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Pulsed LED Light: Exploring the Balance between Energy Use and Nutraceutical Properties in Indoor-Grown Lettuce

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Abstract: In indoor vertical farms, energy consumption represents a bottleneck for both a system's affordability and environmental footprint. Although switching frequency (*sf*) represents a crucial factor in determining the efficacy of light emitting diodes (LED) lighting systems in converting electricity into light, the impact of *sf* is still underexplored. The aim of this work was to investigate the effect of LEDs *sf* on the productive and qualitative responses of lettuce (*Lactuca sativa* L.), also considering the resource use efficiency. Plants were grown for 14 days under red and blue LEDs (215 µmol m^{-2} s^{-1} and 16/8 h light/dark, with a red:blue ratio of 3) characterized by two different *sf* for the blue diode, namely high *sf* (850 kHz) and low *sf* (293 kHz). A fluorescent light (same light intensity and photoperiod) was included. LED *sf* did not alter plant morphological parameters, including fresh or dry biomass, leaf number, leaf area, or water use efficiency. A low *sf* increased the energy use efficiency (EUE) by 40% as compared to high *sf*. The latter enhanced the leaf antioxidant capacity, as a consequence of increased concentrations of caftaric and chicoric acids, isoquercetin, and luteolin, consistent with the upregulation of a few genes related to the biosynthetic pathway of phenolic compounds (4C3H and DFR). The study highlights that different *sf* may significantly affect the EUE as well as crop nutritional properties.

Keywords: lettuce (*Lactuca sativa*); indoor farming; hydroponics; phenolic compounds; antioxidant capacity; LED lighting; pulsed light; flavonoids; antioxidative enzymes; gene expression

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1. Introduction

The use of horticultural LED lighting systems has gained wide relevance in recent years thanks to the potential applications in indoor farming [1]. LED lights are durable, have a long lifetime, high radiant efficiency, and faster switching [2,3]. In addition, they make it possible to select and customize output spectral features, matching the plant's needs and allowing for high-quality crops [4,5], also in terms of nutraceutical properties (e.g., antioxidant capacity). On the other hand, the initial cost of LED lighting technology is higher as compared to other horticultural lighting typologies [6]. Electricity costs in indoor farms—also when LEDs are used—can account for 25 to 30% of the operational costs [7,8]. Improving horticultural LED energy use efficiency (EUE, i.e., plant biomass produced per unit of energy supplied) is, therefore, an imperative research priority for the large-scale adoption of indoor agriculture technologies [9,10]. A possible strategy to increase EUE comes from the substitution of a continuous lighting supply with pulsed

light by modulating both the frequency and the duty ratio (i.e., the ratio between pulse duration (with the light turned on) and length of the light–dark cycle (whole cycle of light on and off) of LED lamps). This fostered the growth parameters in different crops (e.g., *Brassica chinensis*) exposed to intermittent lighting (60:15 min ratio of light:dark) [11]. In basil plants, intermittent lighting with dark periods every 10 min was shown not to alter growth/productive performance, thus allowing for significant increases in EUE [12]. In *Arabidopsis* seedlings, shorter pulse periods (down to 5:10 s of light:dark provided by red, blue, and far-red lights) were also observed to significantly reduce electricity requirements (<50%) while preserving growth at similar values to that measured under continuous lighting (photoperiod of 12:12 h) [13]. Thanks to the advantages provided by LED technology, light pulses can also be extremely short and frequent, since LEDs can be turned fully off and fully on very rapidly, even with an interval of a μs [14].

Lettuce (*Lactuca sativa* L.) is an economically important crop worldwide [15] and one of the most popular species grown in vertical farms [16]. Rapid growth and a short growing cycle have made lettuce a model crop for studying the interaction between plants and light [17]. Lettuce leaves are also a good source of phenolic compounds [18], mostly represented by hydroxycinnamic acid derivatives, with chicoric and caftaric acids as the major representatives of this sub-class, and flavonoids, especially flavonols. Several health benefits have been demonstrated for chicoric and caftaric acids [19–23], while the presence of flavonols in green tissues has been related to the high antioxidative activity in planta, deriving from their chemical structure [24]. In particular, the high ROS scavenging capacity of quercetin and kaempferol glycosides has been confirmed in some species, including lettuce [25] and *Phillyrea latifolia* [26]. Strategies to improve the nutraceutical content of indoor-grown crops are aimed at obtaining highly nutritional crops all year long, an important goal considering that in some lettuce cultivars [27] and in baby leaf lettuce [28], the concentration of phenolic compounds has been shown to change according to seasonality. In lettuce, as in most horticultural crops, red and blue light have been identified as the most efficient spectral regions for enhancing both growth and the concentration of nutraceutical compounds [29,30]. Studies on the optimal red:blue light ratio (RB) for lettuce identified RB = 3 as the combination providing both the highest yield and the highest flavonoid content, as compared to fluorescent light and LED light with other RB ratios. In addition, RB = 3 led to the greatest efficiency in terms of land, water, and energy use [31]. Optimal light intensity (200–250 mmol m^{-2} s^{-1},) and photoperiod (16 h d^{-1}) were also recently identified as critical factors influencing lettuce growth and nutraceutical content, as well as crop EUE [32,33].

Although numerous studies have targeted the photoregulation of plant growth and development, less information is available on the effects of LED light quality on associated gene expression. Blue, red, and white LEDs, individually or in combination, have been reported to influence the expression of key regulatory genes involved in the carotenoid and flavonoid biosynthetic pathways as reported in wheat sprouts [34], and in the antioxidant defense machinery, such as the ascorbate metabolism in post-harvest broccoli [35]. Given that LED lighting can affect the accumulation of non-enzymatic antioxidants, including phenylpropanoids, tocopherols, vitamin C, and ascorbate [36], it may be expected that the activities and the transcript levels of enzymatic antioxidants, such as catalase (CAT) and glutathione S-transferase (GST), may likewise be affected [37].

The present research builds on available information concerning the application of an optimized red and blue LED spectrum (RB = 3) for lettuce cultivation by exploring the potential impact of pulsed light technology. The work defines how growth, the concentration of phenolic compounds, and the antioxidant capacity of hydroponically grown lettuce vary in response to two different switching frequencies (*sf*) of the blue diode in an RB = 3 LED lighting system. The antioxidant capacity of leaf extracts and the expression of genes encoding for enzymes in the biosynthetic pathway of phenolic compounds and antioxidative enzymes were analyzed, with the final aim of increasing our knowledge of

how resource use efficiency and the nutraceutical value of indoor-grown lettuce plants are regulated by LED pulsed light.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Plants were grown at the Department of Agricultural and Food Sciences of the University of Bologna (Italy). Seeds of green lettuce "Gentilina" (*Lactuca sativa* L. cv. Rebelina, Gautier, Eyragues, France) were germinated in polystyrene containers filled with a mixture of peat and vermiculite (70/30 *v/v*), under fluorescent lamps (TL-D90 De Luxe 950, Philips) providing a Photosynthetic Photon Flux Density (PPFD) of 215 μmol m^{-2} s^{-1} and a photoperiod of 16 h d^{-1}. When the plants reached a two true leaf stage (14 days after sowing), the roots were washed and plantlets were transplanted into single-plant hydroponic systems [38] consisting of plastic jars (1 L) filled with a nutrient solution (EC = 1.6, pH = 6.5) of the following composition: NO_3-N, 14 mM; NH_4-N, 4.4 mM; P, 1.0 mM; K, 5.0 mM; S, 2.0 mM; Ca, 1.2 mM; Mg, 5.2 mM; Fe, 17.9 μM; Cu, 2.0 μM; Zn, 3.8 μM; B, 11.6 μM; Mn, 18.2 μM; and Mo, 0.5 μM. The nutrient solution was constantly aerated through air pumps (Airline 3, Haquoss, Turin, Italy, with an air exchange rate of 0.25 L min^{-1} jar^{-1}). After transplanting, the plants were grown in three separate compartments (0.64 m^2 surface and 0.4 m^3 volume) isolated by white opaque walls in a climate-controlled growth chamber (temperature 24 ± 2 °C, RH 55–70%, and 450 ppm CO_2). A planting density of 100 plants m^{-2} and a crop cycle length of 14 days from transplant to harvest were adopted [31].

2.2. Light Treatments

In two of the three compartments, lettuce plants were grown under dimmable LED lamps (Flytech s.r.l., Belluno, Italy) featuring red light (peak at 669 nm) and blue light (peak at 465 nm) emitting diodes. The lamps were set to supply a spectral composition with a red:blue ratio of 3 (RB = 3), a light intensity of 215 μmol m^{-2} s^{-1}, and a photoperiod of 16 h d^{-1}. The spectral distribution was measured using an illuminance spectrophotometer (CL-500A, Konica Minolta, Chiyoda, Tokyo, Japan). A photosynthetic photon flux sensor (with equal sensitivity to red and blue radiation), model QSO (Apogee instruments, Logan, UT, USA) connected with a ProCheck handheld reader (Decagon Devices Inc., Pullman, WA, USA) was used to set a PPFD (μmol m^{-2} s^{-1}) over the plant canopy [31]. The light treatments consisted of the following two different switching frequencies (*sf*) for the blue diode: a high *sf* (850 kHz) and a low *sf* (293 kHz); in the two treatments, the *sf* for the red diode was similar (namely 437 kHz and 443 kHz, respectively). A third light treatment with fluorescent light (FL), one of the traditional artificial light sources for indoor plant cultivation, was applied in the third compartment with the same light intensity and the same photoperiod as the LED light treatment.

2.3. Growth Analysis and Resource Use Efficiency

At harvest (14 days after transplanting), the leaf and root fresh weights per plant (g FW $plant^{-1}$) were measured and the dry weights were quantified after drying the samples at 60 °C for 72 h. Dry matter was calculated as the ratio between leaf dry and fresh weights and expressed as a percent value. Plant leaf area was determined using a leaf area meter (LI-300, LI-COR, Lincoln, NE, USA), and Specific Leaf Area (SLA) was calculated as the ratio between the plant leaf area and the leaf dry weight. The leaf number per plant was also counted.

Water use was quantified for each plant and Water Use Efficiency (WUE) was determined as the ratio between plant fresh weight and the volume of water used and was expressed as g FW L^{-1} H_2O. Lighting Energy Use Efficiency (EUE) was determined as the ratio between the final leaf fresh weight and the lamps' cumulative electricity consumption and was expressed as g FW kWh^{-1}.

2.4. Biochemical Determinations

2.4.1. Extraction Procedure for Phenolic Compounds

Extraction of phenolic compounds from leaf material was performed as described by Llorach et al. [39], with some modifications. After drying, leaves collected from six plants per treatment were pooled and finely ground in a knife mill for 4 × 30 s periods. The fine powder was then subjected to the "coning and quartering" sampling procedure and two technical replicates were carried out. A 1-g aliquot of dried leaves was extracted with 20 mL of a mixture of methanol/water/formic acid (50/48/2 *v/v/v*). The suspension was mixed and sonicated for 15 min at 35 °C. After centrifugation for 30 min at 1400× *g*, the supernatant was transferred into another tube and the pellet was extracted once again with the same procedure. Supernatants were merged, filtered through Grade 44 (3 µm) ashless filter paper, evaporated to dryness, and re-suspended with the same extraction mixture to a 50 mg mL^{-1} concentration. The extract was filtered again through a syringe filter (nylon, 0.22 µm pore diameter, Thermo Fisher Scientific, Carlsbad, CA, USA) and stored at −20 °C until used for analyses.

2.4.2. Determination of Total Phenolic Compounds and Total Flavonoids

The Total Phenolic Content (TPC) of the leaf extracts was evaluated using Folin–Ciocalteu's reagent, as described by Singleton and Rossi [40], with some modifications. A mixture containing 100 µL of diluted extract or standard, respectively, and 440 µL of Folin–Ciocalteu's reagent (diluted 1:10 with distilled water) was incubated for 10 min at room temperature (RT). Then, 440 µL of 7.5% Na_2CO_3 was added and the mixture was incubated in the dark for 60 min at RT. Gallic acid was used as a standard, and a calibration curve was built in the 6.25–100 ppm range. The absorbance was measured at 765 nm using a double-beam spectrophotometer (V-630 Jasco, Jasco Europe S.r.l., Cremella, Italy).

The Total Flavonoid Content (TFC) was evaluated according to the method described by Zhou et al. [41], with some modifications. A mixture containing 100 µL of diluted extract or standard and 440 µL of 0.066 M $NaNO_2$ was left to react for 5 min at RT. Then, 60 µL of 0.75 M $AlCl_3$ was added and the mixture was incubated for 5 min. Finally, 400 mL of 0.5 M NaOH was added and the mixture was incubated for 6 min at RT. The absorbance was measured at 510 nm and the TFC was calculated by interpolating with the calibration curve built with catechin as a standard, in a concentration range of 3.12–200 ppm.

2.4.3. HPLC Analysis of Phenolic Compounds

The extracts were injected into a Jasco (Tokyo, Japan) HPLC-DAD system, which consisted of a PU-4180 pump, an MD-4015 PDA detector, and an AS-4050 autosampler. The stationary phase was an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reversed-phase column (100 mm × 3 mm I.D., 3.5 µm). The injection volume was 20 µL for all of the determinations. The method used for the phenolic acids analysis was adapted from Llorach et al. [39], with some modifications. Elution was carried out with a mixture of solvent A (water/formic acid, 95/5 *v/v*) and solvent B (methanol), with a composition gradient ranging from 95 to 60% of solvent A and flowing at 0.5 mL min^{-1}. The chromatograms were recorded at 329 nm. For flavonoid analysis, the method used was the one described by Milinović et al. [42], with some modifications. Elution was carried out using a mixture of solvent A (water/formic acid, 95.5/4.5 *v/v*) and solvent B (acetonitrile), with a composition gradient ranging from 95 to 36% of solvent A and a flow rate of 0.5 mL min^{-1}. The identification was performed at 360 nm.

2.4.4. In Vitro Antioxidant Assays

The 2,2′-diphenyl-1-picrylhydrazide (DPPH) assay was conducted on methanolic extracts using a Jasco V-630 double beam spectrophotometer, as described by Brand-Williams et al. [43]. An aliquot (950 µL) of a DPPH methanolic solution (0.11 mM) and 50 µL of Trolox (Tx) solutions at different concentrations (in the 50–1000 µM range), were thoroughly mixed in a test tube. For sample analysis, 50 µL of an appropriately diluted

sample solution was used, and a blank was also made using 50 µL of solvent. The vial was incubated in the dark at room temperature for 24 h, then the absorbance of the solution was read at 515 nm. The 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was conducted following the protocol of Thaipong et al. [44], using a Jasco V-630 double beam spectrophotometer. For both the DPPH and the ABTS assays, the calibration curves were set up by plotting the discoloration percentage (i.e., [1 − (ABS Tx/ABS blank)] * 100) as a function of the Tx concentration. Trolox equivalents (TEs) of the samples were calculated by interpolation on the calibration curve. The oxygen radical absorbance capacity (ORAC) assay was conducted using a Perkin Elmer (Turku, Finland) Viktor X3 multilabel plate reader, as described by Ou et al. [45]. The Trolox equivalents were calculated from the relative area under the curve of the emission intensity vs. time plot.

2.5. Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction (RT–qPCR)

Total RNA extraction and RT–qPCR analysis were performed according to Ruiz et al. [46]. Total RNA was extracted from the leaves of three separate plants (i.e., three biological replicates) by performing two separate extractions of 0.1 g FW for each replicate. RNA yield and purity were checked using UV absorption spectra, whereas RNA integrity was determined using electrophoresis on agarose gel. DNA was removed using the TURBO DNA-free ™ (Applied Biosystems, Foster City, CA, USA) from about 10 µg aliquots of total RNA. First-strand cDNA was synthesized from 6 µg of the DNase-treated RNA using the High-Capacity cDNA Kit (Applied Biosystems) with random primers. The reaction mixture for the qPCR analysis was made in a final volume of 25 µL, containing 3 ηg of cDNA, 5 pmol of each primer, and 12.5 µL of the PowerUp SYBR Green PCR master mix (Applied Biosystems), according to the manufacturer's instructions. The actin gene was used as a reference [47] to normalize and estimate the up- or down-regulation of the target genes. The sequences of gene coding for phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), flavonol synthase (FLS), a putative 4-coumaric acid 3′-hydroxylase (4C3H), dihydroflavonol 4-reductase (DFR), glutathione-S-transferase (GST), and catalase (CAT) were obtained from the NCBI database. Primer sequences for all of the analyzed genes are listed in Table S1 (Supplementary Material). PCRs were carried out using a StepOnePlus ^{RM} 7500 Fast (Applied Biosystems) for 2 min at 50 °C, 2 min at 95 °C, and then for 40 cycles as follows: 95 °C for 3 s and 60 °C for 30 s. Fold changes in RNA expression in the LED light-treated samples as compared to FL results were estimated using threshold cycles and analyzed using the comparative threshold cycle method, also known as the $2^{-\Delta\Delta Ct}$ method [48].

2.6. Statistical Analysis

Morphological measurements were performed on six plants per light treatment, while for the biochemical determinations and the RT–qPCR, three plants were considered. Data were analyzed using a one-way ANOVA and means were compared using the LSD test, at a 5% significance level. For the analysis of the transcript level data, a one-way ANOVA was applied followed by Fisher's test, at a 5% significance level.

3. Results and Discussion

3.1. Low Switching Frequencies Improve Energy Use Efficiency

Plant photosynthesis is composed by alternating light and dark reactions. Light reactions, in which light energy is harvested and transformed into chemical energy products, occur very quickly (in the range of nanoseconds to milliseconds) while dark reactions, in which products of the first phase are used to assimilate CO_2, occur more slowly (from seconds to minutes [12]). To date, the main research gap that hinders the use of pulsed light technology, through LED illumination systems that can provide very rapid flashes, is the need for identifying light emission frequencies that improve, or at least do not impair, plant growth and final yield performances. By extending the duration of the dark periods between pulses one can save electricity, thereby reducing the costs of the overall plant

growing system. In this study, the morphological and growth parameters investigated were not significantly affected by the adopted lighting regimes (Table 1). The absence of statistical significance may be due to the high variability among samples (Table 1) and to the low number of samples used in the experiment (n = 6). Therefore, these parameters will need to be explored in future research using a higher number of replicates. Jishi et al. [49] developed and validated a kinetic model for lettuce plants, enabling an estimate net photosynthetic rate under different pulsed light conditions, characterized by different PPFDs, frequencies, and duty ratios. Accordingly, the photosynthetic rate of the leaves grown under low pulsed light frequency and duty ratio was lower than that of the leaves grown under continuous light and was quantitatively comparable to continuous light when high pulsed light and duty ratio were applied. According to former research on lettuce plants performed by Kanechi et al. [14], LED light frequencies from 1.3 to 20 kHz could lead to an increase of up to 20% in shoot FW and leaf area compared to lower frequencies (from 0.5–500 Hz) and continuous light. The effect of LED *sf* on growth and lettuce morphology needs further validation, also considering that, under our experimental conditions, both the high and low *sf* used were much higher than the frequencies tested by Kanechi et al. [14]. The leaf area of the plants grown under LED treatments was significantly lower than in those grown under FL (Table 1), without differences among the LED *sf*. Indeed, a higher leaf area for lettuce plants grown under FL as compared to those grown under LED lights has already been observed, possibly as a strategy to improve light interception when photosynthetic efficiency is lower [31].

Table 1. Effect of different lighting treatments on morphological parameters, water use, and water use efficiency of indoor-grown lettuce plants (n = 6). Different letters indicate significant differences at $p \leq 0.05$. SD = standard deviation.

	High *sf*	**SD**	**Low *sf***	**SD**	**FL**	**SD**	***p*-Value**
Leaf FW (g plant^{-1})	63.6 a	10.9	57.7 a	10.0	58.4 a	8.6	0.53
Root FW (g plant^{-1})	11.3 a	4.7	7.4 a	1.8	7.4 a	1.6	0.06
Leaf DW (g plant^{-1})	3.5 a	1.0	2.7 a	0.6	2.9 a	0.9	0.25
Root DW (g plant^{-1})	0.6 a	0.4	0.3 a	0.1	0.3 a	0.1	0.05
Leaf DM (%)	5.6 a	1.4	4.7 a	0.2	4.9 a	1.0	0.32
Leaf number (n)	12.7 a	3.4	15.2 a	1.2	15.2 a	1.6	0.12
Leaf area (cm^2)	965.4 b	219.5	1062.5 b	167.3	1239.1 a	107.4	0.04
WU (L plant^{-1})	1.2 a	0.1	1.1 a	0.1	1.1 a	0.1	0.19
WUE (g FW L^{-1} H_2O)	54.6 a	10.0	53.9 a	11.3	55.1 a	9.4	0.98

FW = Fresh Weight; DW = Dry Weight; DM = Dry Matter Content; WU = Water Use; WUE = Water Use Efficiency.

Water use efficiency in a closed plant production system can be up to 50 times greater than in a greenhouse [1], a crucial element in environments where access to water is scarce [50], especially considering the water shortage that many urban areas will face in the coming years due to climate change [51]. In our experiments, the tested lighting regimes did not affect either water use (values between 1.1 and 1.2 L H_2O plant^{-1}, Table 1) or WUE (values between 53.9 and 55.1 FW L^{-1} H_2O, Table 1), with comparable values in plants grown with ahigh *sf*, a low *sf*, and under FL. The WUE values are very interesting, especially when compared to the performance in terms of WUE for lettuce production in an open field (3–20 g FW L^{-1} H_2O) or in a greenhouse (5–60 g FW L^{-1} H_2O) [9].

EUE is a critical parameter for indoor cultivation systems, although a high potential for improvement is associated with light spectral features (light quality, intensity, and photoperiod) [9]. With reference to spectral quality, when a red and blue spectrum is used, increasing the percentage of red increases EUE up to a maximum, suggesting an optimal response function, thanks to the enhancement of shoot DW [7,31]. Here, we combined an optimized light spectrum (RB = 3), light intensity (215 µmol m^{-2} s^{-1}), and photoperiod (16 h day^{-1}) and achieved a further improvement in EUE using a low *sf*. Accordingly, in lettuce plants grown under a low *sf*, EUE reached values of up to 123 g FW kWh^{-1}, with a 42% increase compared to plants grown under a high *sf* (86.5 g FW kWh^{-1}) and 2.7-fold compared to plants grown under FL (45 g FW kWh^{-1}) (Figure 1).

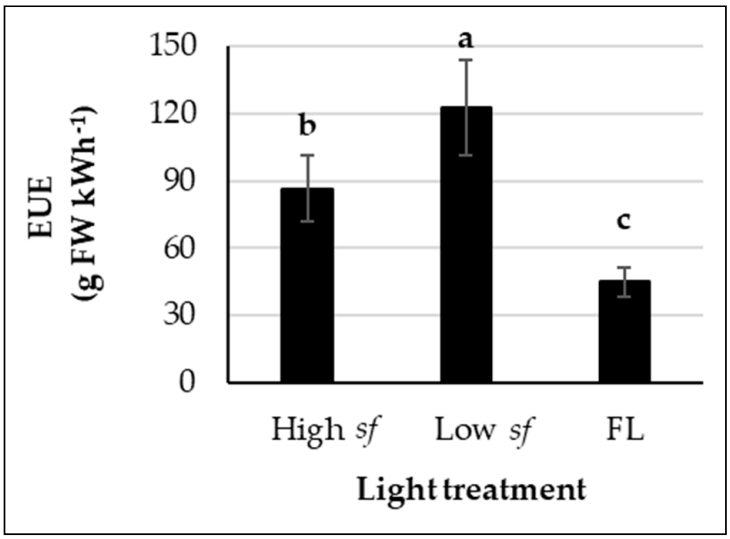

Figure 1. Effect of different lighting treatments on the energy use efficiency (EUE, g FW kWh^{-1}) of indoor-grown lettuce plants ($n = 6$). Vertical bars represent standard deviation. Different letters indicate significant differences at $p \leq 0.05$.

3.2. High Switching Frequencies Enhance Phenolic Compounds and Total Antioxidant Capacity

In recent years, consumer demand for healthy and antioxidant-rich food is increasing [52] in response to a growing awareness of the relevance that functional foods rich in phenolic compounds (e.g., flavonoids and hydroxycinnamic acids) play in preserving human health [53,54]. The concentration of phenolics in plant tissues is the combined result of genetic components, environmental conditions, growing techniques, and the interaction between these factors [55]. Light is one of the environmental factors regulating the synthesis, turnover, and degradation of antioxidant compounds in plants [56]. Different frequencies of supplemental monochromatic LED lighting (2, 32, 256 and 1024 Hz, with a duty cycle of 50%) significantly affected the TPC and the antioxidant activity of several microgreen species, such as basil, pak choi, mustard, and tatsoi, thus improving their nutraceutical properties [57,58]. On the other hand, there is no information regarding the effect of pulsed light on these parameters in lettuce.

In our work, although the TPC and the TFC did not vary significantly when the *sf* was modified (data not shown), changes in the concentrations of specific phenolic compounds were revealed using HPLC-DAD analysis (Figure 2). Possibly, this is due to the scarce specificity of UV–Vis spectrophotometric methods commonly used to assay the TPC or the TFC, as recently emphasized by Granato et al. [59], where the need for more specific measurements when evaluating changes in secondary metabolite patterns was underlined. In lettuce, chicoric acid represents more than 55% of total caffeic acid derivatives [25] and, together with caffeic acid and three more caffeic acid derivatives, constitute 93% of total polyphenols found in greenhouse-grown lettuces. The hereby presented results show that a high *sf* caused a significant increase in the levels of chicoric acid (Figure 2a) and caftaric acid (Figure 2b), while a low *sf* treatment did not affect their concentrations as compared to FL. Chicoric acid levels were about eight-fold higher under a high *sf* treatment as compared to leaves of plants grown with FL, and caftaric acid was two-fold higher. A similar response was observed for flavonoids, amongst which the most abundant compounds were isoquercetin, rutin, and luteolin. In this case, the effect varied depending on the compound. While the rutin levels were comparable under all light treatments (data not shown), high *sf* LED light increased isoquercetin (Figure 2c) and luteolin concentrations (Figure 2d) by more than 15- and 50-fold, respectively, as compared to FL. Considering indoor-grown lettuces, the levels of caftaric acid detected in our lettuce leaves were very similar to those reported by Rouphael et al. [60], while lower levels of chicoric acid were found, if compared to other authors [61]. These discrepancies are not surprising, since

several parameters were shown to have a great impact on the extraction efficiency of these metabolites, such as sampling, sample preparation procedure (freeze-dried vs. oven-dried), solvent-to-solid ratio used for extraction, duration of extraction, and sonication treatment applied, among others [62].

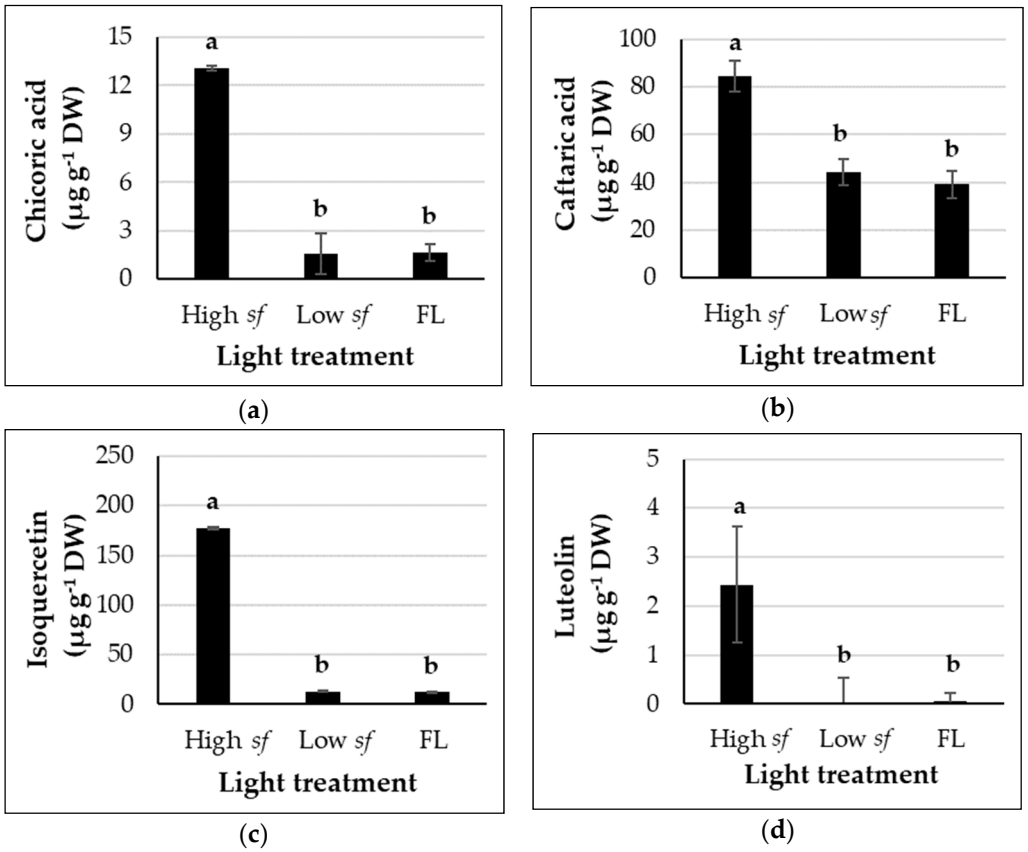

Figure 2. Effect of different lighting treatments on the concentration of the most abundant phenylpropanoids detected in lettuce leaves ($n = 3$), as follows: chicoric acid (**a**), caftaric acid (**b**), isoquercetin (**c**), and luteolin (**d**). Vertical bars represent standard deviations. Different letters indicate significant differences at $p \leq 0.05$.

Phenolic compounds are involved in many processes of plant physiology, being an important component in plant responses to environmental stresses and playing a role as antioxidants and oxygen scavengers [55]. For these reasons, in addition to the nutraceutical aspect, an increase in phenolic compounds in plants is advantageous as they can play a protective role against abiotic and biotic stresses [24,55]. In particular, flavonoids and cinnamic acid derivatives (to which caffeic acid and its derivatives belong) also exert photoprotective functions. In our work, the use of a high *sf* may have acted as an abiotic stressor, resulting in an increased synthesis of flavonoids and cinnamic acid derivatives to protect the photosystems. In order to investigate the mechanisms leading to an enhanced production of phenolic compounds, in future research, measurements of the chlorophyll fluorescence signal could be performed. This would allow for photosynthetic activity to be measured and changes in heat dissipation occurring with different *sf* to be monitored.

Flavonoids and cinnamic acid derivatives are synthetized via the phenylpropanoid pathway (Figure S1, Supplementary Material). Their biosynthesis is controlled by the regulation of genes encoding for key enzymes in the pathway, the first of which is phenylalanine ammonia-lyase (PAL) that catalyzes the first step in the pathway [63]. Here, although the expression of PAL was downregulated under a low *sf* and unaffected by a high *sf* as compared to the FL and FLS, encoding for flavonol synthase, was unaffected by the lighting regimes, the trend observed that the concentration of some phenolic compounds reflected

the transcript levels of the genes involved in the production of hydroxycinnamates and some flavonoid subgroups. Thus, 4C3H, encoding for 4-coumaric acid 3′-hydroxylase, an enzyme further down the biosynthetic pathway of caffeic acid derivatives than PAL [64], was more highly expressed under a high *sf* and this was related to increased amounts of chicoric and caftaric acids. DFR, which leads to the biosynthesis of flavan−4-ols and flavan−3,4-diols (not determined in the hereby presented study), was also up-regulated by a high *sf* (Figure 3).

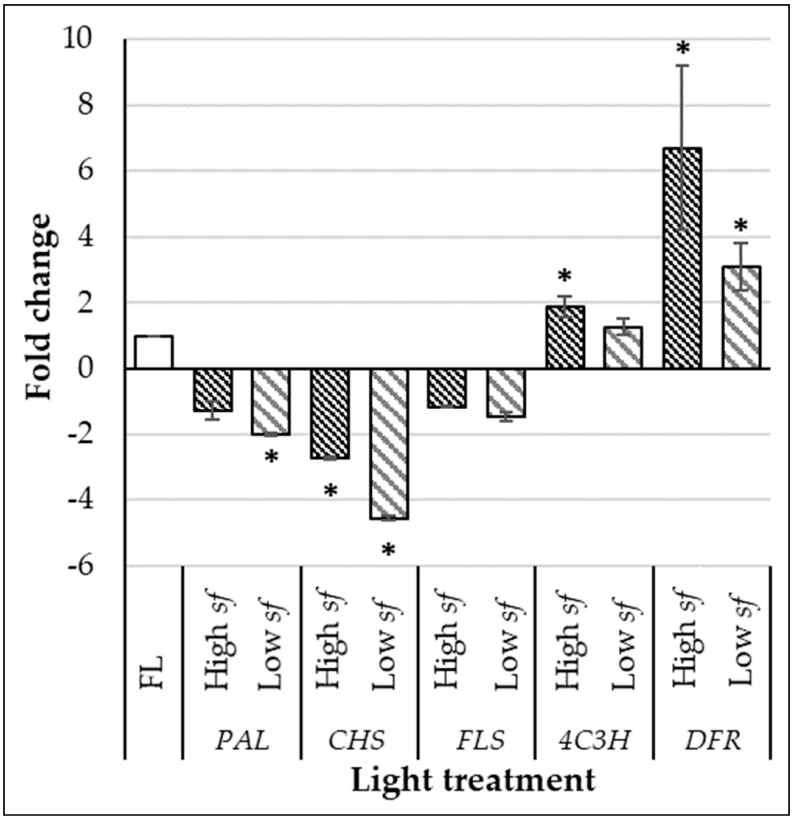

Figure 3. Effect of different lighting treatments on the expression of genes encoding for enzymes in the biosynthetic pathway of phenolic compounds in lettuce leaves (n = 3) expressed as fold-changes relative to the fluorescent light (FL, arbitrarily set at 1). PAL = phenylalanine ammonia-lyase; CHS = chalcone synthase; FLS = flavonol synthase; 4C3H = 4-coumaric acid 3′-hydroxylase; DFR = dihydroflavonol 4-reductase. Vertical bars represent standard errors. * indicates significant differences at $p \leq 0.05$.

Interestingly, leaves grown under high *sf* LED light possessed a significantly higher Total Antioxidant Capacity (TAC) compared to plants grown with FL according to all of the assays used, with increases ranging from 60% (ORAC, DPPH) to 88% (ABTS) (Figure 4). In two varieties of *Brassica rapa*, Vastakaite et al. [58] reported an increase in the antioxidant activity for all of the treatments with supplemental pulsed LED light (ranging from 2 to 1024 Hz). On the contrary, results of the antioxidant assays performed on the extracts of leaves irradiated with a low *sf* were not univocal. According to the DPPH and ORAC tests, no significant changes in the TAC were observed compared to the leaves grown under FL (Figure 4a,c), while according to the ABTS test, the TAC was enhanced (+40%) compared to FL (Figure 4b).

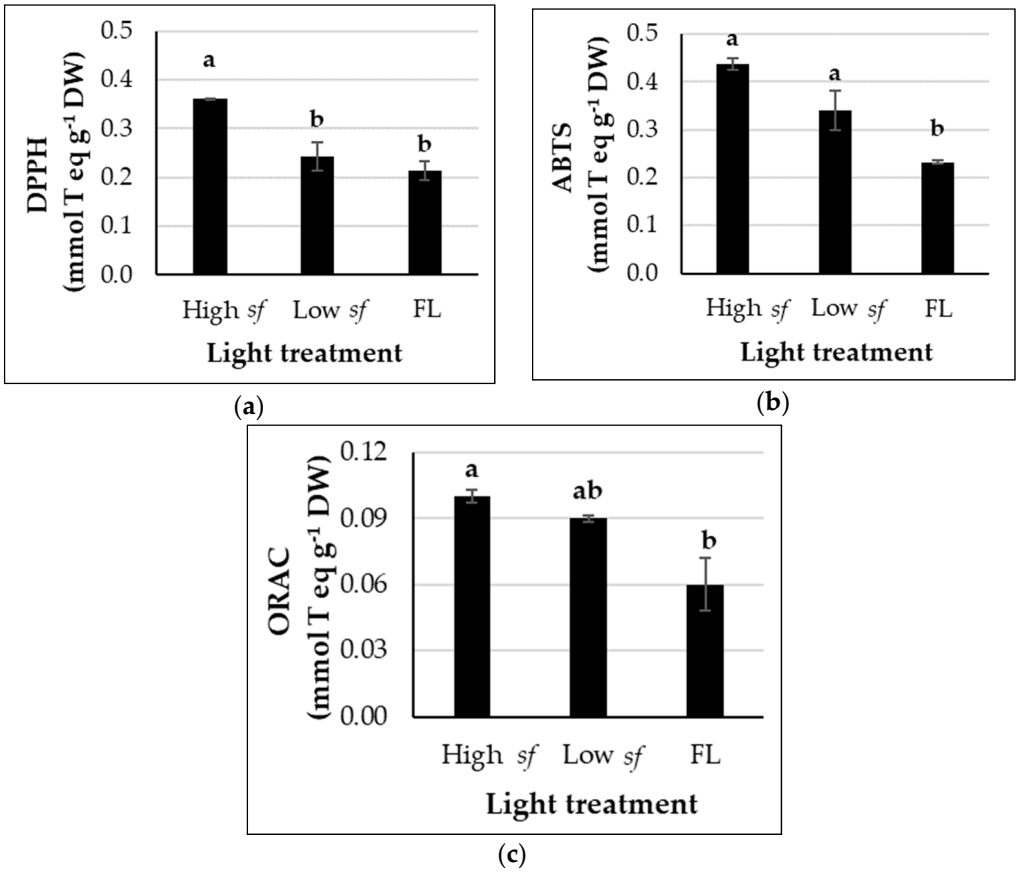

Figure 4. Effect of different lighting treatments on the Total Antioxidant Capacity of leaf extracts (n = 3) measured using three different assays, namely DPPH (**a**), ABTS (**b**), and ORAC (**c**). All of the values are expressed as mmol Trolox equivalents (T eq) g^{-1} DW. Vertical bars represent standard deviations. Different letters indicate significant differences at $p \leq 0.05$.

The antioxidant activity of plant extracts can vary depending on the oxidation conditions and the methods of measurement [65], as shown in the hereby presented study when comparing results on the effect of low *sf* using the DPPH assay with those obtained from the ORAC and ABTS assays. This is not surprising, since these assays differ in their underlying chemical reaction and in the radical source used [66]. According to a comprehensive study on 927 vegetables [67], the ORAC assay is considered to be a more biologically relevant assay compared to DPPH and ABTS, since it is based on the chain-breaking activity against the peroxyl radicals formed in cells during lipid peroxidation. Thus, the increase in the TAC observed in lettuce leaves grown under a low *sf* compared to leaves grown with FL is probably correlated to the increased abundance of some polar components other than those detected by us. To elucidate the nature of these metabolites, a more comprehensive investigation based on untargeted techniques would be needed. Moreover, despite the lack of substantial changes in the overall concentrations of the phenolics and flavonoids (TPC and TFC), a high *sf* was able to induce a significant enhancement in the TAC. This confirms the fact that this parameter also relies on the structure of single molecules, whose contribution depends on their specific antiradical effect [68,69] and may be justified by the fact that our analyses do not include some key antioxidants (e.g., ascorbic acid, and tocopherols).

As highlighted by Fu et al. [70], the activity level of certain antioxidative enzymes, including catalase, is an indicator of whether and to what extent plants are stressed (e.g., light stress). Thus, in order to check for possible light-induced oxidative stress under the different lighting regimes, the expression of the following two genes encoding for antioxidative enzymes were investigated: GST, encoding for glutathione S-transferases, and CAT, encoding for catalase. GST belongs to a superfamily of enzymes catalyzing a

variety of reactions involved in tolerance to abiotic stresses and, indirectly, acts in ROS removal [71]. Transcript levels of GST were unaffected by the different lighting regimes (Figure 5). Instead, CAT, one of the main enzymes metabolizing stress-induced ROS, in particular by converting H_2O_2 to O_2 and water [72], was down-regulated in plants grown under low *sf* LED light compared to FL (Figure 5).

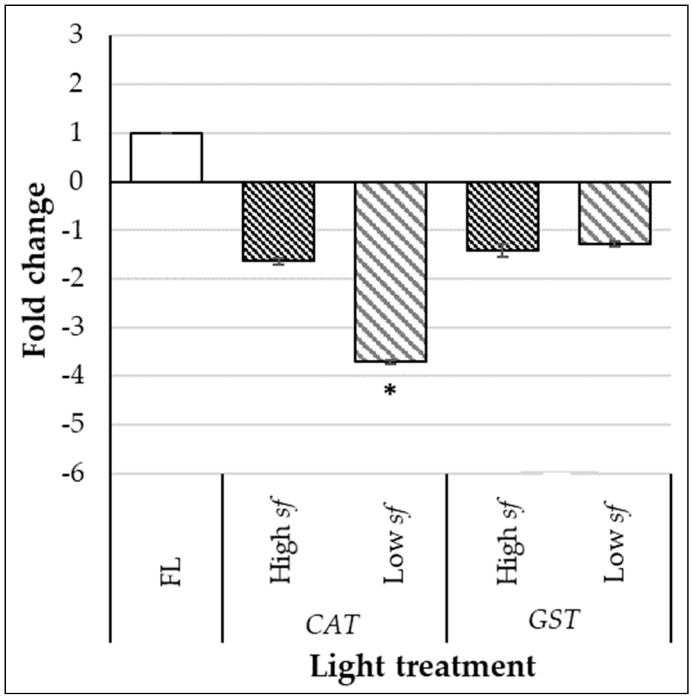

Figure 5. Effect of different lighting treatments on the expression of genes encoding for antioxidant enzymes in lettuce leaves (n = 3) expressed as fold-changes relative to the fluorescent light (FL, arbitrarily set at 1). CAT = catalase; GST = glutathione s-transferase. Vertical bars represent standard errors. * indicates significant differences at $p \leq 0.05$.

Since the plants appeared healthy and their growth was not inhibited (Table 1), the lack of effect or down-regulation of the genes coding for antioxidant enzymes suggest either that under both high and low *sf* LED lighting the plants were not subjected to oxidative stress-inducing conditions, or, that the increased concentrations of non-enzymatic antioxidants, including some phenylpropanoids, may have contributed to protecting the plants from possible light stress.

4. Conclusions

In the present work, the potential impact of pulsed light technology under a previously optimized red and blue LED spectrum (RB = 3) for indoor lettuce cultivation was explored. Under a low *sf*, EUE was significantly improved compared to plants grown under a high *sf* and, especially when compared to plants grown under FL, without compromising the final yield nor WUE, indicating that this could represent an optimal condition in terms of energy costs. In lettuce leaves grown with a low *sf*, the content of the main phenylpropanoids analyzed and the TAC were comparable to those of plants grown under FL. However, plants grown with a high *sf* revealed a significant increase in the main phenolic compounds found in lettuce leaves (i.e., caffeic acid derivatives) as well as in the TAC, thereby contributing to the nutraceutical properties of the crop. This work describes, for the first time, the effect of different *sf* of LED light on the expression of the genes coding for enzymes in the phenylpropanoid pathway. In particular, it reveals that a high *sf* enhanced the transcript abundance of the enzymes closely related to the biosynthesis of caffeic acid derivatives and flavanols. The expression of the genes encoding for the antioxidative enzymes CAT

and GST suggested that the plants, grown with either a high or a low *sf*, did not face any oxidative stress. In summary, a low *sf* can lead to a significant energy saving while obtaining the same yield. On the other hand, a low *sf* does not reinforce the nutraceutical content of lettuce leaves, which is, instead, enhanced by a high *sf*. The present results point to the need for major efforts in achieving a balance between energy use and the nutraceutical properties of indoor-grown crops through a deeper understanding of the physiological, biochemical, and molecular responses of plants to pulsed LED light technology.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11061106/s1: Figure S1, Phenylpropanoid biosynthetic pathway involving anthocyanins and flavonoids. In bold the biosynthetic enzymes. PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4C3H, 4-coumaric acid 3-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; ANR, anthocyanidin reductase; UFGT, UDP-glucose flavonoid 3-O-glucosyltransferase; Table S1, List of oligonucleotides used in this study for RT–qPCR analysis.

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