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Potentiation of the bioavailability of blueberry phenolic compounds by co-ingested grape phenolic compounds in mice, revealed by targeted metabolomic profiling in plasma and feces†

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The low bioavailability of dietary phenolic compounds, resulting from poor absorption and high rates of metabolism and excretion, is a concern as it can limit their potential beneficial effects on health. Targeted metabolomic profiling in plasma and feces of mice supplemented for 15 days with a blueberry extract, a grape extract or their combination revealed significantly increased plasma concentrations (3–5 fold) of blueberry phenolic metabolites in the presence of a co-ingested grape extract, associated with an equivalent decrease in their appearance in feces. Additionally, the repeated daily administration of the blueberry–grape combination significantly increased plasma phenolic concentrations (2–3-fold) compared to animals receiving only a single acute dose, with no such increase being observed with individual extracts. These findings highlight a positive interaction between blueberry and grape constituents, in which the grape extract enhanced the absorption of blueberry phenolic compounds. This study provides for the first time *in vivo* evidence of such an interaction occurring between co-ingested phenolic compounds from fruit extracts leading to their improved bioavailability.

1. Introduction

In contrast to drugs, which have a targeted and well-defined molecular mechanism of action, phytochemicals act in a pleiotropic manner.[1–4] The multiple protective effects of plant bioactives on health are therefore attributed to a combined effect of several interacting compounds, rather than to a single molecule.[5,6] Positive interactions (synergism) between phytochemicals can enhance the potency of a bioactive compound, thereby leading to a combined bioactivity greater than the sum of the individual compounds, while negative interactions (antagonism) result in a reduced bioactivity from what is expected.[2] Synergies between plant bioactives have been widely suggested,[7–9] but few reports have demonstrated this phenomenon or proposed the possible underlying mechanisms. In particular, interactions between phytochemicals have been suggested to affect the way they are absorbed, metabolized and excreted,[10,11] but to date, *in vivo* evidence of an improvement of their bioavailability resulting from such interactions is lacking, owing to methodological limitations. Due to the tremendous diversity of phytochemicals, their variable absorption and their complex biotransformation, it has been almost impossible to comprehensively assess their bioavailability by conventional methods. Metabolomics now provides suitable approaches to analyze changes in metabolite profiles related to synergistic or antagonistic effects, and may thereby improve our understanding of the complex interactions inherent to multi-target and multi-component phytotherapeutics.[12]

Berries are rich sources of phenolic compounds and are receiving growing interest, because of their positive effects on health.[13] Epidemiological, pre-clinical and cell culture studies support the use of phenolic compounds to prevent chronic diseases, such as cardiovascular diseases and certain types of cancers.[14,15] In particular, blueberry and grape are being studied for their capacity to prevent neurodegeneration and cognitive decline.[16–18] The purpose of the present work was to investigate the bioavailability of phenolic compounds from blueberry and grape extracts after oral administration in mice, using targeted metabolomics in plasma and feces, and determine whether acute or chronic co-administration might affect their bioavailability.

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2. Materials and methods

2.1. Plant material, chemicals and phenolic characterization

The Neurophenols Consortium extract is a standardized phenol-rich combination of blueberry (*Vaccinium angustifolium* Ait.) and grape (*Vitis vinifera* L.) extracts. This blend and the individual extracts were provided by Nutra Canada (Canada) and Activ'Inside (France). Phenolic standards and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), with the exception of malvidin 3-glucoside, which was purchased from Extrasynthèse (Genay, France). Liquid chromatography grade solvents and acids were respectively purchased from EMD Millipore Chemicals (Billerica, MA, USA) and Anachemia (Montreal, QC, Canada). Fruit extracts were characterized using a previously described methodology.[19] Briefly, total phenolic content was determined using the Folin–Ciocalteu reagent and quantified as gallic acid equivalent. Anthocyanins were analyzed by reverse-phase HPLC with DAD detection and quantified using malvidin 3-glucoside standard. Proanthocyanidins were separated according to their degree of polymerization (DP) by normal-phase HPLC, and quantified by fluorescence detection using the (−)-epicatechin standard. Flavonols, phenolic acids and resveratrol were analyzed using reverse-phase UHPLC coupled to tandem mass spectrometry. Phenolic compounds were quantified using their high-purity commercial standards when available, aglycone or most similar phenolic structures otherwise.

2.2. Animal study, treatments and sample collection

Seventy-two 4 month-old male C57Bl/6J mice (Jackson laboratory, Bar Harbor, ME, USA) were individually maintained in cages in temperature- and humidity-controlled rooms (21 ± 2 °C, 35–40%) with a daily 12 h–12 h light–dark cycle, and fed a control diet free of phenolic compounds (Teklad 2018, Harlan, KY, USA). Animal facilities and procedures met the guidelines of the Canadian Council on Animal Care, and the protocols were approved by the Animal Care Committee of Laval University (protocol 13-114). Animals were divided into 2 groups to perform separate acute and chronic studies (see experimental design in Fig. 1). For each study, animals were randomly assigned to 4 different groups. The animals of 3 treated groups (n = 10) were supplemented either with a blueberry extract (B), a grape extract (G) or their combination (BG), while the animals of a control group (C, n = 6) received only the vehicle (water), all administered by intragastric gavage in a final volume of 150 µl. BG provided a phenol intake of 297.5 mg per kg body weight (BW), while B and G were individually administered at their exact proportion in the BG blend, leading to phenol intakes of 31.1 and 266.4 mg kg^{-1} respectively. Phenolic contributions of each treatment are detailed in Table 1.

2.2.1. Acute study. After 7 days of acclimation, all the animals were fasted for 2 hours in order to collect baseline blood samples (D0t0, Fig. 1). Thirty minutes following oral gavage with their respective treatment (D0t30), the animals were sacrificed under deep anesthesia with ketamine/xylazine.

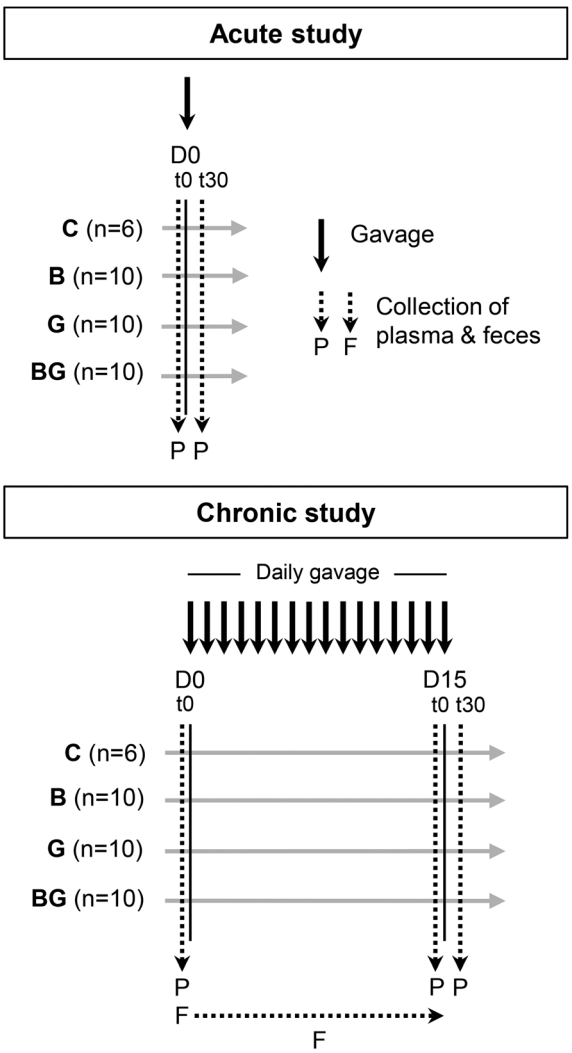

Fig. 1 Experimental design. Mice were administered a single dose (acute study) or a daily dose for 15 days (chronic study) of blueberry (B), grape (G) or blueberry–grape (BG) extracts, or water (control group, C), by oral gavage. Plasma samples were collected before the supplementation in each study (D0t0), 30 minutes after the single oral gavage in the acute study (D0t30), and before and 30 minutes after the last gavage in the chronic study (D15t0 and D15t30 respectively). Feces were collected in the chronic study before (D0) and throughout the 15-day supplementation (D1–D15).

Intracardiac blood samples were collected using EDTA-containing syringes and immediately centrifuged (3000 rpm, 5 min at 4 °C) in order to separate plasma.

2.2.2. Chronic study. After 7 days of acclimation, all the animals were fasted for 2 hours in order to collect baseline blood samples (D0t0, Fig. 1). The animals then received their respective treatments at the same time each day, over the course of 15 days. On the last day of the study, blood samples were collected prior to the last supplementation (D15t0). The animals were then sacrificed thirty minutes after the oral gavage, and intracardiac blood samples were collected (D15t30). Feces were collected for each mouse before and

Table 1 Intake of phenolic compounds in mice

	Dose (mg per kg BW)		
	B	G	BG
Flavan-3-ols/proanthocyanidins	1.5	204.0	205.5
DP 1–3	0.6	170.4	171.0
(+)-Catechin + (−)-epicatechin	*<0.1*	*128.7*	*128.7*
DP >3	0.9	33.6	34.5
Anthocyanins	2.2	—	2.2
Malvidin + glycosides	1.1	—	1.1
Others	1.1	—	1.1
Phenolic acids	13.5	4.7	18.2
Chlorogenic acid	9.8	—	9.8
Gallic acid	0.6	4.3	4.9
Others	3.1	0.4	3.5
Flavonols	4.3	0.2	4.5
Quercetin + glycosides	4.2	0.1	4.3
Others	0.1	0.1	0.2
Stilbenes (resveratrol)	—	0.2	0.2
Total[a]	31.1	266.4	297.5

[a] Total phenolic intake was determined using the Folin–Ciocalteu assay. BW: body weight, DP: polymerization degree. B: blueberry extract, G: grape extract, BG: blueberry–grape extract.

during the whole supplementation period (D0 and D1–15, respectively) and were freeze-dried. Plasma and feces samples were maintained at −80 °C until analysis. No variations in body weight or food consumption were observed between the groups during the 15 days of supplementation.

2.3. Extraction and characterization of phenolic metabolites from plasma and feces

Plasma and dried feces were mixed with 4% phosphoric acid (v/v and with a material–solvent ratio of 1 : 4 respectively) to disrupt phenol–protein binding. Feces were ground with glass beads using a Biospec BeadBeater (Bartlesville, OK, USA) for 15 seconds and the homogenate was centrifuged twice at 15 000 rpm at 4 °C for 15 min. Phenolic metabolites were characterized by UHPLC-MS/MS after micro-extraction on solid phase (μSPE) as previously described,[19] with slight modifications. Acidified plasma or feces supernatants were loaded into preconditioned Waters OASIS HLB micro-elution plates 2 mg – 30 μm. The retained phenolic compounds were eluted with 75 μl of acetone/ultrapure water/acetic acid solution 70/29.5/0.5 v/v/v and directly analyzed by UHPLC-MS/MS, using a Waters TQD mass spectrometer coupled to a Waters Acquity UPLC (Milford, MA, USA). Phenolic acids, flavonols, flavan-3-ols and resveratrol metabolites were separated at 30 °C on a Waters Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm) using 0.2% acetic acid in ultrapure water and acetonitrile as A and B mobile phases respectively, with a gradient elution starting from 5% to 50% B in 8 min, raised to 90% B over 1.10 min and held for 0.90 min, and then reset to initial conditions. The mobile phase flow rate was set at 0.4 ml min^{-1}, and the injection volume was 2.5 μl. Following the separation, the flow was introduced by negative mode electrospray ionization (ESI) into the mass spectrometer with the following parameters: capillary voltage, 2.75 kV; source temperature, 140 °C; cone gas flow rate, 80 l h^{-1} and desolvation gas flow rate, 800 l h^{-1}; desolvation temperature, 400 °C. Anthocyanins were separated at 30 °C on a Supelco Titan C18 column (2.1 × 100 mm, 1.9 μm) using mobile phases A and B (10% acetic acid in ultrapure water and acetonitrile, respectively) by gradient elution starting from 5% to 35% B in 10 min, raised to 80% B over 0.10 min and held for 0.90 min, and then reset to initial conditions. The mobile phase flow rate was set at 0.4 ml min^{-1}, and the injection volume was 2.5 μl. The MS/MS analysis was carried out in the positive ionization mode with the following parameters: capillary voltage, 2.5 kV; source temperature, 150 °C; cone gas flow rate, 50 l h^{-1}; desolvation gas flow rate, 800 l h^{-1}; desolvation temperature, 350 °C. Cone voltage and collision energy were optimized for each compound. Data were acquired through the multiple reaction monitoring (MRM) mode using MassLynx 4.1 software, tracking the transition of a parent/product ion specific for each compound. Phenolic metabolites were identified by comparison with retention time and molecular ions of individual standards, and the quantification was conducted using their calibration curves. Remaining metabolites, for which standards were not available, were identified based on fragmentation information described in the literature and quantified using the calibration curve of their aglycone or the most similar phenolic structure. Parent/product ion pairs (MRM transitions) of identified phenolic metabolites are listed in Table 2.

2.4. Data analysis

Plasma concentrations of phenolic metabolites following acute or chronic administration of fruit extracts were compared using the Welch's *t*-test (correcting for unequal variance) when data were assumed to be normally distributed, or using the Mann–Whitney test otherwise (GraphPad Prism 6.05, La Jolla, CA, USA). Similarly, the effect of treatments on phenolic metabolite concentrations in plasma and feces was analyzed for pair comparison using the Welch's *t*-test or the Mann–Whitney test. Multiple comparisons were performed using one-way analysis of variance (ANOVA) or the non-parametric Kruskal–Wallis test based on whether data followed a normal distribution or not. Differences were considered to be significant at $p < 0.05$. Hierarchical clustering analysis (HCA) of phenolic metabolites detected in plasma and feces was carried out using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) on log-transformed data, with Euclidean distance measure and Ward clustering algorithm.

3. Results

3.1. Identified metabolites of blueberry and grape phenolic compounds

As presented in Table 1, treatment with a blueberry–grape extract provided flavan-3-ols (catechin, epicatechin), proanthocyanidins, gallic acid and resveratrol from the grape extract, as

Table 2 Phenolic metabolites identified in plasma and feces of mice after the administration of fruit extracts

Metabolite		Standard for quantification	MRM	Location
M1	(+)-Catechin	Epicatechin	289 > 109	Plasma (G, BG), feces (G, BG)
M2	(−)-Epicatechin	Epicatechin	289 > 109	Plasma (G, BG), feces (G, BG)
M3	Catechin glucuronide	Epicatechin	465 > 289	Plasma (G, BG), feces (G, BG)
M4	Catechin sulfate	Epicatechin	369 > 289	Plasma (G, BG), feces (G, BG)
M5	Catechin glucuronide sulfate	Epicatechin	545 > 289	Plasma (G,BG), feces (G, BG)
M6	Methyl catechin glucuronide	Epicatechin	479 > 303	Plasma (G,BG), feces (G, BG)
M7	Methyl catechin sulfate	Epicatechin	383 > 303	Plasma (G, BG), feces (B, G, BG)
M8	Methyl catechin glucuronide sulfate	Epicatechin	559 > 289	Feces (G, BG)
M9	B-type procyanidin dimers	Epicatechin	577 > 289	Plasma (G, BG), feces (B, G, BG)
M10	B-type procyanidins trimers	Epicatechin	865 > 578	Feces (G, BG)
M11	Hydroxyphenyl-γ-valerolactone	Gallic acid	191 > 147	Feces (G, BG)
M12	Dihydroxyphenyl-γ-valerolactone	Gallic acid	207 > 163	Plasma (G, BG), feces (B, G, BG)
M13	Resveratrol	Resveratrol	227 > 185	Feces (G, BG)
M14	Resveratrol glucuronide	Resveratrol	403 > 227	Plasma (G, BG)
M15	Resveratrol sulfate	Resveratrol	307 > 227	Feces (G, BG)
M16	Dihydro-resveratrol	Resveratrol	229 > 187	Feces (G, BG)
M17	Quercetin glucuronide	Quercetin	477 > 301	Plasma (B, BG), feces (B, G, BG)
M18	Quercetin sulfate	Quercetin	381 > 301	Feces (B, BG)
M19	Quercetin glucuronide sulfate	Quercetin	557 > 301	Feces (B, G, BG)
M20	Cyanidin 3-glucoside	Malvidin 3-glucoside	449 > 287	Feces (B, BG)
M21	Malvidin 3-arabinoside	Malvidin 3-glucoside	463 > 331	Feces (B, BG)
M22	Delphinidin 3-glucoside	Malvidin 3-glucoside	465 > 303	Feces (B, BG)
M23	Petunidin 3-glucoside	Malvidin 3-glucoside	479 > 317	Plasma (B, BG), feces (B, BG)
M24	Malvidin 3-glucoside	Malvidin 3-glucoside	493 > 331	Plasma (B, BG), feces (B, BG)
M25	3,4-Dihydroxyphenyl propionic acid	3,4-Dihydroxyphenyl propionic acid	181 > 59	Feces (B, G, BG)
M26	3,4-Dihydroxyphenyl acetic acid	3,4-Dihydroxyphenyl acetic acid	167 > 123	Feces (B, G, BG)
M27	Chlorogenic acid	Chlorogenic acid	353 > 191	Plasma (B, BG), feces (B, BG)
M28	Dihydroxycinnamic acid	Caffeic acid	179 > 79	Plasma (B, G, BG)
M29	Ferulic acid	Ferulic acid	193 > 134	Plasma (B, G, BG)
M30	Gallic acid	Gallic acid	169 > 79	Plasma (B, G, BG)
M31	Protocatechuic acid	Protocatechuic acid	153 > 53	Plasma (B, G, BG), feces (B, G, BG)
M32	Vanillic acid	Vanillic acid	167 > 152	Plasma (B, G, BG)

B: blueberry extract, G: grape extract, BG: blueberry–grape extract.

well as anthocyanins, flavonols (quercetin) and chlorogenic acid from the blueberry extract.

Following supplementation of mice with either B, G or BG, a total of 32 phenolic metabolites were identified, 19 of which were detected in plasma and 27 in feces. These metabolites are listed in Table 2. Their concentrations quantified in plasma are reported in ESI Tables S1 and S2† for acute and chronic studies respectively, and their concentrations quantified in feces are reported in ESI Table S3.† Various conjugated metabolites of catechins ((+)-catechin/(−)-epicatechin) and resveratrol were detected in the plasma and/or feces of animals supplemented with G or BG, as well as conjugated metabolites of quercetin in the plasma and/or feces of animals supplemented with B or BG (methylated, glucuronidated and/or sulfated metabolites). Some native phenolic compounds were also identified in plasma and/or feces, such as (+)-catechin, (−)-epicatechin, resveratrol and absorbable dimers/trimers of proanthocyanidins, for animals supplemented with G or BG, as well as chlorogenic acid and glycosides of anthocyanins for animals supplemented with B or BG. Furthermore, several phenolic acids, mostly resulting from the microbial degradation of native phenolic compounds, were identified in the plasma and/or feces of all supplemented animals, such as dihydroxycinnamic acid, ferulic acid, gallic acid, protocatechuic acid, vanillic acid and derivatives of phenylpropionic and phenylacetic acids. Likewise, microbial metabolites derived from catechins and proanthocyanidins (γ-valerolactones) and from resveratrol (dihydro-resveratrol) were mostly identified in the feces of mice supplemented with G or BG.

3.2. Chronic *vs.* acute blueberry–grape co-supplementation: plasma concentrations of phenolic compounds

The total plasma content of phenolic compounds in mice with acute or chronic supplementation (one administration *vs.* 15 daily administrations, respectively) with B, G or BG is presented in Fig. 2. No difference in circulating phenolic concentration was observed between single and repeated administration of individual extracts B (5 and 6 μM, respectively) and G (157 and 198 μM, respectively). In contrast, following the chronic supplementation of mice with their combination (BG), the circulating phenolic concentration was doubled (350 *vs.* 166 μM for acute supplementation, $p = 0.0033$). Among the 19 metabolites identified in the plasma of mice supplemented with BG (Table 3), 9 were found in significantly higher concentration following chronic supplementation compared to acute supplementation (from 1.8- to 3.4-fold): catechin glucuronide, dihydroxycinnamic acid, methyl catechin glucuronide, quercetin glucuronide, methyl catechin sulfate, malvidin 3-glucoside, epicatechin, ferulic acid and catechin glucuronide sulfate.

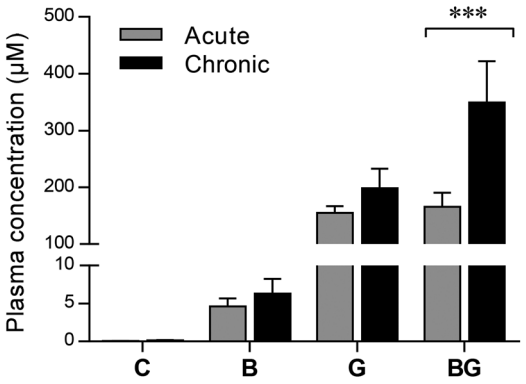

Fig. 2 Total plasma concentration of phenolic compounds following acute or chronic supplementation of mice with fruit extracts. ***p < 0.005. B: blueberry extract, G: grape extract, BG: blueberry–grape extract.

3.3. Blueberry–grape combination *vs.* blueberry or grape supplementation: comparison of plasma concentrations of phenolic compounds

A hierarchical clustering of plasma concentrations of the 19 phenolic metabolites analyzed following the repeated administration of B, G or BG in mice is presented in Fig. 3. Only traces of ferulic and vanillic acids were detected in plasma before supplementation (D0t0). Except for dihydroxyphenyl-γ-valerolactone, no significant concentrations of phenolic metabolites were detected in plasma prior to the last gavage (D15t0), highlighting their complete elimination from circulation in less than 24 h. As visualized on the heatmap, the clustering of phenolic metabolites reflects their origin, *i.e.* conjugated and microbial metabolites of catechins and resveratrol, as well as dimers of proanthocyanidins from G; conjugated quercetin, chlorogenic acid and anthocyanins from B; and phenolic acids from B and G. No difference was observed in circulating concentrations of phenolic metabolites from G, whether G was administered with B or alone. On the other hand, as shown in the inset graphs in Fig. 3, phenolic metabolites from B were found in significantly higher concentrations (from 3.0- to 5.5-fold) in the plasma of mice supplemented with BG in comparison with B alone: quercetin glucuronide (30 *vs.* 10 nM, p = 0.0238), malvidin 3-glucoside (36 *vs.* 8 nM, p = 0.0076), chlorogenic acid (2 *vs.* 0.4 μM, p = 0.0095) and petunidin 3-glucoside (8 *vs.* 2 nM, p = 0.0110). Interestingly, smaller but still significant increases (2.4- to 2.8-fold) in plasma concentrations were also observed following acute supplementation of BG in comparison with B alone for malvidin 3-glucoside (11 *vs.* 5 nM, p = 0.0007), chlorogenic acid (0.9 *vs.* 0.3 μM, p = 0.0219) and petunidin 3-glucoside (3 *vs.* 1 nM, p = 0.0008).

3.4. Blueberry–grape combination *vs.* blueberry or grape supplementation: comparison of the fecal content of phenolic compounds

A hierarchical clustering of fecal concentrations of the 27 phenolic metabolites analyzed following the repeated administration of B, G or BG in mice is presented in Fig. 4. Only traces

Table 3 Comparison of plasma concentrations of phenolic metabolites identified following acute or chronic supplementation of mice with the blueberry–grape extract

Metabolite	Plasma concentration (nM)					
	BG acute administration	BG chronic administration	Fold change (chronic/acute)		Statistical test	p-value
(+)-Catechin	444 ± 79	821 ± 227			T-W	ns
(−)-Epicatechin	1105 ± 186	3701 ± 1058*	3.3	↑	T-W	0.0374
Catechin glucuronide	92 348 ± 14 664	169 357 ± 33 929*	1.8	↑	T-W	0.0334
Catechin sulfate	21 159 ± 6503	54 063 ± 21 491			T-W	ns
Catechin glucuronide sulfate	3293 ± 1454	11 236 ± 3750**	3.4	↑	T-W	0.0060
Methyl catechin glucuronide	27 938 ± 2447	72 475 ± 9901***	2.6	↑	T-W	0.0014
Methyl catechin sulfate	4854 ± 1101	13 432 ± 4035*	2.8	↑	T-W	0.0259
B-type procyanidin dimers	1298 ± 271	2384 ± 797			T-W	ns
Dihydroxyphenyl-γ-valerolactone	174 ± 85	1369 ± 856			M-W	ns
Resveratrol glucuronide	87 ± 18	120 ± 45			T-W	ns
Quercetin glucuronide	12 ± 3	30 ± 7*	2.6	↑	T-W	0.0036
Petunidin 3-glucoside	3 ± 0	8 ± 2			T-W	ns
Malvidin 3-glucoside	11 ± 1	36 ± 11*	3.2	↑	T-W	0.0463
Chlorogenic acid	874 ± 180	1962 ± 784			T-W	ns
Dihydroxycinnamic acid	2541 ± 627	6035 ± 1574*	2.4	↑	T-W	0.0353
Ferulic acid	45 ± 6	151 ± 31***	3.3	↑	T-W	<0.0001
Gallic acid	9324 ± 1581	10 835 ± 2464			T-W	ns
Protocatechuic acid	484 ± 103	965 ± 300			T-W	ns
Vanillic acid	256 ± 49	526 ± 146			M-W	ns
Total (μM)	166 ± 25	350 ± 72***	2.1	↑	T-W	0.0033

Data are displayed as mean of replicates ± SEM (control group: n = 6, treated groups: n = 10). ***p < 0.005, **p < 0.01 and *p < 0.05 *vs.* acute administration of the blueberry–grape extract. Arrows indicate increase fold change in plasma concentration for chronic *vs.* acute exposure to the blueberry–grape extract. T-W: T-test with Welch correction, M-W: Wann–Whitney test, ns: not significant.

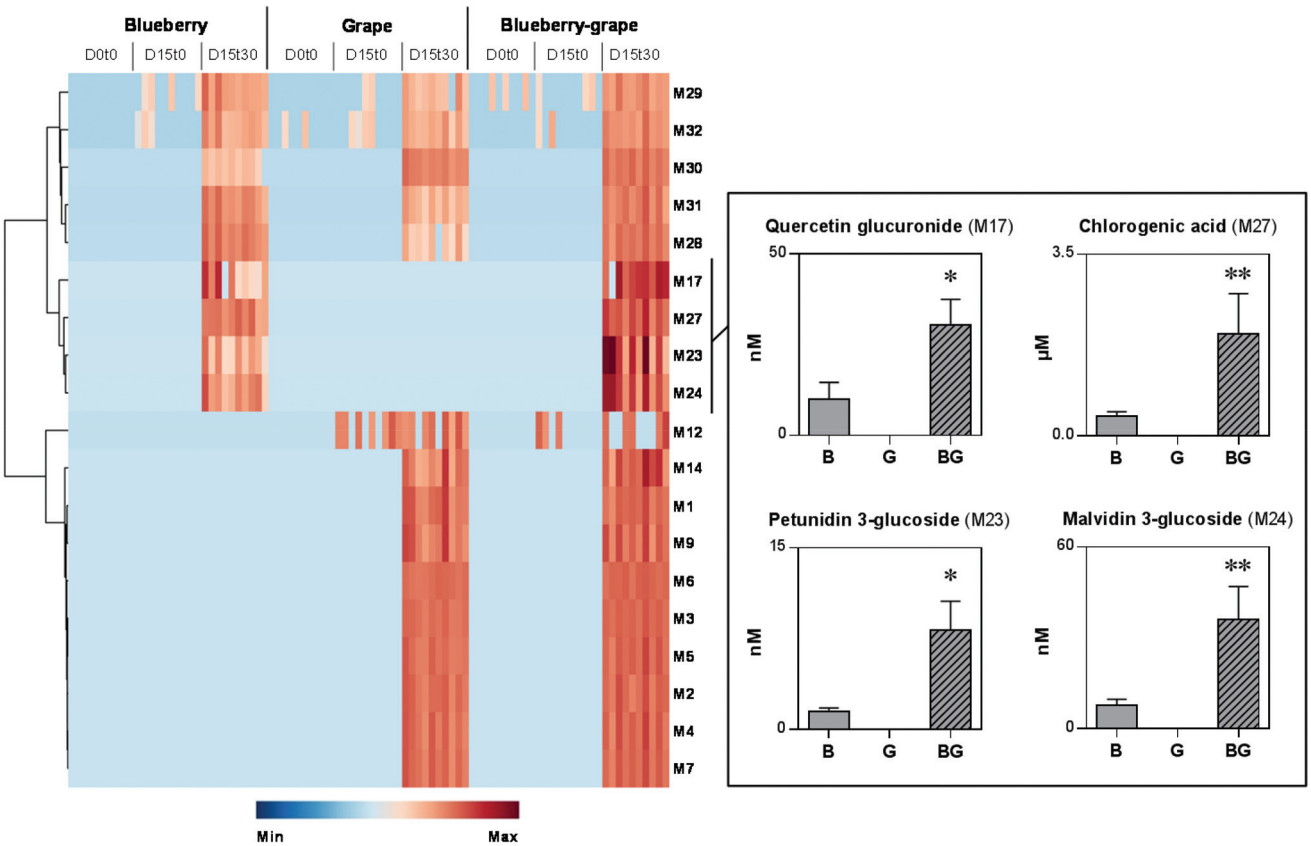

Fig. 3 Hierarchical clustering analysis heatmap of phenolic metabolites identified in plasma of mice following their chronic supplementation with fruit extracts. Each row represents a detected metabolite and each column an animal of the study. Blue and red tiles, respectively, indicate a lower or higher intensity of metabolite concentration in plasma than the mean of all samples. Metabolites assignment (M1–M32) is listed in Table 2. Inset graphs represent blueberry phenolic metabolites whose concentration in plasma was significantly increased following 15 days of blueberry–grape co-supplementation. Data are displayed as a mean of replicates ± SEM (n = 10). B: blueberry extract, G: grape extract, BG: blueberry–grape extract. **p < 0.01 and *p < 0.05 *vs.* blueberry extract alone.

of microbial phenolic metabolites protocatechuic acid, 3,4-dihydroxyphenyl acetic acid and 3,4-dihydroxyphenyl propionic acid were detected in feces prior to the supplementation (D0). The heatmap shows the clustering of phenolic metabolites according to their detection in fecal samples of mice treated with B, G or both extracts. No difference was found in fecal concentrations of phenolic metabolites from G, whether G was administered with B or alone. On the other hand, as shown in the inset graphs in Fig. 4, and in accordance with the previous observations in plasma, phenolic metabolites from B were found in significantly lower concentrations (from 2.9- to 6.3-fold) in the feces of mice supplemented with BG in comparison with B alone: quercetin glucuronide (147 *vs.* 431 pmol g^{-1}, p = 0.0499), petunidin 3-glucoside (50 *vs.* 157 pmol g^{-1}, p = 0.0064), protocatechuic acid (11 *vs.* 35 nmol g^{-1}, p < 0.0001), cyanidin 3-glucoside (14 *vs.* 51 pmol g^{-1}, p = 0.0005), chlorogenic acid (8 *vs.* 38 nmol g^{-1}, p < 0.0001) and delphinidin 3-glucoside (14 *vs.* 87 pmol g^{-1}, p < 0.0001). As an example, comparative MRM profiles of chlorogenic acid analyzed in mouse plasma and feces are presented in Fig. 5, showing different peak intensities between samples from animals supplemented with blueberry or blueberry–grape extracts.

4. Discussion

Numerous factors affect the bioavailability of ingested phenolic compounds, including their poor absorption, extensive metabolism and interactions with gut microbiota.[20–22] Only 5–10% of the total phenolic intake is estimated to be absorbed in the small intestine, while the remaining 90–95% accumulates in the colon and is subjected to microbial degradation.[23] Therefore, phenolic compounds have recently been shown to display prebiotic action by modulating the gut microbial community,[23–25] thereby indirectly affecting their own biodegradation.[19] The bioavailability of phenolic compounds is also strongly affected by phase I, II and III metabolism, taking place both in the intestine and the liver.[5] Phase I reactions (oxidation, reduction and hydrolysis) aim to expose or add a functional group to facilitate phase II reactions, catalyzing conjugation with hydrophilic endogenous molecules, resulting in

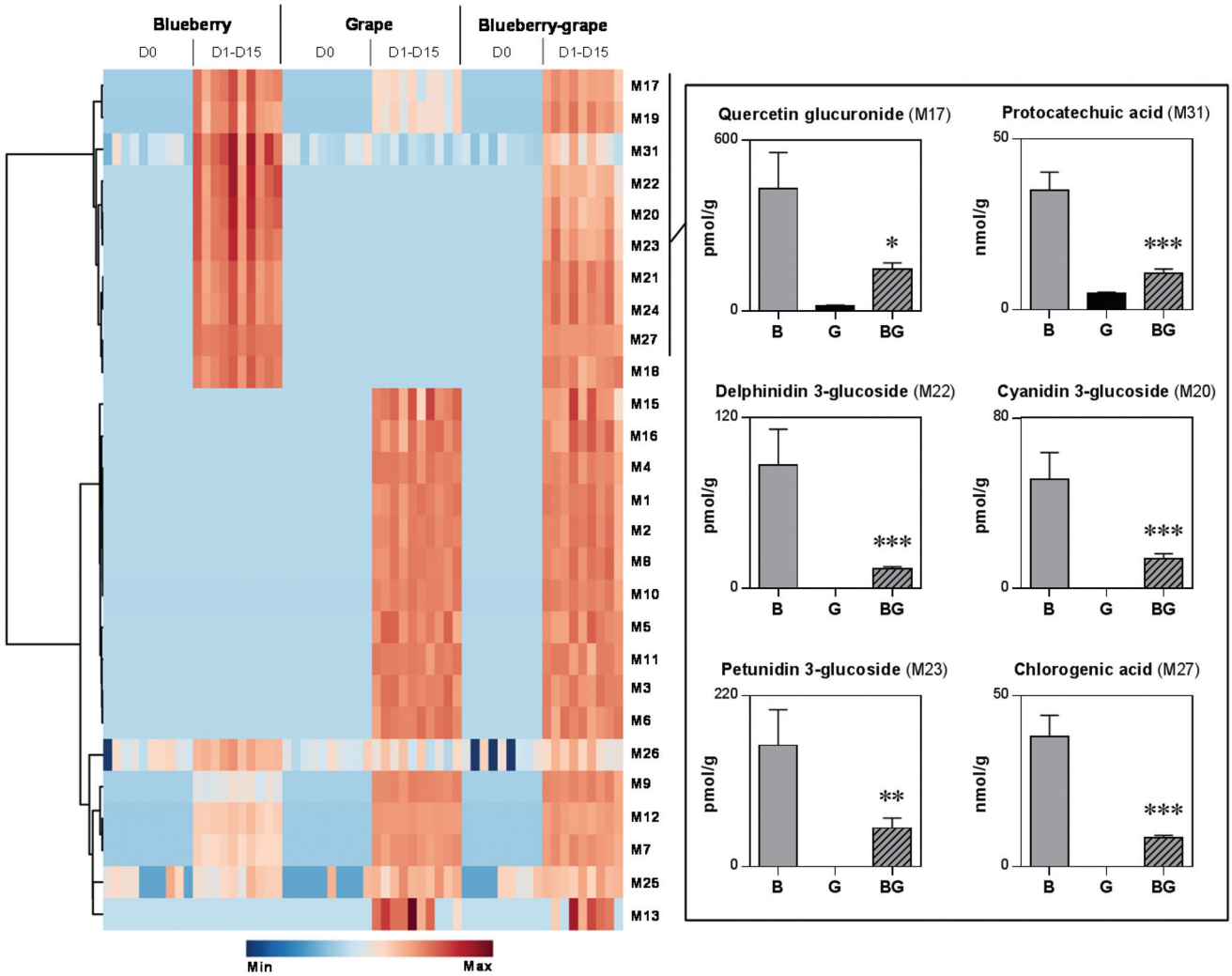

Fig. 4 Hierarchical clustering analysis heatmap of phenolic metabolites identified in feces of mice following their chronic supplementation with fruit extracts. Each row represents a detected metabolite and each column an animal of the study. Blue and red tiles, respectively, indicate a lower or higher intensity of metabolite concentration in feces than the mean of all samples. Metabolites assignment (M1–M32) is listed in Table 2. Inset graphs represent blueberry phenolic metabolites whose concentration in feces was significantly decreased following 15 days of blueberry–grape co-supplementation. Data are displayed as mean of replicates ± SEM (n = 10). B: blueberry extract, G: grape extract, BG: blueberry–grape extract. ***$p < 0.005$, **$p < 0.01$ and *$p < 0.05$ *vs.* blueberry extract alone.

methylated, glucuronidated and/or sulfated conjugates.[20,26] Phenolic compounds and their metabolites are substrates for transmembrane transporters such as the ATP-binding cassette (ABC) transporters, and hence effluxed back into the intestinal lumen for ultimate excretion from the body.[27,28] Enhancing the low bioavailability of phenolic compounds is therefore a major focus of studies, and some strategies have been recently discussed,[11,22,29] such as designing specific synergies between phenolic compounds to favor their absorption.[11]

In the present study, the repeated daily administration of the blueberry–grape extract to mice for 15 days was found to significantly improve the bioavailability (defined as plasma concentrations measured 30 minutes after ingestion) of key phenolic compounds by up to 3.4-fold, in comparison with a single acute administration. This increase in the plasma concentration of phenolic metabolites was not explained by accumulation over time as they were not detected in circulation prior to the last gavage, and thus were completely removed from the circulation in less than 24 h. A similar improvement in the bioavailability of phenolic compounds over time was previously reported following oral administration of a grape seed extract (GSPE) to rats over a period of 10 days with dose escalation.[30] The authors proposed as an explanation for this observation that GSPE either induced a modulation of expression and/or activity of specific intestinal cell transporters, or an alteration of lower intestine microbial ecology (most likely by proanthocyanidins) resulting in a modified capacity for colonic fermentation of phenolic compounds.[30] Interestingly, in the present work, enhancement in the bioavailability of phenolic compounds over time was

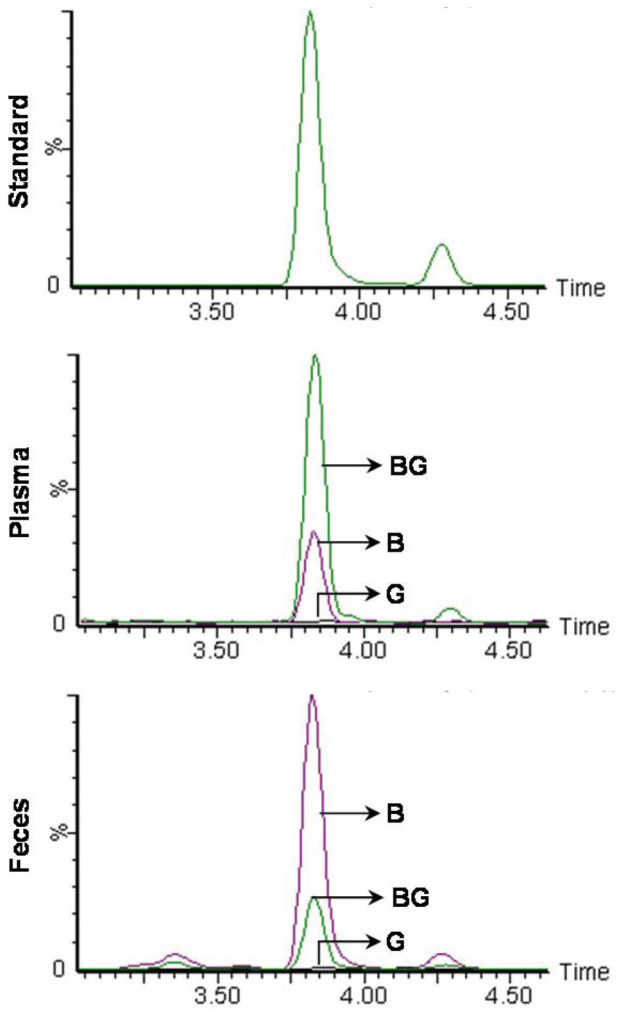

Fig. 5 Representative MRM signals of chlorogenic acid (353 > 191) from the chemical standard, and the plasma/feces specimen from mice supplemented with blueberry (B), grape (G) or blueberry–grape (BG) extracts.

observed with the blueberry–grape combination but not with individual extracts, indicating a positive interaction between blueberry and grape phenolic components. Indeed, blueberry phenolic compounds were more bioavailable when co-ingested with grape phenolic compounds, as indicated by the increase in the plasma concentrations of blueberry phenolic metabolites (up to 5.5-fold) matched by an equivalent decrease in their fecal concentrations (up to 6.3-fold) in mice chronically supplemented with the blueberry–grape extract compared to mice receiving only the blueberry extract. An increase in the plasma concentrations of blueberry phenolic metabolites was also observed in animals acutely supplemented with the blueberry–grape extract, indicating that the potentiation of absorption of blueberry phenolic compounds by grape phenolic compounds occurred at the first administration. However, the bioavailability of grape phenolic compounds was not affected by the blueberry–grape co-supplementation, suggesting that such interactions may not be reciprocal.

Although the notion that interactions may occur between phytochemicals of co-ingested foods is generally well recognized and often cited, it is actually poorly documented and seldom quantified *in vivo*. It has been widely described in regard to food–drug interferences,[10,11,20,31] but only a few studies actually reported such interactions leading to a potentiated bioavailability of phytochemicals *in vivo*. For example, biochanin A, an isoflavone with chemopreventive properties was found to be 3-fold more bioavailable in rats when orally administered together with quercetin and epigallocatechin gallate (EGCG).[32] Curcumin bioavailability was reported to be increased by 154% in rats and by 2000% in humans when co-administered with piperine, an alkaloid from black pepper.[33] Moreover, piperine has been shown to enhance the absorption of EGCG in mice, with a 1.3-fold increase in plasma concentration associated with a slower appearance of the compound in the feces, in comparison with mice treated with EGCG only.[34] In these studies, the improved bioavailability was attributed to a combined inhibition of phase I/II enzymes and ABC efflux transporters. Indeed, phenolic compounds are not only substrates for enzymes taking part in xenobiotic metabolism but also act as their regulators, resulting in an enhancement or limitation in the bioavailability of co-ingested compounds.[28] The mechanistic hypotheses put forward in these few studies using isolated phytochemicals may certainly be transposed to plant extracts and whole foods. Indeed, the present work reports actual interactions between phenolic compounds in complex mixtures, with the grape extract enhancing the absorption of blueberry phenolic compounds. Likewise, our previous study reported an enhanced absorption of phenolic compounds from a blend of strawberry and cranberry extracts when co-administered in rats with a quercetin-rich onion extract.[35] These findings strongly suggest that the presence of a concentrated phenolic compound from the first extract ((+)-catechin/(−)-epicatechin from the grape extract in the present study) may saturate efflux transport and thus favor the absorption of phenolic compounds of low concentration from a co-ingested extract (such as anthocyanins and chlorogenic acid from the blueberry extract). Although the mechanisms underlying this potentiation are not yet elucidated, and in accordance with the previous studies, we suggest that the improved bioavailability observed in the present work results from a probable competition between phenolic compounds as substrates/inhibitors of detoxification enzymes and/or efflux transporters. The modulatory effect of phytochemicals on detoxification processes can occur either directly[26,27] or through an interaction with transcription factors involved in the regulation of an organism's biological responses to xenobiotics such as the aryl hydrocarbon receptor (AHR).[36]

5. Conclusion

Enhancing the low bioavailability of phenolic compounds and their metabolites is believed to be a key strategy to improve their therapeutic effects. In the present work, a grape extract

was found to potentiate the bioavailability of phenolic compounds from a blueberry extract in mice. This study provides for the first time *in vivo* evidence of positive interactions occurring between co-ingested phytochemicals from complex mixtures, resulting in improved bioavailability of phenolic compounds. These findings therefore highlight a new promising strategy for the development of functional foods, *i.e.* the opportunity to design specific combinations of phenolic compounds that could be beneficial for human health.

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