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Gut Microbiota Influences the Photoperiod Effects on Proanthocyanidins Bioavailability in Diet-Induced Obese Rats

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Scope: Polyphenols health effects on obesity are mainly attributed to their metabolites generated after their gastrointestinal digestion, in which gut microbiota plays an important role. Moreover, gut microbiota composition and polyphenols bioavailability are influenced by differences in day light length (photoperiod). Thus, this study evaluates if a grape seed proanthocyanidins (GSPEs) extract bioavailability is influenced by different photoperiod exposure via gut microbiota modulation in an obesogenic context.

Methods and results: Cafeteria diet-induced obese Fischer 344 rats are housed under different photoperiod conditions (6, 12, or 18 h of light per day) during 9 weeks and administered with GSPE (25 mg kg^{-1}) or GSPE and an antibiotic cocktail (ABX) for the last 4 weeks. Serum GSPE-derived metabolites are quantified by HPLC-MS/MS.

Conclusion: A higher bioavailability is observed under 6 h light/18 h darkness (L6) compared to 18 h light/6 h darkness (L18). Individual metabolites, especially those from the gut microbiota, are affected by photoperiods. ABX treatment alters these photoperiod-mediated changes. Therefore, these results suggest that gut microbiota plays a key role in the photoperiod effects on GSPE bioavailability in obese rats.

1. Introduction

Proanthocyanidins (PAs) are among the polyphenols most commonly consumed in the human diet.[1] They are constituted by oligomers or polymers of subunits of flavan-3-ols (catechin and epicatechin) and their gallic acid esters.[2] PAs are present in many plants such as barley, hops, maize, apples, grapes, strawberries, cocoa, almond, cinnamon, peanuts, and tea. Particularly, grape seeds provide a wide variety of PAs, whose consumption has been associated with several beneficial health effects.[3] Indeed, administration of a grape seed proanthocyanidin (GSPE) extract has been reported to ameliorate insulin resistance,[4] hypertension,[5–7] oxidative stress,[8] mitochondrial dysfunctionality,[9] and lipid metabolism,[10–12] among other effects, in rats. Hence, PAs have a significant potential for the prevention of metabolic syndrome (MetS) and cardiovascular diseases.

Several studies have attributed the bioactive effects of polyphenols to their metabolized forms.[13] Thus, they undergo extensively transformation in the small intestine or in the liver by phase-II enzymes to form glucuronide, sulfate, and/or methylate conjugates.[14] Moreover, 90%–95% of dietary polyphenols reach the colon, where they are metabolized by the gut microbiota. Thus, these bacteria are able to hydrolyze, reduce, dehydroxylate, decarboxylate, and demethylate these compounds.[14] As a result, high molecular weight PAs are converted into small microbial-derived metabolites (valerolactone compounds, phenylpropionic acids, phenylacetic acids, benzoic acids, and conjugated phenolic acids).[15,16] Part of these microbial metabolites are absorbed and further metabolized in the liver.[17] Subsequently, both phase II and microbial-derived absorbed metabolites are distributed to different tissues via systemic circulation.[18–20]Additionally, the metabolites in the liver can be reabsorbed through the enterohepatic recirculation. Finally, the metabolites are eliminated in the urine via the bile duct or in the feces.[14]

Taking all the above in consideration, it is important to evaluate the effects of external factors that may affect polyphenols bioavailability as that may have a significant impact in their functionality. In this context, photoperiods are of increasing interest as they have been reported to influence polyphenols functionality.[21,22] Thus, polyphenol-rich fruits provided a higher leptin sensitivity only in cafeteria diet (CAF)-induced obese rats under short winter-like day length (SD).[21] Moreover, a higher bioavailability of phenolic acids was found in normoweight rats treated with

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Table 1. Main phenolic compounds (flavan-3-ols and phenolic acids) of the grape seed proanthocyanidin (GSPE) extract used in this study, analyzed by HPLC-MS/MS.

Phenolic compound	Concentration [mg g^{-1}]
3,4-Dihydroxybenzoic acid	1.40 ± 0.25
(+)-Catechin	51.88 ± 5.56
(−)-Epicatechin	62.86 ± 8.32
3,4,5-Trihydroxybenzoic acid	44.66 ± 7.76
Kaempferol-3-*O*-glucoside	0.50 ± 0.02
Naringenin-7-glucoside	0.64 ± 0.08
p-Coumaric acid	0.09 ± 0.01
Quercetin	0.05 ± 0.01
Quercetin-3-*O*-galactoside	0.43 ± 0.05
4-Hydroxy-3-methoxybenzoic acid	0.09 ± 0.01
Procyanidin dimer	76.84 ± 15.76
Procyanidin trimer	13.04 ± 0.64
Procyanidin tetramer	5.14 ± 0.28
Dimer gallate	15.22 ± 2.72
Epicatechin gallate	14.24 ± 2.76
Epigallocatechin gallate	0.06 ± 0.01

Adapted from Rodríguez et al. 2022.[29] Concentrations are expressed as mg of compound per gram of fresh extract (means ± standard derivation).

red grapes housed under SD in comparison with rats housed under summer-like day length conditions (LD).[22] However, the specific mechanism involved in these photoperiod-mediated effects on polyphenols bioavailability is not yet fully understood. In this regard, gut microbiota may be played an important role. Thus, as above mentioned, these bacteria are significantly involve in polyphenols metabolism and have been shown to be affected by different photoperiod conditions.[23–27] Indeed, it was recently published that mice fed with an isoflavones-based diet and exposure to different photoperiod conditions showed an association between the gut bacterial communities, photoperiod length, and isoflavone compounds.[28]

Hence, the aim of this study was to evaluate if the serum bioavailability of GSPE is influenced by different photoperiod exposure conditions via gut microbiota in cafeteria-induced obese rats.

2. Experimental Section

2.1. Grape Seed Proanthocyanidin Extract

GSPE was obtained from white grape seed and provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). According to the manufacturer, this flavanol-rich extract contained monomeric (25.0%), dimeric and trimeric (43.3%), and oligomeric (<3 units; 31.7%) procyanidin. The main phenolic compounds (flavan-3-ols and phenolic acids) present in the extract are shown in **Table 1**.[29]

2.2. Chemical and Reagents

The chemical and reagents used in the current study had been previously reported by our group.[30] Briefly, acetone, acetonitrile, phosphoric acid (Sigma–Aldrich, Madrid, Spain), glacial acetic acid (Panreac, Barcelona, Spain), and methanol (Scharlab S.L., Barcelona, Spain) were all of HPLC analytical grade. Ultrapure water was obtained from a MilliQ Advantage A10 system (Millipore, Madrid, Spain). Individual standard stock solutions of 2000 mg L^{-1} of (+)-catechin, epigallocatechin gallate (EGCG), 3,4,5-trihydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 3-hydroxybenzoic acid, 3′-hydroxyphenylacetic acid, 3′,4′-dihydroxycinnamic acid, 3-(4′-hydroxyphenyl)propanoic acid, benzoic acid, hippuric acid, 4′-hydroxy-3′-methoxycinnamic acid and benzene-1,2-diol (internal standard; IS) (Fluka, Sigma–Aldrich, Madrid, Spain), and PA B2 (Extrasynthese Lyon, France), were prepared in methanol and stored in dark glass flasks at −20 °C. In addition, a stock solution containing all individual compounds was prepared weekly at a concentration of 2000 ppm in methanol. Standard compounds were mixed to create the calibration curve prepared in acetone/water/acetic acid (70:29.5:0.5 v/v/v). This solution was stored in dark glass containers at −20 °C until chromatographic analysis.

2.3. Experimental Design

Thirteen weeks old male Fischer 344 rats (F344) ($n = 96$) were obtained from Janvier Laboratories (France). Rats were pair-housed at standard conditions (22 ± 1 °C, 50%–55% relative humidity and 12:12 h light/dark cycle) with ad libitum access to water and standard chow diet (72% of carbohydrates, 8% lipid and 19% protein; Safe-A04c, Barcelona, Spain) for 1 week. After this acclimation week, rats were weighted and randomized distributed into three different photoperiods for 9 weeks: short photoperiod (L6, 6 h light/18 h darkness), standard photoperiod (L12, 12 h light/12 h darkness), or long photoperiod (L18, 18 h light/6 h darkness). Into each photoperiod, rats were further randomly divided into four groups ($n = 8$) depending on the treatment administered in the last 4 weeks. During the whole experiment procedure, rats were fed a CAF composed of highly palatable and energy-dense human foods (58% CH, 31% lipid, and 11% protein). CAF was freshly prepared every day and included the following (grams per rat and per day): biscuits with pâté and cheese (15–17 g), bacon (7–10 g), ensaimada (pastry) (10–15 g), carrot (11–12 g), standard chow diet (20–25 g), and milk containing 22% sucrose w/v.

Serum samples were obtained from the blood collected from the neck in nonheparinized tubes. The blood was incubated for 1 h at room temperature and immediately centrifuged at 1200 × *g* for 15 min to collect the serum. The serum samples were stored at −80 °C until chromatographic analysis was performed (**Figure 1**). The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) and the Generalitat de Catalunya approved all the procedures (number reference 9495) in accordance with the EU Directive 2010/63/EU for animal experiments.

2.4. Dosage Information/Dosage Regimen

Animals were administered with a dose of GSPE (25 mg kg^{-1} of body weight) or with the combination of GSPE and antibiotic

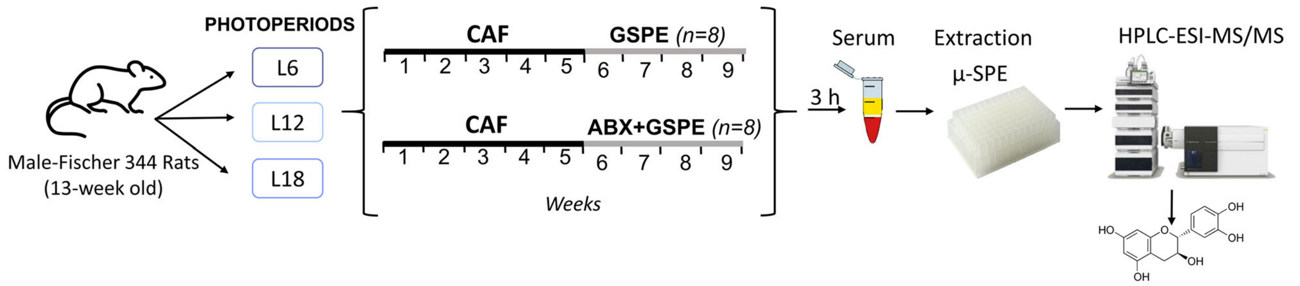

Figure 1. Experimental design. Thirteen-week old male-Fisher 344 rats were housed under three different photoperiods (L6, L12, and L18) for 9 weeks. Into each photoperiod animals were fed a CAF and orally administered with GSPE (25 mg kg^{-1}) or with the combination of GSPE and ABX (0.5 g L^{-1} ampicillin, 0.250 g L^{-1} vancomycin, and 0.125 g L^{-1} imipenem) daily in drinking water during the last 4-week of the experiment. Animals treated with VH or with VH and ABX were included as controls. ABX, antibiotic cocktail; CAF, cafeteria diet; GSPE, grape seed proanthocyanidin; L6, 6 h light/18 h darkness; L12, 12 h light/12 h darkness; L18, 18 h light/6 h darkness; VH, vehicle.

cocktail (ABX) during the last 4 weeks of the experiments. Thus, GSPE was daily and orally administered 1 h after the light was turned on. GSPE was dissolved in a solution of water and condensed milk (vehicle [VH], 5:1 v/v), allowing rats to drink it from the tip of a syringe. The GSPE dose used was within the estimated range of polyphenols intake in humans. This was equivalent to the daily intake of 367 mg for a person of 70 kg,[31] being easy to achieve with a polyphenol-rich diet.[32–34] The ABX (0.5 g L^{-1} ampicillin, 0.250 g L^{-1} vancomycin, and 0.125 g L^{-1} imipenem; Discovery fine chemicals Ltd, United Kingdom) was freshly prepared every day and administered in drinking water ab libitum. This ABX had shown to alter the gut microbiota composition promoting a dysbiosis condition.[35,36] Animals administered with VH or with VH and ABX were used to subtract any phenolic compound in serum no derived from the GSPE. Animals were sacrificed by decapitation, following a fasting period of 3 h after the last dose administration.

2.5. Micro-Solid Phase Serum Phenolic Metabolites Extraction

A microsolid-phase extraction (μSPE) was carried out, as it was previously reported by our group,[30,37] in order to eliminate interferences and quantify the GSPE-derived metabolites. Briefly, the microcartridges were sequentially conditioned with 250 μL of methanol and 250 μL of 0.2% acetic acid. Serum samples (250 μL) were mixed with 300 μL 4% H_3PO_4 and 50 μL IS at 20 ppm. This mixture was loaded into the plates. Subsequently, a washing process was performed to remove interferences that may have been retained in the plates with 200 μL Milli-Q water and then 200 μL acetic 0.2%. Finally, the samples were eluted twice with 50 μL of acetone/water/acetic acid (70:29.5:0.5 v/v/v). The eluted solutions were directly injected into the chromatography equipment.

2.6. Chromatographic Analysis (HPLC-ESI-MS/MS)

Chromatographic separation of phenolic compounds in μ-SPE eluted solutions was performed using an Agilent 1290 LC Series and Zorbax SB-Aq chromatographic column (150 mm × 21 mm i.d., 3.5 μm particle size, Agilent Technologies Palo Alto, CA, USA) at room temperature. The mobile phase consisted of 0.2 % acetic acid in water (solvent A) and 100 % acetonitrile (solvent B) and the gradient program was as follows: the linear gradient was 5% of solvent B to 55% of B in 10 min, 55% B to 80% B in 2 min, maintained 80% solvent B for 3 min, and back to 5% of solvent B for 1 min. A postrun of 10 min was applied for column equilibration. The flow rate was set at 0.4 mL min^{-1} and the injection volume was 2.5 μL for all runs. HPLC system was coupled to a 6410 (MS/MS) tandem mass spectrometer (Agilent Technologies) as it was previously described in detail by Margalef et al. 2014.[37] Negative electrospray (ESI) mode at unit resolution. Electrospray capillary voltage was 3000 V, source temperature was 200 °C, and the flow rate was 14 L min^{-1} with a nebulizer gas pressure of 20 psi. The MS/MS data were acquired in “Multiple Reaction Monitoring” (MRM) mode. Optimized MRM conditions for the analysis were performed as previously reported for the quantification of phase-II and microbial flavan-3-ols metabolites in plasma.[30,37]

2.7. Sample Quantification

For the quantification of the samples, control group blank serum was spiked with standard compounds at eight different concentrations (20–5000 ppb) to obtain calibration curves. Concentrations of metabolites found in GSPE and GSPE + ABX groups were obtained by subtracting the concentrations of compounds quantified in VH and ABX groups, respectively, in each photoperiod. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standards curves. All quality parameters of the HPLC-ESI-MS/MS method are showed in Table S1. Calibration curves were constructed by measuring the peak area for each corresponding standard compound, using the least-squares linear regression analysis. The slope, intercept, and correlation coefficient (R^2) were calculated for each calibration curve. Sensitivity was evaluated by determining the limits of detection (LOD) and quantification (LOQ). The LOD was defined as the lowest concentration corresponding to three times the signal-to-noise ratio, and the LOQ was defined as the lowest concentration corresponding to ten times the signal-to-noise ratio. The accuracy was expressed as relative error (RE) and was evaluated for the three concentrations of each standard within the linearity range: 100, 2.500, and 5.000 ppb. The resulting fluctuations for accuracy were within limits recommended by most of the guidelines (IUPAC, FDA, and SANCO), considering a 15%–20% as an acceptable accuracy. Data acquisition was performed by using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA).

2.8. Statistical Analysis

Data were shown as median (first quartile and third quartile) and were plotted using Graphpad Prism 8.0 software (Graphapad software Inc, San Diego, CA, USA). Statistical analyses were carried out using SPSS software (IBS SPSS statistics 25, Chicago, IL, USA). Normality and homogeneity of variance were evaluated by Shapiro–Wilk and Levene's test, respectively. Data were nonparametric because of the analysis was performed using nonparametric tests: photoperiod effect was analyzed by Kruskal–Wallis test followed by Bonferroni *p*-values adjustment to comparison by pairwise ($p < 0.016$), while the comparison between treatments in each photoperiod was analyzed by Mann–Whitney *U* test ($p < 0.05$).

Principal component analysis (PCA) involving serum phenolic compounds data were analyzed and plotted using MetaboAnalyst v.5.0.[38] Missing values were replaced by the half of the minimum positive values in the data, assuming that most missing values were caused by low abundance metabolites.

Statistical significances were subsequently depicted as follows: * indicating differences between experimental groups (GSPE vs. GSPE + ABX) in each photoperiod ($p \leq 0.05$), and *ab* letters indicating photoperiod effect ($p \leq 0.05$). The statistical test used for individual analysis is provided in the figure legends.

3. Results

To evaluate the role of the gut microbiota in the effect of different photoperiod conditions on the bioavailability of GSPE in an obesogenic context, CAF-fed male F344 exposed to 6, 12, or 18 h of daily light (emulating winter, autumn and spring, or summer light conditions, respectively) were chronically administered with GSPE (25 mg kg^{-1} BW) or with the combination of GSPE and ABX for 4 weeks. The circulating levels of phenolic compounds (flavan-3-ols and phenolic acids, phase II and microbial-derived metabolites) were analyzed in serum.

3.1. Total Serum Phenolic Profile

Different photoperiod effects were observed in total serum phenolic profile (**Figure 2**). Interestingly, rats housed under L6 conditions showed higher levels of metabolites and a different profile compared to those housed under either L12 or L18 conditions. This photoperiod effect was due to the decrease of the glucuronidated metabolites (catechin and epicatechin glucuronide), which were not detected under L12 and L18 conditions. This fact increased the proportion of other metabolites, which included 3,4,5-trihydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 3-*O*-methyl gallic acid, cinnamic acids, hippuric acid, and valerolatone and valeric acid derivates. This group of metabolites accounted for the highest proportion in the serum from rats without ABX housed under L12 and L18 conditions. Thus, under L12 were increased the 3-*O*-methyl gallic acid and the 3-hydroxyphenylacetic acid, while under L18 conditions, these metabolites mainly consisted on hippuric and 3′,4′-dihydroxycinnamic acid. Interestingly, this photoperiod effect was lost when treating with ABX. Moreover, ABX-treated rats showed increased total phenolic metabolites in rats housed under L12 and L18 conditions compared to those rats without ABX. However, rats under L6 administered with ABX showed lower total metabolites than those housed under the same conditions without ABX. Nevertheless, same tendency toward decreased levels of metabolites as the hours of day light increased were observed in ABX-treated rats.

3.2. Flavan-3-ols and Phenolic Acids

Flavan-3-ols and phenolic acids, which are present in the GSPE, were also quantified in serum in their nonmetabolized form (**Figure 3**A and **Table 2**). Remarkably, although the total levels of these compounds were affected by photoperiod conditions, increasing under L6, any photoperiod effect was observed in the individual compounds. Additionally, rats administered with the combination of GSPE and ABX did not show any photoperiod effect in these compounds. Interestingly, most of the phenolic metabolites of this group were significantly increased by the administration of GSPE and ABX independently of the photoperiod conditions compared to GSPE treatment. Thus, overall GSPE + ABX effect was the main factor affecting the flavan-3-ols and phenolic acids. Indeed, rats treated with GSPE + ABX tended to cluster separately from GSPE-treated rats when data were visualized by PCA. However, no cluster was observed by photoperiod effect (Figure 3B).

3.3. Flavan-3-ols-Phase II Metabolites

The concentrations of the different flavan-3-ols-phase II metabolites are shown in the Figure 3C and Table 2. An interesting photoperiod effect was found in this group of metabolites. Rats administered with GSPE (without ABX) and housed under L12 and L18 conditions showed an overall decreased of phase II metabolites levels compared to rats housed under L6. Thus, the concentration of the total phase II metabolites, methyl-epicatechin glucuronide and 3-*O*-methylgallic acid metabolites, was significantly lower under L18 conditions compared to L6 and lower under L12 than under L6. Interestingly, the concentration of both catechin glucuronide and epicatechin glucuronide was higher under L6 conditions while under L12 and L18 conditions, these metabolites were not detected. However, GSPE + ABX-treated rats showed a lower photoperiod effect, being only the most abundant phase-II metabolites (catechin glucuronide and epicatechin glucuronide) decreased under L18 compared to L6 condition ($p < 0.016$) and lower under L12 than under L6. However, this photoperiod effect was not strong enough to cluster differently either GSPE or GSPE + ABX-treated rats according to the photoperiod condition by PCA analysis (Figure 3D).

In addition, the serum GSPE metabolites derived from ABX administration tended to cluster differently from those without ABX administration (Figure 3D). Thus, the ABX administration produced an overall significant increase of total phase II metabolites. In this context, catechin glucuronide increased under both

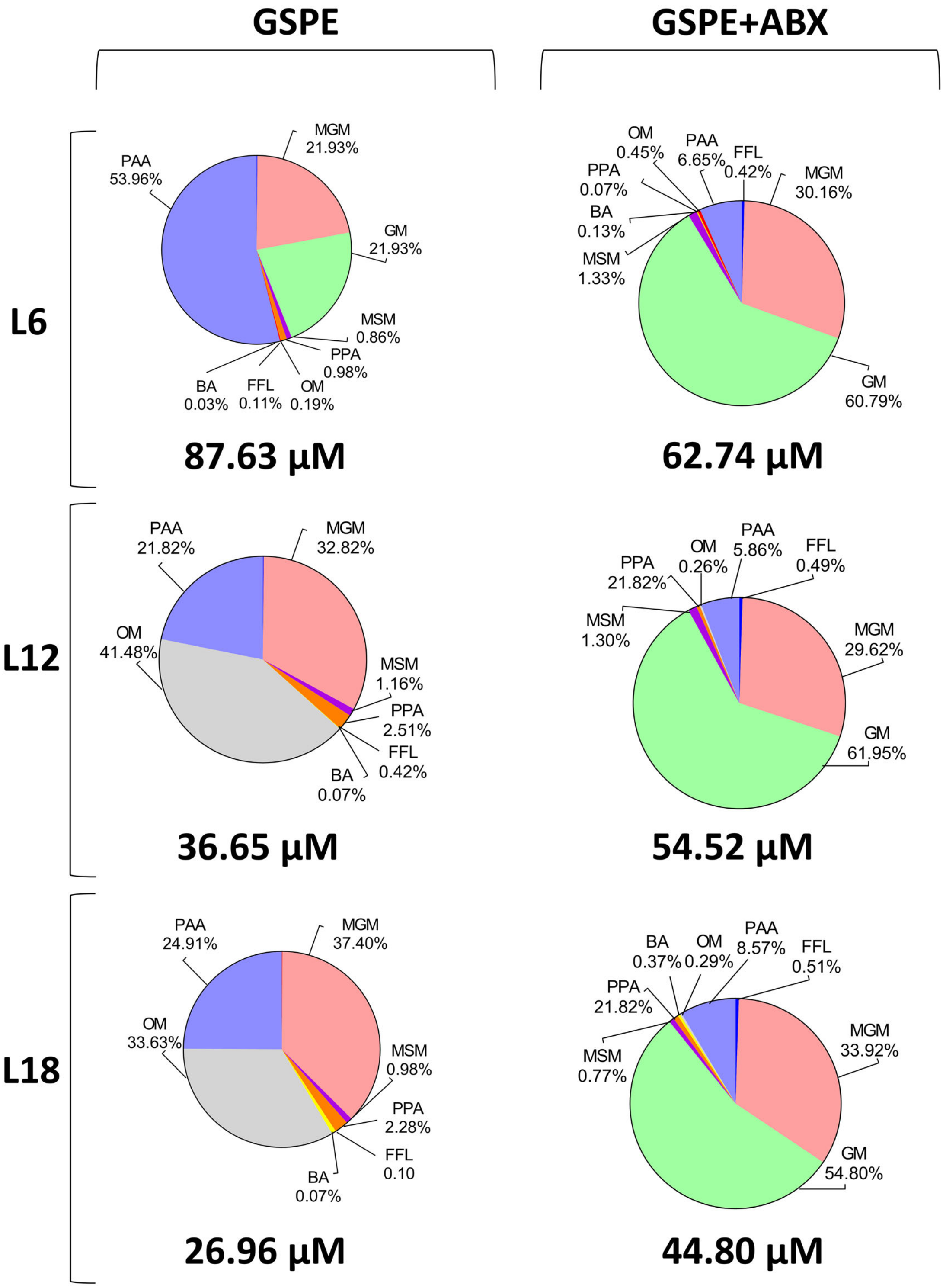

Figure 2. Distribution of phenolic serum metabolites in rats administered with GSPE (25 mg kg^{-1} bw) and exposed to different photoperiods. ABX, antibiotic cocktail; BA, benzoic acids; FFL, free flavonols; GM, glucuronidated metabolites; GSPE, grape seed proanthocyanidin; L6, short photoperiod (6 h light/18 h dark); L12, standard photoperiod (12 h light/12 h dark); L18, long photoperiod (18 h light/6 h dark); MGM, methyl-glucuronidated metabolites; MSM, methyl-sulfated metabolites; OM, other metabolites (4,5-trihydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 3-*O*-methyl gallic acid, cinnamic acids, hippuric acid, and valerolatone and valeric acid derivates); PAA, phenylacetic acids; PPA, phenylpropionic acids.

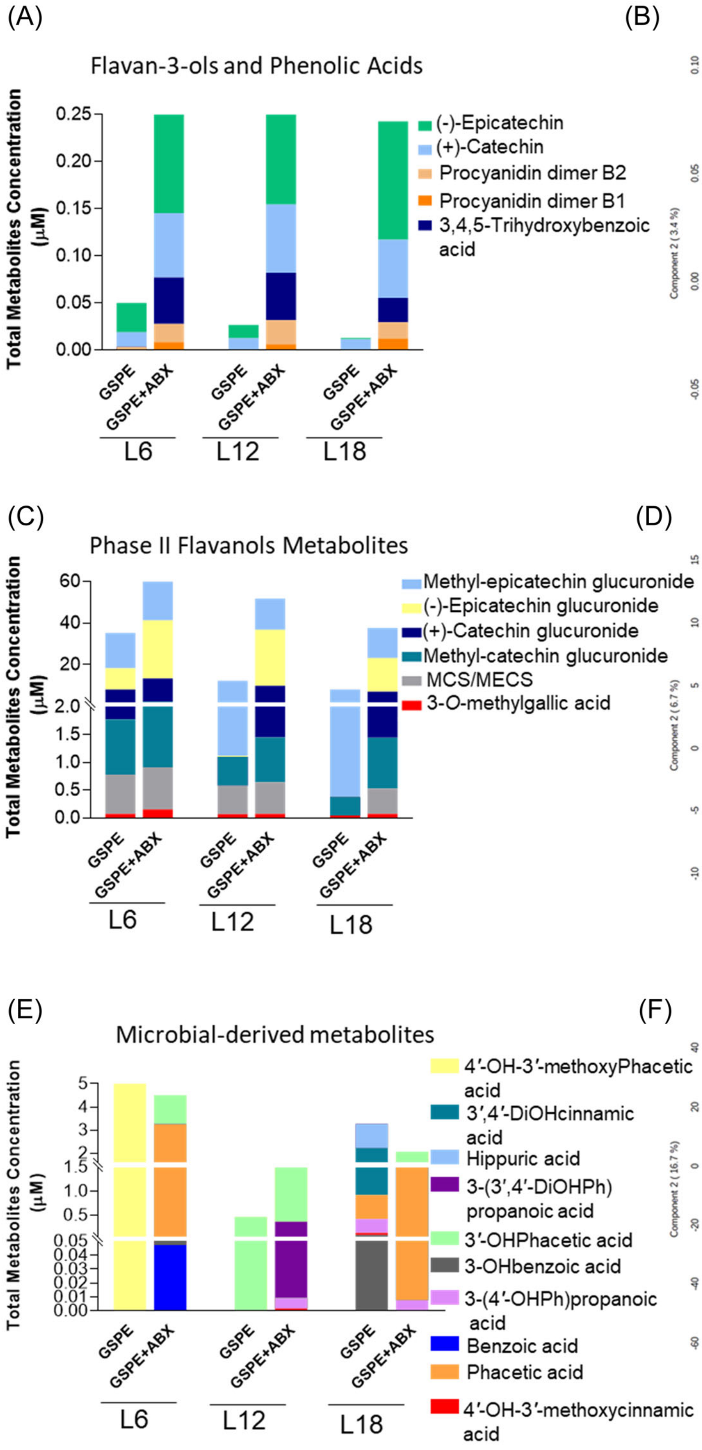

Figure 3. Serum phenolic metabolites quantified 3 h after the last dose of GSPE administered for 4 weeks to CAF-fed rats housed under different photoperiod. A, B) Flavan-3-ols and phenolic acids; C, D) phase II metabolites; and D, E) microbial-derived metabolites. Data are visualized by stacked bar plots showing the median of the different metabolites (A, C, E) and by PCA 2D plot (B, D, F); (*n* = 7–8). ABX, antibiotic cocktail; GSPE, grape seed proanthocyanidin; L6, short photoperiod (6 h light/18 h dark); L12, standard photoperiod (12 h light/12 h dark); L18, long photoperiod (18 h light/6 h dark); MCS/MECS, methyl-catechin sulphate/methyl-epicatechin sulphate; OH, hydroxy; PCA, principal component analysis; Ph, phenyl.

Table 2. GSPE metabolites according to the photoperiod conditions and antibiotic treatment analyzed by HPLC-ESI-MS/MS.

	GSPE metabolites [µM]			P Ph$^{b)}$	GSPE metabolites in rats treated with ABX [µM]			p Ph$^{b)}$	p L6$^{c)}$	p L12$^{c)}$	p L18$^{c)}$
	Median (Q1–Q3)$^{a)}$				Median (Q1–Q3)$^{a)}$						
	L6	L12	L18		L6	L12	L18				
Σ Flavan-3-ols and phenolic acids	**0.19(0.01–0.31)***a*	**0.031(0.018–0.05)***ab*	**0.029(0–0.069)***b*	**0.05**	**0.25(0.20–0.54)**	**0.28(0.13–0.49)** *	**0.26(0.12–0.38)** *	**0.72**	**0.11**	**0.002**	**0.001**
(+)-Catechin	0.02(0–0.08)	0.01(0.003–0.03)	0.01(0–0.03)	0.81	0.07(0.01–0.12)	0.07(0.02–0.10)*	0.06(0.03–0.09)*	0.96	0.16	0.02	0.00
(−)-Epicatechin	0.03(0–0.12)	0.01(0.002–0.04)	0.001(0–0.03)	0.35	0.12(0.09–0.26)*	0.12(0.08–0.26)*	0.13(0.05–0.20)*	0.92	0.04	0.01	0.00
Procyanidin dimer B1	4.56×10^{-4}(0–0.01)	n.d.	n.d.	0.44	0.01(0–0.03)*	0.01(0.002–0.02)*	0.01(0.01–0.03)*	0.75	0.05	0.00	0.00
Procyanidin dimer B2	0.003(0–0.01)	5.56×10^{-4} (0–8.30×10^{-4})	n.d.	0.11	0.02(0.002–0.04)	0.03(0.003–0.06)*	0.02(0.01–0.04)*	0.71	0.12	0.00	0.00
3,4,5-Trihydroxybenzoic acid	n.d.	n.d.	n.d.	0.14	0.05(0.02–0.11)*	0.05(0.02–0.06)*	0.03(0.03–0.03)*	0.24	0.00	0.00	0.000
4-Hydroxy-3-methoxybenzoic acid	n.d.	n.d.	n.d.	0.12	n.d.	n.d.	n.d.	0.11	1.00	1.00	1.00
Σ Phase II Flavan-3-ols	**34.34(13.73–55.96)***a*	**14.77(10.68–27.21)***ab*	**11.67(6.19–20.78)***b*	**0.03**	**60.09(48.161–75)**	**52.64(36.12–57.09)** *	**38.02(34.63–49.09)** *	**0.67**	**0.13**	**0.00**	**0.00**
(+)-Catechin glucuronide$^{d)}$	6.28(0.17–10.96)	n.d.	n.d.	0.06	11.18(7.69–13.53)*a*	8.35(6.63–10.06)**ab*	5.65(5.29–7.25)**b*	0.03	0.20	0.00	0.01
(−)-Epicatechin glucuronide$^{d)}$	10.28(0.07–22.62)	n.d.	n.d.	0.07	27.93(23.08–36.69)**a*	26.9(17.63–28.48)**ab*	16.15(15.10–21.59)**b*	0.04	0.02	0.00	0.00
Methyl catechin glucuronide$^{d)}$	0.99(0.63–1.34)	0.52(0.06–0.94)	0.33(0.03–0.74)	0.08	1.27(0.76–1.66)	0.81(0.49–1.13)	0.92(0.37–1.54)	0.47	0.49	0.29	0.06
3-*O*-methylgallic acid$^{e)}$	0.08(0.05–0.14)*a*	0.07(0.04–0.12)*ab*	0.46(0.02–0.05)*b*	0.03	0.16(0.07–0.29)	0.07(0.06–0.12)	0.07(0.05–0.14)*	0.15	0.13	0.64	0.01
Methyl-epicatechin glucuronide$^{d)}$	16.95(11.37–20.89)*a*	11.09(7.85–15.21)*ab*	7.73(5.96–14.88)*b*	0.03	18.69(14.45–21.62)	15.05(12.71–18.69)	14.45(12.79–16.63)*	0.18	0.64	0.08	0.05
Methyl-catechin sulphate/methyl-epicatechin sulphate$^{d)}$	n.q.	n.q.	n.q.	0.14	n.q.	n.q.	n.q.	0.02	0.64	0.20	0.43
Σ Microbial-derived metabolites	**54.67(5.46–93.24)**	**9.83(1.76–39.55)**	**9.45(2.01–28.10)**	**0.23**	**6.24(3.41–11.63)**	**5.76(0.39–8.61)**	**5.29(2.46–10.95)**	**0.62**	**0.13**	**0.11**	**0.89**
Phenylacetic acid$^{f)}$	n.d.	n.d.	n.d.	0.45	3.19(0.73–4.81)**a*	n.d. *b*	1.50(0.76–5.61)*a*	0.01	0.01	0.14	0.22
3′,4′-Dihydroxyphenylacetic acid	n.d.	n.d.	n.d.	1.00	n.d.	n.d.	n.d.	0.18	0.06	0.06	1.00

(*Continued*)

Table 2. (Continued).

	GSPE metabolites [μM]			P Ph[b)]	GSPE metabolites in rats treated with ABX [μM]			p Ph[b)]	p L6[c)]	p L12[c)]	p L18[c)]
	Median (Q1–Q3)[a)]				Median (Q1–Q3)[a)]						
	L6	L12	L18		L6	L12	L18				
4′-Hydroxy-3′-methoxyphenylacetic acid[g)]	47.44(3.87–86.57)*a*	n.d. *b*	n.d. *b*	0.01	n.d. *	n.d.	n.d.	0.31	0.01	0.63	0.17
3′-Hydroxyphenylacetic acid	n.d. *a*	0.465(0.285–1.637)*b*	n.d. *a*	0.00	1.26(0–2.01)*	1.18(0–2.52)	0.52(0.09–1.33)*	0.71	0.03	0.64	0.01
Hippuric acid	n.d.	n.d.	1.04(0–8.58)	0.13	n.d.	n.d.	n.d.*	1.00	1.00	0.11	0.04
3′,4′-Dihydroxycinnamic acid	n.q.	n.q.	1.31(0–4.99)	0.13	n.q.	n.q.	n.q.*	1.00	1.00	0.11	0.03
4′-Hydroxy-3′-methoxycinnamic acid	n.d. *a*	n.d. *ab*	0.05(0–0.23)*b*	0.05	n.d.	0.002(0–0.006)	n.d.	0.19	0.36	0.68	0.08
Benzoic acid	n.d.	n.d.	n.d.	0.07	0.047(0–0.13)*	n.d.	n.d.	0.12	0.04	1.00	0.69
3-Hydroxybenzoic acid	n.q.	n.q.	n.q.	0.20	n.q.	n.q.	n.q.	0.59	0.57	0.63	0.19
3-(3′,4′-Dihydroxyphenyl)propanoic acid	n.d.	n.d.	n.d.	0.17	n.d.	0.37(0–0.42)*	0.03(0–0.42)*	0.23	0.72	0.03	0.01
Phenylpropionic acid[h)]	n.d.	n.d.	n.d.	0.41	n.d.	n.d.	n.d.	1.00	0.17	0.17	1.00
3-(3′-Hydroxyphenyl)propanoic acid[h)]	n.d.	n.d.	n.d.	0.06	n.d.	n.d.	n.d.	1.00	1.00	0.04	0.32
3-(4′-Hydroxyphenyl)propanoic acid	n.d.	n.d.	0.29(0–0.92)	0.05	n.d.	0.01(0–0.05)*	0.01(0–0.34)	0.09	0.08	0.03	0.38
4-Hydroxyphenylvaleric acid	n.d.	n.d.	n.d.	0.57	n.d.	n.d.	n.d.	1.00	0.02	0.04	0.06
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid	n.d.	n.d.	n.d.	1.00	n.d.	n.d.	n.d.	1.00	1.00	1.00	1.00
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid isomer	n.d.	n.d.	n.d.	1.00	n.d.	n.d.	n.d.	1.00	1.00	1.00	1.00
3,4-Dihydroxyphenylvalerolactone	n.d.	n.d.	n.d.	0.12	n.d.	n.d.	n.d.	1.00	0.14	1.00	1.00
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	n.d.	n.d.	n.d.	0.69	n.d. *a*	0.01(0–0.01)*b*	3.64×10^{-4} (0–0.006)*ab*	0.04	0.11	0.13	0.79

[a)] Data shown as median (first (Q1) and third quartile (Q3)) ($n = 7$–8); [b)] *p*-value by Kruskal–Wallis test comparing photoperiods in each group. *ab* letters indicate significant photoperiods differences analyzed by Kruskal–Wallis test followed by Bonferroni *p*-values adjustment ($p < 0.016$); [c)] *p*-value by Mann–Whitney *U* test comparing experimental groups (GSPE vs. GSPE + ABX) under the different photoperiod. * Indicates significant differences between GSPE and GSPE + ABX in each photoperiod ($p < 0.05$); [d)] Quantified using the calibration curve of (+)-catechin; [e)] Quantified using the calibration curve of 3,4,5-trihydroxybenzoic acid; [f)] Quantified using the calibration curve of 3′-hydroxyphenylacetic acid; [g)] Quantified using the calibration curve of 3′-hydroxyphenylacetic acid; [h)] Quantified using the calibration curve of 3-(4′-hydroxyphenyl)propanoic acid. ABX, antibiotic cocktail; GSPE, grape seed proanthocyanidin extract; L6, short photoperiod (6 h light/18 h dark); L12, standard photoperiod (12 h light/12 h dark); L18, long photoperiod (18 h light/6 h dark); n.d., not detected; n.q., not quantified.

L12 and L18, epicatechin glucuronide increased independently of the photoperiod conditions and of 3-*O*-methylgallic acid and methyl-epicatechin glucuronide were only increased under L18 (Figure 3C; Table 2).

3.4. Microbial-Derived Metabolites

The microbial-derived metabolites group was the second largest group detected in the samples (Figure 3E and Table 2). This group of metabolites was importantly modified by the different photoperiod conditions and by ABX administration. Regarding to photoperiod effect, 4'-hydroxy-3'-methoxyphenylacetic acid was the unique microbial-derived metabolite found under L6 whereas, 3'-hydroxyphenylacetic acid was the unique metabolite found in L12 conditions. Furthermore, these two metabolites were not present in serum of rats exposed to other photoperiod conditions. On the other hand, more metabolites were present under L18 conditions. Thus, hippuric acid, 3′-4′-dihydroxycinnamic acid, 4′-hidroxy-3′-methoxycinnamic acid, and 3-(4′-hydroxyphenyl)propanoic acid metabolites were only found under L18 conditions. In this context, an overall photoperiod effect was observed only in rats not administered with ABX under L6, which cluster separately from rats housed under both L12 and L18 conditions (Figure 3F). However, when rats were administered with ABX, only phenylacetic acid metabolite was affected by photoperiod effect, increasing under both L6 and L18 in comparison with L12 conditions.

Interestingly, the administration of ABX had a different effect on the GSPE microbial-derived metabolites depending on the photoperiod condition. Thus, when rats were treated with ABX, significant higher phenylacetic acid and benzoic acid concentrations were observed under L6 conditions. Furthermore, the 3-(3′,4′-dihydroxyphenyl)propanoic acid, 3-(4′-hydroxyphenyl)propanoic acid, and 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone metabolites were only detected under L12 and L18 conditions, showing that L6 conditions had lower microbial-derived metabolites. In addition, 4′-hydroxy-3′-methoxycinnamic acid was only detected under L12 conditions (Figure 3E; Table 2).

4. Discussion

The consumption of polyphenols has been linked to a wide range of benefits on obesity and MetS development.[39,40] However, their bioactivity depends on its bioavailability, including both its metabolism and absorption.[13] Hence, the study of factors affecting bioavailability and metabolism of polyphenols is of high interest. Gut microbiota is a key factor in the metabolism of polyphenols. Because of this, alteration of gut microbiota causes changes in the bioavailability of polyphenols and in turn in their bioactivity. On the other hand, high fat diets, such as CAF, promote obesity development, alter gut microbiota composition,[41,42] and affect the bioavailability of polyphenols.[43] Additionally, seasonal rhythms have been recently established as crucial factors in the modulation of gut microbiota composition,[44,45] being obese rats more susceptible to photoperiod changes.[23] Moreover, it was reported that changes in photoperiods affected grape seed polyphenols bioavailability.[22] Therefore, the aim of this study was to evaluate if the serum bioavailability of GSPE metabolites is modulated by gut microbiota in a photoperiod dependent manner in an obesogenic context induced by cafeteria feeding.

To evaluate this, a chronic study in CAF-fed rats daily administered 25 mg kg^{-1} of GSPE, with and without an ABX, and exposed to different photoperiod conditions was performed. F344 rats have shown a higher photoperiod sensitivity compared to other strains.[46,47] The administered dose of GSPE was equivalent to the daily intake of 367 mg for a person of 70 kg,[31] which is within the estimated range of polyphenols intake in humans[32–34] and has been demonstrated antiobesity properties.[12,48,49] The ABX used promote a gut microbiota dysbiosis without affecting other physiological parameters such as body composition, biochemical parameters, or glucose tolerance.[36]

GSPE-derived metabolites were quantified in serum after 3 h of the last GSPE dose administration. Phase-II metabolites were the most abundant while microbiota-derived metabolites were detected at lower levels. This was expected since the highest plasma concentration of phase-II metabolites was observed at 2 h after GSPE dosage in kinetic studies, finding a wide variety of phase-II metabolites at 2 and 4 h after GSPE administration.[19] Regarding microbiota-derived metabolites, it has been shown that they reach their maximum levels after 24 h of GSPE administration in adult rats.[19,50] Hence, in this chronic study we are detecting bacterial-derived GSPE metabolites from the dose administered on the previous day (27 h after administration). Nonmetabolized flavan-3-ol and phenolic acids as well as phase II metabolites were increased in rats treated with ABX. It was reported that these metabolites are increased in rats fed a CAF which promote gut microbiota dysbiosis compared to healthy rats.[43] In this context, gut microbiota alterations were linked to higher absorption of small phenolic compounds and flavanol monomers at the level of the small intestine, which can appear in plasma as free and conjugated monomers.[51] The administration of ABX increased the dysbiosis conditions of these rats, reducing gut microbiota diversity.[52] Thus, these findings suggest that intestinal permeability is altered by the dysfunction of tight junctions within the small intestine due to the ABX administration,[53,54] which affects the passive diffusion of flavanols through the enterocytes. In this regard, epicatechin and their glucuronic-derived metabolites are one of the most abundant metabolites shown in the serum of rats treated with ABX. The alteration promoted by ABX administration may increase flavanols absorption in the small intestine, which may explain the reduced microbial derived metabolites detected in serum, since 4-hydroxyphenylvaleric acid, 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid, 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid isomer, and 3,4-dihydroxyphenylvalerolactone were not detected.[55]

Interestingly a photoperiod effect was observed in phase-II metabolites, which decreased their concentration in the longest photoperiods (L12 and L18). In this context, rats without ABX housed under L6 conditions showed higher phase-II metabolites in comparison with those rats housed under both L12 and L18 conditions, which displayed similar concentrations. Interestingly, rats treated with ABX showed a photoperiod effect, which decreased their concentration as the hours of light per day increased. It is known that the phase-II enzymes implicated in the metabolism of these flavan-3-ols-derived metabolites, follow a diurnal rhythmicity.[56] Circadian and seasonal rhythms are related and both of them are wide regulated by light/dark cycles. Thus, photoperiods can modulate seasonal rhythms, but also can influence circadian rhythmicity.[57] In this regard, retinoid-related orphan receptors alpha and gamma (RORα and γ) expressed in liver as a component of the circadian clock, play an important role in the regulation of genes encoding several phase II metabolic enzymes.[58,59] Moreover, it has been recently reported that hepatic UDP-glucuronosyltrasnferases (UGTs) phase II enzymes, which carried out metabolites glucuronidation, show a diurnal rhythmicity regulated by Rev-erbα (a circadian clock component).[60,61] Both RORα and REV-erbα are linked to regulate many metabolism pathways to ensure that metabolic enzymes are produced at appropriate times (i.e., stimulation of catabolic pathways during the wake period to match fuel demands). Along these lines, metabolism and absorption processes variate according to the time of the day, being most optimal and reaching the highest levels of intermediate metabolites during the active phase of the organism.[62,63] In this context, our results showed lower concentration of phase II metabolites under L18 conditions, in which rats have a longer resting phase. Thus, bioavailability and metabolism of these phenolic compounds could be influenced by diurnal rhythmicity of phase II enzymes, being more active in the dark phase, in which rats are in their active phase. In concordance, Iglesias-Carres et al. also showed that the bioavailability of grapes seed was higher in rats exposed to L6 conditions compared to rats under L18 conditions.[22] In fact, grapes may present a higher bioavailability under L6 conditions due to the fact that they are normally consumed in winter season. This could be linked with a major bioactivity according to the Xenohormesis theory.[64,65] This theory stablished that the heterotroph organisms (i.e., humans) are able to sense singling stress-induced molecules from other species (i.e., plants). Hence, heterotrophs take these phytochemicals cues (polyphenols) from other species through the diet to get information on the environmental status, adjusting their metabolism in anticipation of adverse conditions to increase their chances of survival.[66] Thus, the consumption of grapes out of their natural conditions season would provide chemical cues that do not correspond to the current environmental situation to the consuming organism, and thus would result in nonefficient metabolic processes.[65]

Regarding microbial-derived metabolites, although their concentrations in serum were low, results showed interesting photoperiod effects. Thus, rats administered with GSPE without ABX showed an overall photoperiod effect mainly due to an increase of the hippuric acid and the 3′-4′-dihidroxycinnamic acid (classify as other metabolites in the total serum phenolic profile) under L18 conditions, being not detected under L6 or L12 condition. Both, hippuric acid and the 3′-4′-dihidroxycinnamic acid have been reported to be increased as a consequence of consumption of food with a great amount of polyphenols such as vegetables, grapes, coffee, or tea.[67,68] In fact, hippuric acid has been shown as a potential biomarker of the polyphenol catabolism, being of great interest to detect the healthy state of children and adolescents.[69] Moreover, the 3′-4′-dihidroxycinnamic acid has been reported to have antioxidant, antiinflammatory, antitumor, and antibacterial properties.[70] In addition, several evidences showed that the plasma or urine concentration of these two microbial-derived metabolites can change according to the gut microbiota composition.[71] Another interesting metabolite which lead the increase of the proportion of other metabolites, was the 3-hydroxyphenylacetic acid, which only was increased under L12 conditions. This microbial-derived metabolite has showed antihypertensive and anti-cytotoxic effects, depending on the gut dysbiosis condition.[72,73] Therefore, the levels of these two interesting metabolites can be affected by the photoperiod via gut microbiota composition, as it can be influenced by the photoperiod.[23,36] Interestingly, these effects were significantly less prominent when treating with ABX. These findings reinforce the important role of gut microbiota in phenolic compounds metabolites bioavailability and suggest that these bacteria are involved in the photoperiod-mediated changes observed in PAs bioavailability, which may impact in their bioactivity. Indeed, in our previous work we observed that the GSPE affected gut microbiota composition differently depending on the photoperiod conditions, as well as if the rats were or not treated with ABX.[36] Thus, rats administered with GSPE without ABX showed higher levels of *Butyrivibrio* and *Coprococcus* and lower levels of *Parabacteroides* under L6. Rats housed under L12 presented higher abundance of *Lactobacillales* and *Lactococcus* and rats housed under L18 showed higher abundance of *Bifidobacterium* and *Kleibsella*. On the other hand, rats administered with GSPE and ABX showed a strong photoperiod effect on gut microbiota composition, especially under L18. In this context, rats housed under L6 showed lower levels of *Lactobacillus* and *Bacteroides*. Rats under L12 showed a decreased of *Coprococcus*, *Ruminococcus*, and *Prevotella*, and an increase of *Bacteroides*, and *Bilophila* relative abundance genera, while rats under L18 conditions, showed higher relative abundance of *Parabacteroides*, *Bifidobacterium*, *Bilophila*, and *Blautia* genera. Along these lines, these changes on the gut microbiota composition promoted by the different photoperiod conditions may explain the differences on the bioavailability of GSPE. For instance, *Butirivibrio* genera has been linked to lactone fission reaction and the strains of *Bifidobacterium lactis*, *Lactobacillus gasseri*, and *Ruminococcus productus* have been associated with hydrolysis reaction by esterases enzymes.[74] Thus, these changes in the abundance relative of certain gut bacteria promoted by GSPE and photoperiod could be related to the differences metabolites observed. However, further investigations are needed to stablish an association between these bacteria and the metabolites derived from GSPE metabolism.

Therefore, this study shows that photoperiod-mediated changes in the bioavailability of phenolic compounds are significantly influenced by gut microbiota in obese rats. Thus, these results contribute to a better understanding of polyphenols metabolism and bioavailability and its application in the chronotherapy field.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

V. A.-G. and I. E.-M. contributed equally to this work. V.A.-G.: conceptualization, formal analysis, data curation, investigation, methodology, software, visualization, writing original draft; I.E.-M.: conceptualization, formal analysis, data curation, investigation, methodology, software; M.M.: investigation, funding acquisition, project administration, and resources; B.M.: investigation, funding acquisition, project administration, and resources; M.S.: investigation, funding acquisition, project administration, and resources; A.A.-A.: conceptualization, investigation, methodology, funding acquisition, project administration, resources, supervision, and writing review & editing; C.T.-F.: conceptualization, investigation, methodology, funding acquisition, project administration, resources, supervision, and writing review & editing.

Data Availability Statement

Data available on request from the authors.

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