ARTICLE

# The effects of Grape Seed Proanthocyanidins in cafeteria diet-induced obese Fischer 344 rats are influenced by faecal microbiota in a photoperiod dependent manner.

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Verónica Arreaza-Gil, Iván Escobar-Martínez, Begoña Muguerza, Gerard Aragonès, Manuel Suárez, Cristina Torres-Fuentes† and Anna Arola-Arnal.

Polyphenols are of high interest due to their beneficial health effects, including anti-obesity properties. Gut microbiota may play an important role in polyphenols-mediated effects as these bacteria are significantly involved in their metabolism. Moreover, seasonal rhythms have been demonstrated to influence both gut microbiota composition and polyphenols bioavailability. Thus, the goal of this study was to evaluate the impact of photoperiods and microbiota on polyphenols functionality under an obesogenic context. To this aim, cafeteria diet-fed Fischer 344 rats were housed under three different photoperiod conditions (L6: 6 h of light, L12: 12 h of light and L18: 18 h of light) for 9 weeks. During the last 4 weeks of the experiment, rats were daily administered with an oral dose of a grape seed proanthocyanidin extract (GSPE) (25 mg/kg body weight). Additionally, rats treated with GSPE and an antibiotic cocktail (ABX) in their drinking water were included for a better understanding of the gut microbiota role in GSPE functionality. Vehicle and non-ABX treated rats were included as controls. GSPE decreased body weight gain and fat depots only under L18 conditions. Interestingly, gut microbiota composition was strongly altered in this photoperiod. GSPE+ABX-treated rats gained significantly less body weight compared to the rest of treatments under L18 conditions. These results suggest that GSPE functionality is modulated by gut microbiota in a photoperiod dependent manner. These novel findings corroborate seasonal rhythms as key factors that must be taken into account when investigating the effects of polyphenols in the treatment or prevention of chronic diseases.

## Introduction

Polyphenols have attracted increasing scientific attention due to their beneficial role in human health. They are the most common plant-derived bioactive component in our diet, being widely present in a variety of food such as fruits, vegetables, cereals, tea, coffee and wine.[1,2] In this context, grape seed proanthocyanidins (GSPE) have shown several beneficial effects against obesity and metabolic syndrome (MetS) development.[3] Thus, it was found that GSPE can improve lipid metabolism by reducing fat depots[4] and improving insulin resistance,[5] preventing the increase in blood of glucose, triglycerides, and insulin levels by increasing adipokine secretion and decreasing oxidative stress pathways.[6,7] Furthermore, GSPE can reduce lipid accumulation through inhibiting preadipocyte differentiation, reducing the formation of new adipocytes and regulating white adipose tissue (WAT) hypertrophy and proliferation, improving therefore WAT function.[4,8,9] Hence, GSPE can effectively prevent obesity through different mechanisms. [10]

In this context, it was also reported that the administration of GSPE improved the structural diversity of gut microbiota in high fat diet (HFD)-fed mice, increasing the relative abundance of *Faecalibaculum*, *Bacteroides* and *Akkermansia* as well as, the levels of butyric and propionic acids, changes that correlated with reduced final body weight, decreased insulin resistance and elevated levels of adiponectin and leptin.[11] Furthermore, GSPE ameliorated plasma levels of inflammatory factors such as interleukin-6 or tumour necrosis factor-alpha, reduced epididymal fat mass and improved insulin sensitivity by modulating bacterial content such as *Clostridium*, *Roseburia* and *Prevotella* in HFD mice.[12] This fact is of relevance as gut microbiota has been described as critical for the maintenance of the homeostasis and metabolic functions in the host[13] and alteration in its composition, also known as dysbiosis, can contribute to the development of metabolic diseases such as obesity and MetS.[14] Thus, gut microbiota composition may be another of the mechanisms by which GPSE affects metabolism preventing obesity. Additionally, it is worth mentioning that about 90-95% of the total ingested polyphenols reach the colon and undergo extensive transformation by gut microbiota.[15,16] Thus, alterations in gut bacteria composition may significantly impact polyphenols functionality since it is believed that the effects of polyphenols are basically driven by its metabolized forms.[17] Therefore, evidence suggests that polyphenols can modulate gut microbiota composition and, at the same time, intestinal bacteria can modulate the metabolism of phenolic compounds, showing a bidirectional interaction that may have a significant impact on phenolic compounds bioactivity.

[a] *Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Nutrigenomics Research Group, 43007 Tarragona, Spain.*
† Corresponding author: cristina.torres@urv.cat

Seasonal rhythms have been recently reported as a key factor affecting the bioavailability and functionality of polyphenols[18], but also gut microbiota composition.[19–21] In this context, fluctuations in environmental cues, mainly driven by the length of the daylight phase, have been linked to changes in gut microbiota.[22–24] In fact, a study carried out in Siberian hamsters revealed that intestinal microbiota is responsive to changes in photoperiods. These changes in bacterial abundance were associated with the elevated body and fat masses observed in animals housed under long summer-like day length conditions (LD) compared to animals housed under short winter-like day length (SD).[19] Regarding seasonal rhythms effects on polyphenols functionality, cafeteria diet-induced obese rats supplemented with polyphenol-rich fruits showed higher leptin sensitivity only under SD photoperiod.[25] Moreover, normoweight rats housed under different photoperiods and treated with red grapes presented a higher bioavailability of grape phenolic acids under SD photoperiod.[18] In addition, it was recently published that animals fed with a diet containing isoflavones and housed under different photoperiod conditions showed an association between the gut bacterial communities, photoperiod length and isoflavone compounds. [26]

Therefore, photoperiods seem to be a key factor in modulating the bidirectional interaction between dietary polyphenols and gut microbiota. However, this association is still poorly understood and further investigations are needed. Therefore, the aim of this study was to evaluate if GSPE anti-obesity effects can be modulated by gut microbiota in a photoperiod dependent manner.

## Material and Methods

### Grape seed proanthocyanidins extract

GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer this phenolic-rich extract is mainly composed by monomers of flavan-3-ols (15.98%), dimers (13.05%), trimers (12.22%), tetramers (9.97%) and oligomers (5–13 units; 23.77%) of proanthocyanidin. In addition, the phenolic acids and flavan-3-ols present in the GSPE contained gallic acid (3.11%), protocatechuic acid (0.13%), epicatechin (9.34%), epicatechin gallate (2.12%), catechin (12.13%), proanthocyanidin dimers B1 (8.88%), B2 (3.32%) and B3 (4.61%), dimer gallate (0.89%) and trimeric proanthocyanidins (0.49%).[27]

### Experimental design

Thirteen weeks-old male F344 rats (n=96) were obtained from Janvier Laboratories, France. Initially rats were pair-housed at standard conditions (22ºC, 65% relative humidity and 12:12 hour light/dark cycle) with ad libitum access to water and standard chow diet for one week. After this acclimation period, rats were weighted and randomized distributed into three different light-dark cycles (photoperiods) conditions to mimic seasonal day lengths 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]. Rats were fed a cafeteria diet (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 (20-25 g) and milk containing 22% sucrose (w/v).

Rats were further randomly distributed in four groups (n=8) for each photoperiod condition according to the treatment administrated in the last-4 weeks of the experiment (weeks 5-9) (Fig. 1): 1) vehicle (VH, condensed milk diluted with water in 1:5 proportion), 2) grape seed proanthocyanidin extract (GSPE) (25 mg/kg body weight dissolved in VH), 3) VH and an antibiotic cocktail (ABX), and 4) combination of GSPE and ABX. VH and GSPE were daily administered orally one hour after the light was turned on. The antibiotic cocktail (0.5 g/l ampicillin, 0.250 g/l vancomycin and 0.125 g/l imipenem; Discovery fine chemicals Ltd, United Kingdom) was freshly prepared every day and administered in drinking water. Body weight (BW) was recorded weekly during the whole experimental procedure.

Animals were sacrificed by decapitation following 3 h fasting after the administration of the last dose. Decapitation was carried out without anaesthesia by trained staff to avoid any interference with the results. Faecal samples for microbiota analysis were freshly collected from the colon and immediately snap-frozen until further analysis. The cecum as well as WAT depots including mesenteric (mWAT), retroperitoneal (RWAT), inguinal (iWAT), epididymal (eWAT) and subcutaneous were collected, weighed and immediately frozen in liquid nitrogen. The visceral mass was calculated as the sum of visceral adipose tissue depots (mWAT, RWAT and eWAT). Total body fat mass was measured as the sum of the visceral fat and subcutaneous fat (iWAT and Subcutaneous). The Adiposity Index was expressed as total body fat mass/final body weight. All the samples were stored at -80°C until further analyses. 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.

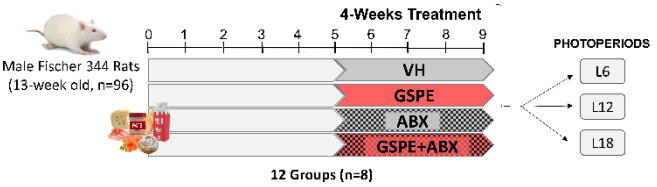


**Fig. 1** Animal experimental design. CAF-fed 13-week-old male Fischer 344 rats were pair-housed under three different photoperiods (6, 12 or 18 h of light per day) for 9 weeks. During the last 4 weeks, animals were daily administered with either an oral dose of GSPE (grape seed proanthocyanidin extract) (25mg/kg) dissolved in a solution of water and condensed milk (5:1, VH), or a combination of GSPE and an antibiotic cocktail (ABX) in drinking water (ampicillin: 0.5g/l, vancomycin: 0.25 g/l, imipenem: 0.125 g/l). Vehicle and ABX-treated animals were included as controls. L6: 6h light/18h darkness; L12: 12h light/12h darkness; L18: 18h light/6h darkness; CAF: cafeteria diet; VH: vehicle.

**Biochemical serum parameters Analysis.**

Serum samples were obtained from the blood collected from the neck, in non-heparinized tubes. The blood was incubated for 1 h at room temperature and immediately centrifuged at 1200x g for 15 min to collect the serum. Levels of triglycerides, total cholesterol, glucose (QCA, Barcelona, Spain), and non-esterified free fatty acids (NEFAs) (WAKO, Neuss, Germany) were analysed in serum samples by enzymatic colorimetric assays. Serum insulin levels were analysed using a rat insulin ELISA kit (Millipore, Barcelona, Spain) according to the manufacturers' instructions.

**Oral Glucose Tolerance Test**

Oral Glucose tolerance test (OGTT) were performed at the last week. Glucose was orally administered after 6 h fasting (2 g/kg body weight, 50% w/v) and blood glucose levels were measured with a glucometer (Glucocard SM, Menarini Diagnostics, Italy) at 0, 15, 30, 60 and 120 min after glucose administration.

**16S rRNA analysis.**

Faecal DNA was isolated using a QiAamp Fast DNA Stool mini kit (Qiagen Inc., Hilden, Germany) and kept at -20°C until further analysis. Isolated DNA was quantified using a NanoDrop ND2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and used for 16S ribosomal RNA sequencing by Ion S5 system (Life Technologies, California, USA) as described previously. [28]

**Statistical Analysis.**

BW gain and OGTT data were plotted using Graphpad Prism 8.0 software (Graphapad software Inc, San Diego, CA, USA) showing mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS software (IBS SPSS statistics 25). Normality as well as homogeneity of variance were tested by Shapiro-Wilk test and Levene test respectively for BW gain, OGTT, biochemical serum parameters and fats depots data. BW gain and OGTT over time was analysed using repeated-measured ANOVA followed by Tukey HSD/Kramer post hot test at each individual time point. The correspondence area under the curve (AUC) of BW gain and the OGTT, biochemical serum parameters and fats depots data were analysed by two way ANOVA (factors: GSPE and ABX) followed by Tukey HSD/kramer to evaluate the treatments effect in each photoperiod and by one-way ANOVA followed by Tukey HSD/kramer post hot test to evaluate the photoperiod effect in each group Additionally, when Tukey HSD/kramer post hoc test showed tendency (0.05>p<0.1), effects of treatments were analysed by Student's t-test.

MicrobiomeAnalyst web-based tool[29,30] was used for faecal microbiota analysis. Features remaining was 34257, after filter features that no contained at least 2 count in 10% of the samples. α-diversity was calculated by Chao1 index and analysed by Kruskal-Wallis test to elucidate differences between groups. β-diversity was measured based on Bray-Curtis distances and analysed by permutational multivariate analysis of variance (PERMANOVA) to assess the dissimilarity of faecal microbiota composition among different groups. Afterwards, the nonparametric Mann-Whitney/Kruskal-Wallis test followed by Dunn's multiple comparison and Bonferroni adjustment of P values was performed to elucidate pairwise differences in specific bacteria relative abundance between groups.

The statistical test used for individual analysis is provided in the figure legends.

## Results

**Grape Seed Proanthocyanidins effects on biometrics and biochemical serum parameters levels were photoperiod dependent**

BW gain, fat depots and biochemical serum parameters were analysed to elucidate the impact of GSPE treatment and photoperiod exposure on obesity development. GSPE treatment led to a significant reduction of BW gain only when rats were housed under L18 conditions ($p<0.05$) (Fig. 2). Furthermore, GSPE-treated rats showed a non-significant decrease in fat depots accumulation compared to VH-treated rats only under L18 conditions ($p>0.05$) (Table 1). In contrast, GSPE-treated rats showed higher glucose tolerance after 60 min of glucose administration only under L6 conditions (Fig. 3A, D).

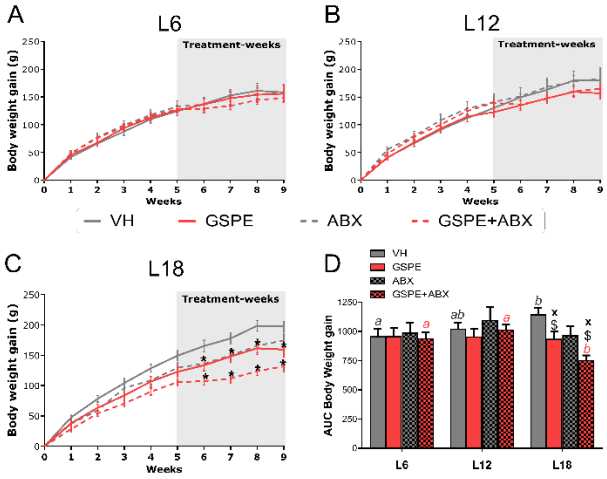


**Fig. 2** Effects of photoperiods and treatments on body weight gain. (A to C): Body weight gain under (A) short (L6), (B) standard (L12), and (C) long (L18) photoperiod conditions. * indicates significant GSPE effect, analysed by measured-repeated ANOVA followed by Tukey HSD/kramer post hoc test ($p<0.05$); (D) Area under the curve (AUC) of body weight gain. $ indicate tendency analysed by 2-way ANOVA followed by Tukey HSD/kramer post hoc test ($0.05>p<0.1$); x indicate significant GSPE effect analysed by Student's t-test ($p<0.05$); and ab letters indicate photoperiod effect analysed by one-way ANOVA followed by Tukey HSD/kramer post hoc test ($p<0.05$).Data are plotted as the mean ± SEM (*n=8*, except in L6.VH rats *n=7*). L6: 6h light/18h darkness; L12: 12h light/12h darkness; L18: 18h light/6h darkness; CAF: cafeteria diet; VH: vehicle; GSPE: grape seed proanthocyanidin extract; ABX: antibiotic cocktail.

Interestingly, GSPE treatment abolished the photoperiod effect observed on BW and fat depots in VH-treated rats, which showed higher BW gain and fat depots under L18 and higher glucose intolerance under L6 conditions (Fig. 2D; Fig. 3D; Table

1). In addition, GSPE-treated rats showed higher cholesterol levels under L18 conditions compared to VH-treated rats. GSPE-treated rats showed significant higher levels of total cholesterol and NEFAs when rats were housed under L18 compared to rats housed under L6 conditions. In contrast, VH-treated rats showed significant lower levels of total cholesterol and NEFAs under L18 compared to L6 conditions. Glucose and insulin levels did not change due to photoperiod conditions in any treated group (Table 1).

**Table 1** Fat parameters and biochemical serum levels of different CAF-treated groups under the three-photoperiod conditions.

| | VH | | | GSPE | | | ABX | | | GSPE+ABX | | | 2-way ANOVA (ABX / GSPE) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L6 | L12 | L18 | L6 | L12 | L18 | L6 | L12 | L18 | L6 | L12 | L18 | | | |
| Biometric Parameters (g) | | | | | | | | | | | | | L6 | L12 | L18 |
| MWAT | 12.2 ± 1.8 | 14.2 ± 1.6 | 15.43 ± 0.25 | 12.72±1.29 | 15.59±1.68 | 14.15±1.33 | 12.75 ± 1.4 | 13.17 ± 1.7 | 16.43 ± 1.6 | 10.25±0.80 | 13.65±1.48 | 12.09±1.29* | ns | ns | G |
| eWAT | 16.03 ± 1.8 | 18.9 ± 0.1 | 19.69 ± 1.59 | 18.65±1.10 | 18.10±1.01 | 16.47±1.75 | 17.77 ± 1.2 | 18.59 ± 1.9 | 18.63 ± 1.62 | 15.72±0.78 | 18.75±0.76 | 14.86±0.54 | ns | ns | ns |
| iWAT | 8.27 ± 1.5 | 8.64 ± 0.8 | 9.24 ± 1.39 | 7.28±0.90 | 9.44±1.06 | 7.05±1.00 | 8.77 ± 0.9 | 8.41 ± 0.9 | 11.71 ± 1.2 | 8.71±0.58 | 8.98±1.36 | 7.56±1.23 | ns | ns | ns |
| RWAT | 16.81 ± 1.7*a* | 21.1 ± 1.2*ab* | 22.01 ± 1.5*b* | 18.49±1.50 | 18.44±1.48 | 19.67±1.44 | 20.24 ± 1.2 | 20.91 ± 1.9 | 23.75 ± 1.4 | 17.65±0.96 | 20.58±1.14 | 16.39±1.53* | ns | ns | G |
| Subcutaneous | 22.50 ± 4.8 | 19.59 ± 2.6 | 27.73 ± 3.28 | 17.98±2.75 | 19.70±2.21 | 24.20±2.69 | 17.95 ± 1.2 | 18.65 ± 2.8 | 24.31 ± 2.58 | 22.61±2.47 | 19.52±1.93 | 16.60±1.72* | ns | ns | G |
| Fat Mass | 73.45 ± 9.4*a* | 82.56 ± 5.3*ab* | 94.09 ± 5.3*b* | 75.12±5.85 | 81.28±4.82 | 81.54±6.87 | 75.25 ± 4.6 | 79.73 ± 7.5 | 94.84 ± 5.28 | 74.94±4.34 | 81.48±5.21 | 67.50±5.10* | ns | ns | G |
| Visceral Fat | 42.67 ± 4.9*a* | 54.33 ± 3.4*ab* | 57.13 ± 3.96*b* | 49.86±3.61 | 52.13±3.43 | 50.29±4.24 | 50.77 ± 3.1 | 52.68 ± 5.3 | 58.82 ± 2.91 | 43.62±2.01 | 52.97±2.98 | 43.34±3.19* | ns | ns | G |
| Adiposity Index | 0.13 ± 0.01*a* | 0.14 ± 0.01*ab* | 0.16 ± 0.01*b* | 0.14±0.01 | 0.15±0.01 | 0.14±0.01 | 0.13 ± 0.01 | 0.14 ± 0.01 | 0.16 ± 0.01 | 0.14±0.01 | 0.14±0.01 | 0.12±0.01* | ns | ns | G |
| Cecum | 4.45 ± 0.3 | 5.08 ± 0.3 | 5.15 ± 0.38 | 4.49±0.45 | 4.52±0.31 | 4.02±0.24 | 7.68 ± 0.5# | 8.12 ± 0.4# | 7.28 ± 0.53# | 8.82±0.55# | 7.54±0.64# | 8.60±0.84# | ns | ns | G |
| Serum levels (mg/dl) | | | | | | | | | | | | | L6 | L12 | L18 |
| Triglycerides | 214.77 ± 24.9 | 201.75 ± 15.7 | 166.73 ± 22.9 | 132.49 ± 18.07 | 161.55±14.54 | 193.54±21.21 | 184.31 ± 19.2 | 215.27 ± 26.5 | 176.08 ± 12.9 | 179.59± 11.41 | 196.46±26.92 | 163.16±15.09 | ns | ns | ns |
| Total Chol. | 121.21 ± 16.5*a* | 137.03 ± 24.9*a* | 104.9 ± 8.9*b* | 102.09 ± 12.43*a* | 109.12±8.02*ab* | 139.29±7.21**b* | 113.70 ± 10.3 | 138.18 ± 12.3 | 142.66 ± 13.4 | 116.84 ± 6.77 | 131.14±17.81 | 114.19±5.53 | ns | ns | G*A |
| Glucose | 135 ± 7.2 | 144.76 ± 6.2 | 129.13 ± 4.2 | 125.53± 8.06 | 127.91±8.52 | 137.57±2.96 | 146.28 ± 4.9 | 130.09 ± 7.3 | 140.50 ± 4.6 | 133.43 ± 4.91 | 139.18±8.91 | 129.85±5.20 | ns | ns | ns |
| NEFAs | 26.36 ± 2.7*a* | 33.17 ± 3.4*a* | 20.69 ± 0.7*b* | 19.38 ± 1.69*a* | 23.37±1.92*ab* | 29.26±2.91*b* | 25.98 ± 3.6 | 25.04 ± 3.03 | 25.98 ± 3.7 | 24.51 ± 2.07 | 24.57±1.98 | 25.75±4.17 | ns | ns | ns |
| Insulin (ng/ml) | 13.06 ± 2.8 | 12.61 ± 1.9 | 10.17 ± 2.2 | 13.62 ± 2.49 | 14.05±2.54 | 8.52±1.92 | 17.35 ± 3.9 | 14.81 ± 3.7 | 10.95 ± 1.6 | 11.06± 3.01 | 11.02±1.21 | 10.99± 1.61 | ns | ns | ns |

Data are expressed as the mean ± SEM (n= 8, except L6.CAF-VH n=7). * and # indicate significant GSPE and ABX effects respectively in each photoperiod condition, analysed by two-way ANOVA (factors: ABX and GSPE) followed by Tukey-kramer post hoc test. ns: no significant effect, G: GSPE effect, G*A, interaction between GSPE and ABX, *p<0.05, #p<0.05; *ab* letters indicate significant photoperiod effect in each treated group, analysed by one-way ANOVA followed by Tukey-kramer post hoc test (p<0.05); mWAT, mesenteric white adipose tissue; eWAT, epididymal white adipose tissue; iWAT, inguinal white adipose tissue; RWAT, retroperitoneal white adipose tissue; Chol, cholesterol; NEFAs, non-esterified free fatty acid; L6: 6h light/18h darkness; L12: 12h light/12h darkness; L18: 18h light/6h darkness; CAF: cafeteria diet; VH: vehicle; GSPE: grape seed proanthocyanidin extract; ABX: antibiotic cocktail; Ph: photoperiod.

Additionally, to evaluate the impact of the gut microbiota on GSPE effects and its interaction with photoperiods, rats were treated with ABX for the last 4 weeks. Rats treated with the combination of GSPE and ABX showed a significant decreased in BW gain by 21% (p<0.05) and lower fat depots (p<0.05) compared to ABX-treated rats only under L18 conditions. In fact, under L18 photoperiod, ABX-treated rats trended to decrease BW gain by 15% (p=0.065) compared to VH-treated rats. Besides this decrease in BW gain, ABX-treated rats did not show any change in fat depots accumulation compared to VH-treated rats (Fig. 2C-D; Table 1). Moreover, GSPE+ABX-treated rats showed higher glucose tolerance after 60 min of glucose administration compared to ABX-treated rats only under L6 photoperiods (Fig. 3A). No significant effects were found in biochemical parameters in GSPE+ABX rats compared to ABX-treated rats. However, significant higher levels of total cholesterol were observed in ABX-treated rats compared to VH-treated rats when housed under L18 conditions (Table 1).

Interestingly, photoperiod effect was found in BW gain in rats treated with GSPE+ABX. Thus, GSPE+ABX-treated rats housed under L18 showed lower BW gain compared to those housed under both L6 and L12 conditions. In contrast, ABX-treated rats did not show any photoperiod effect on BW gain (Fig. 2D). Furthermore, ABX-treated rats housed under L18 conditions showed significantly higher iWAT, fat mass and adiposity index compared to those housed under L6 (p<0.05) (Table 1). Interestingly, GSPE treatment abolished this photoperiod effect in ABX-treated rats. Finally, a significant interaction effect was observed between photoperiod and GPSE in most of the fat depots parameters (iWAT, RWAT, fat mass, adiposity index and visceral mass) (Photoperiod*GSPE, p<0.05).

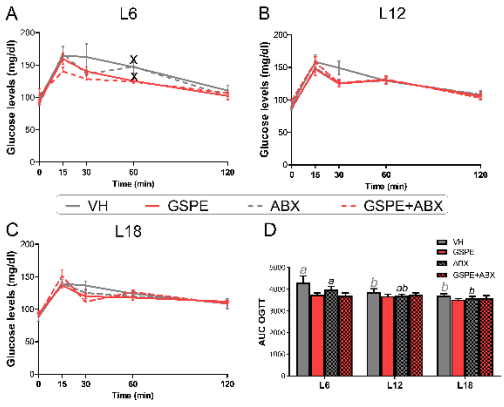


**Fig. 3** Effects of GSPE and photoperiods on glucose levels. (A to C): Oral glucose tolerance test under (A) short (L6), (B) standard (L12), and (D) long (L18) photoperiod conditions. Measured-repeated ANOVA followed by Tukey-kramer post hoc test (p>0.05); x indicates GSPE effect analysed by Student's t-test at each point (p<0.05); (D) Area under the curve (AUC) of glucose tolerance test. ab indicate significant GSPE and Photoperiod effect respectively, analysed by one-way ANOVA followed by Tukey-kramer post hoc test (p<0.05). Data are plotted as the mean ± SEM (*n=8*, except in L6.VH *n=7*). L6: 6h light/18h darkness; L12: 12h light/12h darkness; L18: 18h light/6h darkness; CAF: cafeteria diet; VH: vehicle; GSPE: grape seed proanthocyanidin extract; ABX: antibiotic cocktail.

**Grape Seed Proanthocyanidins affects faecal microbiota composition in a photoperiod-dependent manner**

Faecal microbiota composition of the different experimental groups was analysed in order to evaluate if GSPE modulates it differently depending on the photoperiod conditions, which may explain the photoperiod dependent GSEP effects presented above.

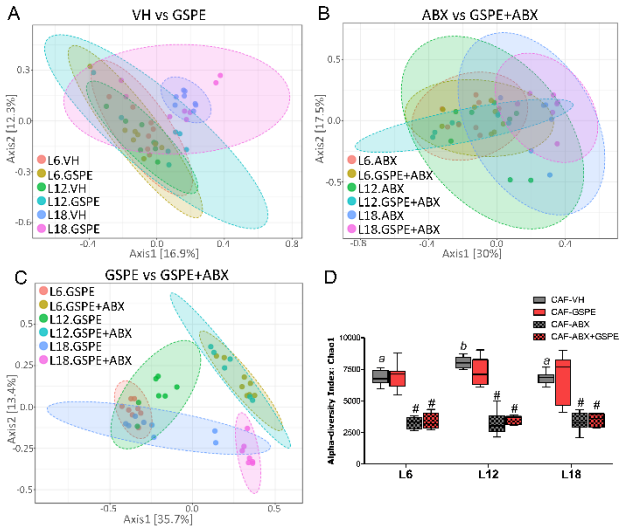


**Fig. 4** Photoperiod effects on β- and α-diversity. (A to B): β-diversity based on Bray-Curtis distances and visualized by a Principal Coordinates Analysis (PCoA) 3D plot (PERMANOVA, p<0.001) in rats treated with (A) VH or GSPE and (B) ABX or GSPE+ABX under different photoperiod (L6, L12 and L18). (C) β-diversity to compare GSPE and GSPE+ABX treatments under different photoperiods (L6, L12 and L18); (D): Alpha diversity calculated by chao-1 index in all CAF-groups under different photoperiod conditions. Data are plotted as box and whiskers (median with interquartile ranges). # indicates significant ABX effect between VH- and ABX-treated rats under same photoperiod conditions, analysed by U-Mann Whitney (p<0.05); ab letters indicate significant photoperiod effect analysed by Kruskal-Wallis test followed by Bonferroni correction for multiple comparisons (p<0.016). *(n=8*, except L6.CAF-VH *n=7*). L6: short photoperiod (6h light/18h dark); L12: standard photoperiod (12h light/12h dark); L18: long photoperiod (18h light/6h dark); L6: 6h light/18h darkness; L12: 12h light/12h darkness; L18: 18h light/6h darkness; CAF: cafeteria diet; VH: vehicle; GSPE: grape seed proanthocyanidin extract; ABX: antibiotic cocktail.

Overall changes in faecal microbiota communities were observed by assessing microbial beta-diversity using a principal component analysis (PCoA) based on Bray Curtis dissimilarity. Regarding GSPE treatment, it did not result in significant clusters (PERMANOVA, p>0.05) in any photoperiod conditions (Fig. 4A-B). However, when analysing each photoperiod independently, rats treated with GSPE tended to cluster differently from rats treated with VH when housed under L12 conditions (PERMANOVA, p<0.063) and L18 conditions (PERMANOVA, p-value<0.077) (Fig. 4A; Fig. S1), suggesting that GSPE may alter gut microbiota depending on photoperiod exposure. Moreover, when comparing GSPE-treated rats with GSPE+ABX-treated rats, a cluster according to ABX treatment was observed, indicating an ABX effect in GSPE-treated rats under the different photoperiod conditions (Fig. 4C). In addition, GSPE treatment did not alter the α-diversity, Firmicutes-to-Bacteroidetes (F/B) ratio or Proteobacteria-to-Firmicutes (P/F) ratio, which were decreased only by ABX effect (Fig. 4D; Fig. 5C-D).

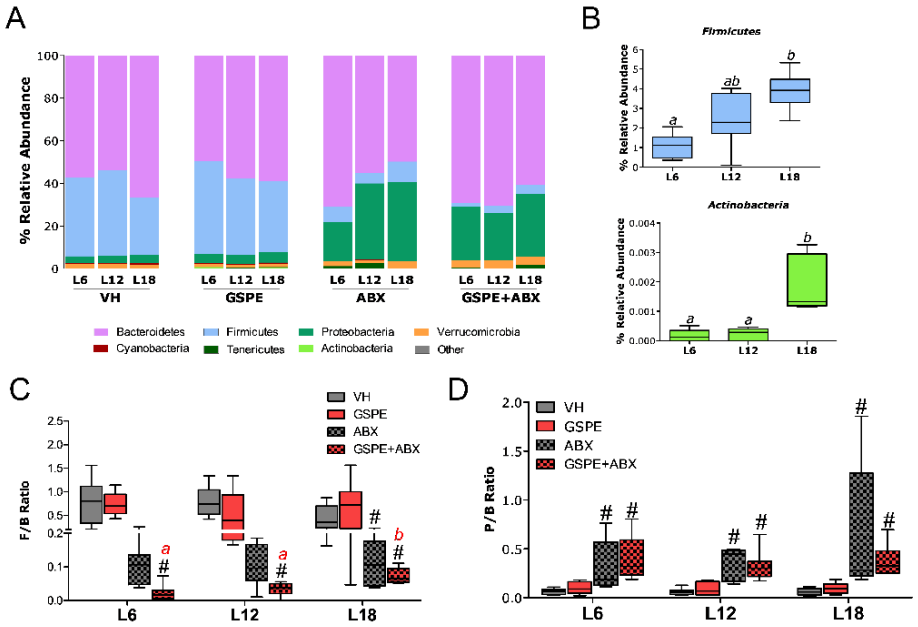


**Fig. 5** Effect of photoperiods on bacteria phyla relative abundance. (A) Stacked bar plots showing the relative abundance of taxa at phylum level. (B) Box and whiskers plots with interquartile ranges for Firmicutes and Actinobacteria relative abundance of GSPE+ABX rats under different photoperiods; (C) Box and whiskers plots (median with interquartile ranges) for Firmicutes-to-Bacteroidetes (F/B) ratio and (D) for Proteobacteria-to-Bacteroidetes (P/B) ratio; # and ab letters indicate ABX and photoperiod significant effects respectively, analysed by Kruskal-Wallis followed by Bonferroni correction for multiple comparisons (p<0.016). (*n=8*, except L6.CAF-VH *n=7*). L6: short photoperiod (6h light/18h dark); L12: standard photoperiod (12h light/12h dark); L18: long photoperiod (18h light/6h dark). L6: 6h light/18h darkness; L12: 12h light/12h darkness; L18: 18h light/6h darkness; CAF: cafeteria diet; VH: vehicle; GSPE: grape seed proanthocyanidin extract; ABX: antibiotic cocktail.

Remarkably, photoperiod effects were observed in microbial beta-diversity of VH-treated rats. Thus, VH-treated rats housed under L18 conditions clustered differently from rats housed under either L6 or L12 conditions, which clustered

together (Fig. 4A). Interestingly, this marked L18 photoperiod effect was not observed in GSPE-treated rats which did not cluster differently from rats housed under L6 or L12 conditions (Fig. 4A), indicating that GSPE may be able to modulate photoperiod effects on gut microbiota composition. This L18 photoperiod effect was less obvious in rats treated with ABX, although certain degree of separation could be observed along the axis 1 independently of VH or GSPE treatment (Fig. 4B). When analysing microbial beta-diversity of GSPE-treated rats versus GSPE+ABX-treated rats, a strong L18 photoperiod effect was observed for those receiving GSPE+ABX co-treatment (Fig. 4C). Thus, GSPE+ABX-treated rats housed under L18 conditions clustered differently from both GSPE- and GSPE+ABX-treated rats housed under L6 and L12 conditions (Fig. 4C). In addition, the photoperiod effect observed in CAF-fed rats under L12, which showed a significant higher alpha diversity than rats under L6 and L18, was abolished by GSPE, GSPE+ABX, but also by ABX treatments (Fig. 4D).

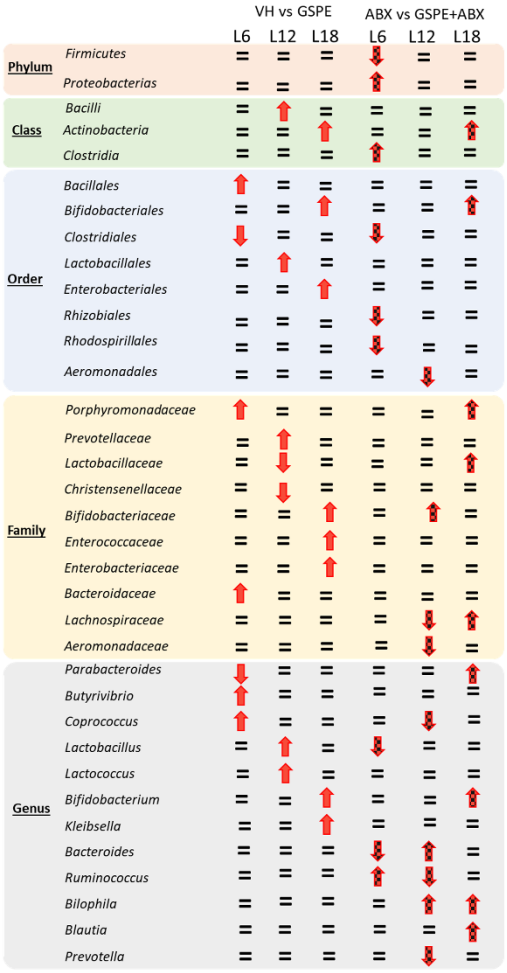


**Fig. 6** GSPE effects depended on photoperiod conditions. Summary of significant GSPE effects in the different photoperiod conditions, when comparing VH versus GSPE-treated rats and ABX versus GSPE+ABX-treated rats.

Faecal composition was also analysed at different taxonomic levels. GSPE effect on faecal microbiota composition was different depending on photoperiod conditions (Fig. 6; Table S1). Thus, although GSPE did not cause changes at phylum level (Fig. 5A) and at class level only Bacilli ($p<0.05$) under L12 and *Actinobacteria* ($p<0.05$) under L18 were affected, several bacteria at order, family and genera levels were significantly altered. For instance, GSPE treatment significantly increased *Bacillales* under L6, *Lactobacillales* under L12 and *Bifidobacteriales* and *Enterobacteriales* orders under L18 condition (Table S1). At family level, GSPE treatment significantly decreased *Porphyromonadaceae* under L6 and *Lactobacillaceae* and *Christensenellaceae* under L12 while increased *Prevotellaceae* under L6, and *Bifidobacteriaceae*, *Enterococcaceae* and *Enterobacteriaceae* under L18 conditions. Finally, among bacteria genera levels, GSPE significantly decreased *Parabectoroides* and significantly increased Bacillus, Butyrivibrio and *Coprococcus* under L6, while significantly decreased *Lactobacillus*, *Lactococcus* and *Ruminococcus* under L12, and increased *Bifidobacterium* and decreased *Kleibsella* under L18 conditions.

Interestingly, photoperiod housing conditions also significantly affected several bacterial genera relative abundance in GSPE-treated rats (Fig. 7; Table S1). In CAF-fed rats receiving GSPE, photoperiod effects were observed for low abundance genera belonging to the Firmicutes phyla such as *Butyricimonas*, *Coprobacillus*, *Rummelibacillus* and *Lachnobacterium*, which showed higher levels in rats housed under L18 compared to those housed under L6 conditions. GSPE treatment also altered genera belonging to *Proteobacteria* phyla such as *Enterobacter*, *Erwinia* and *Kleibsella*, increasing their levels under L18 compared to L6. Interestingly, GSPE treatment abolished the photoperiod effect observed in the most abundant bacterial genera compared to VH rats. Thus, *Bacteroides*, *Oscillospira*, *Sutterella*, *Coprococcus* and *Ruminococcus* did not show photoperiod effect in these GSPE-treated rats.

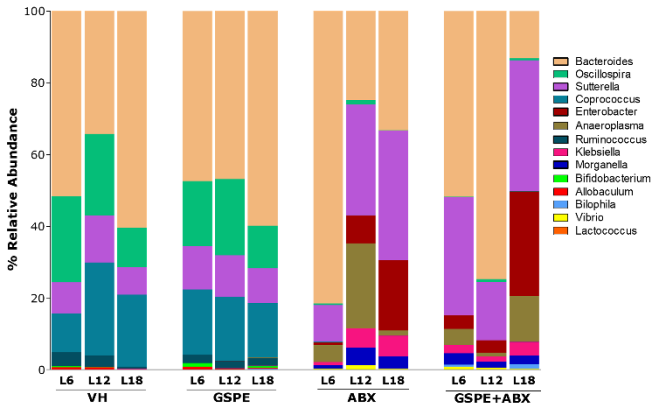


**Fig. 7** Relative abundance at genus level of the 14 most abundant genera. Stacked bar plots showing the relative abundance of taxa at genus level (*n=8*, except L6.CAF-VH *n=7*); L6: 6h light/18h darkness; L12: 12h light/12h darkness; L18: 18h light/6h darkness; CAF: cafeteria diet; VH: vehicle; GSPE: grape seed proanthocyanidin extract; ABX: antibiotic cocktail.

On the other hand, several bacteria at the different taxonomic levels were altered by the combination of GSPE and ABX treatments compared to ABX-treated rats, also depending on photoperiod conditions (Fig. 6; Table S1).Thus, when

analysing GSPE effects in ABX-treated rats (GSPE+ABX compared to ABX group) a significant decrease in *Firmicutes* relative abundance ($p<0.05$) and a tendency to increase *Proteobacteria* relative abundance ($p=0.059$) were observed only when rats were housed under L6 conditions (Fig. 5A). In addition, GSPE treatment increased the relative abundance of *Clostridia* class under L6 and of *Actinobacteria* class under L18, while no bacteria class was affected under L12 conditions. At order level, *Bacillales* was significantly increased and *Clostridiales*, *Rhizobiales* and *Rhodospirillaled* were significantly decreased under L6, *Aeromonadales* was decreased under L12 and *Bifidobacteriales* were increased under L18. At family level, *Lactobacillaceae* were increased under L6, Lachnospiraceae and *Aeromonadaceae* were decreased under L12, and *Bifidobacteriaceae*, *Porphyromonadaceae* and *Lachnospiraceae* were increased under L18 conditions. Finally, among bacteria genera, GSPE+ABX decreased *Bacteroides* and *Lactobacillus* and increased *Ruminococcus* under L6, decreased *Coprococcus*, *Prevotella* and *Ruminococcus* and increased *Bacteroides* and *Bilophila* under L12, and increased *Bifidobacterium*, *Parabacteroides*, *Blautia* and *Bilophila* under L18 conditions (Fig. 6; Table S1).

## Discussion

Dietary polyphenols naturally present in fruits and vegetables are gaining increasing attention due to their beneficial health effects on obesity and MetS development. [31,32] Hence, the study of factors affecting polyphenols bioactivities is of high interest nowadays. Gut microbiota is one of the main factors that may be playing an important role on polyphenols functionality. Thus, gut bacteria are significantly involved in polyphenols metabolism. Moreover, recent studies indicate that dietary polyphenols are relevant in the modulation of gut microbiota composition, which are crucial for the maintenance of homeostasis and metabolic function in the host. [33,34] Additionally, seasonal rhythms have recently been established as key factors in the modulation of the gut microbial and, interestingly, also in the metabolism of polyphenols.[19,21] However, the interaction between polyphenols, gut microbiota and photoperiods is poorly understood and further investigations are needed. Thus, the purpose of this study was to evaluate if the beneficial anti-obesity effects of GSPE are modulated by gut microbiota in a photoperiod dependent manner.

Different photoperiod conditions were used to mimic seasonal rhythms. The short photoperiod conditions emulated the hours of light in short days typical of the winter season, while the long photoperiod conditions simulated the long days typical of the summer season. GSPE was orally administered at 25 mg/kg/day which is within the estimated range of polyphenols intake in humans. This is equivalent to the daily intake of 367 mg for a person of 70 kg, [35] which is easy to achieve with a polyphenol-rich diet. [36–38] Furthermore, the use of low doses of GSPE have shown beneficial effects on obesity development, not being necessary the administration of high doses in order to get a positive effect. [5] Thus, GSPE has been proven to have lipolytic properties and to be an effective anti-obesity agent when administered at this low dose. [39] Moreover, administration of 25 mg/kg/day of GSPE to CAF-fed Wistars rats during 3 weeks decreased body weight gain and adipose depots accumulation. [40] Furthermore, the same doses administered for 2 weeks significantly reduced adiposity index in hamster fed with a high fat diet, although BW gain was not affected.[6] However, GSPE administration at the same dose for 30 days did not significant decrease body weight or fat depots in diet-induced obese female Wistars rats.[5] In this study, the results did not show a significant reduction of BW gain or fat depots in standard conditions (L12 photoperiod) by GSPE treatment. These discrepancies regarding the GSPE effect on BW gain and fat depots may result from the use of animals of different age, sex and strains as well as, from the duration of the treatment.

In our study, rats housed under L18 conditions gained more BW compared to rats housed under L6 or L12 conditions. Interestingly, GSPE treatment decreased significantly this BW gain observed in this long photoperiod. The higher BW gain under long photoperiod conditions is in accordance with other studies reporting increased BW gain in rats held under photoperiods of more than 12 h of light/day compared to rats held under photoperiods of less than 10 h of light/day.[41] Indeed, increased BW under long photoperiods is common in mammals, who are able to adapt to changes in the light and dark cycle during the different seasons, and it is associated with a more efficient pattern of energy harvesting from the consumed food.[42,43]

Interestingly, the GSPE effects observed in L18 can be associated with changes in fecal microbiota composition. Hence, GSPE altered gut microbiota composition differently depending on the photoperiod conditions. Thus, GSPE increased levels of *Bifidobacterium* and *Coprobacillus* and decreased *Kleibsella* genera only under L18. These bacteria genera levels are linked to lower body weight gain and non-obese phenotypes.[44,45] In contrast, decreased *Parabacteroides* under L6 and *Lactobacillus, Lactococcus* and *Ruminococcus* under L12. Strains belonging to *Parabacteroides* genera such as *P. goldsteinii* and *P. distasonis* have been described as important bacteria with anti-obesity effects.[46,47] Furthermore, strains of *Lactobacillus, Lactococcus* and *Ruminococcus* genera, belonging to Firmicutes phyla, have been associated with lower BW gain. Thus, *Lactobacillus* strains are able to alleviate obesity in mice induced by high-fat diet via relieving fat accumulation, lipid metabolism and regulating the content of leptin and adiponectin,[48] *Lactococcus* reduce weight gain and lipid accumulation and regulating leptin and adipokine secretion,[49] and *Ruminococcu*s genera has been shown to decreased in obese subjects and is known as potential butyrate producer, which exert beneficial effects against obesity by increasing lipid oxidation.[50,51] Therefore, these changes in the gut microbiota composition by GSPE depending on photoperiod may help to explain the difference of BW gain in GSPE-treated rats under L18 compared to both L6 and L12 conditions.

In addition, the differences in BW gain depending on photoperiod conditions have been linked with changes in gut microbiota composition correlated with higher capacity to

harvest energy from diet and increase glucose and fatty acid absorption.[52] Thus, evidence in Siberian hamster,[53] broiler roosters,[54] Sprague-Dawley rats[22] and humans[55] have shown different gut microbiota composition depending on photoperiod exposure. In this context, GSPE decreased the photoperiod effect observed on fecal microbiota composition in VH-treated rats, showing similar beta- and alpha-diversity independently of photoperiod conditions, and modulating the most abundant genera. Accordingly, *Bacteroides*, *Oscillospira*, *Coprococcus* and *Ruminococcu*s genera levels, which were associated with higher BW gain and fat depots accumulation under L18 in VH-treated rats,[28] did not show any photoperiod effect in GSPE-treated rats. Therefore, GSPE affected BW gain and gut microbiota composition in a photoperiod dependent manner, suggesting a potential interaction among GSPE, gut microbiota and photoperiod conditions.

To further evaluate the role of gut microbiota on GSPE effects in the different photoperiod conditions, rats were treated with an ABX cocktail. The effects of this cocktail on gut microbiota were consistent with previous studies.[56,57] Remarkably, the effect of GSPE on BW gain observed in L18 conditions was potentiated in ABX-treated rats. Furthermore, GSPE also significant decreased fat depots accumulation only under L18 in ABX-treated rats. Moreover, GSPE+ABX-treated rats showed a significant L18 effect on fecal microbiota composition. Therefore, the differences in GSPE anti-obesity effects observed in this group under L18 could be due to changes in the bioavailability of GPSE mediated by more pronounced changes in gut microbiota observed in these conditions.[12] Thus, it has been estimated that the 90-95% of the total polyphenol intake remain unabsorbed in the small intestine and they are metabolized by the gut microbiota in the colon.[15,16] The gut microbiota therefore play an important role in the extensive breakdown of polyphenols into a low-molecular-weight phenolic acids and other microbial-derived metabolites that can be absorbed into the circulatory system and confer health benefits.[17] Thus, changes in the gut microbiota composition mediated by ABX treatment and photoperiod conditions, may change microbial-derived GSPE metabolites, altering the bioactivities of this polyphenol extract. Indeed, ABX+GSPE treatment led to significant photoperiod effect on *Firmicutes* and *Actinobacteria* phyla, increasing both of them under L18. These high levels of *Firmicutes* under L18 promoted an increase in F/B ratio, which could be associated to lower body mass in these rats. In fact, other studies using cafeteria diet reported that the F/B ratio is decreased in these induced-obese rats due to the differences in the type of fat present in this diet, mainly lard and milk-derived fat, in comparison with other high fat diets induced obesity models [58,59]. In addition, the increase of *Actinobacteria* in L18 led to significant higher levels of *Bifidobacterium* genera in this photoperiod. *Bifidobacterium* is the most studied genus of *Actinobacteria* phylum, showing a high versatility.[60,61] These bacteria metabolize dietary compounds and ferment non-digestible dietary foods resulting in the formation of SCFAs, and are able to displace harmful bacteria by competing with pathogens for nutrients.[62] Likewise, *Bifidobacterium* has been reported to exert anti-obesity effects,[63–65] which is in concordance with our previous results where this bacterium taxon was associated with decreased body mass. Therefore, we suggest that the strongest effect of GSPE in decreasing body weight gain in ABX-treated rats housed under L18 conditions may be mediated by these changes in gut microbiota observed in these photoperiod conditions.

It is also important to consider the effect of ABX administration on the body composition, since several studied have provided increasing evidence for an association between the ABX consumption and weight gain and fat accumulation.[66–68] However, our results did not show ABX effects on body weight gain, body composition nor serum parameters, which could be due to differences in the doses employed and/or the duration of the treatment.

## Conclusions

GSPE administered together with ABX potentiated the decrease of BW gain and body composition only under L18 compared to when GSPE was administered alone, which suggests an interaction between polyphenols, gut microbiota, and photoperiods. Thus, GSPE anti-obesity effects were modulated by gut microbiota in a photoperiod dependent manner. Therefore, seasonal rhythms and gut microbiota are key factors that must be taken into account when investigating the benefits of polyphenols in the organism.

## Author Contributions

Conceptualization: V.A.-G., A.A.-A. and C.T.-F.; Formal analysis: V.A.-G. and I.E.-M.; Data curation: V.A.-G; Investigation: V.A.-G., I.E.-M., M.S., B.M., G.A., A.A.-A. and C.T.-F.; Methodology: V.A.-G., A.A.-A. and C.T.-F.; Funding acquisition: B.M., M.S., G.A., A.A.-A. and C.T-F.; Project administration: B.M., M.S., G.A.,A.A.-A. and C.T-F.; Resources: B.M., M.S., G.A., A.A.-A. and C.T-F.; Software: V.A-G; Visualization: V.A.-G.; Supervision: A.A.-A. and C.T-F; Validation: V.A.-G., A.A.-A. and C.T.-F.; Writing original draft: V.A.-G.; Writing review & editing: A.A.-A. and C.T.-F.

## Conflict of interest

There are no conflicts to declare.

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