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# Continuous and pulsed LED applications on red and green lettuce (*Lactuca sativa* L. var. *capitata*) for pre- and post-harvest quality and energy cost assessments

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ABSTRACT

Light manipulation for plant cultivation is a critical area of study in controlled environment agriculture (CEA), where a variety of artificial lighting conditions are often employed in plant factories, growth chambers and greenhouses. With this aim of manipulation, two lettuce cultivars, green and red, were treated with two different LED treatments: a continuous LED in which an average photosynthetic photon flux density (PPFD) at the seedling level was maintained at 228 μmole $s^{-1}$ $m^{-2}$, a photoperiod of 16 h was set for a growing cycle of 30 days, and a dynamic mode pulsed LED treatment with a pulsed frequency set at 1 kHz with a duty cycle of 50 % along with the PPFD at 228 μmole $s^{-1}$ $m^{-2}$, a 16-hour photoperiod, and a growing cycle of 30 days. Sampling for quality assessment was done at harvesting (T0) and the effect of pre-harvest LED application was analyzed after 7 days of cold storage (T7). A significant reduction in the average fresh weight of both cultivars was seen under pulsed LED while a significant increase in leaf length was noticed among the treatments in red lettuce. Both treatments resulted in non-significant variations for photosynthetic pigments: total chlorophyll and carotenoids, while no significant differences were seen in terms of phenolic index and anthocyanin production in green lettuce. Red lettuce, however, yielded a significantly higher phenolic index for continuous LED at T0, which significantly declined at T7. In green lettuce, nitrate production underwent no significant differences under both treatments and time points; however, pulsed LED in red lettuce yielded significantly higher nitrate than continuous LED at T0. At both timepoints, no marked changes were seen in terms of total sugars in green lettuce, while a significant reduction in sugar was recorded under pulsed LED treatment. On the other hand, a significant decline in total sugars was noticed between the timepoints for red lettuce under continuous LED treatment, while no such variations were seen in red lettuce. Similarly, at T0 both green and red lettuce showed no remarkable increment or decline for sucrose, while it significantly declined between timepoints for red lettuce. Non-destructive analysis was carried out to investigate the health status of lettuce plants where the green lettuce under pulsed LED accumulated higher anthocyanins at both T0 and T7 and higher chlorophyll at T7 than red lettuce under the same LED application. Significantly higher anthocyanins were also seen at T7 between the two treatments in green lettuce. Non-significant differences, however, were found between the treatments and timepoints for both the photochemical maximum quantum efficiency of photosystem II (Fv/Fm ratio) and the overall performance index (PI) of leaves. This research demonstrated the significance of artificial light modification from continuous to pulsed LED to save energy costs and a step forward towards retaining the quality of the produce in this dynamic mode.

## 1. Introduction

Seasonal light variations assist numerous organisms in shaping their complex life cycles and associated activity patterns (Flynn and Wolkovich, 2018). Likewise, for plant and agricultural productions, it directly affects photosynthesis as it serves as a powerhouse for carbon fixation.

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Enhanced light captures by photosystems ensure an increased photosynthesis that ultimately leads to an increased agricultural yield, a dire need of today's rising population (Parry et al., 2011). Light quality, quantity and photoperiod influence plant growth and development. To harvest the useful spectrum from sunlight or artificial sources for photosynthesis, plant posses' pigments such as chlorophylls and carotenoids. Carotenoids are the accessory photosynthetic pigments which apart from protecting the chlorophylls, absorb sunlight in 400–500 nm, harvest and transfer it to chlorophylls (Armbruster et al., 2017). On the other hand, chlorophylls such as a and b absorb the red (600–700 nm) and blue (400–500 nm) portions of sunlight (Ouzounis et al., 2015).

Plants have photoreceptors which do not only help them in detection but are responsive to light variations. For ultraviolet B radiation, plants contain UV RESISTANCE LOCUS 8 (UVR8) receptor that detects wavelengths in (280–315 nm) ranges (Heijde and Ulm, 2012). To monitor blue light (390–500 nm), plants contain blue light receptors including phytotropin's (PHOTs), cryptochromes (CRYs) and ZEITLUPE proteins (ZTL, LKP2 AND FKF1) (Suetsugu and Wada, 2013). Moreover, phytochromes (PHY's) serves a purpose to perceive red (600–700 nm) and far-red (700–800 nm) light which upon absorption of photons can interconvert between: Pfr (FR-absorbing and biologically active; at 730 nm) and Pr (R-absorbing and biologically inactive; at 670 nm), an interconversion which is termed as 'photoconversions' of PHY's (Jeong et al., 2024).

Different spectral components of lights are diversely effective and influential to physiological metabolism in plants. For example, it is known that R and B lights are most potent light spectra for the plants to perform photosynthesis yet the leaves exhibit green color due to the greater reflection of green (G) light which apart from the greenish appearance helps to regulate the stomatal movement in the leaves against drought stress and assists plants in efficient photosynthesis by increasing carbon gains (Smith et al., 2017; Bian et al., 2019). Upregulation of auxin biosynthesis under an increase FR perception, helps plants inducing shade avoidance response (SAR) which includes petioles reorientation/ elongation and increased stem elongation (Kalaitzoglou et al., 2019). In addition, significant changes in the level of UV-B due to the changes in day time, season and weather, effect plant's morphological, physiological and molecular mechanisms that altered root: shoot ratio, produced thicker leaves, decreased photosynthesis in results to lesser chlorophyll and active reaction centers (RCs), lipid peroxidation of membranes and an increased accumulation of reactive oxygen species (ROS) (Ali et al., 2023a, 2023b). Likewise, UV-B induces the accumulation of phytohormones involved in defense mechanisms such as salicyclic acid, jasmonic acid and ethylene apart from controlling the genes involved in phenylpropanoid pathway (Heijde and Ulm, 2012). In contrast to UV-B, B-light facilitates photosynthesis by actively regulating the stomatal opening and chlorophyl biosynthesis (Hogewoning et al., 2010). Previously in pea sprouts, it has been observed that B-light is responsible for an increased productions of phenolic compounds such as phenolic acids, gallic acid, chlorogenic acid and phthalic acid (Liu et al., 2016). Monochromatic B-light, in submerged macrophyte *Ottelia alismoides* however, discouraged leaf elongation, dry weight and starch synthesis compared to monochromatic R-light (Wang et al., 2022). Although monochromatic R-light is believed to improve leaf development, elongation and photosynthesis, prolong exposures can be dangerous in terms of flowering inhibition and promoted leaf curling (Seif et al., 2021; Hogewoning et al., 2010).

In recent years, as an approach to eradicate the deleterious effects of monochromatic lights, numerous studies on the use of different combinations of R, B, G, and FR lights have been carried out. For example, in pea sprouts, the ratio of R4: B1 is helpful for accumulating higher phenols and total flavonoid, optimal growth and improved morphological profile (Zhao et al., 2024). Moreover, a combination of 40 % B, 20 % G, 40 % R in two radish microgreens resulted in an increased biomass and yield (Garegnani et al., 2024). Similarly, an increase in the rate of photosynthesis and pigments content, higher number of leaves, leaf area, shoot number, root number, root length, dry, and fresh mass was recorded in tomato plantlets under the R: $B = 10{:}01$ LED combination (Naznin et al., 2019). In lettuce, soluble protein, free amino acids, and vitamin C (VC) contents were found relatively higher under the 4R:1B light condition (Zhang et al., 2018). The combination of B2:R8: FR1.2 promoted root and shoot growth, increased total phenolic levels, enhanced antioxidant activity, chlorogenic acid and caffeic acid contents in lettuce (Lee et al., 2016). Notably, by installing 10 % G-LED in combination with 72 % R-LED and 18 % B-LED resulted in higher fresh masses for lettuce cultivation in hydroponic plant factories compared to those grown under only R and B LED's (Razzak et al., 2022). Moreover, increased shoot fresh weight at the final harvest resulted in the lettuce at seedling stage while treating with photon ratio of R:G: B = 1:4:1 treatment (Lim et al., 2023). In another study, baby-leaf lettuce 'Rouxai' under a PFD of 200 μmol $m^{-2}$ $s^{-1}$ and a combination of warm-white +B50 resulted in an increased total phenolic content (TPC) and anthocyanin content (TAC) by 25 % and 2.0-fold respectively (Vaštakaitė-Kairienė et al., 2021).

Numerous research is available about the effect of artificial lighting on crops at harvest but the impact of pre-harvest LED lighting on the nutritional quality of leafy greens during post-harvest storage is unclear and less explored. It has been documented that two important factors such as cultivation condition and age of plants during harvest are pivotal to determining the quality attributes of leafy green during post-harvest stage (Witkowska, 2013). High metabolic rates and lesser reserves are usually attributed to plants at early developmental stages such as baby greens. Harvesting at this stage will likely lead to rapid deterioration of quality. On the other hand, matured plants, if harvested will surely exhibit longer shelf life due to higher storage reserves and less metabolic rate (Vaštakaitė-Kairienė et al., 2021).

Despite of benefits LED technology offers, one major challenge these days is the higher energy cost of the indoor LED lighting operations. One possible solution to this problem is the introduction of pulsed irradiation, generated through pulsed width modulation (PWM) method, which is beneficial in provision of various frequencies and duties in lighting system (Son et al., 2018). In one of our previous experiments with pulsed LED in lettuce, we found that with 2 Hz pulsed frequency, lettuce yielded less than continuous LED apart from the reduced length and width of leaves (Ali et al., 2023c). Availability of few studies on pulsed irradiation especially to investigate the appropriate pulsed frequency, complicates the understanding of this mode of LED operation in the controlled environment agriculture (CEA). Therefore, to better understand whether this mode is useful to regulate plant growth and morphology in addition to lowering the energy costs, further studies with dynamic pulsed modes (frequency, exposure, distancing from the canopy) and energy costs data are needed.

Considering previous results with 2 Hz pulsed frequency, we hypothesized for this experiment that pre-treatment with pulsed frequency of 1 KHz could be beneficial for contributing or retaining quality attributes in lettuce, both at harvest and post-harvest stages. In addition, apart from the enhanced nutritional profile, this pulsed frequency will compensate for the energy costs of the lighting chamber used in the experiment for lettuce production. Two lettuce varieties with contrasting morphology and physiology were used in this study. The objectives of this study were (1) to investigate the energy costs of the 1 KHz pulsed LED operation compared to the continuous LED for photoperiod of 16 h, and (2) evaluate the morpho-physiological parameters of both lettuces at harvest under two LED modes and to examine the pre-treatment effect during post-harvest stage.

## 2. Materials and methods

### 2.1. Plant material

The experiment was conducted at Department of Agricultural and Environmental Sciences, University of Milan, Italy. Two lettuce varieties

(*Lactuca sativa* L. var. *capitata*) with distinct green and red phenotypes were used as planting material. Seedlings were obtained from the Ingegnoli plant nursery Milan, Italy and were transferred to the individual pots containing substrate (VIGOR PLANT SUPERNUTRIENTE, Italy) when seedlings were at 5th - 6th leaf stage of former and 9th - 10th leaf stage of later lettuce variety respectively. The plants were watered regularly and were fertilized once during the experiment after 15 days of transplanting. After the acclimatization period of 3 days, the pots were transferred from the glasshouse to the LED growing chambers.

### 2.2. Experimental setup

Two LED growing chambers (MEG Science™, Italy) were used for the experiment where each growing chamber consisted of two horizontally separated shelves with an aim to create two light sealed environments for independent LED operations. Each shelf is supported by a planar shape light engine, a water-cooling system for temperature regulation and assisted with 8 independent LED's channels to produce wavelength peaks ranging from 450 nm to 730 nm. Digital λ®, a Web App based control software, was installed that allowed producing dynamic lighting recipes for both continuous and pulsed operational modes. The spectral composition used is as follows: 23 % blue, 12 % green-yellow, 56 % red and 6 % far-red as shown in Fig. 1.

By using spectroradiometer (CSS-45, Gigahertz-Optik, Germany) irradiance distribution and light intensity were monitored to understand the light distribution inside the growth chambers. The measurements were taken at a sensing height of 77 mm from the top of each shelf. For irradiance, the average of all values at 77 mm corresponded to 46.05 W/$m^2$ with a uniformity value (min/max) equal to 19.46 % as shown in Fig. S1.

Similar measurements were made to evaluate the distribution of PPFD in the growth chamber. The average PPFD among all values at 77 mm corresponds to 228 μmol/$m^2$·s with a uniformity value (min/max) equal to 19.84 % as shown in Fig. S2. The bottom shelves of each chamber, continuous light recipe with an average photosynthetic photon flux density PPFD at the seedling level of 228 μmole $s^{-1}$ $m^{-2}$, a photoperiod of 16 h was set for a growing cycle of 30 days while the top shelves had pulsed LED light, a dynamic mode in which the light had a pulsed frequency of 1 kHz and a duty cycle of 50 % that means the pulse time of 50 % light on, 50 % light off, while the spectral power distribution (SPD), photoperiod and PPFD was maintained the same as continuous LED. The average temperature in the growing chamber was maintained at 22 ± 1 °C throughout the experiment. Twelve pots per treatment per lettuce variety were used in total for the two chambers while six pots per variety per treatment was used for each shelf of the chambers used. The overall experimental setup of the chamber and plants is shown in Fig. S3.

On the harvesting day, six plants per treatment per variety were selected randomly as a representative for the time point T0. Moreover, to observe the effects of continuous and pulsed LED preharvest application on the post-harvest quality attributes, six plants each from both treatments and varieties were kept in polyethene bags for cold storage at 6 °C. After 7 days of cold storage, plants were analyzed, both non-destructively and through analytical analyses to investigate quality differences between the two time points: harvest (T0) and post-harvest (T7).

To quantify the energy consumption under two different lighting treatments used, an electricity consumption meter was used. Continuous lighting was activated for a day and the energy consumption was multiplied by the total duration of the growing cycle. The same procedure was adopted to evaluate energy consumption related to pulsed LED lighting.

### 2.3. Non-destructive analyses

#### 2.3.1. Non-destructive estimations of chlorophyll, flavanols and anthocyanins by using MPM-100 multi-pigment-meter

After moving the plants into chambers, adaxial leaf surfaces were chosen from week-old seedlings for simultaneous in vivo estimation of chlorophyll, flavanols and anthocyanins contents by MPM-100 Multi-Pigment-Meter (ADC Bioscientific Ltd, UK) for three consecutive weeks (Cerovic et al., 2008). The ratio fluorescence method was used to

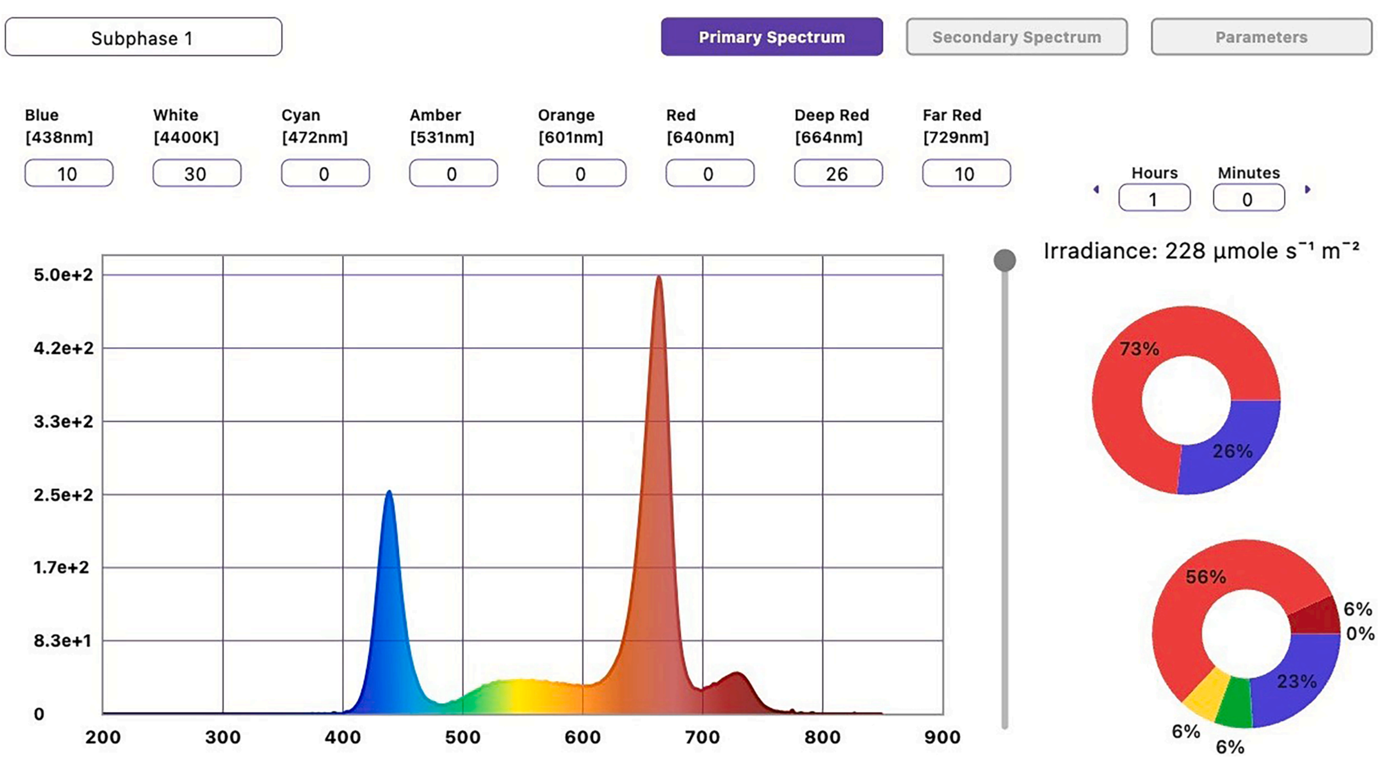


**Fig. 1.** Spectral quantum distribution used for both continuous and pulsed LED.

measure anthocyanin content and flavanol content while measuring the chlorophyll content leaf transmission in the far red and near infra-red was assessed. However, to compare the effect of continuous and pulsed LED effects in green and red lettuce, measurements taken only at T0 and T7 are presented.

#### 2.3.2. Non-destructive estimation of FV/FM and PI by using Handy PEA+ fluorimeter

A portable fluorimeter (Handy PEA+; Hansatech, Kings Lynn, UK) was used to measure the leaves light utilization and health status of photosystem II for three consecutive weeks after moving the plants from glasshouse to growing chamber. A week-old lettuce leaves from both cultivars were chosen and clipped (4mm diameter) to ensure the dark adaptation period for the samples: a vital step to monitor the chlorophyll *a* fluorescence. The instrument uses a sensor unit which is comprise of 3 ultra-bright red LEDs (peak wavelength: 650 nm) and a maximum intensity of up to 3500 μmol $m^{-2}$ $s^{-1}$ for a uniform illumination over the exposed leaf area. The Fv/Fm and PI were evaluated for an insight on relative leaf functionality under tested treatments and between the varieties. Measurements however taken at T0 and T7 are presented for Fv/Fm and PI of the red and green lettuce varieties under continuous and pulsed LED treatments.

### 2.4. Morphological analyses

#### 2.4.1. Determination of morphological traits

The average fresh weight was recorded by harvesting the total produce from each treatment using a digital scale (Mettler PM480 Delta Range). To measure the dry weight. water loss percentage of the samples were considered after drying the lettuce samples in an oven (VENTICELL, VC 707 ECO) at 110 °C for three days. For leaf morphological measurements such as leaf length and leaf width, a ruler was used, and measurements were done in centimetres. Leaf length measurements were taken from the tip of the lettuce leaves to the point where it was attached to the petiole however, for the leaf width measurements, the longest extension of two points on the leaf blade perpendicular to the leaf length axis were considered. A week-old leaf was selected one per pot for the leaf morphological measurements.

### 2.5. Analytical measurements

#### 2.5.1. Determination of total chlorophylls and carotenoid

From the fresh leaf tissues (around 50 mg), total chlorophylls and carotenoids were extracted in 5 mL of methanol 99.9 %. After adding the methanol, samples were placed for 24 h in a dark room at 4 °C. By using spectrophotometer, absorbance readings were measured for chlorophyll at 665.2 nm and 652.4 nm and for total carotenoids at 470 nm with the help of Lichtenthaler's formula (Lichtenthaler, 1987).

#### 2.5.2. Determination of phenolic index and total anthocyanin contents

For the extraction of the phenolic compounds, around 50 mg of lettuce leaves disks were collected and dipped in 5 mL of acidified methanol (1 % HCl v/v) for an overnight extraction in the dark. The phenolic index was used as an indication of the total phenolics content where absorbance was measured at 320 nm using an UV–Vis spectrophotometer (Ke and Saltveit, 1989).

The same extracts (the one used for phenolic index) were used to measure the total anthocyanins contents. The concentration of anthocyanins was expressed as cyanidin-3-glucoside equivalents and determined spectrophotometrically at 535 nm using an extinction coefficient εM of 29,600 (Ferrante et al., 2004).

#### 2.5.3. Determination of sucrose

Around 1 g of leaves was ground in 4 mL of distilled water. The extract was centrifuged (ALC centrifuge-model PK130R) at 4000 rpm for 15 min and the supernatant was recovered and used for the colorimetric determination of sucrose. To perform sucrose assay, 0.2 mL of leaf extract was mixed with 0.2 mL 2 M NaOH. The mixture was incubated for in a Dubnoff (PID) bath at 100 °C for 10 min. After removing from the water bath, 1.5 mL hot resorcinol buffer (containing 30 % hydrochloric acid, 1.2 mM resorcinol, 4.1 mM thiourea 1.5 M acetic acid) was added into the samples and incubated at 80 °C for another 10 min. After cooling at room temperature, the O.D. was determined at 500 nm. For the calculation of final concentration of sucrose, sucrose standard curve (0–2 mM) was used (Rorem et al., 1960).

#### 2.5.4. Determination of total sugars

Anthrone method with minor modifications is used for the colorimetric determination of total sugars (Yemm and Willis, 1954). The anthrone reagent (10.3 mM) was prepared dissolving anthrone in ice-cold 95 % $H_2SO_4$. Later, 0.5 mL of extract (previously prepared for sucrose) was placed on top of 2.5 mL anthrone reagent. The resulting mix was kept in ice for 5 min and later vortexed vigorously following heating at 95 °C for 10 min. Remove from the hot bath and cool in ice. Based on a glucose calibration curve (0–4 mM), total sugar contents were measured at 620 nm.

#### 2.5.5. Determination of reducing sugars

The same extract that was prepared earlier for sucrose analyses was used for the reducing sugar analyses. Around 0.2 mL of extract was taken and added to 0.2 mL of a solution containing 62.6 mM dinitrosalicylic acid (DNS) and 1.52 M potassium sodium tartrate (Miller, 1959). By using Dubnoff bath, the reaction mixture was heated at 100 °C for 5 min. With the addition of 1.5 mL of distilled water, absorbance was measured at 530 nm. By using a glucose standard curve (0–4 mM), reducing sugars were expressed as glucose equivalent and calculated.

#### 2.5.6. Determination of nitrate

Cataldo's method was used for the determination of nitrates (Cataldo et al., 1975). The extract used was the same previously prepared for sucrose. Twenty μL of the sample was added to 80 μL of 5 % salicylic acid in sulphuric acid. Later, 3 mL of 1.5 N NaOH was added into the mixture. After cooling the samples at room temperature, spectrophotometric readings at 410 nm were carried out. By using $KNO_3$ standard calibration curve (0–10 mM), nitrate concentration was measured for the samples.

#### 2.5.7. Determination of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) assay was used to assess any possible photodamage to the membranes (lipid peroxidation) (Heath and Packer, 1968). Around one gram of leaf samples was harvested and stored at −80 °C. Later, sample grinding was done in 5 mL of trichloroacetic acid (TCA) of 0.1 % w/v followed by the centrifugation (ALC centrifuge-model PK130R) of resultant mixture at 4000 rpm for 10 min. After supernatant collection, one mL of the extract was mixed with 4 mL of 20 % (w/v) TCA, 25 L of 0.5 % thiobarbituric acid (TBA) including distilled water. The resultant mixture was moved to Dubnoff bath (PID) which was set at 95 °C. Samples remained there for 30 min and later cooled in ice. For measuring the TBARS values, absorbance was measured at 600 nm and was subtracted from the reading taken at 532 nm (as an index of non-specific turbidity). The concentration of TBARS were expressed as malondialdehyde (MDA) equivalents (nmol $g^{-1}$ F.W.), with the extinction coefficient εM = 155 $mM^{-1}$ $cm^{-1}$. However, due to malfunctioning of the −80 °C refrigerator and transferring samples to safe storage, the samples for T0 were misplaced and could not be found, hence the TBARS data presented here only represents T7 (Fig. S4).

Evolution 300 UV–Vis spectrophotometer (Thermo-Scientific) was used for all the spectrophotometric determinations.

### 2.6. Data analyses

All the analyses were performed using GraphPad Prism version 6 for

Windows (GraphPad Software; La Jolla, California, USA, www.graphpad.com). The data were subjected to a two-way analysis of variance (two-way ANOVA) followed by Tukey multiple comparisons test except for the dry weight which was analysed using the sample *t*-test. Different alphabetics were labelled in all the analyses to represents the significant differences ($p < 0.05$) while the similar alphabets show non-significant differences between the treatments.

## 3. Results

The estimation of chlorophyll, flavanols, and anthocyanins through MPM-100 while the Fv/Fm and PI through fluorimeter were carried out non-destructively at T0 and T7 as shown in Tables 1 and 2. Significant differences for Fv/Fm and PI were noticed in red lettuce between the two LED treatments but no significant changes in the PI and Fv/Fm were observed for the green lettuce among the treatments. Moreover, a significant change in anthocyanin estimated values was observed between the two varieties for the pulsed treatment in which the green lettuce under pulsed LED showed higher anthocyanins than the red lettuce under pulsed LED. In terms of chlorophyll, red lettuce under continuous LED showed a higher estimated chlorophyll values than both continuous and pulsed LED treatments in green lettuce. The significant differences observed at T0 diminished largely at T7 yet in terms of anthocyanins, green lettuce under pulsed LED showed a significant higher anthocyanin compared to red lettuce under the pulsed LED and green lettuce under continuous LED treatment. Another significance was observed in chlorophyll estimation at T7 between the red and green lettuce for pulsed LED treatments in which chlorophyll for red lettuce under pulsed LED was significantly higher than its green counterpart.

According to Fig. 2a, significant increased leaf length in red lettuce was observed for pulsed LED however, in green lettuce no significant changes were noticed. Nevertheless, no significant differences were found in terms of leaf width for both green and red lettuce as shown in Fig. 2b. A significant decreased average fresh weight was recorded in both lettuces grown under pulsed LED compared to the ones grown under continuous LED as per Fig. 3a. However, in terms of dry weight, no significant differences were observed in both green and red lettuce varieties for the pulsed and continuous LED treatments.

Both T0 and T7 for Fig. 4a, b, c, and d, total chlorophyll and total carotenoids in red and green lettuce did not show any significant variations among the treatments and timepoints. The same trend was seen for total phenolic index as well as anthocyanins in green lettuce as shown in Fig. 5a and b, respectively. However, according to Fig. 5c, a significant reduction in phenolic accumulation was noticed in red lettuce for pulsed LED compared to the continuous LED. Significant reduction for anthocyanin accumulations between T0 and T7 has also been noticed in red lettuce grown under continuous LED where the lettuce grown under pulsed LED displayed no significant differences between the timepoints.

For nitrate accumulation as in Fig. 6a, green lettuce at both timepoints did not show any significant changes among the treatments while a significant increase of nitrates was observed in red lettuce grown under pulsed LED at T0 compared to the continuous counterpart. The non-significant differences in nitrate accumulations were however, noticed between the timepoints as evident from Fig. 6b.

For total sugars, no significant differences were found in green lettuce grown under pulsed and continuous LED (Fig. 7a), however, in terms of reducing sugars, green lettuce grown under continuous LED produced significantly higher than pulsed LED at T0 as per Fig. 7b. At T7, no significant results however, were observed among the treatments and timepoints. Notably, the treatments were non-significant in production of sucrose at both timepoints while at T0 the continuous LED resulted in an increased significant accumulation of sucrose compared to the pulsed LED at T7 as shown in Fig. 7c.

In red lettuce, at T0, both pulsed and continuous LED accumulated non-significant total sugars while during the storage phase at T7, the lettuce produced under continuous LED showed a significant decline in total sugars productions compared to the lettuce of continuous LED at T0 as seen in Fig. 8a. For reducing sugars according to Fig. 8b, no significant variations were found in red lettuce for both treatments and timepoints respectively. For sucrose as for Fig. 8c, a significant increment was seen in red lettuce grown under continuous and pulsed LED at T0 with respect to the lettuces under both treatments at T7. Values were, however, non-significant among treatments for both timepoints.

In addition to the morpho-physiological analyses, total consumption of energy was recorded in the growth chamber throughout the growing period as shown in Table 3. It was found that during the 30 days of experiment using the continuous and pulsed LED treatments for 16 h a day, consumption under pulsed LED was 17.76 kWh against the 24.81 kWh for continuous LED. Energy use efficiency was measured to examine the production in gram per kilo watt hours. The results indicated that the continuous LED treatment for both green and red lettuce performed better than pulsed LED as shown in Table S1.

## 4. Discussion

The quantity and quality of light that reaches chloroplasts are essential for photosynthesis. Although photosynthetic photon flux density (PPFD), the most widely used indicator of photosynthetic radiation measures photons between 400 and 700 nm in wavelength, the response of photosynthesis to light varies with wavelength (Zhen and van Iersel, 2017). In the present study, green and red lettuce for both pulsed and continuous LED treatments at T0 and T7 showed no significant variations in the accumulation of chl a and chl b: the pigments which are necessary to perform photosynthesis and eventually the deciding factor for the overall growth and yield of the produce. Similarly, carotenoids, the pigment commonly known for photoprotection and assisting chlorophyll with the energy transfer through singlet-singlet excitation transfer (Maoka, 2020), did not show any changes in either varieties or treatments. Although, we conclude that non-significant variations in photosynthetic pigment accumulations are a better indicator of using the pulsed LED of higher frequency such as 1 kHz employed in this study instead of the lower 2 Hz frequency previously used (Ali et al., 2023c), these physiological findings did not translate into the better production performance of either green or red lettuce. This was quite clear from the average fresh weight production in both green and red lettuce varieties,

**Table 1**
Non-destructive estimation of chlorophyll, flavanols and anthocyanins recorded by MPM-100 Multipigment meter at T0 while photochemical maximum quantum efficiency of photosystem II (Fv/Fm) and performance index (PI) measured through Fluorimeter. Values are means ($n = 10 \pm S.E.$). Different alphabetic letters indicate statistically significant differences among the treatments and timepoints followed by two-way analysis of variance followed by Tuckey multiple comparison test ($p < 0.05$).

| Treatments | Non-destructive Analyses T0 | | | | |
|---|---|---|---|---|---|
| | Chlorophyll | Flavanols | Anthocyanins | Fv/Fm | PI |
| **Green lettuce (continuous LED)** | $0.45\pm0.028^{a}$ | $0.078\pm0.02^{ab}$ | $0.05\pm0.006^{ab}$ | $0.86\pm0.003^{a}$ | $0.46\pm0.03^{a}$ |
| **Green lettuce (pulsed LED)** | $0.41\pm0.04^{a}$ | $0.025\pm0.07^{a}$ | $0.067\pm0.007^{a}$ | $0.83\pm0.009^{a}$ | $0.42\pm0.06^{a}$ |
| **Red lettuce (continuous LED)** | $0.63\pm0.06^{b}$ | $0.11\pm0.036^{b}$ | $0.035\pm0.012^{ab}$ | $0.81\pm0.012^{a}$ | $0.39\pm0.08^{a}$ |
| **Red lettuce (pulsed LED)** | $0.55\pm0.02^{ab}$ | $0.06\pm0.015^{ab}$ | $0.03\pm0.008^{b}$ | $0.82\pm0.03^{a}$ | $0.29\pm0.06^{b}$ |

**Table 2**
Non-destructive estimation of chlorophyll, flavanols and anthocyanins recorded by MPM-100 Multipigment meter at T7 while photochemical maximum quantum efficiency of photosystem II (Fv/Fm) and performance index (PI) measured through fluorimeter. Values are means ($n = 10 \pm S$.E.). Different alphabetic letters indicate statistically significant differences among the treatments and timepoints followed by two-way analysis of variance followed by Tuckey multiple comparison test ($p <$ 0.05).

| Treatments | Non-destructive Analyses T7 | | | | |
|---|---|---|---|---|---|
| | Chlorophyll | Flavanols | Anthocyanins | Fv/Fm | PI |
| **Green lettuce (continuous LED)** | $0.33\pm0.046^{ab}$ | $0.05\pm0.01^{a}$ | $0.09\pm0.009^{a}$ | $0.81\pm0.023^{a}$ | $0.42\pm0.07^{a}$ |
| **Green lettuce (pulsed LED)** | $0.32\pm0.05^{a}$ | $0.023\pm0.005^{a}$ | $0.14\pm0.007^{b}$ | $0.74\pm0.04^{a}$ | $0.34\pm0.09^{a}$ |
| **Red lettuce (continuous LED)** | $0.58\pm0.04^{ab}$ | $0.07\pm0.018^{a}$ | $0.07\pm0.010^{ac}$ | $0.76\pm0.04^{a}$ | $0.43\pm0.05^{a}$ |
| **Red lettuce (pulsed LED)** | $0.59\pm0.03^{b}$ | $0.05\pm0.014^{a}$ | $0.07\pm0.02^{c}$ | $0.81\pm0.02^{a}$ | $0.32\pm0.034^{a}$ |

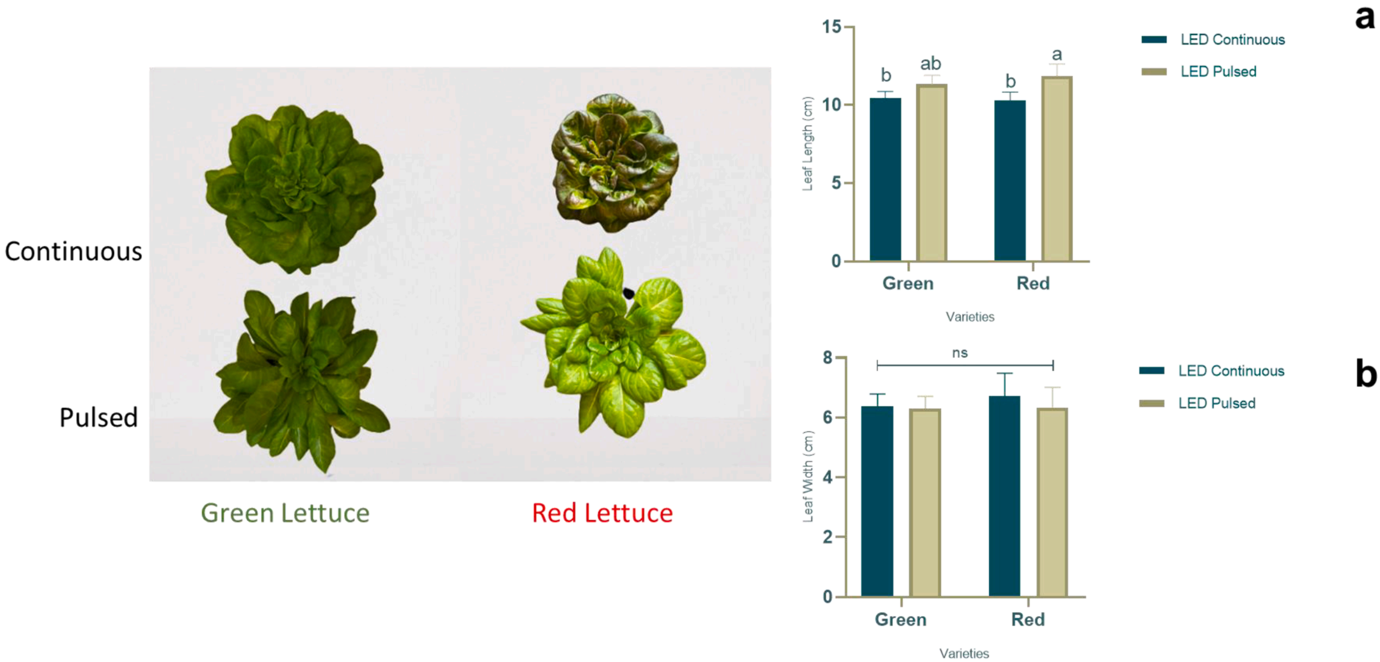


**Fig. 2.** (a) Leaf length (b) Leaf width of the red and green lettuce cultivars treated with pulsed and continuous LED treatments. On the left side are the images taken before harvest, where the top row represents both green and red lettuce grown under continuous LED while the bottom row represents the lettuces grown under pulsed LED treatment. Values recorded for the leaf morphology are means ($n = 10 \pm S$.E). Different alphabetic letters indicate statistically significant differences among the treatments and timepoints followed by two-way analysis of variance followed by Tuckey multiple comparison test ($p < 0.05$).

where the pulsed LED produced significantly less weight than the continuous LED. One possible reason could be since R/B, R/B/G LED combinations, especially with the pulsed mode resulted in significant stalk lengthening, a trait that reduced the lettuce production and marketability (Alrajhi et al., 2023). In addition, Son et al. (2018) found no differences in the fresh shoot weight and leaf area of the lettuce grown under the pulsed irradiation with the LED recipe of R: W: $B = 7:2:1$ and the control (continuous LED). Moreover, due to the rapid growing cycling under the LED combination approach, we can deduce that the lettuce production under continuous LED performed well than the pulsed LED. In line with our results regarding non-significant variations for total chlorophyll, Camejo et al. (2020) found that W-LED or RB-LED combinations are not significant reason for the chlorophyll productions or alterations in lettuce. Although, chlorophyll established itself as the prime component for the photosynthesis, numerous reports suggested that higher illumination yield lesser chlorophyll but results in coherent photosynthetic performance against higher chlorophyll biosynthesis for lower illumination, the result for which is less efficient photosynthesis and reduced quality of lettuce (*Lactuca sativa* L. Var. youmaicai) (Fu et al., 2017). Better light absorption, control over reactive oxygen species and improved shoot development are indications of a positive production of photosynthetic pigments including chlorophyll and carotenoids. Previously, higher accumulation of carotenoids was achieved in lettuce by Amoozgar et al. (2017) while working with the combination of 70R:30B LEDs that resulted in higher photosynthesis and maximal quantum yield of PSII photochemistry. In the same research he also noticed an overall improved nutritive profile of lettuce. The LED combination of B-W is known to upregulate the expression of certain genes responsible for the biosynthesis of carotenoids such as phytoene synthase (PSY), β-cyclase (β-LCY), and β-carotene hydroxylase (β-$OHASE_1$).

Plants experienced various biotic and abiotic stresses, as a response, plants have a self-defense mechanism based on the production of various kinds and levels of secondary metabolites to fight against diseases, harmful ultraviolet radiation, parasites and for producing specific and distinct hues (Appolloni et al., 2022). CEA production of leafy vegetables leads to a lesser prone agriculture regarding the above-mentioned problems, yet being a living entity, plants face problems and undergo changes to compensate for the loss of quality and quantity (Ampim et al., 2022). For this study, it was observed that pulsed and continuous LED were no different in phenolic index as well as anthocyanins accumulation for green lettuce at T0 and T7 but for the red lettuce, a significant higher phenol was recorded in plants grown under continuous LED compared to the pulsed, that ultimately dropped significantly at T7. Moreover, anthocyanins accumulations were non-significant among the treatments but likewise phenolic index, red lettuce produced under

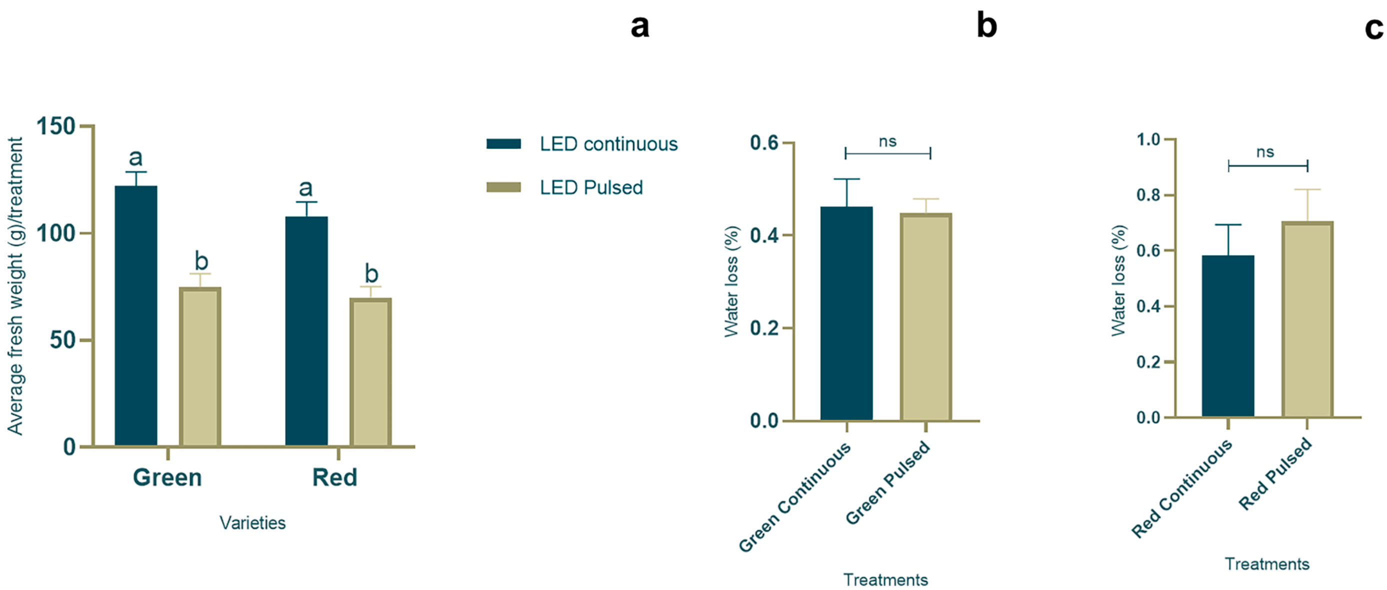


**Fig. 3.** (a) Average fresh weight (g) (b) Water loss (%): green lettuce (c) Water loss %: red lettuce, treated with pulsed and continuous LED treatments. Values are means ($n = 12 \pm S$.E. for average fresh weight and $n = 6 \pm S$.E. for water loss %). Different alphabetic letters indicate statistically significant differences among the treatments followed by two-way analysis of variance in average fresh weight and *t*-test for weight loss followed by Tuckey multiple comparison test ($p < 0.05$).

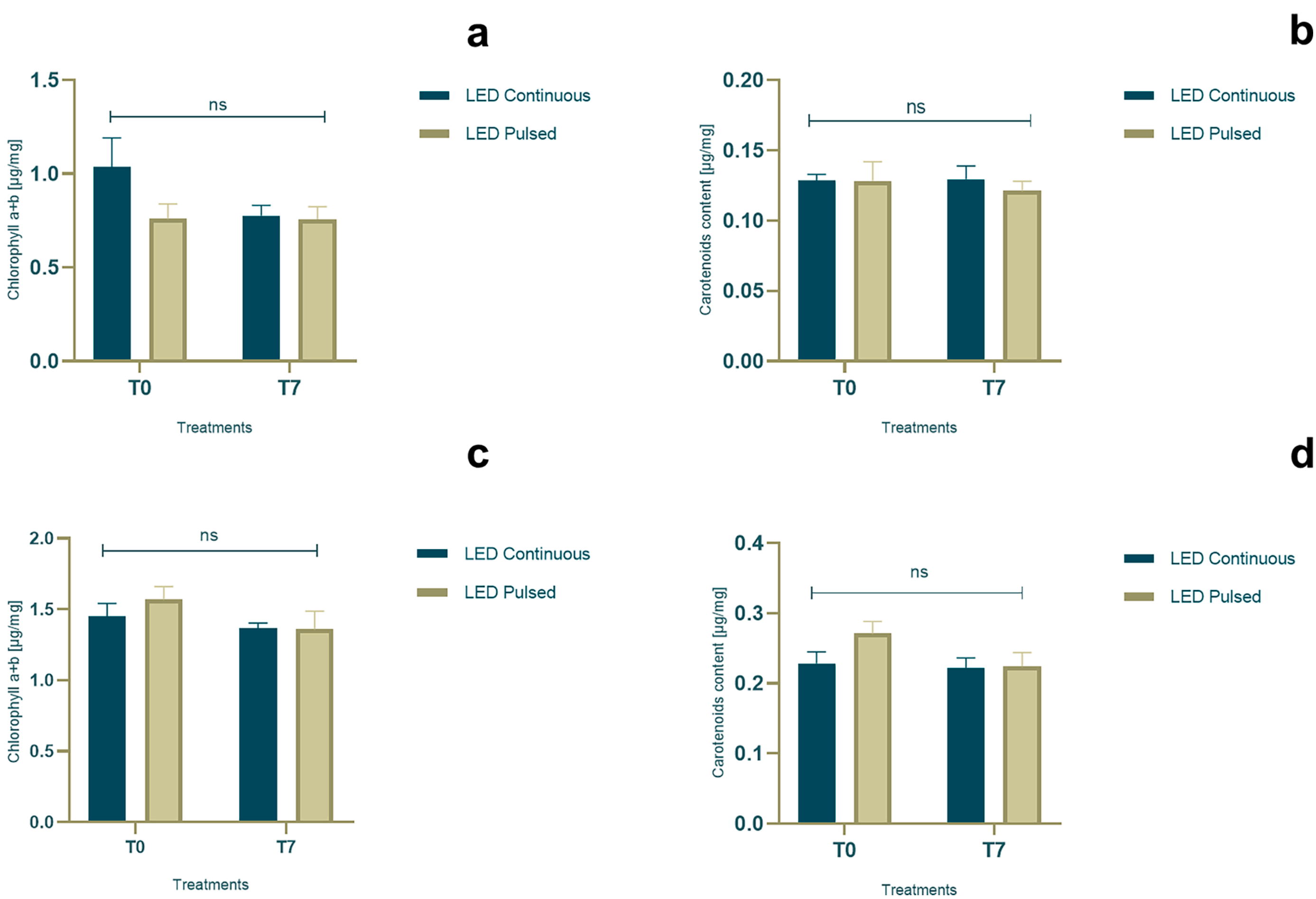


**Fig. 4.** (a) Chlorophyll $a + b$: green lettuce (b) Carotenoids: green lettuce (c) Chlorophyll $a + b$: red lettuce (d) Carotenoids: red lettuce, treated with pulsed and continuous LED treatments. T0 refers to the harvesting point while T7 refers to the point of cold storage after 7 days of harvesting. Values are means ($n = 6 \pm S$.E). Different alphabetic letters indicate statistically significant differences among the treatments and timepoints followed by two-way analysis of variance followed by Tuckey multiple comparison test ($p < 0.05$).

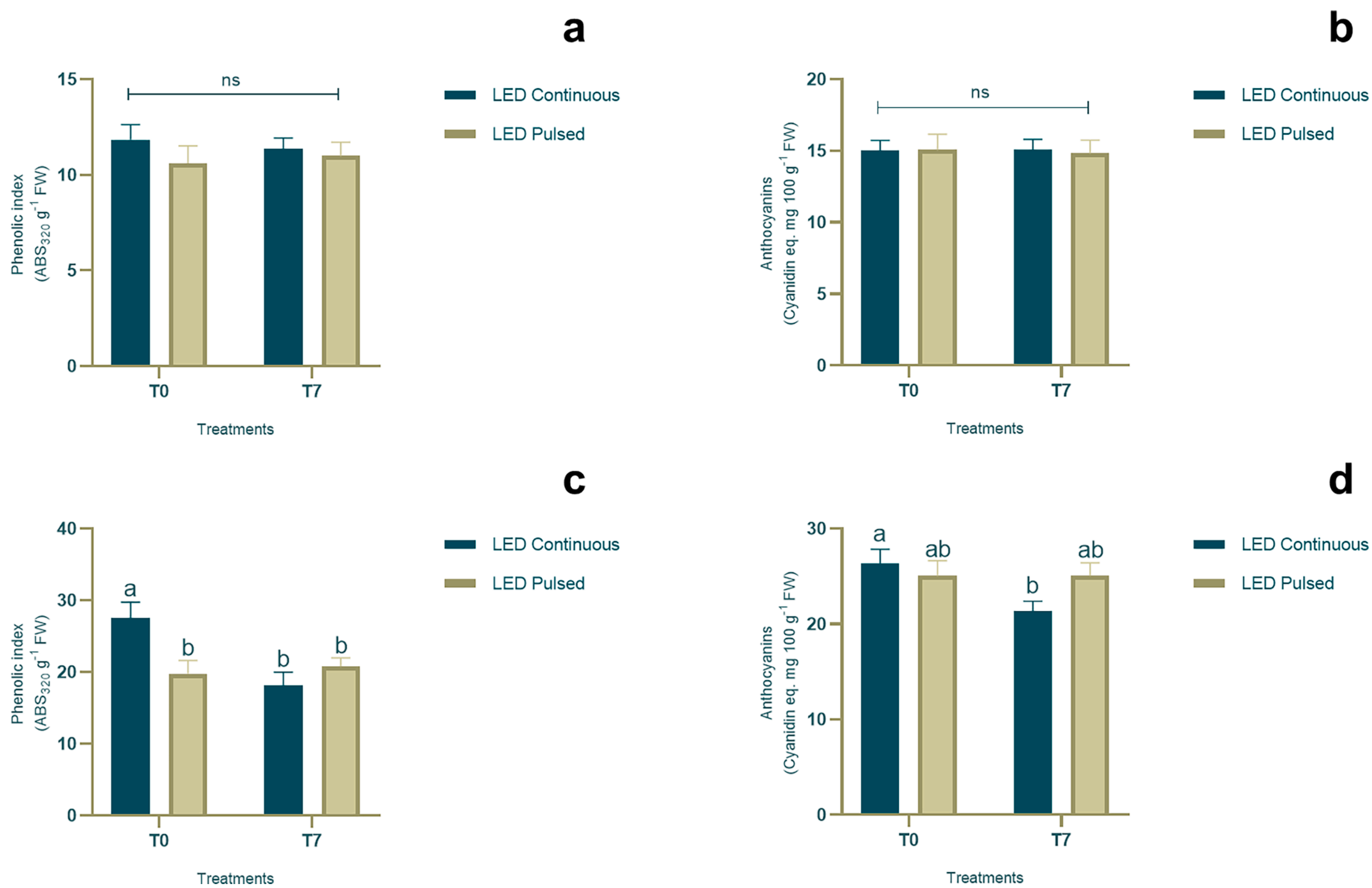


**Fig. 5.** (a) Phenolic index: green lettuce (b) Anthocyanins: green lettuce (c) Phenolic index: red lettuce (d) Anthocyanins: red lettuce, treated with pulsed and continuous LED treatments. T0 refers to the harvesting point while T7 refers to the point of cold storage after 7 days of harvesting. Values are means ($n = 6 \pm S$.E). Different alphabetic letters indicate statistically significant differences among the treatments and timepoints followed by two-way analysis of variance followed by Tuckey multiple comparison test ($p < 0.05$).

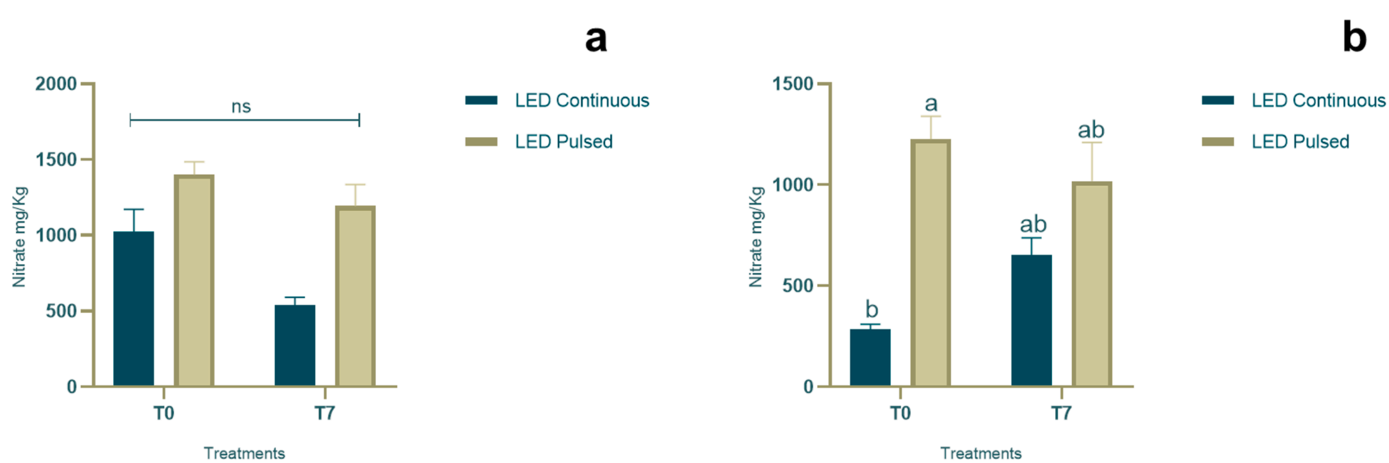


**Fig. 6.** (a) Nitrate: green lettuce (b) Nitrate: red lettuce, treated with pulsed and continuous LED treatments. T0 refers to the harvesting point while T7 refers to the point of cold storage after 7 days of harvesting. Values are means ($n = 6 \pm S$.E). Different alphabetic letters indicate statistically significant differences among the treatments and timepoints followed by two-way analysis of variance followed by Tuckey multiple comparison test ($p < 0.05$).

continuous LED significantly drop anthocyanins during storage possibly be due to the physiological changes where plants spend most of the reserve food and energy for performing respiration rather than producing secondary metabolites to ensure prolong shelf life. It is known that certain genes such as phenylalaninie ammonia-lyase (PAL), chalcone synthase (CHS) and dihydroflavanol-4-reductase (DFR) play significant roles in the production of phenols and anthocyanins. The expression of these genes can be triggered by monochromatic blue LED or the LED combinations with a defined share of blue LED (Giliberto et al., 2005). For the present study, the exact reason is unknown why the red lettuce under pulsed and continuous LED behaved differently than green lettuce in terms of phenolic index and anthocyanin accumulations, one thing is clear that the cultivar' sensitivity in terms of responsiveness to the applied spectra is dependent of the cultivar metabolic plasticity

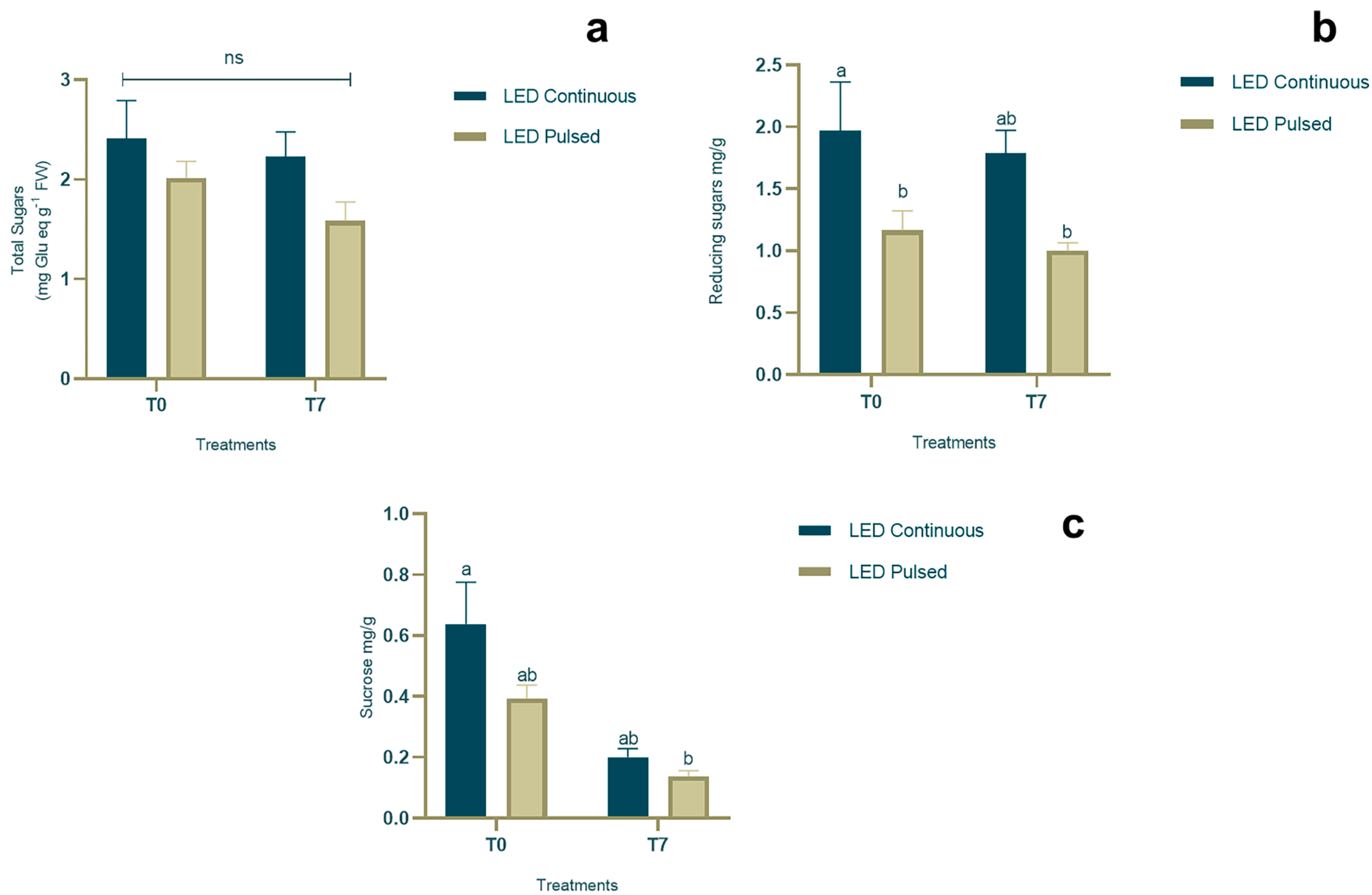


**Fig. 7.** (a) Total sugars: green lettuce (b) Reducing sugars: green lettuce (c) Sucrose: green lettuce, treated with pulsed and continuous LED treatments. T0 refers to the harvesting point while T7 refers to the point of cold storage after 7 days of harvesting. Values are means ($n = 6 \pm S$.E). Different alphabetic letters indicate statistically significant differences among the treatments and timepoints followed by two-way analysis of variance followed by Tuckey multiple comparison test ($p < 0.05$).

(Alrajhi et al., 2023). During postharvest storage, Chinese kale *(Brassica oleracea* var. *alboglabra* 'Bailey') pre-treated with R light (peak = 660 nm, 80 μmol $m^{-2}$ $s^{-1}$) retained higher aliphatic, indole and glucosinolates compared to the kale grown under white light (Deng et al., 2017). Under both studied LED treatments, B and R lights contributed the major proportions of the spectrum and possibly influenced the expression of flavonoid-3-O-glucosyltransferase (UFGT), CHS, rubisco smaller subunit genes and eventually anthocyanins under continuous LED than pulsed treatment, a finding previously discussed by Soufi et al. (2023). Furthermore, the production of secondary metabolites is prone to the leaf growth phases other than the quality and quantity of light exposure. Since leaf morphology for the red lettuce was significantly affected in terms of leaf thinning, increased length and less density under pulsed LED, all these factors might contribute to the difference in secondary metabolites productions among the pulsed and continuous LED treatments. However, as no such leaf morphological fluctuations were observed among the treatments in green lettuce, no significant variations occurred to produce these potential secondary metabolites in green lettuce.

To regulate the nitrate content of vegetable-based foods, a European regulation (EU:1258/2011) exists to ensure the provision of food with lesser nitrates to avoid any harmful consumption of leafy vegetables. The present study found that green and red lettuce behaved differently to the pulsed and continuous LED for nitrate accumulation as the former did not show any significant variations for the tested treatments. Red lettuce, however, accumulated significant nitrate for pulsed LED than continuous. Nitrate accumulation usually occurs in the vegetative parts of plants and according to a USDA report, factors such as stress either, drought, frost, salinity or the application of 2,4-D (2,4 Dichlorophenoxyacetic acid) cause plants to accumulate more nitrate in the leaves. Due to the difference in metabolic plasticity of tested cultivars, red lettuce possibly experienced pulsed LED as a stress, resulting in a reduced nitrate reductase (NR) expression, that led to an enhanced nitrate accumulation. Numerous research in the past on leafy vegetables such as basil, spinach, rocket, green lettuce and tatsoi clearly showed that LED in general is responsible for containing less nitrate in vegetables (Piovene et al., 2015; Ohashi-Kaneko et al., 2007; Signore et al., 2020; Bian et al., 2020; Simanavičius and Viršilė, 2018). Moreover, RB LED is known to enhance the vital metabolic and physiological activities of plants. The provision of the LED combination in this study might result in an increase in the uptake of nitrates, as nitrates serve as a primary source of nitrogen for chlorophyll formation, which was seen in the results of non-destructive estimation of chlorophyll where red lettuce exhibited higher chlorophyll estimated values than green lettuce for the given time. Similarly, pre-treatment of wild rocket (*Diplotaxis tenuifolia*) plant and lettuce 'Lollo Bionda' treated with $R + W$ supplementary lights or monochromatic B, showed lesser nitrates during post-harvest (Marques, 2016). Sugars serve plants in defense, structural and functional processes, apart from being the desirable component of the food quality. Modulating lights to a spectrum which is better perceived by plants. can be an approach to trigger the photosynthetic process of a crop that possibly leads to higher production in terms of yield. Our results suggested that the total sugars were nonsignificant among the treatments and also timepoints for green lettuce which was the only case between the timepoints in continuous LED treatment of red lettuce variety. For reducing sugar, non-significance occurred among the

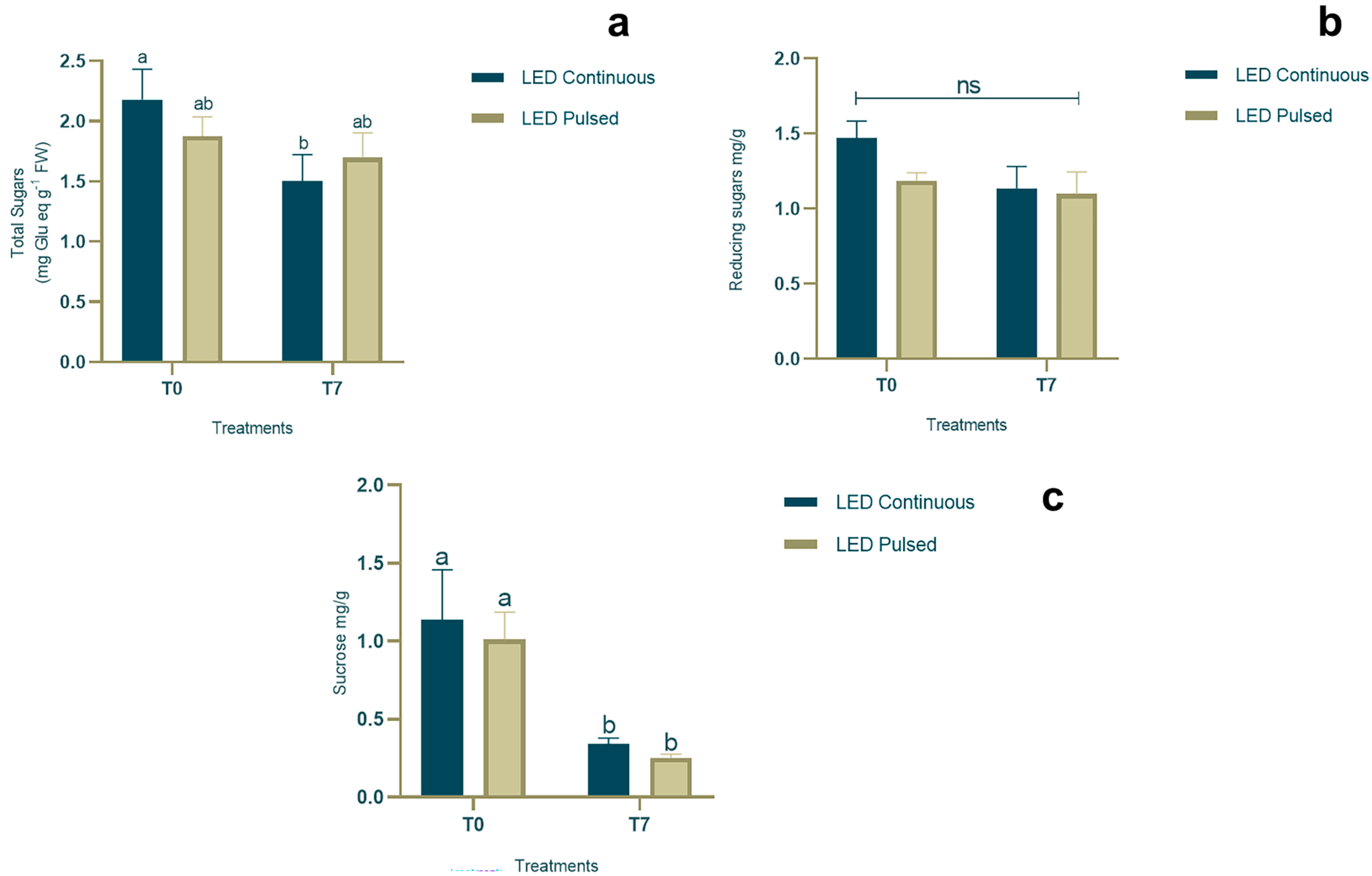


**Fig. 8.** (a) Total sugars: red lettuce (b) Reducing sugars: red lettuce (c) Sucrose: red lettuce, treated with pulsed and continuous LED treatments. T0 refers to the harvesting point while T7 refers to the point of cold storage after 7 days of harvesting. Values are means ($n = 6 \pm S$.E). Different alphabetic letters indicate statistically significant differences among the treatments and timepoints followed by two-way analysis of variance followed by Tuckey multiple comparison test ($p < 0.05$).

**Table 3**
Measurement of energy consumption under continuous LED and pulsed LED treatments operated for 30 days and 16 h a day. Total consumption is represented as kilo watt hours (kWh).

| Treatments | Energy consumption (W) | Total operational time (h/day) | Total number of days | Total consumption (kWh) |
|---|---|---|---|---|
| **Pulsed 1 kHz LED** | 37 | 16 | 30 | 17.76 |
| **Continuous LED** | 51.7 | 16 | 30 | 24.81 |

treatments and time points for red lettuce. However, in green lettuce, continuous LED was responsible for a significant increment than pulsed LED, where at T7 the concentration non-significantly dropped among the treatments, Sugars are known to impact all phases of plant life cycle which directly influence the growth and development (Ciereszko, 2018). The decrease in the sugars at post-harvest storage clearly depicted the fact that plants started utilizing the stored sugars for respiration. Previous studies concluded that both monochromatic R and combination of R: B LEDs are known to induce higher production of sugars in the lettuce (Soufi et al., 2023). Moreover, an increased carbohydrates and starch production was seen in the tomato plants exposed to 3R: 1B combination (Li et al., 2017). For sucrose, red and green cultivars for both LED treatments did not show any significant changes, however the values significantly dropped from the T0 to T7. Increased sucrose metabolism in plants is associated with an increase activity of certain enzymes such as sucrose-phosphate synthase (SPS) and sucrose synthase (SS). The pulsed frequency of 1KHz appeared prominent in accumulation of total sugars, reducing sugars as well as sucrose in line with the production under continuous LED for this study. Notably, in our previous experiment, which was conducted with a pulsed frequency of 2 Hz, continuous LED was significant in the accumulation of total sugars as well as sucrose production (Ali et al., 2023c).

To observe the interactive effect caused by various light sources on photochemistry, chlorophyll fluorescence is an innovative approach where an increased thermal dissipation of absorbed light energy (estimated as non-photochemical quenching (NPQ)) or the efficiency in photochemistry cause decrease in overall chlorophyll fluorescence value. Our results indicated that both red and green lettuce varieties did not significantly differ for Fv/Fm values at both T0 and T7. In an experiment by Son et al. (2018), similar results were recorded in lettuce where no significant variations in the pulsed and continuous irradiated lettuce with different monochromatic and LED recipes, were observed which concluded that the selection of duty cycle, choosing an appropriate pulsed frequency and the species under experiment all play a key role in defining the Fv/Fm values. Kanechi et al. (2016) observed similar non-significant Fv/Fm values in the lettuce leaves subjected to pulsed and continuous LED application except for the 2.5 Hz pulsed frequency in which a noticeable decrease in chlorophyll fluorescence was observed. However, in our previous experiment with a frequency of 2 Hz, we did not observe any differences in Fv/Fm values which clearly show that pulsed LED in general does not affect the Fv/Fm values. However, a selection of species might influence the Fv/Fm values. Apart from Fv/Fm, pulsed LED in red lettuce resulted in a significantly lower performance index of leaves among varieties at T0 however, no significant differences were observed between the treatments and among varieties at T7. Generally, the observed PI provides information on the intensity of active reaction centres (RCs), the electron transport efficiency and the probability that an absorbed photon is trapped by the RCs. Although, a lower performance index was observed under pulsed LED for red lettuce, we assumed that the pulsing effect might disturbed the efficiency of electron transport or suffered from lesser active reaction centres which eventually lead to an inefficiency of cyclic phosphorylation and hence resulted in an overall decreased performance index.

## 5. Conclusion

Our results suggest that for indoor lettuce production, pulsed LED can be an alternative to continuous LED in terms of retaining the quality of the produce. Pulsed light is a safe option to reduce the consumption of resources considering the higher energy demands under continuous LED for commercial productions. Moreover, both green and red lettuce responded differently to the tested LED treatments. In terms of leaf morphology, pulsed LED in red lettuce significantly resulted in longer and thinner leaves. The photosynthetic pigment accumulation such as chlorophyll and carotenoids, for secondary metabolites production such as phenolic index and anthocyanins and for the total sugars accumulation, the pulsed frequency of 1 kHz did not differ significantly to continuous LED in green lettuce however, continuous LED for red lettuce resulted in significant accumulation of secondary metabolites between treatments. A significant reduction was noticed in reducing sugars for green lettuce between the treatments while in red lettuce a significant drop in the sucrose was recorded between the timepoints. Although, majority of the quality attributes did not suffer a significant decline under 1 kHz pulsed application, yet we are far from deciding 1 kHz pulsed as a final frequency in the commercial production of lettuce where the prime goal is yield and increased fresh weight of the produce. Detailed research is required to investigate the potential of multiple higher frequencies than 1 kHz, a change in duty cycle, LED recipe and a possible prolonged photoperiod by keeping the energy consumption in check, after which a conclusion about the replacement of continuous LED with the pulsed LED can be made.

## CRediT authorship contribution statement

**Awais Ali:** Writing – original draft, Investigation, Data curation, Formal analysis. **Piero Santoro:** Resources, Conceptualization. **Antonio Ferrante:** Writing – review & editing, Conceptualization. **Giacomo Cocetta:** Writing – review & editing, Validation, Methodology, Supervision, Project administration, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2024.113785.

## Data availability

Data will be made available on request.

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