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Optimizing *Pleurotus ostreatus* cultivation: the role of light wavelengths and substrate composition on yield and nutritional value

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Introduction: The productive and morphological effects of monocramatic light applied during *Pleurotus* cultivation are not well known; a deep understanding of the light's effect could be suitable for a production protocol of this crop in a protected environment.
Methods: This study investigates the effects of different light wavelengths and substrates on the production and nutritional values of *Pleurotus ostreatus*. Using two substrates—wheat straw (WS) and a mixture of wheat straw and cottonseed hulls (WS+CH)—the experiment evaluated productive traits, morphological characteristics, and biofortification potential under five lighting conditions: red, blue, red-blue, white, and dark.
Results and Discussion: The results demonstrated significant influences of both light and substrate treatments on yield and quality. Higher production was observed under blue, red-blue and white light treatments (> 0.300 kg kg^{-1}) compared to red and dark lights. Blue light also enhanced cap size and yield. In contrast, red light was less effective in improving production but increased vitamin D2 biosynthesis. The WS+CH substrate under red light achieved the highest vitamin D_2 content (123 µg kg^{-1} dry weight). Nutritional analysis revealed that protein content ranged from 19.8% to 27.3%, with WS+CH outperforming WS (25.4% vs 19.8%). Glutamic and aspartic acids were the prevalent amino acids, which may contribute to the umami flavor, whereas histidine and valine levels were significantly increased by blue light treatments. In conclusion, controlled application of specific light wavelengths during cultivation significantly enhances both the morphological development and nutritional quality of *Pleurotus ostreatus*, demonstrating benefits beyond post-harvest treatment. Blue and red light play complementary roles in promoting fruiting body growth and biofortification. Additionally, the choice of substrate substantially influences yield and nutritional composition, underscoring its critical role in optimizing mushroom production systems.

KEYWORDS

vitamin D2, amino acid profile, oyster mushrooms, nutritional quality, LED lighting

1 Introduction

Pleurotus spp. is one of the most important genera in cultivated mushroom groups (Zied and Pardo-Giménez, 2017). Among *Pleurotus* genera *P. ostreatus* is one of the most widely cultivated (Zied and Pardo-Giménez, 2017), foremost for its flavor and taste and its high nutritional value: it is rich in vitamins, minerals and proteins. Protein content usually ranges between 17 and 40% (Deepalakshmi and Mirunalini, 2014), less than in meat but higher than in eggs and milk (Corrêa et al., 2016). The presence of all essential amino acids (Devi et al., 2024) makes *P. ostreatus* a suitable protein source for vegetarian and vegan diets (Corrêa et al., 2016; Diamantopoulou et al., 2023). Amino acids (AAs) profile is fundamental for protein synthesis and is correlated with important health-related effects (Tagkouli et al., 2020). The amount of some Aas (as Glutamic acid and Aspartic acid), belonging to free amino acids (FAAs) category, provide umami flavor, the typical mushrooms taste, and sweet flavors (Donglu et al., 2017). The AAs and protein content is correlated with mushrooms strain, cultivation substrate and harvest time (Mendez et al., 2005). *P. ostreatus* can be easily cultivated in a large range of lignocellulosic substrates derived from agricultural residues (Girmay et al., 2016). Every geographical area of production finds a different type of substrate optimal for mushroom production, available locally and cheap (Gebru et al., 2024). For instance, in the USA cottonseed hulls are a major component of *Pleurotus*'s substrate, whereas in Europe wheat straw is more common. Besides the substrate, the effect of environmental condition such as CO_2 concentration, humidity and temperature on growth and morphology of this species has already been studied in depth (Bellettini et al., 2019). At the same time the effect of light on mushroom growth is not clear yet. Several studies demonstrate that light's effect can be different among species. *Agaricus bisporus* doesn't need any light during fruiting body development and can grow in complete darkness (Baars et al., 2020), whereas in *Pleurotus* spp. cultivation, light is an important factor for fruiting body development (Baars et al., 2020). Usually, edible mushrooms are cultivated in a protected environment with little radiation coming from outside or in a growing room with artificial white lighting. Only 200–640 lux (corresponding to 3.7-12 μmol m^{-2} s^{-1}) for 8–12 h day^{-1} are necessary to promote fruiting bodies in *Pleurotus* spp (Bellettini et al., 2019). In recent years, studies have detected photoreceptors for red and blue light in fungi (Kojima et al., 2015), and it has been reported that blue and ultraviolet light induces fruiting bodies formation (Wang et al., 2020). Moreover, light effect can differ among *Pleurotus* species and phenological phases: mostly between mycelium development and fruiting bodies growth. For instance, red light enhances and inhibits fruiting bodies growth in *Pleurotus eryngii* (Yue et al., 2022) and *P. ostreatus*, respectively (Zhu et al., 2024), whereas during *P. ostreatus* mycelium development red and dark light are more favorable than white or blue light (Zhu et al., 2024). A clear knowledge of which wavelengths, intensity of radiation and photoperiod will be more suitable to enhance *P. ostreatus* cultivation is missing and a clear protocol for artificial lighting of this crop in a protected environment is not established. Most of the literature about the application of artificial light in edible mushrooms regards post-harvest treatments and its effect on shelf life and vitamin D_2 levels (Cardwell et al., 2018; Feng et al., 2023; Nölle et al., 2017; Taofiq et al., 2017) mostly on *A. bisporus* (Blumfield et al., 2020; Guan et al., 2016; Taofiq et al., 2017; Urbain and Jakobsen, 2015), *P. ostreatus* (Huang et al., 2015; Jasinghe and Perera, 2005; Wittig et al., 2013; Wu and Ahn, 2014) and *P. eryngii* (Huang et al., 2015). Vitamin D is a fundamental nutritional compound; it can be produced by the human body if exposed to UV radiation from sunlight. However, Cardwell et al. (2018) calculated that almost 1 billion people of the world's population suffer from vitamin D deficiency. Vitamin D can also be obtained from dietary sources: fungi, yeast, and animal products (such as meat or milk) are the main sources of vitamin D. The former contains vitamin D2 (the most common form of vitamin D), while the latter contains vitamin D3; in addition other forms as vitamin D_3 and D_4 are present also in fungi, even if in less quantity (Taofiq et al., 2017). The dietary recommended intake ranges between 5 - 15 μg day^{-1} worldwide; in EU, across all ages, the recommended intake is 15 μg day^{-1} (European Food Safety Authority (EFSA), 2017). Biofortified food source with a higher amount of vitamin D can be a solution to solve dietary deficiency, and mushrooms are a good solution for improving vitamin D_2 especially in vegetarian and vegan diets where animal food is not permitted (Cardwell et al., 2018). There is a lack of information about the application of visible light spectrum during *Pleurotus* cultivation and its effect on nutritional values. Research by Yue et al. (2022) indicated the effect of blue and red light on fruiting bodies morphology and quality of *P. eryngii*. They reported that red light increased the percentage of most amino acids compared to white and other light treatments, except for methionine, tyrosine, serine, and glutamic acid, which increased under sun-like and blue light (Yue et al., 2022). De Bonis et al. (2024), moreover, found that artificial light treatments, specifically blue light applied during the cultivation cycle, can enhance vitamin D_2 content as well as the cap diameter.

This study aimed to evaluate the effects of single-wavelength light on *Pleurotus ostreatus* cultivation using two commonly adopted substrate formulations: wheat straw (widely used in Europe) and a mixture of cottonseed hulls and wheat straw (the predominant formulation in the United States). The impact of light and substrate on productive performance and morphological traits was assessed, together with their biofortification potential in terms of amino acid profile, protein content, and vitamin D_2 levels. The overall objective was to generate deeper insight into how monochromatic light influences mushroom productivity and quality, with the final aim of supporting the development of an artificial lighting protocol applicable across substrates commonly used worldwide.

2 Materials and methods

The experiment started on 20th December 2023, in a growing room at the Mushroom Research Center at Penn State University (State College, PA, USA). During the incubation phase the air temperature was set to 23°C, whereas during the 1st and 2nd flush, air temperature was dropped to 18°C.

Six racks (1.5 m × 0.5 m × 2.0 m) with two shelves each were arranged in the room, covered with a black plastic sheet to avoid any light contamination.

10.3389/fhort.2025.1720226

2.1 Light treatments

Strip LED lights (C-LED company - Bologna, IT) were used for this experiment. Lamps measured 1.5 m and were placed in the middle of each shelf; each light was equipped with a dimmer to change radiation's intensity, and a timer to set up the photoperiod (8 hours of light day^{-1}). Lights were set to enlighten the upper surface of the bags with an intensity of 26-28 μmol m^{-2} s^{-1} (Figure 1). Each rack was set up with one wavelength treatment: red light, blue light, white light, and a control without any light (Dark). Table 1 describes the main characteristics of each light treatment.

Lighting was provided from incubation through fruit body development in all illuminated treatments except for the Red-Blue one, in which red light was provided during incubation and primordia development, and blue light during the morphological growth of fruiting bodies. Blue lighting started when more than half of total holes were covered with primordia.

2.2 Substrates

Under each light treatment, 12 bags of 3 kg each were placed on a rack. Six bags were prepared with wheat straw and nitrogen supplementation (Promycel gold (54% protein), Amycel, CA, USA), the most used mixture for the European commercial substrate, and 6 bags were composed of 75% cottonseed hulls and 25% wheat straw (Table 2) according to the mixture commonly adopted for *Pleurotus* spp. substrate in the US (Royse, 2004).

2.3 Bag preparation

Sixty bags were prepared the day before the inoculation. Straw (chopped to max 2 cm length) and cottonseed hulls were mixed with water to reach 65-70% of moisture and a commercial protein supplement, Promycel Gold, was added to reach 30–40 C/N ratio in both substrates. Then bags were sterilized in autoclave for 20 min at 121°C and then let cool overnight. Then both substrates were inoculated with 3% w/w of *Pleurotus ostreatus* 123 spawn from LF Lambert Spawn Co. (PA, USA), the inoculation rate was similar in all bags under all lights treatments. After inoculation, 6 holes were cut on the top of the bag to allow the same amount of radiation exposure during cultivation steps.

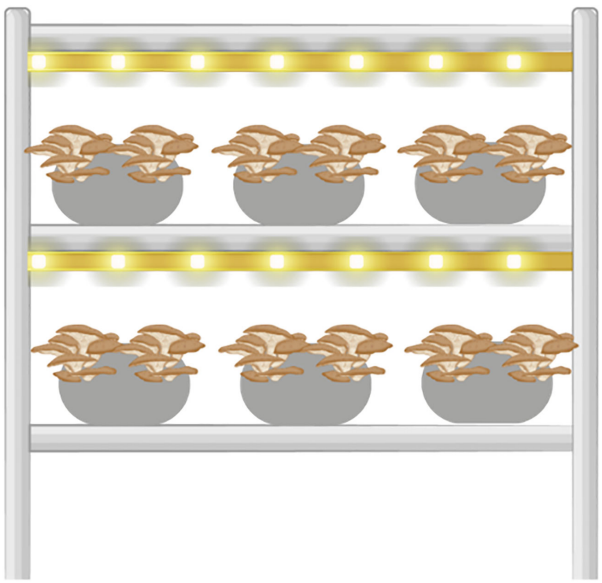

FIGURE 1
Illustration of the arrangement of LED lights and mushrooms' bags on the shelves during the experiment.

2.4 Substrate analysis

At the beginning of the experiment, two samples of each substrate were taken for chemical characterization. Total nitrogen, and total carbon contents (TMECC 4.02-D), pH (TMECC 4.11-A), and organic matter (TMECC 05.07-A) were measured following analytical methodology from Test Methods for the Examination of Composting and Compost, USDA and U.S. Composting Council (2002). Samples of the fresh substrate were also put in oven at 60°C until weight stabilization, to assess the moisture content.

2.5 Productive traits collection

The effect of light treatments and substrates was observed among two flushes. After the incubation and at the end of the first flush primordia appearance was monitored and calculated as percentage of primordia for total bag's holes. Then, during each flush, fruiting bodies were harvested at commercial maturity (the edges start to be parallel to the ground but before turning upward) and weighed. For each fruiting body, cap number, cap diameter and thickness were measured together with stipe length. Biological efficiency (BE) is one of the most commonly used indices to evaluate mushroom performance across different substrates or treatments, allowing production results to be compared among various studies. BE was calculated with the following formula (Royse, 1985):

$$BE(\%) = \frac{\text{weight of the fresh mushrooms harvested (kg bag}^{-1})}{\text{dry substrate weight (kg bag}^{-1})} \times 100$$

2.6 Qualitative traits collection

For each pair bags, during both flushes, fruiting body samples were taken on the day of maximum production to assess the nutritional values, i.e. amino acid and vitamin D_2 content. In total, three replicates per treatment were obtained for each flush. The samples were frozen at -20°C and then freeze-dried and ground into powder before the assay.

TABLE 1 Wavelengths and intensity applied in the light treatments under study.

Light treatment	Wavelength	Intensity
Red	650 nm	26-28 μmol m^{-2} s^{-1}
Blue	450 nm	
White	450 nm (Blue) 23% + 500 nm (Green) 17% + 650 nm (Red) 56% + 730 nm (Far Red) 4%	
Red——Blue	(Red) 650 nm during incubation and primordia appearance ——(Blue) 450 nm during fruiting bodies development	
Dark	/	0.05 μmol m^{-2} s^{-1}

2.6.1 Vitamin D_2 determination

The extraction of vitamin D_2 from *P. ostreatus* mushrooms followed the procedure described by Hu et al. (2020) with some modifications. Briefly, 0.15 – 0.2 g of dried and powdered mushrooms were subjected to extraction with 2 mL of ethanol using an ultrasonic bath (Ultrasonic cleaner, VEVOR) operating at 70W and 40 kHz for 20 min at 25°C. Samples were then centrifugated at 3500 rpm at 4°C for 15 min (Thermo Sorvall Legend XTR), and the resulting supernatants were carefully transferred to a vial. The collected samples were then dried in a centriVap concentrator (Labconco, USA) and resuspended in 0.5 mL of methanol HPLC grade. The extract was filtered through a 0.2 μm nylon filter before the injection for HPLC analysis. The quantification of vitamin D_2in was then performed by reverse-phase HPLC through the injection of 50 μL of the mushroom extract into the system (Agilent Technologies 1260 Infinity Series1260, Mainz, Germany). The instrument control, data acquisition, and analyses were performed using Agilent Chemstation software. The separation of the compounds was achieved using an analytical reverse phase column Zorbax Eclipse XDB-C18 (3.5 μm, 4.6 × 150 mm, Agilent Technologies). Throughout the experiments, the column oven temperature was maintained at 30°C. The mobile phase consisted of methanol:water composition (95:5), with a flow rate set at 1 mL min^{-1}. Vitamin D_2 was detected at 265 nm and identified by comparing the retention time with the standard (5.8 min). The concentration of D_2 in the samples was quantified by using a calibration curve as a reference. Values were reported as μg g^{-1} on a dry weight basis (Hu et al., 2020).

2.6.2 Amino acids determination

An Agilent 1260 Infinity HPLC (Agilent Technologies, Santa Clara, CA, USA), which was equipped with a reversed-phase column C18 (CORTECS C18, 2.7 μm, 2.1 × 150 mm) kept at 45°C and a diode array detector (Agilent 1260 Series, DAD VL+), was used for the separation and quantification of amino acids in mushrooms samples. Amino acids were analyzed after acid hydrolysis and pre-column derivatization with AQC, separated by RP-HPLC and analyzed by UV detection according to the method described by Bosch et al. (2006). An multielements AccQTag Ultra Derivatization Kit (Agilent 5061-3330) was used for HPLC standards. Briefly, for the determination of alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine, and protein content the sample was hydrolyzed with hydrochloride acid 6 M at 105°C for 24 h. Total cysteine (Cys) was determined as the sum of cysteine and cystine after the reaction with 3,30-dithiodipropionic acid, producing a mixed disulfide, which then underwent acid hydrolysis accordingly (Tuan and Phillips, 1997). After hydrolysis, the samples were neutralized with sodium hydroxide 8 M, adjusted to volume, and filtered with 0.45 μm filters. The derivatization step was conducted (for samples and standard) by adding 70 μL of AccQ-Tag Ultra borate buffer and 10 μL of the filtered sample to a vial. Then, the solution was mixed by means of a vortex for several seconds. Next, 20 μL of the derivatization agent (dissolved in acetonitrile) was added, and the mixture was heated for 10 min at 55°C. The sample was further diluted with 900 μL borate buffer and 5 μL was injected. Tryptophan (Try) was determined following the method adapted from CD 2000/45/EC (European Union Commission Directive, 2000). First, 100 mg of the sample was placed in a Teflon hydrolysis tube, then 8 g barium hydroxide and 16 mL water were added, and the tubes were incubated at 105°C for 24 h. Hydrolysates were cooled, neutralized to pH 7 using 6 N HCl, and diluted to 100 mL with 1 M sodium borate buffer. Aliquots of these solutions were filtered through a 0.22 μm syringe filter. A total of 20 μL of the sample was injected into the column (Xselect HSS T3, 5 μm; 4.6 × 250 mm), and the separation was performed by an isocratic elution system consisting of 25 mM sodium acetate:acetonitrile (91:9) delivered at 0.9 mL min-1 and detected with a DAD at 280 nm.

To understand protein's quality the AA content was calculated also as μg g^{-1} protein to be compared with a chemical level recommended by FAO/WHO report for adults (WHO, 2007).

TABLE 2 Substrate composition: WS (wheat straw), WS+CH (wheat straw + cottonseed hulls).

Substrate	Composition	Moisture	pH	Organic matter	Total nitrogen	Total carbon	C/N
WS	100% wheat straw	70%	6.1	96.9%	1.28%	50.4%	39.2
WS+CH	25% wheat straw + 75% cottonseed hulls	66%	6.3	96.7%	1.78%	50.4%	28.4

2.7 Statistical analysis

The experimental plan used in this study was a completely randomized design. For productive and morphological traits, each treatment included six replicates (represented by bags), while for qualitative analysis, fruiting body samples were obtained by combining two bags to form three replicates per treatment for each flush. Data of the flushes separately were statistically analyzed using a two-way analysis of variance (ANOVA), and means were separated with Tukey's HSD test at $\alpha = 0.05$. The normality and homoscedasticity of the data were tested using the Levene and Shapiro–Wilk tests. Interaction effect were tested and reported or mentioned only when significant. Statistical analyses and principal component analysis (PCA) were performed in RStudio version 4.3.3.

3 Results

3.1 Productive trials

Figures 2 demonstrates the evolution of primordia appearance for both light treatments and substrates in the 1st and 2nd flush respectively. In the 1st flush, WS appeared to hasten production and showed significant differences at 19 and 20 days after spawning (DAS). However, the final production of primordia was different only for the 2nd flush for substrate treatments where WS+CH reached 77.2% of holes occupied by primordia compared to WS which obtained only 62.2%. Light treatments did not affect total primordia production for both flushes. Red-Blue light showed higher primordia production compared to White light at 20 DAS. The interaction between substrate and light treatments was significant at 17 and 18 DAS in the 1st flush (data not showed) where substrate influenced primordia appearance under black light and WS × Dark light outperforms WS +CH × Dark light. In addition, at 33 DAS in the 2nd flush, WS+CH substrate improved primordia appearance (91%) under R-B light compared to WS substrate (30.6%) (data not showed). Light treatments influenced two flushes production yield (Figures 3A, B). During the 1st flush (3.a) Red-Blue light had the highest production from 25 to 29 DAS compared to Dark and Red light. In addition, Red-Blue light appeared to have a shorter cycle as the harvest ended 3 days before the other light treatments. During the 2nd flush (3.b) light treatments also affected cumulative production: from 39 to 44 DAS Blue light had the highest yield, particularly compared to Red and Dark treatments at 44 DAS. However, at the end of the 2nd flush, the yield of Blue and White treatments was similar and higher than the Dark treatment. Also, substrate treatments significantly impacted cumulative yield (Figures 3C, D) in both flushes: in 1st flush, from 23 to 27 DAS, WS yielded higher compared to WS+CH but the total yield was not significantly different (0.21 kg kg^{-1}, on average). However, during the 2nd flush WS+CH had the best performance from 40 DAS to the end of the trial, with a final yield of 0.11 kg kg^{-1} substrate compared to 0.07 kg kg^{-1} of WS.

Table 3 displays the total yield and the biological efficiency obtained from the sum of the 1st and the 2nd flush. The WS+CH treatment displayed a higher total yield compared to WS, but the biological efficiency was not statistically different with 91.2% on average. Light treatments affected total yield and biological efficiency: Blue, Red-Blue and White treatments showed the highest total yield with an average value above 0.30 kg kg^{-1} and 98.9%, while Red and Dark treatments had the lowest value with 0.25 kg kg^{-1} and an average biological efficiency of 79.7%. A significant interaction between substrate and light was observed on total yield and BE. Regarding the total yield the effect of the substrate differed across light. Specifically, WS+CH significantly increased the response in R-B, B, and W light compared to WS alone. BE interaction showed an increase in WS x Dark (91.5%) compared to WS+CH x Dark BE (67.7%).

In Table 4 and Figures 4, 5 the morphological characteristics of the fruiting bodies are described. The cap number was affected by the light treatments during the production cycle in the 1st flush (Figure 5A). Red and Red-Blue treatments promoted higher values (25.4 and 28.1, respectively) compared to Blue treatment (16.0), whereas in the 2nd flush (Figure 5B) only Red-Blue treatment was significantly different from all the other light treatments with a cap number of 11.3 per bag, compared to an average of 7.15. The interaction substrate × light treatments among the two flushes observed was not significant. The cap diameter, shown in Table 4, was influenced by the substrate with a larger value in WS (+6.93%) and WS+CH (+7.18%) respectively in the 1st and 2nd flush. The diameter was also affected by the light treatments: Blue and White light had a positive effect on the diameter, whereas under Red light and Dark conditions caps expressed a smaller diameter. Cap thickness (Table 4) was also affected by light treatments. For both flushes, Blue light obtained the highest thickness (3.98 and 4.10 mm respectively), whereas White light resulted in thicker caps (4.02 mm) in the 1st flush and was less thick in the 2nd (3.43 mm). Lastly, stipe length was affected by both substrate and light treatments: WS +CH substrate obtained longer stipes than WS, whereas Red light and Dark treatments promoted longer stipe growth with a length greater than 3.6 cm (Table 4).

The interaction substrate × light treatment was significant in the 1st flush for cap diameter (Figure 6A) where WS resulted in larger cap than WS+CH under Blue and Red light. Cap thickness was not significant (Figure 6B) whereas for stipe length (Figure 6C) in both flushes Red and Dark had longer stipe in WS+CH than the same light treatment with WS substrate.

3.2 Nutritional value: amino acids and vitamin D_2 contents

The vitamin D_2 content shown in Figure 7 was significantly different in both flushes and for both substrate and light treatments. WS+CH achieved the highest vitamin D_2content, with 123 µg kg^{-1} dw and 92.6 µg kg^{-1} dw for the 1st and 2nd flushes, respectively, whereas WS had lower values: 70.2 µg kg^{-1} dw and 57.4 µg kg^{-1} dw. Under different light treatments, the amount of vitamin D_2 in the fruiting bodies varied significantly. Red light was the most effective treatment, with 139 µg kg^{-1} dw, compared to all other treatments, which had Vitamin D_2 content below 100 µg kg^{-1} dw. During the 2nd flush, Red light resulted in values different only from those under Blue, Dark, and

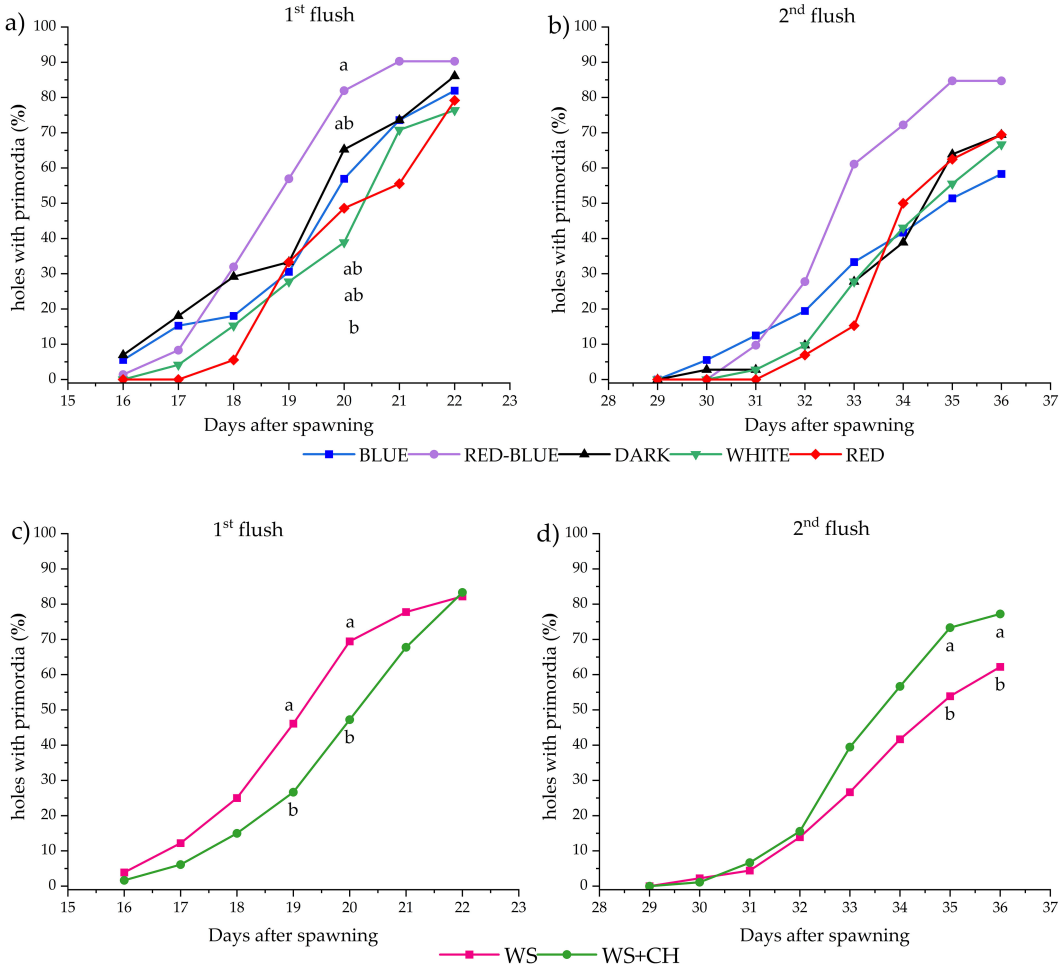

FIGURE 2
Effects of light during the 1st and the 2nd flush **(A, B)** and substrate (WS: Wheat straw, WS+CH: Wheat Straw+Cotton seed hulls) in 1st and the 2nd flush (c and d) on the cumulative percentage of primordia appearance for total number of bag's holes. Different letters indicate significant differences among treatments for p ≤0.05 according to Tukey's HSD test.

Red-Blue light. In Table 5 is showed the interaction effect of Substrate × Light treatments on vitamin D_2 content on dry and fresh weight, in both flushes the combination WS+CH × Red light obtained the higher concentration of vitamin D_2, the lower vitamin D_2 values was observed in almost all light treatments with WS substrate, particularly under Dark and Blue light in the 1st and 2nd flush, respectively.

Supplementary Table 1 shows the effects of substrate and light treatments on crude protein content (CP) and amino acid content (AA) (mg g^{-1}), expressed as a percentage of freeze-dried samples. The percentage of crude protein in the fruiting bodies was significantly different only in the 1st flush for substrate treatments: WS+CH had 25.4% of CP compared to 19.8% in WS. Glutamic acid was the prevalent amino acid in all treatments under study, ranging from 33.5 to 41.2 mg g^{-1} in the 1st flush and from 35.3 to 45.3 mg g^{-1} in the 2nd flush. For essential amino acids (EAA), lysine was the most prevalent (21.5-17.4 mg g^{-1} and 23.9-28.9 mg g^{-1}).

The most significant factor affecting amino acid content was substrate: for both flushes, the amount of AAs was higher with WS +CH compared to WS substrate. Light treatments only affected the content of valine and isoleucine in the 1st flush, with higher values under Blue compared to Red-Blue treatment. In the 2nd flush, histidine had higher values under Blue and Red-Blue light compared to Red, White, and Dark treatments.

In Figure 8 the Principal Component Analysis (PCA) of the amino acids content revealed that the 1st two principal components (Dim1 and Dim2) accounted for approximately 94.7% of the total variability. The analysis identified two distinct clusters corresponding to the different substrates with 95% confidence. Histidine, Alanine,

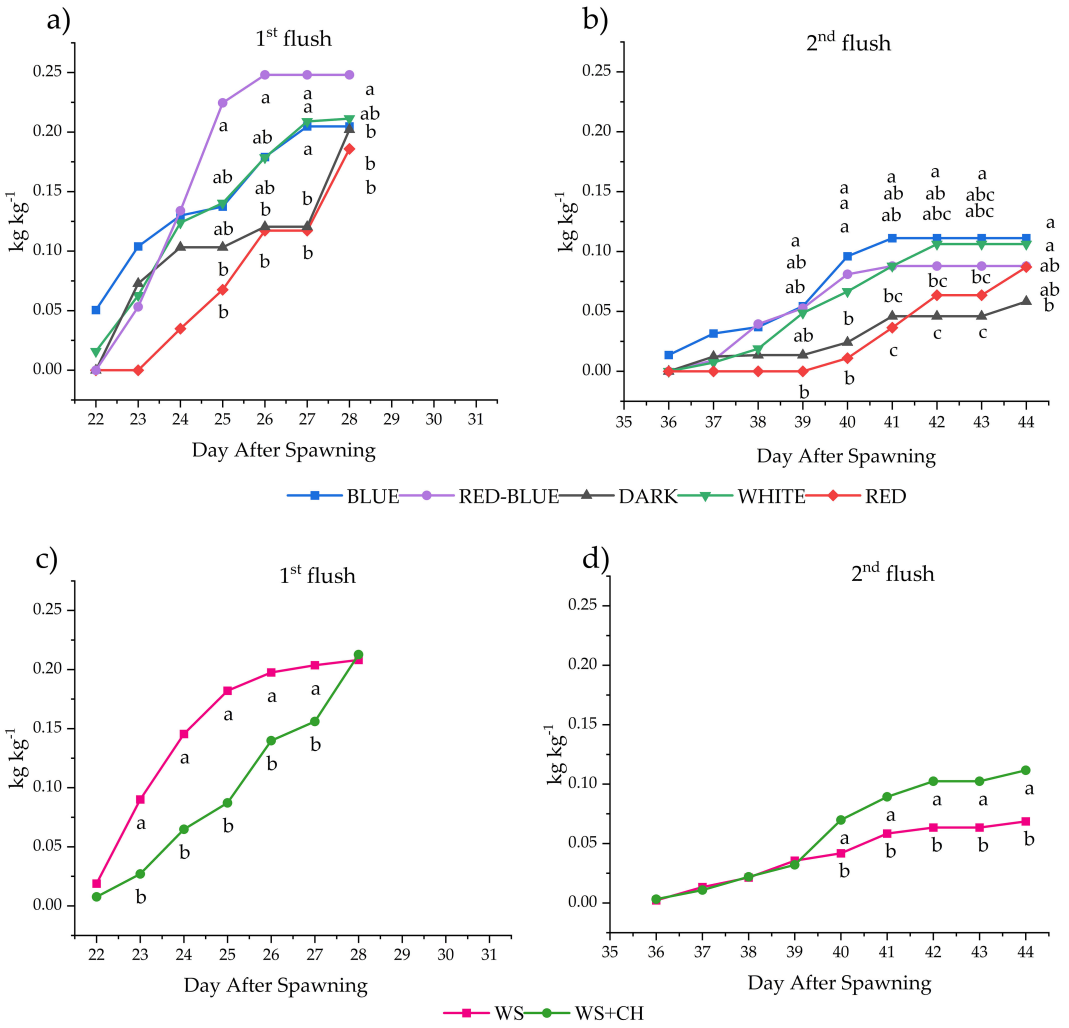

FIGURE 3
Effects of light during the 1st flush **(A)** and the 2nd flush **(B)** and substrate (WS: Wheat straw, WS+CH: Wheat Straw+Cotton seed hulls) during the 1st flush **(C)** and the 2nd flush **(D)** on cumulative yield (kg kg^{-1} of substrate). Different letters indicate significant differences among treatments for $p \leq 0.05$ according to Tukey's HSD test.

and Glycine were the most representative contributors to the variability captured by the first principal component. No distinct clustering was observed for the light treatments.

Comparing our results with the reference composition of protein for meeting adult requirements indicated by the FAO/WHO (WHO, 2007), all essential amino acids met the reference composition parameters except for sulfur amino acids (the sum of methionine and cysteine), which had a value below 20 for Red and White light in the 1st flush and is slightly under 22 mg g^{-1} protein in several cases (Table 6).

4 Discussion

4.1 Production and morphology

4.1.1 Light treatment effect

During the experiment, light treatments did not affect primordia initiation, but no measurements were conducted to monitor mycelium colonization of the substrate. Previous studies on mycelial growth during the incubation have reported contrasting effects of light: Nakano et al. (2010) found that blue light, particularly at higher intensities, negatively affected colonization, whereas Arjona et al. (2009) observed accelerated mycelium development under red light and in darkness. However, our results suggest that light exposure does not promote an earlier onset of the reproductive phase and the use of artificial lighting can thus be avoided, resulting in potential economic savings. Light is an important factor, along with decreasing temperature, for stimulating primordia differentiation and the start of a productive flush (Bellettini et al., 2019). Among all visible light treatments blue light is indicated to promote primordia induction (Feng et al., 2023), however, in this study Blue light did not seems to affect primordia appearance (Figure 2). On the other hand Blue light positively affected yield in both flushes and total yield compared with Dark or Red light (Table 3), in agreement with other studies (Wang et al., 2020; Zhu et al., 2024). In this study, all treatments containing Blue light (Blue, White and Red-Blue) displayed higher productive results compared with Red and Dark light

10.3389/fhort.2025.1720226

TABLE 3 Effects of the substrate and light treatment on total yield (kg kg^{-1}) and biological efficiency (%).

Treatments		Total yield	Biological efficiency
		kg kg^{-1}	%
Substrate (S)			
Wheat straw (WS)		0.273 ± 0.01 b	91.0 ± 2.78
Wheat straw + Cottonseed hulls (WS+CH)		0.311 ± 0.01 a	91.4 ± 3.34
Light treatment (L)			
Red (R)		0.256 ± 0.02 b	79.8 ± 4.39 b
Blue (B)		0.306 ± 0.01 a	95.7 ± 4.21 a
Red-Blue (R-B)		0.324 ± 0.01 a	101 ± 2.27 a
White (W)		0.322 ± 0.01 a	100 ± 3.23 a
Dark (D)		0.252 ± 0.02 b	79.6 ± 5.43 b
Substrate × Light treatment (S × L)			
WS ×	R	0.229 ± 0.02 d	76.5 ± 6.61 abc
	B	0.291 ± 0.03 abcd	96.9 ± 8.29 ab
	R-B	0.287 ± 0.01 abcd	95.8 ± 2.83 ab
	W	0.284 ± 0.01 abcd	94.5 ± 4.78 ab
	D	0.275 ± 0.02 cd	91.5 ± 4.90 abc
WS +CH ×	R	0.283 ± 0.02 bcd	83.1 ± 6.00 abc
	B	0.321 ± 0.01 abc	94.5 ± 3.07 ab
	R-B	0.360 ± 0.01 ab	106 ± 2.11 a
	W	0.361 ± 0.01 a	106 ± 3.13 a
	D	0.230 ± 0.02 d	67.7 ± 7.02 c
Significance			
S		***	ns
L		***	***
S × L		**	**

Means are followed by standard error; different letters indicate significant differences for p ≤0.05 according to HSD Tukey's test.
ns: not significant, "**": p-value<0.01, "***": p-value<0.001.

treatments. This is not in agreement with Yue et al. (2022) who found that Red light enhanced *P. eryngii* yield, whereas Roshita and Goh (2018) confirmed that Blue light improves *Pleurotus sajor-caju* and *Pleurotus florida* production. These contrasting results indicate a high variability of light effects within *Pleurotus* species. Zhu et al. (2024) sought to explain the increased production of *P. ostreatus* under Blue light compared to Red. They found that Blue and White light led to an upregulation of protein involved in ATP synthesis pathway during both the vegetative and reproductive phases, ultimately boosting the yield of fruiting bodies. In contrast, Red light was shown to induce lower expression of these proteins lead to an inadequate ATP supply. Lastly, the application of Blue light only during fruiting bodies development promoted production as the application of Blue during both incubation and production indicating that Blue light can enhance *Pleurotus* yield even if the application occurred only during production stage.

P. ostreatus morphology is typically described by cap number per fruiting body, their diameter and thickness and, ultimately, stipe length. In this study, the cap number decreased under Blue light, whereas Red and Dark light treatments increased the average cap number. In particular, as Red-Blue treatments indicated, the effect of Red radiation was more effective during primordia development and differentiation and not during fruiting bodies development. Instead, Blue light appeared to be a limiting factor for the cap number, as even White light resulted in fewer caps than Red or Dark light treatments. This trend suggests that using Red light or a Dark environment during the early stages of primordia development could enhance the cap number after incubation and between flushes. Worldwide, *P. ostreatus* crops are typically sold with a high number of small caps, whereas the Italian market prefers fewer but larger caps. For this reason, depending on different market requirements, Blue or Red/Dark radiation may be required.

10.3389/fhort.2025.1720226

TABLE 4 Fruiting bodies characteristics. Number of caps for fruiting bodies, dimension of fruiting bodies: diameter (cm), thickness (mm) and stipe length (cm).

Treatments	Cap diameter (cm)				Cap thickness (mm)				Stipe length (cm)			
	1st flush		2nd flush		1st flush		2nd flush		1st flush		2nd flush	
Substrate (S)												
Wheat straw (WS)	6.33	± 0.09 a	7.66	± 0.14 b	3.93	± 0.06 a	4.00	± 0.10	2.66	± 0.06 b	2.40	± 0.06 b
Wheat straw + Cottonseed hulls (WS+CH)	5.92	± 0.09 b	8.21	± 0.15 a	3.53	± 0.06 b	3.91	± 0.09	2.92	± 0.07 a	3.37	± 0.11 a
Light treatment (L)												
Red (R)	5.44	± 0.14 c	7.43	± 0.22 c	3.45	± 0.08 bc	3.56	± 0.09 c	3.66	± 0.09 a	4.02	± 0.14 a
Blue (B)	6.86	± 0.20 a	9.14	± 0.24 a	3.98	± 0.11 a	4.70	± 0.14 a	2.40	± 0.10 b	2.29	± 0.08 bc
Red-Blue (R-B)	6.01	± 0.11 b	7.63	± 0.19 bc	3.76	± 0.09 ab	4.27	± 0.13 ab	2.03	± 0.06 c	1.91	± 0.10 c
White (W)	6.63	± 0.11 a	8.36	± 0.21 ab	4.02	± 0.09 a	3.43	± 0.19 c	2.52	± 0.07 b	2.54	± 0.10 b
Dark (D)	5.35	± 0.14 c	7.12	± 0.24 c	3.24	± 0.09 c	3.96	± 0.11 bc	3.79	± 0.09 a	3.69	± 0.17 a
Significance												
S	***		**		***		***		***		***	
L	***		***		***		***		***		***	
S × L	**		ns		ns		ns		***		***	

Effect of substrate, light wavelength (Means ± standard error); different letters indicate significant differences for p ≤0.05 for HSD Tukey's test.
ns: not significant, "**": p-value<0.01, "***": p-value<0.001.

Blue light limited the cap number but enhanced their diameter and thickness. Several studies reported a larger cap size as the prominent effect of blue light in *Pleurotus* species (Roshita and Goh, 2018; Wang et al., 2020; Yue et al., 2022; Zhu et al., 2024). The reason for this morphological effect was explained by the upregulation under blue light of genes involved in glycolysis/gluconeogenesis and the pentose phosphate pathway both involved in generating reducing equivalents and ATP (Wang et al., 2020) and consequently enhance mushrooms growing. This upregulation was observed in the cap under Blue light, and in the stipe under Red light and Dark

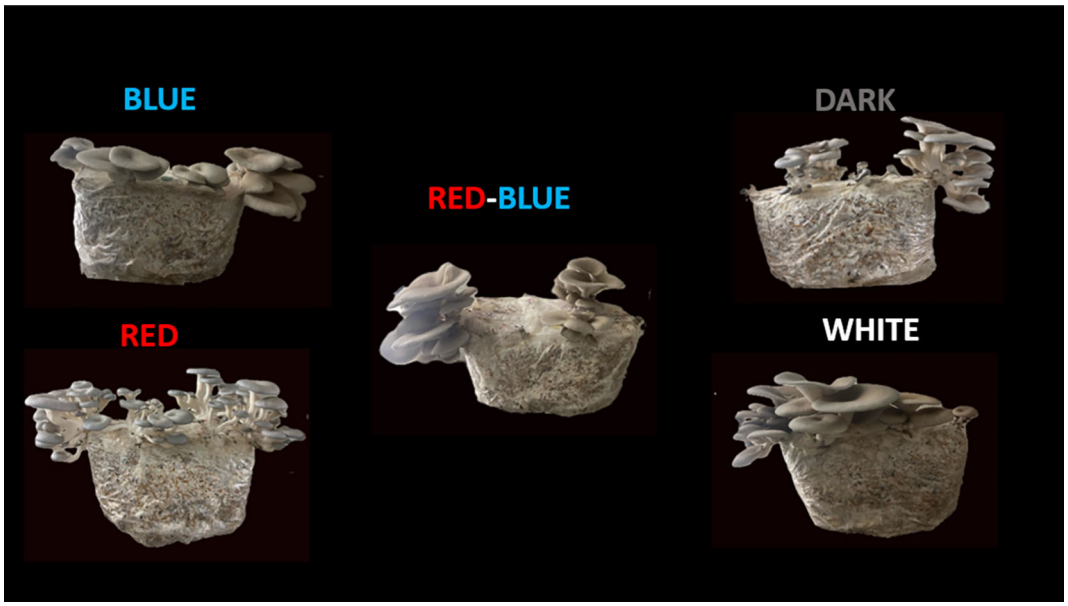

FIGURE 4
Representative photos of the fruiting bodies morphology according to light treatments.

10.3389/fhort.2025.1720226

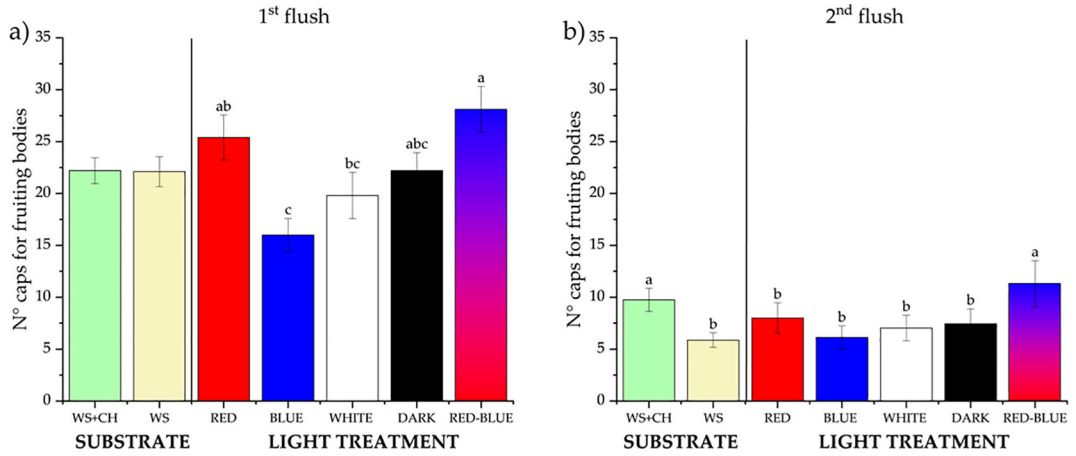

FIGURE 5
Effects of substrate (WS: wheat straw and WS+CH: wheat straw + cottonseed hulls) and light treatment on the number of caps for each fruiting body during 1st **(A)** and 2nd flush **(B)**. Within each parameter, values without common letters differ for $p \leq 0.05$ according to Tukey's HSD test. Bars indicate the standard errors.

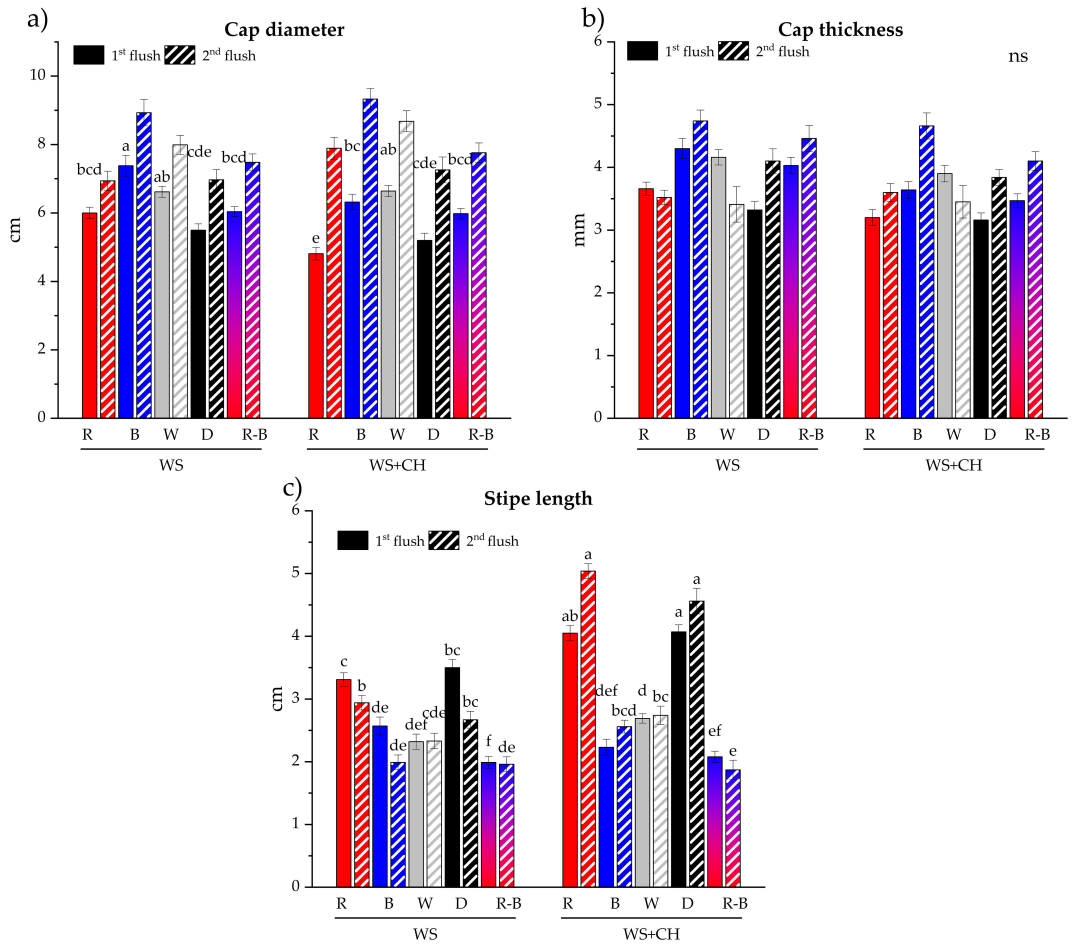

FIGURE 6
Effect of interaction between substrates and light wavelengths during 1st and 2nd flush (full and stripe colors respectively) on cap diameter **(A)** and thickness **(B)** and stipe length **(C)**. Within each treatment, values without common letters differ at p-value ≤ 0.05 according to Tukey's HSD test. Bars indicate standard error.

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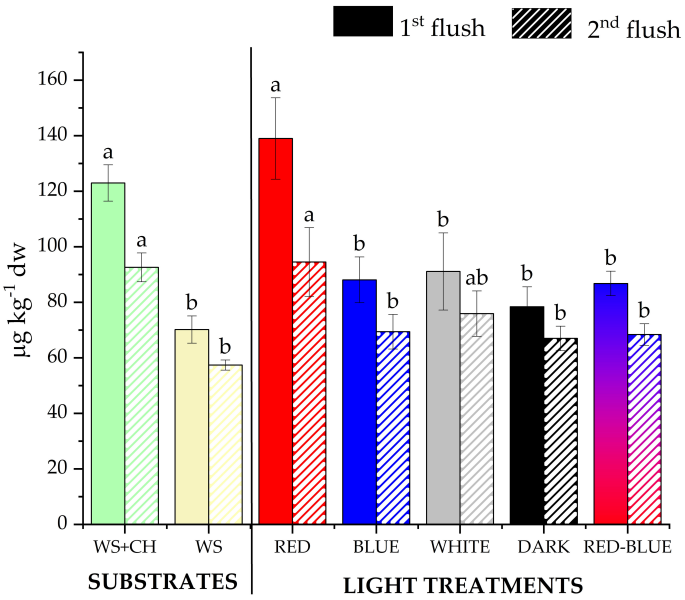

FIGURE 7
Effect of substrate (WS: Wheat straw and WS+CH: Wheat straw + Cottonseed hulls) and light treatment on vitamin D_2 content during the 1st (full color) and 2nd flush (stripe color). Within each parameter, columns without common letters differ for $p \leq 0.05$ according to Tukey's HSD test. Bars indicate the standard error.

condition (Wang et al., 2020). This result supports our findings and may explain the higher elongation of the stipe observed under Red and Dark light treatments.

Most of the morphological findings from this study revealed similar trends in mushrooms exposed to Red and Dark light. Research has identified Red light photoreceptors in mushrooms and basidiomycetes (Corrochano, 2019), indicating that *Pleurotus* can detect red radiation. However, the higher elongation of the stipe under Red light is not beneficial for mushroom production, since in the market there is a greater demand for mushrooms with a large cap rather than a long stipe. In addition, from an agronomic perspective, since Red light caused elongation levels similar to those observed in complete darkness, it appears unsuitable as a wavelength for artificial lighting in mushroom cultivation.

4.1.2 Substrate effect

Substrate had a significant effect on primordia and fruiting bodies production: in the 1st flush, WS hastened primordia appearance and in the 2nd flush WS+CH obtained a higher production of primordia and, consequently, of fruiting bodies and yield. Substrate is one of the most important factors for *Pleurotus* production and its chemical and physical characteristics can have a strong impact on timing and quality of the production. Several studies already underline the different performances of *P. ostreatus* cultivated with cottonseed hulls and wheat straw. For instance, Girmay et al. (2016) obtained a higher biological efficiency on cottonseed hulls compare to wheat straw and sawdust thanks to the faster decomposition rate which make cottonseed hulls a superior substrate than others lignocellulosic wastes. Yang et al. (2013) found a lower yield and BE on wheat straw compared to cottonseed hulls, thanks to the physical composition of straw and C/N ratio. In our study, the difference of C/N ratio between substrates was limited with the addition of nitrogen supplementation which probably caused a faster development of primordia and fruiting bodies in the 1st flush, then its effect ended at the beginning of the 2nd flush where WS+CH outperformed the WS substrate. Nitrogen supplementation was also added in WS+CH substrate but in a lower amount (to account for differences in starting nitrogen in the mixes). For this reason, probably the production of the 1st flush slowed down without affecting the final yield. Even if the different results occurred in 2nd flush WS and WS+CH displayed similar total BE above 50% (91-101%), indicating a good performance of both substrates in *P. ostreatus* production (Jayaraman et al., 2024). The nitrogen supplementation effect is also noticeable in cap diameter and thickness which were larger in the 1st flush for WS and for WS +CH, respectively. The enhancement in cap number on cotton seed hulls substrate was also noticed by Girmay et al. (2016) but, in contrast with our results, Yang et al. (2013) observed that *Pleurotus ostreatus* cultivated on wheat straw exhibited longer stipe length compared to substrate containing cotton seed hulls, likely due to the higher carbon-to-nitrogen (C/N) ratio of the wheat straw used in their study. Finally, the utilization of WS as a *P. ostreatus* substrate appeared to be significantly associated with the quantity and nature of nitrogen supplementation. The rapid degradation of cotton seed hulls by *Pleurotus* renders this waste a superior option when compared with other lignocellulosic materials.

10.3389/fhort.2025.1720226

TABLE 5 Effect of interaction substrate (WS: Wheat straw and WS+CH: Wheat straw + Cottonseed hulls) × light treatment on vitamin D2 content expressed on dry weight and on fresh weight in both flushes; different letters indicate significant differences for p ≤0.05 according to Tukey's HSD test.

		Vitamin D_2			
		µg kg^{-1} dw	µg kg^{-1} fw	µg kg^{-1} dw	µg kg^{-1} fw
		1st flush		2nd flush	
WS ×	Red	93.3 bcde	6.30	65.2 bcd	4.41
	Blue	61.9 de	3.84	51.1 d	3.17
	White	64.3 de	4.49	53.8 cd	3.76
	Dark	57.4 e	4.33	54.6 cd	4.12
	Red-Blue	73.9 cde	5.05	62.6 cd	4.28
WS+CH ×	Red	185.2 a	22.95	123.8 a	15.34
	Blue	114.3 bc	10.61	87.7 bc	8.14
	White	117.8 b	9.38	98.1 ab	7.81
	Dark	99.3 bcd	7.50	79.4 bcd	6.00
	Red-Blue	99.7 bcd	7.87	74.1 bcd	5.85

4.2 Nutritional values

4.2.1 Vitamin D_2

Vitamin D_2 content was affected by light treatments during both flushes. In this study, in contrast with other findings, different wavelengths belonging to visible light spectrum increased the amount of vitamin D_2. Previously, it was widely assumed that UV radiation increased vitamin D_2 content during post-harvest treatment, but the application of this type of radiation during cultivation is not recommended as it can cause DNA damages and oxidative stress in fungi cells (Fuller et al., 2015). In a recent article by De Bonis et al. (2024) it was observed that Blue light increased vitamin D_2 content

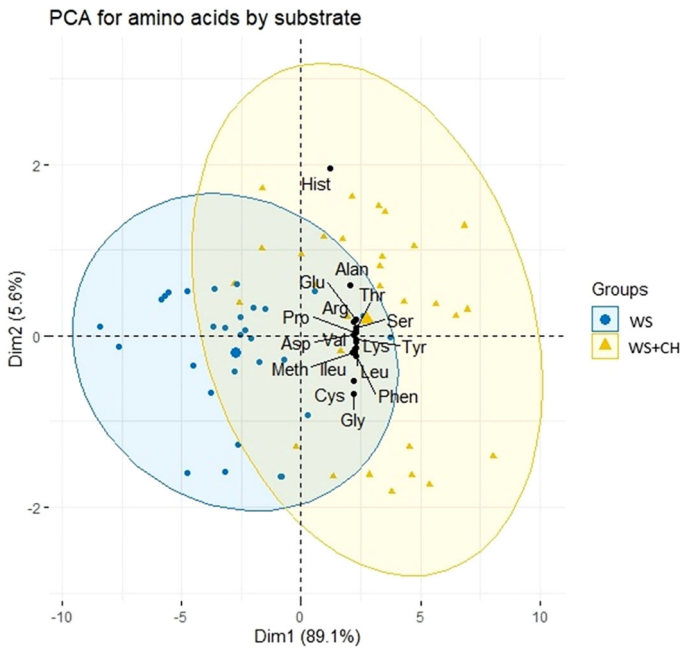

FIGURE 8
Principal component analysis (PCA) of amino acids content in Pleurotus ostreatus fruiting bodies cultivated in different substrates: Wheat straw (WS) and Wheat straw + Cottonseed hulls (WS+CH). Hist: Histidine, Thr: Threonine, Lys: Lysine, Meth: Methionine, Val: Valine, Ileu: Isoleucine, Leu; Leucine, Phen: Phenylalanine, Tryp: Tryptophan, Arg: Arginine, Ser: Serine, Gly: Glycine, Asp: Aspartic acid, Glu: Glutammic acid, Alan: Alanine, Pro: Proline, Tyr: Tyrosine, Cys: Cysteine.

10.3389/fhort.2025.1720226

TABLE 6 Recommended essential amino acids scoring patterns for adults (mg g^{-1} protein) and essential amino acid content (mg g-1 protein) of fruiting bodies cultivated on different substrates: Wheat straw + Cottonseed Hulls (WS+CH), Wheat straw (WS) and under different light wavelength (Blue, Red, White, Dark, Red-Blue).

Reference composition		Hist	Thr	Lys	SAA	Val	Ileu	Leu	AAA	Tryp
		mg g^{-1} protein								
		15	*23*	*45*	*22*	*39*	*30*	*59*	*38*	*6*
WS+CH	1st	35	47	85	21	44	36	66	60	12
	2nd	26	48	88	23	44	37	69	62	11
WS	1st	34	49	88	23	46	39	69	61	11
	2nd	27	48	87	24	44	37	68	62	11
BLUE	1st	36	51	91	23	48	40	71	65	12
	2nd	32	48	88	21	43	35	68	61	11
RED	1st	35	49	87	15	46	38	69	61	11
	2nd	22	49	92	22	48	40	72	65	11
WHITE	1st	36	47	85	15	43	36	66	59	11
	2nd	23	49	91	22	47	40	71	66	11
DARK	1st	33	48	86	22	46	38	67	60	11
	2nd	25	45	79	20	40	33	63	60	10
RED-BLUE	1st	34	46	81	21	41	34	63	57	11
	2nd	32	48	87	21	45	37	69	62	11

Hist, Histidine; Thr, Threonine; Lys, Lysine; SAA, Sulphur Amino acids :Methionine + Cysteine, Val, Valine; Ileu, Isoleucine; Leu, Leucine; AAA,Aromatic Amino Acids Phenylalanine+ Tyrosine.

compared to natural light in a mushrooms facility, suggesting that the application of wavelengths close to UV light during cultivation could promote vitamin D_2 biosynthesis as the post-harvest application of UV. In this study, Red light was the most effective to increase vitamin D_2 during both flushes. Red-Blue treatment did not obtain the same result, so the effect of Red light in this case was prominent during the last phase of fruiting bodies development. The most likely explanation of these contrasting results can be related to the different strain of *P. ostreatus* used which reacts differently under different light radiation. This hypothesis needs to be confirmed with further studies but other differences in vitamin D_2 biosynthesis among different mushrooms strains has been reported by Vieira Junior et al. (2022) for *Agaricus subrufescent* and by Ahlawat et al. (2016) for *Agaricus bisporus*. Another important result, reported in this experiment is the effect of substrate on vitamin D_2 biosynthesis. For both flushes, WS+CH increased the amount of vitamin D_2 compared to only WS underlining the importance of cultivation substrate in *P. ostreatus* vitamin D_2 biosynthesis. To the best of the author's knowledge, no studies have specifically addressed the effect of substrate on vitamin D_2 production in edible mushrooms. According to the recommended daily intake (RDI) amount of Vitamin D_2 indicated by the (European Food Safety Authority (EFSA), 2017), the consumption of 150 g of fresh *P. ostreatus* cultivated in WS+CH substrate under Red light in the 1st and 2nd flush can provide the 22.95% and 15.34% of RDI of vitamin D_2 respectively.

4.2.2 Crude protein and amino acid contents

Proteins and amino acids content in cultivated edible mushrooms is strongly influenced by the substrate used (Cueva et al., 2017; Salami et al., 2016), as confirmed by the results of this experiment. According to the literature, protein content in *Pleurotus* species typically ranges between 17% and 40% (Khan and Tania, 2012) and is also affected by mushroom strains (Manzi et al., 1999). In this study, the protein content across both flushes fell within 25% and 19%, and was strongly correlated with the C/N ratio, as also reported by Cueva et al. (2017).

The predominant AA's identified (i.e. lysine, threonine, glutamic acid, and aspartic acid) are consistent with findings on other *Pleurotus* species (Manzi et al., 1999; Tagkouli et al., 2020). Notably, glutamic and aspartic acids are associated with the umami flavor, characteristic of mushrooms taste (Bach et al., 2017). Principal component analysis (PCA) highlights that substrate composition significantly influences the amino acid profile. The distinct separation along Dim1, combined with the unique contributions of individual amino acids, suggests that substrate modification alters the biochemical composition in measurable ways.

Light treatments had limited effects on the overall amino acid content. However, specific AAs, such as valine, isoleucine, and histidine, were affected by light conditions. Valine and isoleucine levels increased under Blue light but not under Red-Blue treatments, suggesting a positive effect of blue wavelength specifically during mycelium development. In contrast, histidine content was enhanced

under both Blue and Red-Blue treatments, indicating a beneficial effect during fruiting body growth. Blue light demonstrated a positive impact on some AAs as Valine and Isoleucine in this study. Conversely, Yue et al. (2022), who worked with *P. eryngii*, reported more substantial differences across all AAs under varying light conditions, particularly with red light treatment. The comparison between these two discrepant outcomes suggests a differential response of *Pleurotus* species to light. Therefore, further studies are necessary to gain a deeper understanding of the amino acid-related light response mechanisms in *Pleurotus* spp.

To evaluate the quality of mushroom protein and its amino acid profile, the chemical score of each AA was calculated as the ratio of AA content (mg g^{-1} of protein) to the reference value for essential AAs provided by WHO (2007). The limiting amino acids were sulfur-containing amino acids (methionine + cysteine), which exceeded the WHO reference for adults only on the WS substrate but fell below the reference values in most other cases. The limiting AAs varied among different studies, mostly for the differences in the strain used: Manzi et al. (1999) indicated leucine and lysine as limiting AAs dependent by *P. ostreatus* strains, whereas Bach et al. (2017) observed a limiting amount of leucine in their trial.

5 Conclusion

Blue light appears to be the most promising light wavelength to apply in *P. ostreatus* cultivation to improve yield and cap's dimension. The higher productive outcomes observed in the Red-Blue treatments suggest that Blue light could be applied selectively during specific stages of fruiting body development, thereby possibly reducing energy consumption and operational costs.

Red light, while not significantly influencing the morphological or productive traits of *P. ostreatus*, played an important role in vitamin D_2 biosynthesis. This finding highlights the need for further investigation into the potential applications of Red light in enhancing vitamin D_2 content. Moreover, the significant effect of the substrate observed in this study suggests that vitamin D_2 levels could potentially be enhanced not only through artificial lighting but also via substrate optimization. If strain and substrate play such a fundamental role in vitamin D_2 biofortification, further research is required to elucidate the underlying biological pathways involved in vitamin D_2 biosynthesis and to investigate the genetic variability among different strains.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MD: Conceptualization, Methodology, Writing – review & editing, Data curation, Investigation, Writing – original draft, Visualization, Formal Analysis. JP: Writing – review & editing, Investigation, Supervision, Validation, Conceptualization, Methodology. CN: Conceptualization, Resources, Validation, Project administration, Writing – review & editing, Methodology, Investigation, Software, Funding acquisition, Supervision.

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Conflict of interest

The authors declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer PP declared a shared parent affiliation with the author JP to the handling editor at the time of review.

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