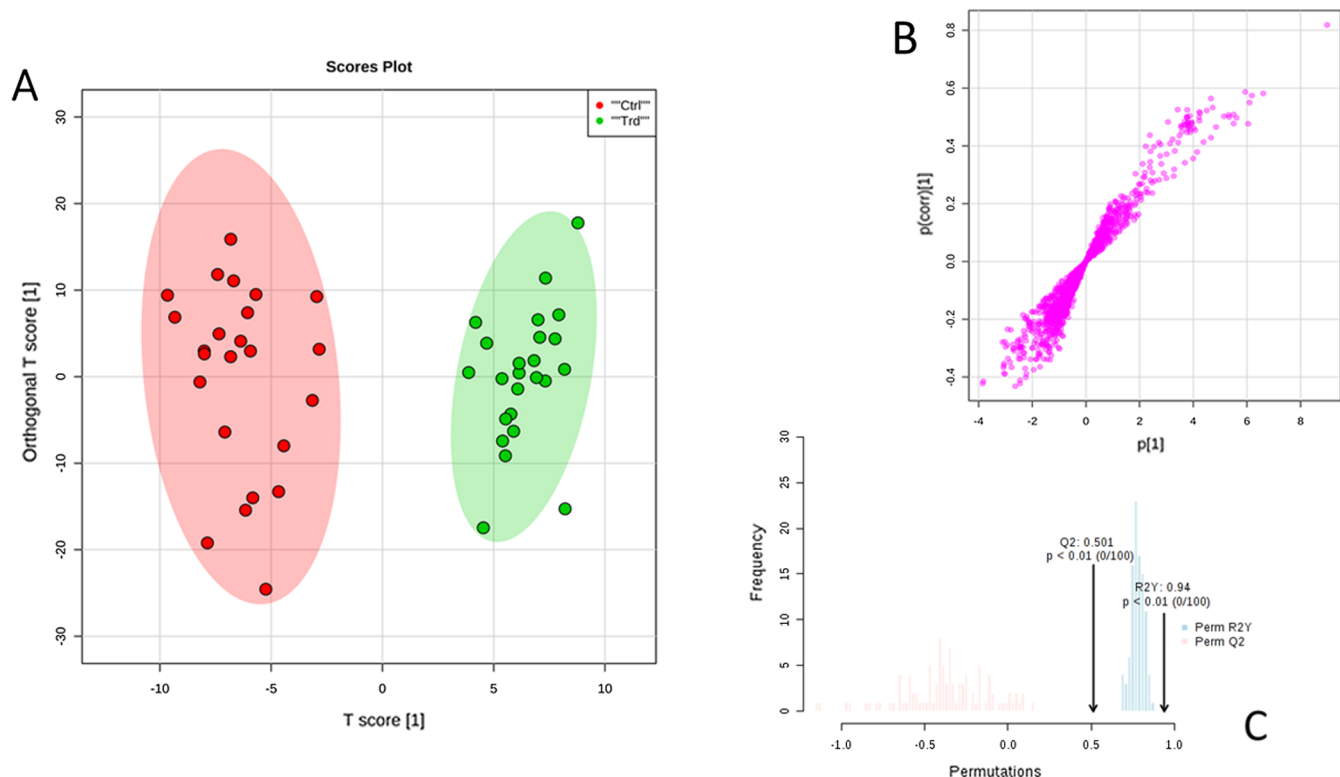


Fig. 3. A) Score scatter plot obtained from OPLS-DA of the entire dataset, including the samples collected at the 1st, 2nd and 4th weeks of control and treatment. Control urine samples are represented by red circles while samples collected during supplementation with *C. longa* are represented by green circles. The circles around observation groups in the score plot display the 95% confidence regions; B) Loadings plot of the same OPLS-DA model; C) Validation parameters obtained from permutation test, setting 100 random permutations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Variables positively correlated to supplementation with *C. longa* ($p < 0.05$; $p(\text{corr})[1] > 0.4$). The features are divided in classes based on the metabolic pathway in which they are involved. Tentative identification (level 2 annotation) was performed searching for accurate m/z values in freely available web databases (HMDB and Metlin) and on the basis of the MS^c spectra.

	$p(\text{corr})[1]$	$p[1]$	$\log_2(\text{fold change})$	m/z	Main fragments (m/z)	RT (min)	Tentative ID (*)	METLIN ID
Curcuminoids and metabolites/ <i>C. longa</i> constituents	0.818	9.49	3.34	357.1689	177.0537, 122.0378	10.9	Hexahydrocurcumin($[M + H - H_2O]^+$)	71,920
	0.550	6.02	2.54	501.1005	291.0854, 177.0586, 153.0529	11.3	Hesperetin 7-O-glucuronide	96,103
	0.504	3.78	1.34	163.0748	127.532, 91.0567, 77.0400	12.4	4-Hydroxycinnamoylmethane	69,300
	0.497	3.75	1.77	153.0542	65.0372	7.9	Vanillin	62,927
	0.487	4.19	2.09	395.0943	197.0764, 149.0576	8.8	Dihydroferulic acid 4-O-glucuronide**	96,081
	0.485	3.68	1.70	195.0650	145.0277, 117.0351	7.9	Ferulic acid	4156
	0.477	6.44	2.81	409.0963	177.0545, 122.0368	9.7	Dihydrocurcumin($[M + K]^+$)	87,760
	0.457	3.77	2.15	369.1519	177.0535, 145.0285, 117.0348	10.8	Curcumin	44,447
	0.413	4.60	1.75	377.1065	195.0034	10.2	Ferulic acid 4-O-glucuronide($[M + Li]^+$)	96,091
	0.401	4.16	1.95	239.1387	119.0858, 91.0569	11.2	<i>ar</i> -Turmerone($[M + Na]^+$)	90,885
Prostaglandines related to inflammation	0.500	5.12	1.32	311.1484	229.1218, 211.1087, 191.0674	10.7	tetranor-PGJM	96,506
	0.479	3.79	1.29	329.1594	275.1256, 229.1221, 211.1089	10.6	tetranor-PGDM	45,422
Metabolism of fatty acids	0.499	4.72	1.77	146.0806	87.0419	3.4	3-Hydroxybutyric acid($[M + ACN + H]^+$)	85,159
	0.483	3.06	1.70	253.1432	213.1465, 167.1461	12.3	Dodecanedioic acid	5596
Activity of gut microbiota	0.473	4.23	1.41	365.1202	183.0643	12.3	Hydroxyphenyllactic acid($[2M + H]^+$)	34,515
	0.469	3.72	1.29	144.1016	98.0993, 68.0468	2.4	Proline-betaine***	7089
Other	0.581	6.72	3.37	230.0484	91.0567	2.6	Benzyl sulfate($[M + ACN + H]^+$)	90,204
	0.497	5.12	2.04	693.3329	339.3236	10.7	PI(22:1(11Z)/0:0)	81,187

*In brackets are indicated the adducts ion, when different to $[M + H]^+$.

**The feature showing $p(\text{corr})[1] = 0.448$, $m/z = 390.1392$ and $RT = 8.8$ min was identified as the $[M + NH_4]^+$ adduct of the same metabolite.

***The feature showing $p(\text{corr})[1] = 0.574$, $m/z = 287.1965$ and $RT = 2.2$ min was identified as the $[2M + H]^+$ adduct of the same metabolite.

Table 3

Variables negatively correlated to supplementation with *C. longa* ($p < 0.05$; $-p(\text{corr})[1] < -0.3$). The features are divided in classes based on the metabolic pathway in which they are involved. Tentative identification (level 2 annotation) was performed searching for accurate m/z values in freely available web databases (HMDB and Metlin) and on the basis of the MS^e spectra.

	$-p(\text{corr})[1]$	$-p[1]$	$\log_2(\text{fold change})$	m/z	Main fragments (m/z)	RT (min)	Tentative ID (*)	METLIN ID
Acylcarnitines	-0.356	-2.79	-1.61	314.2325	85.0284	13.9	9-Decenoylcarnitine**	0013205***
	-0.356	-2.33	-1.55	312.2169	144.1024, 85.0288, 60.0795	13.6	2-trans,4-cis-Decadienoylcarnitine	0013325***
	-0.346	-2.69	-1.50	338.2323	144.1864, 85.0285, 60.4659	14	Decanoylcarnitine([M + Na] ⁺)	0000651***
Metabolism of nicotinic acid	-0.341	-2.74	-1.81	286.2000	85.0280	11.4	3-hydroxyoctanoyl carnitine ([M - H ₂ O + H] ⁺)	0061634***
	-0.332	-1.33	-1.24	256.0926	85.0288, 57.0345	2.4	Propionylcarnitine ([M + K] ⁺)	85,176
	-0.378	-1.41	-1.74	235.1187	153.0648, 135.0563, 110.0654	3.8	N1-Methyl-2-pyridone-5-carboxamide ([M + 2ACN + H] ⁺)	0004193***
	-0.358	-2.74	-1.73	155.0810	94.0661, 65.0390	2.3	Trigonelline ([M + NH ₄] ⁺)	273
	-0.329	-1.25	-1.54	198.0869	135.0543, 102.9981, 79.0424	2.2	Nicotinuric acid ([M + NH ₄] ⁺)	1499
Gut microbial activity	-0.401	-2.39	-2.68	194.0810	91.0534, 65.0390	10	Phenylacetylglutamine	4237
	-0.381	-3.05	-2.48	227.1024	107.0484, 65.0391	6.5	Hydroxyphenylacetylglutamine ([M + NH ₄] ⁺)	5703
Metabolism of amino acids	-0.354	-1.55	-1.70	196.0712	87.0549, 74.0238	3.1	L-Asparagine	14
	-0.306	-2.39	-2.02	150.0182	136.1905, 105.0987	2.6	Quinolinic acid ([M - H ₂ O + H] ⁺)	330
Bile acids and steroids	-0.423	-2.50	-1.23	463.2444	389.2694, 347.2571, 333.2411	12.4	3a,7a,12a,19-Tetrahydroxy-5b-cholanoic acid ([M + K] ⁺)	5329
	-0.326	-2.7	-1.20	377.1793	297.2213, 253.1963	8.5	17-Hydroxypregnenolone sulfate ([M - 2H ₂ O + H] ⁺)	57,973
Folate metabolism	-0.329	-2.95	-1.18	477.2235	313.1400, 194.1049	12.4	5-Methyltetrahydrofolic acid ([M + NH ₄] ⁺)	320,456
Purine metabolism	-0.361	-2.58	-1.44	151.0609	96.0548, 69.0438	6	1-Methylhypoxanthine	3779
	-0.347	-2.69	-1.25	238.0931	140.0450, 69.0441	3.9	Dimethyluric acid ([M + ACN + H] ⁺)	2822
	-0.312	-1.66	-3.08	312.1303	180.0883, 131.0345	6.7	1,7-Dimethylguanosine	58,069

*In brackets are indicated the adducts ion, when different to [M + H]⁺.

**The feature showing $-p(\text{corr})[1] = -0.450$, $m/z = 336.2168$ and RT = 13.3 min was identified as the [M + Na]⁺ adduct of the same metabolite.

***Record from HMDB.

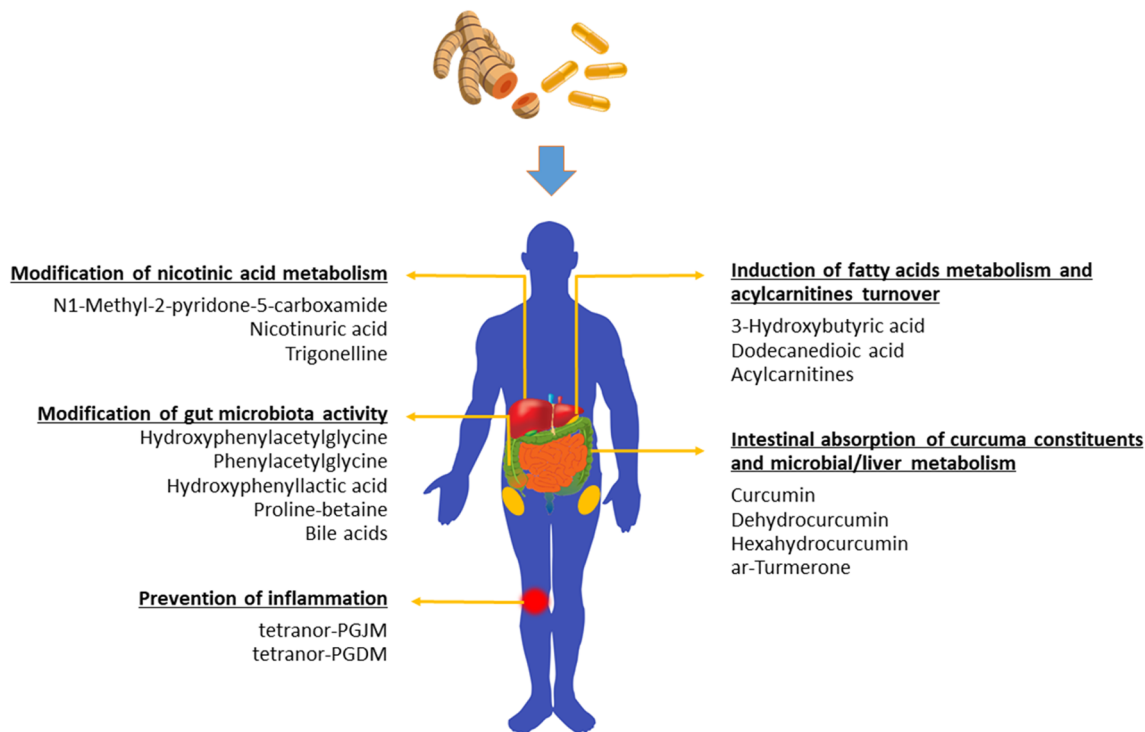


Fig. 4. Summary of the main effects of the consumption of *C. longa* dried extract observed in the study. The main metabolites identified from metabolomics analysis of 24-h urine and associated to each effect are reported.

ferulic acid and its glucuronide derivative, dihydroferulic acid 4-O-glucuronide, and vanillin (Nelson et al., 2017), although they could derive also from the metabolism of other polyphenols contained in the extract. On the other hand, 4-hydroxycinnamoylmethane was related to the degradation of bisdemethoxycurcumin.

Finally, the main constituent of the apolar fraction of curcuma extract, *ar*-turmerone, was also tentatively identified among the descriptive variables positively correlated to curcuma consumption. Its tentative identification was confirmed by comparison with reference standard through GC-MS analysis (Fig. 5). Yue and coll. showed that

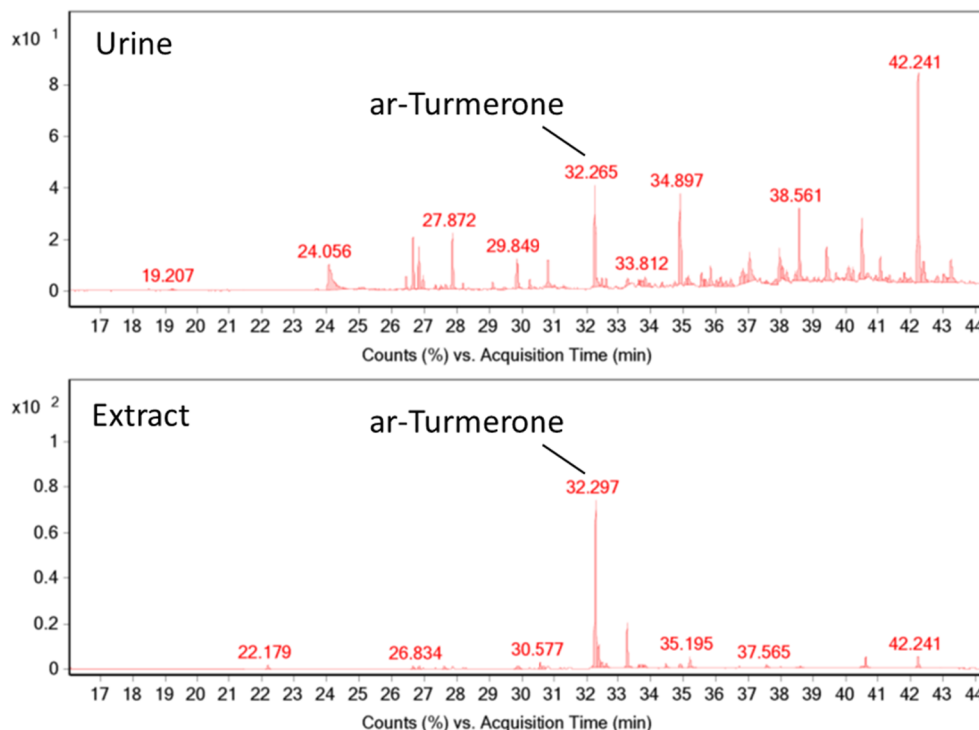


Fig. 5. GC-MS chromatograms of urine sample (upper panel) and apolar constituents from dried curcuma extract (lower panel).

the association of curcumin and *ar*-turmerone could significantly increase the permeation of the former through a Caco-2 cell monolayer, if compared to curcumin alone, hence acting as a permeation enhancer (Yue et al., 2012). To the best of our knowledge, our results are the first to show the urinary excretion of *ar*-turmerone following *C. longa* consumption, and they point out the need to consider also the apolar fraction of curcuma extracts as potential source of active constituents, in addition to curcuminoids.

3.1.2. Metabolites related to inflammation

One of the major findings of this work is the observation that the consumption of curcuma induces an increase of the urinary excretion of metabolites related to inflammation in healthy volunteers. Two variables positively correlated to *C. longa* intake were tentatively identified as tetranor-PGDM and tetranor-PGJM (Table 2), two metabolites of prostaglandin-D2 (PGD2) (Song et al., 2008).

Literature data report that the levels of PGD2 are significantly increased during inflammatory processes at the resolution phase (Trivedi et al., 2006; Zayed, Li, et al., 2008), suggesting that it could exert an anti-inflammatory activity. Other authors showed that PGD2 plays a role in the resolution of osteoarthritis (OA) (Jakob, Démarteau, Suetterlin, Heberer, & Martin, 2004; Relić et al., 2004), enhancing the expression of collagen type II and aggrecan, two cartilage-specific matrix components (Jakob et al., 2004), preventing chondrocyte apoptosis (Relić et al., 2004), and inhibiting specific matrix metalloproteinases, whose play an important role in cartilage damage (Zayed, Afif, et al., 2008).

Belcaro and coll. reported the efficacy of a daily treatment with 100 mg of curcuminoids (75% curcumin, 15% demethoxycurcumin and 10% bisdemethoxycurcumin) for 8-months in reducing both pain and stiffness associated to OA and in significantly reducing the amounts of several markers of inflammation (IL-1 β , IL-6, sCD40L, sVCAM-1 and ESR) in the joints of human patients, supporting the efficacy of curcuma extract as anti-inflammatory treatment (Belcaro et al., 2010). Our results show that the urinary excretion of PGD2 metabolites is increased after a prolonged supplementation with curcuma, thus suggesting increased levels of PGD2 and the induction of the anti-inflammatory

PGD2 pathway. However, it should be taken in account that our study does not involve any inflammation model, being the enrolled volunteers healthy. Hence, the results that we obtained can be considered as a starting point for further research aimed at investigating more in depth the role of curcuma in the prevention of inflammation and the mechanisms of action involved.

3.1.3. Metabolites related to modification of gut microbiota

The supplementation with *C. longa* induced changes to urinary metabolites that can be indirectly correlated to an effect on gut microbiota. The amounts of hydroxyphenylacetylglutamine (HPAG) and phenylacetylglutamine (PAG), two tyrosine metabolites that have been previously related to microbial activity in the intestinal lumen (Li et al., 2008; Sokol et al., 2008), were decreased in 24-h urine collected during *C. longa* consumption (Table 3). The urinary excretion of PAG, produced by *Erysipelotrichaceae* and *Allobaculum* (Barouei et al., 2017), has been found altered in animals and volunteers receiving either high fat diets (HFD) or treatments to manage obesity, as a consequence of altered gut microbiota activity. In HFD rats, for example, the urinary levels of PAG were reported to be higher compared to animals receiving a normal diet (Jambocus, 2016). In elderly subjects, urinary amounts of PAG were reduced after 4-weeks of Mediterranean diet supplemented with coenzyme Q10, in comparison with volunteers receiving a diet rich in saturated fatty acids (González-Guardia et al., 2015). Other authors reported lower urinary excretion of PAG following fecal microbial transplantation, as a consequence of increased beneficial bacteria (*Bifidobacteria* and *Lactobacilli*) (Wiest, Albillos, Trauner, Bajaj, & Jalan, 2017). On the other hand, previous studies have reported that the increased excretion of HPAG is positively correlated to the abundance of *Faecalibacterium*, *Bacteroides* and *Escherichia* genera in the intestine of patients with Crohn's disease (Jansson et al., 2009; Rojo et al., 2017).

Other metabolites correlated to the effects of curcuma on gut microbiota were tentatively identified as proline-betaine and hydroxyphenyllactic acid (Table 2) (Fletcher, Erwin, Lanzas, & Theriot, 2018; Mishima et al., 2017; Rosas et al., 2015), whose amounts were increased in urine after the intervention. Conversely, the amounts of 3a,7a,12a,19-tetrahydroxy-5b-cholanoic acid, 17-hydroxypregnenolone sulfate and 5-

methyltetrahydrofolic acid, were reduced after supplementation. They could be also related to the activity of *C. longa* extract on gut microbiota, being the colonic bacteria involved in the transformation of bile acids and steroids and in the synthesis of folic acid (LeBlanc et al., 2013; Nicholson et al., 2012).

Overall, the results suggest that curcuma constituents interact with gut microbiota and can influence its activity, and this could contribute to the health-promoting effects of this natural product in healthy subjects. It is known that dietary polyphenols from food or natural extracts are prone to metabolism by gut microbes, due to their scarce intestinal absorption that increase their permanence in the intestinal lumen. This interaction leads to the formation of low molecular weight metabolites that, interacting with the gut microbiota, could lead to effects on host health (Filosa, Di Meo, & Crispi, 2018; Peron et al., 2019). Hence, not only curcuminoids, but also other polyphenols and apolar constituents from curcuma could exert an effect on gut microbiota. However, future studies involving metabolomics and metagenomics approaches will be necessary to confirm these observations, and to better understand the effects of curcuma on the composition and the activity of the intestinal microbiota.

3.1.4. Metabolites related to increased fatty acids metabolism

Increased urinary levels of 3-hydroxybutyrate (3-HB) and dodecanedioic acid (Table 2), two metabolites correlated to mitochondrial β -oxidation of fatty acids (Benjamin, Wada, Grundy, Szuszkiewicz-Garcia, & Vega, 2016; Mingrone, Castagneto-Gissey, & Macé, 2013), were observed during supplementation with *C. longa*. Furthermore, the amounts of small- and medium-chain acyl-carnitines (C3, C8 and C10) were decreased in urine after treatment (Table 3).

An increased urinary excretion of 3-HB has been already reported in one of our previous works involving *P. cuspidatum*, in which this metabolite has been associated to the activity of resveratrol as mimetic of caloric restriction, and to the induction of fatty acids β -oxidation following its consumption (Peron et al., 2017). On the other hand, Tranchida and coll. recently reported that a treatment with curcumin led to decreased levels of 3-HB in serum (Tranchida et al., 2015) and liver (Tranchida et al., 2017) of hyperlipidemic rats, as a result of impaired fatty acids β -oxidation and ketone bodies production after curcumin consumption. However, in this case, the effects of treatment were assessed in animals affected by a pathological condition, with altered carbohydrate and lipid metabolisms (Tranchida et al., 2015; Tranchida et al., 2017), hence the two results could not be comparable, since the volunteers enrolled in our study had healthy BMI and were not affected by metabolic disorders.

Carnitine is used by the cells as a shuttle to transport activated fatty acids from the cytosol into the mitochondria for subsequent β -oxidation, aimed at energy production (Schooneman, Vaz, Houten, & Soeters, 2013). Variations of the circulating and urinary levels of acylcarnitines could give a reflection on how efficiently the organism uses its resources or if a metabolic pathway (TCA-cycle or β -oxidation) is preferred depending on the availability of certain energy substrates (carbohydrates or lipids) or in the case other metabolic pathways are impaired (Schooneman et al., 2013). Raised amounts of plasma acyl-carnitines have been associated to the deregulation of fatty acid oxidation and to an increased risk of metabolic diseases, such as diabetes (Mihalik et al., 2010). In our study, we observed that the supplementation with *C. longa* led to a reduced excretion of a pool of medium and short-chain acylcarnitines (C3, C8 and C10), and, as was already reported in animals undergoing fasting state, this could reflect an increased utilization of fatty acids by mitochondria for subsequent β -oxidation and energy production (Flanagan, Simmons, Vehige, Willcox, & Garrett, 2010; Luci et al., 2006). From these experiments in rats, the authors observed that caloric restriction causes a reduction of acetyl-CoA in the mitochondrion available for esterification of free carnitine, in order to minimize the use of carbohydrates as source of energy. The final observed effect is an increased tissue carnitine concentration and a

reduced acetylcarnitine amount (Flanagan et al., 2010; Luci et al., 2006). Overall, our results suggest that curcuma could induce the mitochondrial β -oxidation of fatty acids for energy production in healthy subjects (Benjamin et al., 2016; Flanagan et al., 2010; Mingrone et al., 2013).

3.1.5. Metabolites related to the metabolism of nicotinic acid and nicotinamide

Trigonelline, nicotinuric acid (glycine conjugate of nicotinic acid) and N1-methyl-2-pyridone-5-carboxamide (2PY) (Table 3) derive from the metabolism of nicotinic acid, and their amount were decreased after supplementation with *C. longa*. Variations of the urinary excretion of trigonelline and nicotinuric acid have been previously correlated to altered energy metabolisms in humans (Huang, Cheng, Fan, Hong, & Shiao, 2013; Palau-Rodriguez et al., 2015). In the study of Huang and coll., the urinary amounts of nicotinuric acid were reported to be increased in overweight subjects, in diabetic patients and in subjects with high levels of triglycerides, LDL cholesterol and total cholesterol (Huang et al., 2013). On the other hand, low levels of trigonelline have been reported in the urine of diabetic subjects, as an indication of an altered energy metabolism (Calvani et al., 2010). Trigonelline could be produced by gut microbiota through different pathways, among which the metabolism of niacin (Palau-Rodriguez et al., 2015) and the conversion of S-adenosylmethionine to S-adenosylhomocysteine (methionine cycle) (Calvani et al., 2010). Considering these data, lower amounts of both the markers after supplementation with *C. longa* could reflect both the effects of this natural product on lipid metabolism and its interaction with the activity of gut microbiota.

2PY is a metabolic product of N-methyl-nicotinamide (MNA), formed by the enzyme aldehyde oxidase as terminal step of the degradation of nicotinamide adenine dinucleotide (NAD) in the liver (Lenglet et al., 2016). NAD, in turn, could be synthesized in humans from dietary nicotinic acid, nicotinamide or tryptophan, through different pathways (Lenglet et al., 2016). Studies in mice showed an increased production and urinary excretion of 2PY as a response to oxidative stress *in vivo* (D. Li et al., 2015; Yang et al., 2017), correlating the urinary amounts of this metabolite to the oxidative status of the animal. However, changes in the excretion of 2PY were also reported due to variations in dietary tryptophan and due to the amount of nicotinamide biosynthesized from this latter (Fukuwatari & Shibata, 2013). Hence, the decreased urinary excretion of 2PY observed after consumption of *C. longa* extract could reflect either a modification of the metabolism of tryptophan and nicotinamide induced by treatment or an antioxidant effect exerted by the extract. However, as reported below (paragraph 3.2), we monitored the urinary excretion of three oxidative stress markers related to protein (nitrotyrosine) (Leeuwenburgh, Hansen, Shaish, Holloszy, & Heinecke, 1998), nucleic acid (8-hydroxydeoxyguanosine (8-OHdG)) (Cooke, Lunec, & Evans, 2002) and lipid (8-iso prostaglandin F2 α (8-isoPGF2 α)) (Ryu et al., 2016) oxidation in the urine samples collected during and after supplementation with curcuma, without detecting significant changes compared to control samples. Nevertheless, we cannot exclude that curcuma could act as antioxidant interfering with other oxidative pathways, hence, for a better elucidation of alternative antioxidant effects, further studies are required.

3.1.6. Purine derivatives

Other three variables negatively correlated to supplementation were tentatively identified as methylated purine derivatives, namely 1-methylhypoxanthine, dimethyluric acid and 1,7-dimethylguanosine. Purines have several biological roles and are implicated in cellular energy systems (ATP, NAD), signaling (GTP, cAMP and cGMP) and in the production of nucleic acids (Law et al., 2017). Previous studies showed an impaired metabolism of energetic purines in diabetes, as an indication of serious energy metabolism disruptions (Dudzinska, 2014). Dudzinska reported increased erythrocyte levels of hypoxanthine (an

Table 4

Urinary amounts of three oxidative stress markers monitored at the end of the 1st and 4th weeks of control and at the end of the 1st and 4th weeks of supplementation with *C. longa*, namely 8-iso prostaglandin F2 α (8-isoPGF2 α), 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine. Values are reported as AUC of the respective chromatographic peak normalized with creatinine $\pm$ SD. No significant differences were detected (p-value > 0.05).

Marker	Control		Treated	
	Day 7	Day 28	Day 7	Day 28
8-isoPGF2 α	0.15 $\pm$ 0.10	0.14 $\pm$ 0.13	0.13 $\pm$ 0.08	0.14 $\pm$ 0.06
8-OHdG	0.15 $\pm$ 0.07	0.16 $\pm$ 0.13	0.15 $\pm$ 0.09	0.17 $\pm$ 0.11
Nitrotyrosine	1.24 $\pm$ 1.27	1.38 $\pm$ 1.29	1.59 $\pm$ 0.99	1.45 $\pm$ 0.19

end product of the adenosine metabolism) as an indication of increased dephosphorylation of adenine nucleotide in type 2 diabetes (Dudzinska, 2014). An increased urinary excretion of some methylated purines was reported in pregnant women affected by gestational diabetes, among whom 1-methylhypoxanthine and 1,7-dimethylguanosine, indicating a correlation between the metabolic imbalance characteristic of diabetes and an increased metabolism of purines involved in energetic processes (Law et al., 2017). In the case of our intervention, the reduced urinary excretion of both 1-methylhypoxanthine and 1,7-dimethylguanosine could reflect the effects on energetic metabolism exerted by *C. longa*, as already described above.

3.2. Analysis of urinary oxidative stress markers

Curcuma and curcumin have been previously investigated for their antioxidant activity, and several studies report its effectiveness *in vitro* (Ak & Gülçin, 2008; Tanvir et al., 2017). However, as for many other polyphenols that show an antioxidant activity *in vitro*, there are many issues to observe the same *in vivo*. In our study, three urinary oxidative stress biomarkers were monitored, namely 8-isoPGF2 α ([M + H]⁺ *m/z* = 355.2480), 8-OHdG ([M + H]⁺ *m/z* = 284.0993) and nitrotyrosine ([M + H]⁺ *m/z* = 227.0672). These were identified in the dataset obtained from the untargeted analysis by means of their *m/z* and MS^e spectra, and their excretion throughout the experimental period was monitored using the AUC of the relative chromatographic peaks normalized with creatinine. The results (Table 4 and Fig. S3) showed no statistical difference between the amounts of the considered markers at the 1st week of supplementation and at the last week, as well as between the amounts in control samples and at the end of treatment, indicating a negligible scavenging activity of curcuma against the oxidation of lipids, nucleic acids and proteins when consumed by healthy subjects.

4. Conclusion

Urinary metabolomics revealed that the supplementation with curcuma induced the following changes in urine composition of healthy subjects. First, curcuma constituents and derivatives produced by host metabolism were detected in urine, indicating the absorption of phytoconstituents from the extract and their extensive metabolism by liver and gut microbiota. Ar-turmerone was reported here for the first time in urine after dried curcuma extract consumption. This suggests the need for considering also the sesquiterpene fraction for performing studies on this natural product.

In a previous work on animal model, we observed that different metabolic pathways are modified by curcuma administration. With this pilot trial on volunteers, we demonstrated that the consumption of curcuma leads to changes of the metabolic composition of urine also in healthy humans. In detail, compounds related to inflammation and metabolites related to fatty acid metabolism suggest a complex role of this extract on inflammatory and metabolic pathways involved in

energy production, suggesting the need for further studies in this area. Furthermore, changes of several microbial metabolites (hydroxyphenyllactic acid, proline-betaine, HPAG and PAG) suggest a role of curcuma constituents in the modulation of the microbial population in the gut.

4.1. Study limitations

The aim of our study was to perform a pilot trial to assess the effects of supplementation with a dried curcuma extract in healthy subjects. For this reason, we chose to involve the smallest number of volunteers required to obtain reliable data, considering the intrinsic metabolic variability among the subjects. Furthermore, we focused the attention on the urinary output, without considering other biological matrices as plasma or feces, whose could afford other information about the absorption, metabolism and excretion of curcuminoids from *C. longa* extracts. Untargeted metabolomics is known to be an “hypothesis generating” and not an “hypothesis driven” approach. Nevertheless, our preliminary data, as well as supporting the observations regarding the activity of *C. longa* that have been already published, could be a starting point for further investigations aimed at exploring more in depth the mechanism(s) of action of this complex natural product and its biological effects *in vivo*.

Declaration of Competing Interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2019.108730>.

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