A HIGH-FAT HIGH-SUCROSE DIET AFFECTS THE LONG-TERM METABOLIC FATE OF GRAPE PROANTHOCYANIDINS IN RATS

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List of abbreviations used

EC: (epi)catechin

EGC: (epi)gallocatechin

Gluc: glucuronyl group

GSE: grape seed extract

HFHS: high-fat high-sucrose diet

Me: methyl group

MetS: metabolic syndrome

MRM: multiple reaction monitoring

MS: mass spectrometry

PA: proanthocyanidin

STD: standard

Sulf: sulfate group

Abstract

Purpose. Polyphenol metabolites are key mediators of the biological activities of polyphenols. This study aimed to evaluate the long-term effects of a high-fat high-sucrose (HFHS) diet on the metabolism of proanthocyanidins (PAs) from grape seed extract (GSE).

Methods. Adult female Wistar Kyoto rats were fed a standard (STD) or HFHS diet supplemented or not with GSE for 16 weeks. PA metabolites were determined by targeted HPLC-MS/MS analysis.

Results. A lower concentration of total microbial-derived PA metabolites was present in urine and the aqueous fraction of faeces in the HFHS + GSE group than in the STD + GSE group. In contrast, a tendency towards the formation of conjugated (epi)catechin metabolites in the HFHS + GSE group was observed.

Conclusions. These results show that a HFHS diet significantly modifies PA metabolism, probably via: i) a shift in microbial communities not counteracted by the polyphenols themselves; and ii) an up-regulation of hepatic enzymes.

Keywords: Polyphenols, proanthocyanidins, high-fat high-sucrose diet, bioavailability, metabolites

1. Introduction

Metabolic syndrome (MetS) is a cluster of risk factors (abdominal obesity, hypertension, hyperglycaemia, hypertriglyceridaemia) that increases the risk of developing type 2 diabetes or cardiovascular diseases [1]. MetS is increasingly becoming a public health problem, affecting some 20%-30% of the population in developed countries [2]. MetS has been shown to result from factors that are common in current Western lifestyles: sedentariness and unhealthy dietary patterns including an excess of fat and simple carbohydrates, i.e., high-fat high-sucrose (HFHS) diets. The metabolic alterations caused by a HFHS dietary pattern have been thoroughly studied in animal models, showing that it triggers insulin resistance, hyperinsulinaemia, hyperlipidaemia, elevated blood pressure, hepatic steatosis and both endothelial-dependent and endothelial-independent arterial dysfunction, among other effects [3-5]. HFHS diets have been used in several animal models to evaluate the potential role of different bioactive food compounds in the modulation of MetS; for instance, a HFHS diet has been supplemented with ω-3 polyunsaturated fatty acids of marine origin [6], the iminosugar D-fagomine [7] or with polyphenols [8].

Proanthocyanidins (PAs) constitute a class of polyphenols; a broad group of dietary phytochemicals. The members of this class range from dimers to high-molecular-weight polymers of different constituent flavanol units and are notably present in certain foodstuffs such as cocoa, grapes and nuts [9]. In recent years, several studies in animal models have shown that PAs may play a beneficial role in modulating MetS through a combination of mechanisms, i.e., direct inhibition of enzymes involved in the metabolism of carbohydrates, improvement in insulin sensitivity, repression of intestinal lipid absorption, activation of endogenous antioxidant systems and reduction of the overexpression of certain cytokines [10-14]. A key factor in the health effects of

polyphenols is their metabolic fate since, once ingested, they are extensively transformed by phase I and phase II enzymes, as well as by the gut microbiota; PA-derived metabolites may ultimately be responsible for the biological effects of PAs [15-16]. Other components present in the diet, e.g., milk or oil, may affect the bioavailability of polyphenols [17-18]. Other physiological aspects, such as age, do not seem to play a relevant role in the metabolism of PAs [19]. Therefore, to properly ascertain the role of supplemented polyphenols, the effect of the overall diet on the profile and amount of potentially active circulating metabolites must be evaluated.

Several studies in animal models have supplemented HFHS diets with different polyphenols in order to determine how they modulate MetS [20-22]. However, the levels of polyphenol-derived metabolites after a HFHS diet were not assessed. Also, the effect of combined supplementation with polyphenols and a probiotic on circulating phenolic metabolites was evaluated in animals fed a HFHS diet [23], but no comparison was provided of the effects of this supplementation on animals fed a standard (STD) diet.

Therefore, the aim of this study was to compare the levels of metabolites derived from grape PAs in rats fed a HFHS diet with those in rats fed a STD diet; which may provide useful information for understanding the reported effects of the addition of polyphenols to HFHS diets.

2. Materials and methods

2.1 Chemicals and reagents

The STD diet, Global 2014, and HFHS diet, TD 08811, were from Teklad Global 2014 (Harlan Teklad Inc., Indianapolis, IN, USA). Fine Grajfnol® powder 98% (grape seed extract; GSE) from grape seed was from JF-Natural Product (Tianjin, China).

According to the manufacturer, this extract contained 95% PAs (UV) of which 60% was B2 procyanidin dimer (HPLC), with a mean degree of polymerization of 2. So the extract contained mainly dimmers, with some amounts of monomers and trimers. Ash content was ≤ 1.5% and loss on drying was ≤ 5.0%. Porcine gelatin type A 240/260 was from Juncà (Girona, Spain) and soybean lecithin Topcithin 50 from Cargill (Barcelona, Spain). Organic unrefined soybean oil (first cold pressing) was from Clearspring Ltd. (London, UK).

Ketamine chlorhydrate was purchased from Merial Laboratorios (Barcelona, Spain) and xylacine from Química Farmacéutica (Barcelona, Spain). Standards of (−)-(epi)catechin (EC) (≥ 98%), (−)-(epi)gallocatechin (EGC) (≥ 95%), 3-hydroxyphenylacetic acid (≥ 99%), 4-hydroxyphenylacetic acid (≥ 98%), 3,4-dihydroxyphenylacetic acid (≥ 98%), 3-hydroxybenzoic acid (≥ 99%), 4-hydroxybenzoic acid (≥ 99%), homovanillic acid (≥ 98%), vanillic acid (≥ 97%), caffeic acid (≥ 98%), 3,4-dihydroxyphenylpropionic acid (≥ 98%), 3-(4-hydroxyphenyl)propionic acid (≥ 98%), 3,4-dihydroxybenzoic acid (≥ 97%), benzoic acid (≥ 99%), hippuric acid (≥ 98%), ferulic acid (≥ 99%), isoferulic acid (≥ 97%), *p*-coumaric acid (≥ 98%), *m*-coumaric acid (≥ 98%), gallic acid (≥ 97%), enterodiol (≥ 95%), phenylacetic acid (≥ 99%), taxifolin (≥ 85%), and tert-butylhydroquinone and formic acid (analytical grade) were obtained from Sigma Chemical (St Louis, MO, USA). Methanol (analytical grade) and hydrochloric acid (≥ 85%) were from Panreac (Castellar del Vallès, Spain). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Water for the assay solutions was obtained using a Milli-Q water purification system (Millipore Corporation, Billerica, MA, USA).

2.2 Animals

A total of twenty female Wistar Kyoto (WKY) rats (Janvier, Le Genest-St-Isle, France), aged 8-9 weeks were housed in cages (*n* = 2-3/cage) with a 12 h light/12 h dark cycle, at

22ºC ± 2ºC and a relative humidity of 50% ± 10%. All the procedures adhered strictly to the European Union guidelines for the care and management of laboratory animals, and were approved by the CSIC Bioethical Issues Subcommittee (ref. AGL2009-12 374-C03-03). Thus they were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

2.3 Experimental design

The rats were randomly divided into four groups, each (n = 5) fed a different diet: control (STD diet); HFHS diet; STD diet supplemented with GSE (STD + GSE); HFHS diet supplemented with GSE (HFHS + GSE). The animals were given access to feed and water *ad libitum*. The composition of each diet is provided in **Table 1**.

The diets were prepared in-house and pelletized by lyophilization. To prevent oxidation and contamination by fungi, the dry pellets were vacuum packed and stored at 4ºC until used. To guarantee the proper mixture of the different components and an adequate consistency of the final pellet, soybean lecithin and porcine gelatin were added. *tert*-Butylhydroxiquinone was added as an antioxidant.

The animals received water and the pelleted feed for 18 weeks after being randomly assigned to the four dietary groups. Between weeks 14 and 16 of the experiment, the rats were placed in metabolic cages for urine and faeces collection, and deprived of food for 24 h.

2.4 Sample processing

The biological samples were prepared according to previously described procedures for the extraction of phenolic metabolites [24-26]. On collection, urine samples were acidified with HCl (1 mM, 5 μL) then urine and faeces were frozen in liquid nitrogen and stored at −80ºC. For analysis, the whole urine samples were freeze dried and re-

suspended in 1 mL of acid water (water acidified to pH 3 with formic acid). Then, taxifolin (100 μL of a 50 ppm solution) was added to each sample as an internal standard, to obtain a final concentration of 5 ppm. The samples were then subjected to solid-phase extraction in Oasis HLB (60 mg) cartridges from Waters Corporation (Mildford, MA, USA). The cartridges were activated with methanol (1 mL) and acid water (2 mL) and the samples loaded. To remove interfering components, the samples were washed with acid water (9 mL) and then the phenolic compounds were eluted with methanol (1 mL). The eluate was evaporated under nitrogen and the residue reconstituted with 500 μL of the initial HPLC mobile phase ([A], see below). The temperature of evaporation was kept under 37ºC to avoid deterioration of the phenolic compounds. The samples were filtered through a polytetrafluoroethylene 0.45 μm membrane from Waters Corp. into amber vials for HPLC-MS/MS analysis.

Faeces samples were re-suspended in acid water (1:1 w/w) and homogenized using a vortex. Then, after adding the internal standard (taxifolin, 5 ppm) the mixtures were centrifuged (10000 *g*, 10 min at 4ºC), and the supernatant was freeze dried and re-suspended in 1 mL of acid water and homogenized using a vortex, and later subjected to SPE and the workup process as described for the urine samples.

2.5 HPLC-ESI-MS/MS analysis of polyphenol metabolites

An Applied Biosystems (PE Sciex, Concord, Ontario, Canada) API 3000 triple quadrupole mass spectrometer with a TurboIon spray source was used in negative mode to obtain MS and MS/MS data, according to procedures described previously [24-26]. Liquid chromatography separations were performed using an Agilent 1100 series liquid chromatograph system (Agilent, Waldbronn, Germany) equipped with a Phenomenex (Torrance, CA, USA) Luna C18 (50 x 2.0 mm i.d.) 3.0 μm particle size column and a

Phenomenex Securityguard C18 (4 x 2.0 mm i.d.) column. Gradient elution was performed with a binary system consisting of: [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in CH_3CN. An increasing linear gradient (v/v) of [B] was used, [t(min), % B]: 0,8; 10,23; 15,50; 20,50; 21,100; followed by a re-equilibration step. Each metabolite in the urine samples was first identified by multiple reaction monitoring (MRM) of the transitions of the putative metabolites using a dwell time of 100 ms and then confirmed either by comparison with a standard when available, repeat MRM with a second characteristic transition and posterior comparison of the results with the retention time obtained in the first MRM, or neutral-loss and product ion scan experiments. The cycle time used was 2 s. The list of metabolites to be searched for was compiled from the literature on the bioavailability of grape polyphenols [24-27]. Analyst 1.4.2 software from AB Sciex was used for data acquisition and processing. Calibration curves for each metabolite were plotted using between 4 and 11 standards at different concentrations (ranging from 0.001 to 60 ppm). The concentrations obtained from the calibration curves were further corrected by the internal standard. When no commercial standard was available, the metabolites were quantified using a structurally related compound. The standard may still show a different response from that of the metabolite, so this method cannot be considered to provide proper quantification and should therefore be used mainly for comparative purposes. Details of the MRM transitions used, the conditions of the MS experiments, the standards used and the strategy employed for the identification of each metabolite (comparison with commercial standard or analysis of MS/MS fragmentation pattern) are provided in **Table S1**.

2.6 Statistics

Results are expressed as mean concentrations (μM) with standard error of the mean (SEM). Also, to facilitate comparisons between groups, the values corresponding to the HFHS, STD + GSE and HFHS + GSE groups were divided by those of the STD group, to obtain the relative incremental factor or x-fold increase. The non-parametric Kruskal-Wallis and Mann-Whitney U tests were applied to analyse significant differences ($P < 0.05$) between groups. The Kruskal-Wallis test was applied to determine any significant difference between the treatments and, if any were detected, the Mann-Whitney U test was used to compare all the different pairs of the treatments. The SPSS IBM 19 package for Windows was used throughout.

3. Results

3.1 Microbial-derived metabolites in urine

Forty-eight transitions, corresponding to microbial metabolites reported to be formed during the intestinal fermentation of PAs (25) were searched for in the samples. **Table 2** shows the concentration data for the metabolites detected, as well as the x-fold incremental factors compared to the STD group.

As expected, in most cases the metabolite concentrations in the STD + GSE group were significantly higher than in the STD control and HFHS groups. Meanwhile, the concentrations of PA metabolites when the high-dense-energy diet was supplemented with GSE (HFHS + GSE group) were significantly lower than those recorded for the STD + GSE group. This was observed for metabolites belonging to all the steps along the PA fermentation pathways (valerolactones, lignans, phenylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acids, cinnamic acids and glyccinated benzoic acids), and was particularly marked for phenylvaleric acids and phenylpropionic acids. The total concentrations of metabolites belonging to these two

classes in the HFHS + GSE group were seven-fold and nearly twenty-fold lower, respectively, than in the STD + GSE group.

3.2 Microbial-derived metabolites in faeces

Ten microbial-derived metabolites were identified in faeces; **Table 3** shows the concentration data of the metabolites and the x-fold incremental factors compared to the STD diet. The same tendencies as observed for microbial-derived metabolites in urine were found in faeces: a) there was a significant increase in the overall concentration of these compounds in the STD + GSE group, compared to the non-supplemented groups; b) the formation of microbial-derived metabolites was lower in the HFHS + GSE group. Thus, for most of the compounds detected, the concentrations in the HFHS + GSE group were significantly lower than in the STD + GSE group.

3.3 Conjugated metabolites of (epi)catechin and (epi)gallocatechin in urine

A total of 39 transitions were searched for in urine, corresponding to monoconjugated, diconjugated and triconjugated (derived from the combination of methylated or Me, sulfated or Sulf and glucuronidated or Gluc forms) metabolites of EC and EGC. Among them, 15 metabolites were detected in the samples: 5 monoconjugated (EC glucuronidated in different positions), 7 diconjugated (five of EC and two of EGC) and three triconjugated (two of EC and one of EGC) (**Table 4)**. They were identified using further MRM or their MS/MS fragmentation patterns (**Table S1**).

Five EC monoglucuronides were detected; one exhibited its highest concentrations in the STD + GSE group, while three others were detected at their highest concentrations in the HFHS + GSE group. With regards to the diconjugated EC metabolites, a tendency towards significantly higher concentrations in the HFHS + GSE group than in the STD + GSE group was observed. No significant difference was observed between the groups for either EC triconjugated metabolites or EGC conjugated metabolites. Total excretion

of conjugated metabolites in urine was significantly higher in the HFHS + GSE group than in the other three groups.

4. Discussion

In this study, we explored the effects of a HFHS diet on the metabolic fate of supplemented grape PAs, compared to a STD diet with or without this supplementation. Much effort has been devoted to properly characterizing the transformation of PAs after their intake, based on animal studies, in which they were fed a STD diet, or in human studies, involving either acute PA intake or a controlled and balanced diet [27]. However, since Western diets typically present an excess of fat and simple carbohydrates with respect to health recommendations, and it is known that other dietary components may affect the bioavailability of polyphenols, the potential effect of this dietary pattern on the transformation of polyphenols needs to be evaluated. Specifically, we determined here the levels of PA-derived metabolites after supplementing rats on a HFHS diet for a period of 16 weeks; representative of long-term adherence to a high-energy-dense diet.

Overall, the profiles of metabolites detected were similar to those previously reported in urine and faeces after supplementation with grape PAs [24-25], and they were within the same ranges as those reported in studies with similar supplementation over shorter periods [28]. Regarding the apparently paradoxical detection of valerolactones in the HFHS group which was fed a synthetic diet that did not contain polyphenols, it should be remarked that several studies in humans have found basal concentrations of these compounds after as long as 72 h of a polyphenol-free diet [26,29], despite the fact that their renal excretion takes place 8-24 h after intake [30-31]. This seems to indicate that, although PAs are the main precursors of valerolactones [26], a minor fraction of these metabolites may be originated from other precursors, as proposed in the Human

Metabolome Database [32].

The most remarkable effect we observed was that many microbial metabolites were significantly decreased in the HFHS-GSE group, as compared to the STD-GSE group, in both urine and the aqueous fraction of faeces- representative of those in contact with the intestinal epithelium [33]. Therefore, the high-energy-dense diet reduces the amount of polyphenol metabolites bioavailable and bioaccessible in the gut. Overall, this is probably due more to a decrease in their formation more than in their absorption, since the same tendency was observed in urine (post-absorption) that in faeces (not absorbed). Nevertheless, for some specific compounds, such as 4-hydroxyphenylpropionic acid, a decrease in their absorption should not be discarded, since the HFHS-GSE group showed the highest concentration values in the faeces. The present results may have implications for the potential beneficial effects of GSE supplementation when following a HFHS diet, since increasing evidence shows that the microbial metabolites of polyphenols play a key role in their health-related effects [16]. Along these lines, it was recently reported that 3,4-dihydroxybenzoic acid (one of the compounds whose circulation was found to be reduced when following the HFHS diet in this study) has the capacity to activate components within the insulin signalling pathway [34]. Similarly, the circulating levels of urolithin A glucuronide, a microbial metabolite of ellagitannins, another class of polyphenols, were inversely associated with impaired glycaemic control [35].

Another important implication of the effects we observed in the microbial transformation of PAs when following a HFHS diet are the modifications to the microbiota responsible for that very transformation. We previously reported that a HFHS diet induces a shift in bacterial species towards a higher prevalence of Enterobacteriales, including *Escherichia coli*, probably related to weight gain in rats

[36]. Moreover, in both rodents and humans, a shift towards lower values of the ratio Bacteroidetes/Firmicutes, with a loss of diversity in rodents, has been related with a fat phenotype [37-38]; although this has not been convincingly confirmed and some authors report contradictory results in humans [39]. Information on the bacterial species involved in the transformation of polyphenols is still limited [40], as is overall knowledge of the two-way interaction polyphenols–gut microbiota [41]. However, it seems plausible that the modifications to the microbiota caused by a HFHS diet selectively affect species capable of transforming polyphenols. At the same time, polyphenols may be capable of modifying the composition of gut microbiota, as suggested by the increase in the population of *Bifidobacterium* and decrease in Enterobacteriales after the supplementation of healthy humans with PA-rich GSE [42]. Opposite effects on the same bacterial types have also been attributed to high-energy-dense diets and obesity in rodents [43] and humans [44]. GSE might counteract a putative decrease in PA-processing bacteria caused by a HFHS diet, but our results suggest that this is not the case as the concentration of microbial-derived metabolites was lower in the HFHS + GSE group than in the STD + GSE group- nevertheless, it kept higher than in the HFHS group. Similarly, supplementation of a HFHS diet with resveratrol did not counteract the dysbiosis triggered by a HFHS diet; while, in contrast, quercetin supplementation did compensate for the diet-induced changes. These results suggest differential effects depending on the type of polyphenol [22]. Interestingly, when animals fed a HFHS diet received a combined supplementation with cranberry polyphenols and a probiotic, the latter increased the circulation of phenolic metabolites [23].

Additionally, a tendency towards increased formation of conjugated EC metabolites in the HFHS + GSE group was observed. This suggests higher activity of the detoxifying

enzymes in enterocytes and liver. It has been reported that the activity of the liver cytochrome P450 2E1 is increased during non-alcoholic steatohepatitis [45]; a pathology linked to high-fat diets. It has also been reported that the hepatic expression of uridine 5'-diphosphate glucuronosyltransferase (the enzyme responsible for the glucuronidation of polyphenols) is up-regulated in male rats fed a HFHS diet, which is related, among other things, to increased expression of the proliferator-activated receptor α (PPARα), which appears when consuming such a diet [46]. Although those authors did not find the same effects in female rats, our results indirectly seem to indicate a similar up-regulation of this enzyme or those involved in the sulfation or methylation of EC after long-term exposure to a HFHS diet. Another possible explanation for the increase in EC conjugates would be delayed exposure to phase II enzymes associated with longer digestion times in animals fed a HFHS diet.

The dose used in this study (30 mg PA/kg body weight of rat) would be equivalent to a daily dose of 4.9 mg/kg body weight in humans [47], i.e., 340 mg/d for a 70 kg adult. Since median daily polyphenol intake in humans is spread over a wide range, from about 150 to nearly 500 mg/p/day [48], significantly large subpopulations consume more polyphenols than the amount equivalent to the dose used in the present study. As no adverse effects have ever been reported, this dose could certainly be considered safe. Indeed, toxicological studies in rats report no adverse effect at doses much higher than that used in this study [49]. Similar doses of GSE have been shown to have beneficial effects on variables related to metabolic syndrome, such as lipidaemia—in rats and humans—or insulin metabolism—in rats [10,12].

This study has some limitations. First, a higher number of animals would have strengthened the statistical significance of the differences detected in some metabolites. However, we consider that the resulting lack of significance in some of the observations

does not invalidate our overall conclusions. Second, due to the limited number of commercial standards of PA metabolites that are currently available, the results had to be expressed as equivalents of other metabolites; thus, the values provided here should only be used for comparative purposes.

In summary, a HFHS diet significantly decreased the production of microbial-derived PA metabolites in GSE-supplemented rats, with respect to PA metabolism in animals fed the STD diet. At the same time, an increase in conjugated EC metabolites was observed in the HFHS group; probably due to up-regulation of hepatic enzymes. Our results seem to indicate a shift in the microbial populations triggered by a HFHS diet that is not reversed by the polyphenols in GSE. This effect should be further studied-nevertheless, the concentrations of microbial-derived PA metabolites kept higher in the HFHS + GSE group than in the HFHS group. Since microbial metabolites seem to be key mediators of the biological activities of polyphenols, a decrease in their formation when following a HFHS diet would presumably affect the health-related properties of polyphenols.

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Conflict of interest

The authors declare that they have no conflict of interest.

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TABLES

Table 1. Composition of experimental diets

	Diet			
	STD	**HFHS**	**STD-GSE**	**HFHS-GSE**
Ingredients (g)				
Flour	1000.0[1]	1000.0[2]	1000.0[1]	1000.0[2]
TBHQ	0.08	0.08	0.08	0.08
Porcine gelatin	25.0	25.0	25.0	25.0
Soybean lecithin	6.0	22.0	6.0	22.0
Soybean oil	17.4	22.0	17.4	22.0
Grajfnol®[3]	-	-	0.88	1.09
Macronutrients[4]				
Protein (% by weight)	16.0	20.9	16.0	20.9
Carbohydrate (% by weight)	66.8	47.4	66.8	47.4
Fat (% by weight)	6.0	25.6	6.0	25.6
Energy from protein (%)	16.5	16.5	16.5	16.5
Energy from carbohydrate (%)	69.4	37.7	69.4	37.7
Energy from fat (%)	14.1	45.8	14.1	45.8
Total energy density (Kcal/g)[7]	3.9	5.1	3.9	5.1

[1] Standard flour (Teklad Global 2014), containing wheat middlings, ground wheat, ground corn, corn gluten meal, calcium carbonate, soybean oil, dicalcium phosphate, iodized salt, L-lysine, vitamin E acetate, DL-methionine, magnesium oxide, choline chloride, manganous oxide, ferrous sulfate, menadione sodium bisulfite complex (source of vitamin K activity), zinc oxide, copper sulfate, niacin, calcium pantothenate, calcium iodate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, vitamin B12 supplement, folic acid, cobalt carbonate, biotin and vitamin D3 supplement.

[2] High-fat high-sucrose diet (Tekland TD 08811), containing sucrose, anhydrous milkfat, casein, maltodextrin, corn starch, cellulose, mineral mix AIN-93G-MX, soybean oil, vitamin mix AIN-93G-VX, L-cystine, choline, bitartrate, green food colour, tert-butylhydroquinone

[3] Grajfnol® dose was adjusteded to provide a daily proanthocyanidin dose of 30 mg/kg body weight (body weight was higher in rats following a HFHS diet).

[4] Energy density is estimated as *metabolizable energy* based on the Atwater factors, assigning 4kcal/g to protein, 9kcal/g to fat, and 4kcal/g to carbohydrate, including dietary fiber.

STD, standard; HFHS, high-fat high-sucrose diet; GSE, grape seed extract; n.s., not specified

Table 2. Microbial-derived proanthocyanidin metabolites in urine from rats fed a standard (STD) diet or a high-fat high-sucrose (HFHS) diet without or with grape seed extract (GSE). Results expressed as µM, after quantification with structurally similar commercial standards (see**Table S1**).

Metabolite	STD	HFHS		STD+ GSE		HFHS + GSE	
	mean ± s.e.m.	mean ± s.e.m.	x-fold[1]	mean ± s.e.m.	x-fold[1]	mean ± s.e.m.	x-fold[1]
Valerolactones							
3- or 4-Hydroxyphenylvalerolactone	1.43 ± 0.43	2.34 ± 0.54	1.6	21.39 ± 7.31$^{**\&\&}$	15.0	0.94 ± 0.52$^{\$\$}$	0.6
3,4-Dihydroxyphenylvalerolactone	0.35 ± 0.24	n.d.	-	12.38 ± 4.14$^{**\&\&}$	35.4	n.d.$^{\$\$}$	-
Gluc-3,4-dihydroxyphenylvalerolactone	3.05 ± 0.93	14.97 ± 5.56	4.9	9.3 ± 1.66*	3.0	8.72 ± 3.04	2.9
Sulf-3,4-dihydroxyphenylvalerolactone	0.94 ±0.58	n.d.	-	77.49 ± 6.63$^{**\&\&}$	82.4	51.41 ± 26.57$^{*\$\$}$	54.5
3-Hydroxyphenylmethylvalerolactone	1.43 ± 0.43	7.99 ± 2.12	5.6	5.26 ± 1.49**	3.7	5.00 ± 2.48	3.5
4-Hydroxyphenylmethylvalerolactone	12.75 ± 4.36	46.49 ± 1.81	3.6	35.43 ± 9.84	2.8	35.07 ± 14.14	2.7
Gluc-3-hydroxymethylphenylvalerolactone	6.68 ± 2.52	17.25 ± 6.14	2.6	9.66 ± 1.60	1.4	5.66 ± 1.82	0.8
Sulf-3- ó 4-hydroxymethylphenylvalerolactone	4.12 ± 1.37	21.11 ± 9.35	5.1	5.17 ± 0.93	1.3	18.55 ± 4.61*	4.5
Total	30.66 ± 6.43	116.12 ± 34.87	3.6	175.93 ± 25.19**	5.7	126.43 ± 46.58	4.2
Lignans							
Enterolactone[2]	> 60	> 60		> 60		> 60	
Phenylvaleric acids							
3-Hydroxyphenylvaleric acid	1.84 ± 0.80	n.d.**	-	5.76 ± 0.87$^{*\&\&}$	3.1	3.06 ± 1.22$^{\$\$}$	1.7
4-Hydroxyphenylvaleric acid	0.27 ± 0.10	0.40 ± 0.10	1.5	2.67 ± 0.90$^{**\&\&}$	10.0	2.06 ± 0.69$^{*\&}$	7.5
3,4-Dihydroxyphenylvaleric acid	0.52 ± 0.15	6.14 ± 1.83	11.8	2.66 ± 0.69*	5.1	4.18 ± 1.30	8.1
Sulf-3,4-dihydroxyphenylvaleric acid	1.66 ± 0.67	2.52 ± 0.87	1.5	59.43 ± 12.46$^{**\&\&}$	35.8	1.01 ± 0.50$^{\$\$}$	0.6
Total	4.30 ± 1.37	9.06 ± 1.87	2.1	70.51 ± 12.63$^{**\&\&}$	16.4	10.30 ± 3.44$^{\$\$}$	2.4
Phenylpropionic acids							
3-Hydroxyphenylpropionic acid	393.66 ± 162.88	13.36 ± 7.15*	0.03	801.14 ± 523.07$^{\&\&}$	2.0	24.13 ± 8.71$^{\$\$}$	0.06
Gluc-3- or- 4hydroxyphenylpropionic acid	1.27 ± 0.77	24.31 ± 9.33	19.2	0.96 ± 0.12	0.8	15.31 ± 6.51$^{*\$\$}$	12.1
Dihydrocaffeic acid (3,4-Dihydroxyphenylpropionic acid)	0.22 ± 0.10	0.67 ± 0.24	3.1	3.21 ± 2.59*	14.8	0.32 ± 0.17	1.5
Sulf-3,4-dihydrocaffeic acid	2.19 ± 0.91	2.91 ± 0.97	1.3	6.80 ± 4.34	3.1	2.87 ± 0.96	1.3

Total	397.34 ± 163.18	41.25 ± 8.72	0.1	815.12 ± 530.01$^{\&\&}$	2.1	42.63 ± 15.65$^{\$\$}$	0.1
Phenylacetic acids							
3-Hydroxyphenylacetic acid	3.74 ± 1.40	4.71 ± 1.57	1.3	24.10 ± 3.79$^{**\&\&}$	6.4	7.23 ± 3.11$^{\$}$	1.9
4-Hydroxyphenylacetic acid	3.62 ± 1.40	80.49 ± 20.14	22.3	102.44 ± 17.61$^{**\&\&}$	28.3	37.99 ± 17.26$^{\$}$	10.5
3,4-Dihydroxyphenylacetic acid	0.05 ± 0.02	0.56 ± 0.22	10.9	0.58 ± 0.28**	11.6	0.35 ± 0.16	6.8
Sulf-3,4-dihydroxyphenylacetic acid	0.46 ± 0.26	0.76 ± 0.30	1.6	0.47 ± 0.11	1.0	0.53 ± 0.18	1.1
Total	7.87 ± 2.74	86.51 ± 21.92**	11.0	127.6 ± 16.2**	16.2	46.09 ± 20.49$^{\$}$	5.8
Benzoic acids							
4-hydroxybenzoic acid	0.82 ± 0.33	4.40 ± 1.13	5.3	3.60 ± 1.13*	4.4	2.06 ± 0.57	2.5
3,4-Dihydroxybenzoic acid	0.02 ± 0.01	0.10 ± 0.03	4.7	1.20 ± 0.55$^{**\&\&}$	56.9	0.04 ± 0.02$^{\$\$}$	2.0
Gluc-3-hydroxybenzoic acid	0.01 ± 0.01	0.005 ± 0.002	0.4	0.15 ± 0.07$^{**\&\&}$	10.6	0.003 ± 0.002$^{\$\$}$	0.3
Gluc-4-hydroxybenzoic acid	n.d.	0.04 ± 0.02**	> 31	0.02 ± 0.005*	> 7	0.01 ± 0.01	> 10
Sulf-3,4-dihydroxybenzoic acid	0.39 ± 0.12	2.43 ± 0.68	5.0	3.90 ± 2.12**	5.1	1.82 ± 0.72	3.8
Sulf-vanillic-acid	19.43 ± 3.83	12.04 ± 3.92	0.6	25.92 ± 4.41	1.3	9.98 ± 3.60$^{\$}$	0.5
Total	20.68 ± 3.57	19.01 ± 5.17	0.9	34.79 ± 1.70	1.7	13.92 ± 4.53	0.7
Cinnamic acids							
Caffeic acid	0.06 ± 0.03	n.d.*	-	0.17 ± 0.10$^{\&\&}$	3.0	n.d.$^{*\$\$}$	-
m-coumaric acid	6.92 ± 3.26	0.37 ± 0.12*	0.05	13.96 ± 1.74$^{\&\&}$	2.0	3.73 ± 1.11$^{\&\&\$\$}$	0.5
p-coumaric acid	1.43 ± 0.53	0.16 ± 0.04	0.1	1.86 ± 0.53$^{\&\&}$	1.3	0.10 ± 0.04$^{\$\$}$	0.07
Sulf-coumaric acid-1	n.d.	0.38 ± 0.16**	> 38	0.79 ± 0.27**	> 790	0.14 ± 0.13*	> 14
Sulf-coumaric acid-2	0.002 ± 0.001	0.35 ± 0.15**	169.3	0.75 ± 0.24**	375.0	0.08 ± 0.08$^{\$}$	38.0
Ferulic acid	0.91 ± 0.37	n.d.**	-	1.14 ± 0.43$^{\&\&}$	1.2	n.d.$^{**\$\$}$	-
Total	9.31 ± 3.90	1.26 ± 0.33	0.1	18.68 ± 2.00$^{\&\&}$	2.0	4.05 ± 1.27$^{\&\$\$}$	0.4
Glycinated benzoic acids							
Hippuric acid	2.85 ± 1.05	167.85 ± 73.30**	58.9	125.18 ± 73.97	43.9	105.48 ± 75.36	37.0
Hydroxyhippuric acid	0.02 ± 0.02	0.22 ± 0.07*	12.7	0.96 ± 0.20$^{**\&\&}$	48.0	0.12 ± 0.06$^{\$\$}$	7.2
Me-hippuric acid-1	0.01 ± 0.01	0.33 ± 0.13**	37.3	5.32 ± 2.02**	532.0	0.13 ± 0.12	14.3
Me-hippuric acid-2	0.17 ± 0.07	n.d.**	-	0.79 ± 0.42$^{\&\&}$	4.6	n.d. $^{**\$\$}$	-
Total	3.05 ± 1.10	168.40 ± 73.41**	55.3	132.25 ± 74.10*	43.4	105.73 ± 75.51	34.7
Total of microbial-derived metabolites[3]	473.21 ± 174.23	441.61 ± 123.04	0.9	1375.03 ± 534.83	2.9	355.49 ± 164.69	0.7

n.d., non-detected; Gluc, glucuronide; Me, methyl; Sulf, sulfated

[1] Values generated by dividing metabolite concentration by the concentration of the same metabolite in the STD group. When the compound was n.d. in the STD group, the limit of detection was used to calculate x-fold value.

[2] Enterolactone occurred in all groups at concentrations above the highest point in the calibration curve..
[3] Enterolactone was not included in the calculation of the total microbial metabolites because its actual concentration could not be determined.
[*] P < 0.05 vs STD group. [**]P< 0.01 vs STD group. [&] P < 0.05 vs HFHS group. [&&] P< 0.01 vs HFHS group. [$] P < 0.05 vs STD + GSE group. [$$] P < 0.01 vs STD + GSE group. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney U tests

Table 3. Microbial-derived proanthocyanidin metabolites in faeces from rats fed a standard (STD) diet or a high-fat high-sucrose (HFHS) diet without or with grape seed extract (GSE). Results expressed as µmol/g dried faeces, after quantification with structurally similar commercial standards (see **Table S1**).

Metabolite	STD	HFHS		STD + GSE		HFHS + GSE	
	mean ± s.e.m.	mean ± s.e.m.	x-fold[1]	mean ± s.e.m.	x-fold[1]	mean ± s.e.m.	x-fold[1]
Phenylvaleric acids							
3-Hydroxyphenylvaleric acid	0.19 ± 0.09	n.d.**	-	55.59 ± 49.19$^{*\&\&}$	292.6	27.44 ± 22.72$^{*\&\&}$	147.4
Phenylpropionic acids							
3-Hydroxyphenylpropionic acid	2.67 ± 2.30	n.d.**	-	5.75 ± 5.25$^{\&\&}$	2.2	0.35 ± 0.07$^{\&\&}$	0.13
4-Hydroxyphenylpropionic acid	0.65 ± 0.16	0.20 ± 0.11	0.3	2.32 ± 1.82	3.6	39.63 ± 33.96$^{\&\&}$	61.3
Total	3.32 ± 2.47	0.20 ± 0.11*	0.3	8.07 ± 5.10$^{\&\&}$	2.4	39.99 ± 34.00$^{\&}$	12.0
Benzoic acids							
4-hydroxybenzoic acid	n.d.	n.d.*	-	0.06 ± 0.04$^{*\&}$	> 615	n.d.$^{*\$}$	-
3,4-Dihydroxybenzoic acid	n.d.	n.d.		0.01 ± 0.01$^{**\&\&}$	> 9	n.d.$^{\$\$}$	-
Total	n.d.	n.d.	-	0.07 ± 0.05$^{**\&\&}$	> 48	n.d.$^{\$\$}$	-
Cinnamic acids							
Caffeic acid	0.001 ± 0.0004	n.d.	-	0.03 ± 0.02$^{**\&\&}$	30.0	n.d.$^{**\ \$\$}$	-
p-coumaric acid	0.002 ± 0.001	0.001 ± 0.0002	0.5	0.15 ± 0.12$^{*\&}$	75.0	0.005 ± 0.003	2.2
Total	0.003 ± 0.0020	0.001 ± 0.0002	0.3	0.18 ± 0.14$^{*\&\&}$	60.0	0.005 ± 0.003$^{\$}$	2.2
Glycinated benzoic acids							
Hippuric acid	0.003 ± 0.002	n.d.	-	0.003 ± 0.002	1.0	0.02 ± 0.02	5.3
Me-hippuric acid-1	0.39 ± 0.31	n.d.**	-	629.57 ± 646.52$^{\&\&}$	1614.3	n.d.$^{**\&\&\$\$}$	-
Me-hippuric acid-2	n.d.	n.d.**	-	354.38 ± 324.50$^{**\&\&}$	> 1,000	n.d.$^{\$\$}$	-
Total	0.39 ± 0.31	n.d.	-	1046.95 ± 970.73$^{**\&\&}$	2664.0	0.02 ± 0.02$^{*\$\$}$	0.04
Total of microbial-derived metabolites	3.90 ± 2.49	0.20 ± 0.11*	0.03	1110.86 ± 1018.35$^{**\&\&}$	284.8	67.46 ± 56.73$^{\&\&}$	17.3

n.d., non-detected; Gluc, glucuronide; Me, methyl; Sulf, sulphated

[1] Values generated by dividing metabolite concentration by the concentration of the same metabolite in the STD group . When the compound was n.d. in the STD group, the limit of detection was used to calculate the x-fold value.

* P < 0.05 vs STD group. **P< 0.01 vs STD group. $^{\&}$ P < 0.05 vs HFHS group. $^{\&\&}$ P< 0.01 vs HFHS group. $^{\$}$ P < 0.05 vs STD + GSE group. $^{\$\$}$ P < 0.01 vs STD + GSE group. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney U tests

Table 4. (Epi)catechin and (epi)gallocatechin conjugated metabolites in urine from rats fed a standard (STD) diet or a high-fat high-sucrose (HFHS) diet without or with grape seed extract (GSE). Results expressed as µM, after quantification with structurally similar commercial standard (see**Table S1**).

Metabolite	STD	HFHS		STD + GSE		HFHS+ GSE	
	mean ± s.e.m.	mean ± s.e.m.	x-fold[1]	mean ± s.e.m.	x-fold[1]	mean ± s.e.m.	x-fold[1]
EC monoconjugated							
Gluc-EC-1	n.d.	n.d.	-	0.32 ± 0.16$^{**\&\&}$	> 62	0.26 ± 0.11$^{**\&\&}$	> 52
Gluc-EC-2	n.d.	n.d.	-	0.22 ± 0.11$^{*\&}$	> 43	0.32 ± 0.17$^{*\&}$	> 64
Gluc-EC-3	0.04 ± 0.02	0.20 ± 0.06	5.4	0.17 ± 0.06	4.3	0.24 ± 0.13*	6.4
Gluc-EC-4	0.02 ± 0.001	0.11 ± 0.03	5.6	0.05 ± 0.02	2.9	0.05 ± 0.01**	2.8
Gluc-EC-5	0.05 ± 0.03	0.69 ± 0.29	13.2	0.11 ± 0.06	2.2	0.55 ± 0.26**	10.6
Total	0.11 ± 0.04	1.00 ± 0.38	9.2	0.88 ± 0.35*	8.1	1.44 ± 0.64**	19.8
EC diconjugated							
Gluc-Sulf-EC	0.07 ± 0.02	0.02 ± 0.01	0.3	0.19 ± 0.09	2.8	0.03 ± 0.01$^{\&\$\$}$	0.4
Me-Gluc-EC-1	n.d.	n.d.	-	0.05 ± 0.02$^{**\&\&}$	> 10	0.05 ± 0.01$^{**\&\&}$	> 10
Me-Gluc-EC-2	n.d.	n.d.	-	0.83 ± 0.27$^{**\&\&}$	> 167	1.67 ± 0.52$^{**\&\&}$	> 333
Me-Gluc-EC-3	n.d.	n.d.	-	0.30 ± 0.10$^{**\&\&}$	> 59	0.96 ± 0.34$^{**\&\&}$	> 190
Me-Sulf-EC	0.15 ± 0.05	0.15 ± 0.02	1.0	0.16 ± 0.03	1.1	0.15 ± 0.04	1.0
Total	0.20 ± 0.07	0.17 ± 0.07	0.8	1.55 ± 0.46$^{**\&\&}$	7.8	2.85 ± 0.86$^{**\&\&}$	14.2
EC triconjugated							
3Me-EC	0.06 ± 0.02	0.09 ± 0.03	1.4	0.06 ± 0.02	1.0	0.06 ± 0.01	1.0
2Me-Gluc-EC	0.05 ± 0.01	0.06 ± 0.02	1.6	0.09 ± 0.02	1.8	0.03 ± 0.01	0.6
Total	0.10 ± 0.03	0.15 ± 0.05	1.5	0.15 ± 0.03	1.5	0.10 ± 0.02	1.0
EGC diconjugated							
2Sulf-EGC	0.84 ± 0.17	1.54 ± 0.46	1.8	1.22 ± 0.24	1.1	0.89 ± 0.25	1.1
EGC triconjugated							
Me-Gluc-Sulf-EGC	0.23 ± 0.07	0.13 ± 0.13	0.5	0.46 ± 0.06	2.0	0.26 ± 0.10	1.1
Total of conjugated metabolites	1.49 ± 0.33	2.99 ± 1.06	2.3	4.25 ± 0.94	2.9	8.62 ± 2.46$^{**\&\&\$}$	5.8

n.d., non-detected; Gluc, glucuronide; Me, methyl; Sulf, sulphated

[1] Values generated by dividing metabolite concentration by the concentration of the same metabolite in the STD group . When the compound was n.d. in the STD group, the limit of detection was used to calculate the x-fold value.

* P < 0.05 vs STD group. **P< 0.01 vs STD group. $^{\&}$ P < 0.05 vs HFHS group. $^{\&\&}$ P< 0.01 vs HFHS group. $^{\$}$ P < 0.05 vs STD + GSE group. $^{\$\$}$ P < 0.01 vs STD + GSE group. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney U tests