Food Chemistry 463 (2025) 141487

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Fruit-specific effects of tryptophan and melatonin as active components to extend the functionality of red fruits during post-harvest processing

Alba Arabia [a,b], Paula Muñoz [a,b], Sergi Munné-Bosch [a,b,*]

[a] *Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona, Barcelona, Spain*
[b] *Research Institute of Nutrition and Food Safety, University of Barcelona, Barcelona, Spain*

ARTICLE INFO

Keywords:
Melatonin
Tryptophan
Red fruits
Quality
Shelf life
Phytohormones

ABSTRACT

Preserving quality attributes in the distribution chain is a challenging task, particularly in fruits with a brief shelf life. The application of melatonin in cherries, raspberries, strawberries and blueberries stored at room temperature was evaluated, as well as the effects of its precursor (tryptophan) to determine their specificity and interchangeable feasibility for post-harvest applications. The results demonstrated that melatonin is effective in all tested fruits, reducing deterioration rate and its severity, preserving fruit firmness and reducing darkening and weight loss. Furthermore, tryptophan applications incremented melatonin contents in strawberries and blueberries and delayed decay in both fruits. Melatonin reduced postharvest losses in all studied fruits related to its antisenescent properties, while the beneficial impact of tryptophan in extending shelf life was fruit-specific and appeared to be partly mediated by melatonin. Melatonin and tryptophan must be considered as active components of new formulations for extending the shelf life of red fruits during post-harvest processing.

1. Introduction

Red fruits include, among others, strawberries (*Fragaria x ananassa*), raspberries (*Rubus idaeus*), blueberries (*Vaccinium* spp.), and sweet cherries (*Prunus avium*). These fruits are considered important sources of micro- and macronutrients, sugars, dietary fiber, vitamins and minerals (Kumar et al., 2018). However, most of their health-promoting properties have been largely associated with their high levels of bioactive compounds (ascorbic acid, flavonoids, anthocyanins, and tannins) with known antioxidant capacity, making them functional foods (Battino et al., 2009; Giampieri et al., 2013; McCune et al., 2011). Nevertheless, the perishability of red fruits is a striking drawback for commercialization amid they are highly prone to mechanical damage during harvesting and transportation, with a shelf life under ambient conditions limited to just 2 to 7 days (Piljac-Žegarac & Šamec, 2011; Shah et al., 2024). Likewise, they are easily spoiled and do not store very well for long periods, challenging their handling and distribution (Huynh et al., 2019; Vu et al., 2011). Moreover, it is not always possible to preserve fruit under cold conditions, such as during transport from fields to processing plants or storage chambers, at distribution sites in warehouses, and at various stages of technological fruit processing (Shoji et al., 2022). An additional challenge to this equation is that red fruits must be harvested at their optimum ripening point to obtain the best eating quality, which should be preserved during the postharvest period. After harvest, overripening is one of the main causes of red fruit decay inducing short shelf life and senescence. It encompasses different physiological changes such as water loss, excessive softening, color darkening and rot susceptibility (Kumar et al., 2018). That, in turn, will negatively affect the quality parameters of fruits and result in a general loss of nutritional and organoleptic attributes, leading to products being discarded (Pott et al., 2020). Although none of the changes that occur during overripening can be stopped, it is urgently needed the development of new sustainable postharvest technologies for a wide variety of fruits to delay these events at different points of the supply chain. In this regard, overripening is well-regulated by a synergistic and dynamic hormonal interplay that determines postharvest fruit quality (Kumar et al., 2014; Perotti et al., 2023). Therefore, the modulation of contents and activity of distinct plant hormones and regulatory molecules throughout postharvest might be crucial for controlling and prolonging fruit shelf life.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a natural, multifunctional and ubiquitous indolamine in plant species, including fruits. Melatonin is synthesized from the amino acid tryptophan, one of the least abundant amino acids in plant cells. This limited presence is

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* Corresponding author at: Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona, Barcelona, Spain.
E-mail address: smunne@ub.edu (S. Munné-Bosch).

https://doi.org/10.1016/j.foodchem.2024.141487
Received 22 July 2024; Received in revised form 18 September 2024; Accepted 28 September 2024

explained because tryptophan is essential as a N source for synthesizing proteins and N compounds and acts as a biosynthesis precursor of phytohormones and secondary metabolites (Mukherjee, 2018). Even since the melatonin discovery in plants, there has been a great interest in the functional, biotechnological, nutraceutical, and evolutionary aspects of this bioactive molecule. It is found in varying amounts in a wide range of species in all plant organs including fruits (Arabia et al., 2023). Moreover, its pathway is highly coordinated and well-regulated (Mukherjee, 2018). Tryptophan is converted into tryptamine by tryptophan decarboxylase (TDC) and then into serotonin through the enzyme tryptamine 5-hydroxylase (T5H). Further, melatonin is synthesized from serotonin in two reactions including 3 enzymes: serotonin *N*-acetyltransferase (SNAT), *N*-acetylserotonin *O*-methyltransferase (ASMT), and caffeic acid *O*-methyltransferase (COMT) (Back et al., 2016; Liu et al., 2022; Negri et al., 2021). Additionally, melatonin can also be a precursor for metabolites being rapidly converted to 2-hydroxymelatonin (2-HM) by melatonin 2-hydroxylase (M2H) (Byeon et al., 2015). Besides being an excellent antioxidant, there is increasing evidence that melatonin can act as a signaling molecule performing a multitude of regulatory functions in plants (Arnao & Hernández-Ruiz, 2019; Chen et al., 2022; Verde et al., 2023; Yang et al., 2021).

The multiple effects of melatonin in fruits have been extensively reviewed over the past 10 years. It has been reported that melatonin can be involved in fruit ripening enhancing the efficiency and biosynthesis of other antioxidants, regulatory molecules and phytohormones that will determine the fruit defense system and final quality (Liu et al., 2019; Mansouri et al., 2021; Qu et al., 2022; Shan et al., 2022; Sun et al., 2015; Wang et al., 2020). The treatment of exogenous melatonin to extend postharvest shelf life has been studied in a wide variety of fruits (Arabia et al., 2022; Onik et al., 2021; Shah et al., 2023; Wang et al., 2019). Moreover, in the case of red fruits, melatonin maintains quality in cold-stored blueberries (Magri & Petriccione, 2022), delays senescence in sweet cherries stored at both 4 °C and 20 °C (Miranda et al., 2020; Pang et al., 2023), and prevents decay in strawberries by enhancing antioxidant systems and specific metabolic pathways both at cold and room temperature (Liu et al., 2018; Promyou et al., 2023). For red raspberries, pre-harvest melatonin treatment helps preserving nutritional quality and reducing postharvest oxidative stress (Shah et al., 2024). Therefore, melatonin treatment can be a worthwhile and health-conscious alternative to consider for alleviating postharvest deterioration. Nonetheless, despite all the previous reports on melatonin, there is a lack of consistency on doses, methods of application and experimental conditions in the literature that lead to very different results and therefore, making it difficult to recommend its implementation as a universal postharvest strategy for a wide range of fruits. In this study, we aimed to evaluate the effectivity and mode of action of applying exogenous melatonin and tryptophan (as a highly cost-effective alternative) on four different red fruits under the same postharvest conditions to determine the uniformity of their effects on postharvest shelf life. The specific objective was to determine the impact of exogenous applications of melatonin and tryptophan on the shelf life and organoleptic and nutritional quality of red fruits, while assessing how these treatments influence the content of various derived-indolamines and ripening-related phytohormones.

2. Materials and methods

2.1. Fruit materials, treatments and samplings

Different red fruits with botanical differences according to origin and composition, being simple or aggregate fruits, and true or false fruits were used to understand the effects of melatonin and tryptophan for broad spectrum applications. Red raspberries (*Rubus idaeus* Driscoll's variety) were harvested from an orchard in Huelva (Spain, provided by Driscoll's) at the end of May 2023, blueberries (*Vaccinium corymbosum* cv. Duke) were harvested from an orchard in Cantabria (Spain) in the middle of June 2023, strawberries (*Fragaria x ananassa* cv. Albion) from an orchard in Sant Cebrià de Vallalta (Catalonia, Spain) at the end of June 2023 and cherries (*Prunus avium* cv. Lapins) from an orchard in Zaragoza (Spain) in July 2023 (Fig. S1). All of them were collected at commercial harvest maturity and were immediately transported to a laboratory at the University of Barcelona (Barcelona, Spain). Fruits of uniform size that were free from mechanical damage, plant diseases, and insect pests were selected for the experiment. Then, fruits were randomly divided in three groups and were sprayed by nebulization with melatonin solution (100 μM), L-tryptophan solution (100 μM) and distilled water (control). To facilitate proper solution absorption, 0.1 % Tween 20 was used as a surfactant in treatments and control solutions. Subsequently, fruits were allowed to dry naturally at room temperature (25 °C). Finally, fruits were distributed in 20 commercial plastic boxes of 125 g for raspberries and blueberries (28 and 50 fruits in each box, respectively) and in 0.5 kg boxes for strawberries (40 fruits in each) and 0.4 kg boxes for cherries (50 fruits in each). Fruits remained at room temperature, in facilities equipped with temperature controllers to minimize fluctuations, until control fruit boxes reached 70 % decay. For each species, 10 boxes were used as replicates for daily monitoring of fruit decay and weight loss. On the other hand, the other 10 boxes were used as replicates for each day's analysis of firmness, hydration and color. Moreover, 3 fruits of each replicate were frozen in liquid N_2 at 0 % decay (start point) and when control boxes reached 25 % decay and 70 % decay (end point). Fruits were kept at −80 °C until biochemistry analysis.

2.2. Determination of decay incidence, severity and weight loss

Fruit decay was monitored daily until control fruits reached 70 % deterioration. The incidence of fruit decay was determined by the number of fruits showing decay symptoms (such as lesions, overripening symptoms or visible fungal growth) relative to the total number of fruits in each replicate and expressed in percentage (%). The severity of the decay was scored as follows: 0: no decay, 1 = 0–25 % surface damage, 2 = 25–50 % surface damage, 3 = 50–75 % surface damage, 4 = 75–100 % surface damage. The decay rate was calculated with the following equation:

$$decay\ rate(\%) = \sum \frac{AxB}{CxD}\ x\ 100$$

where A is the decay scale, B is the number of fruits in that scale, C is the number of total fruits (varies according to fruit type) and D is the highest scale of decay (D = 4). The weight loss rate of the fruits in each plastic box was calculated by the percentage of difference between initial weight and final weight compared to the initial weight.

2.3. Analysis of color, firmness and fruit hydration

Fruit color, firmness and hydration were measured daily on 3 fruits from each replicate ($n = 10$). Peel color was determined by measurements of the basic parameters of L*(lightness), a* (redness), b*(yellowness), C* (chroma) and Hue angle using a colorimeter (Konica Minolta Chroma Meter CR400–410) and taking two measurements per fruit, one on each side of the fruit. Firmness (N) was assessed in strawberries and cherries using a PCE-FM200 penetrometer (PCE Group, UK) with a motorized force test stand (PCE-VTS 50) equipped with 8 mm diameter cone probe under the condition of 5 mm depth and at a speed of 500 mm·min^{-1}. In the case of blueberries and raspberries, the penetrometer was equipped with a compression probe of 24 mm diameter under the same conditions. Finally, the hydration of the fruits was analyzed using the formula [(Fresh Weight-Dry Weight / Dry Weight] after incubating the samples at 70 °C for two weeks.

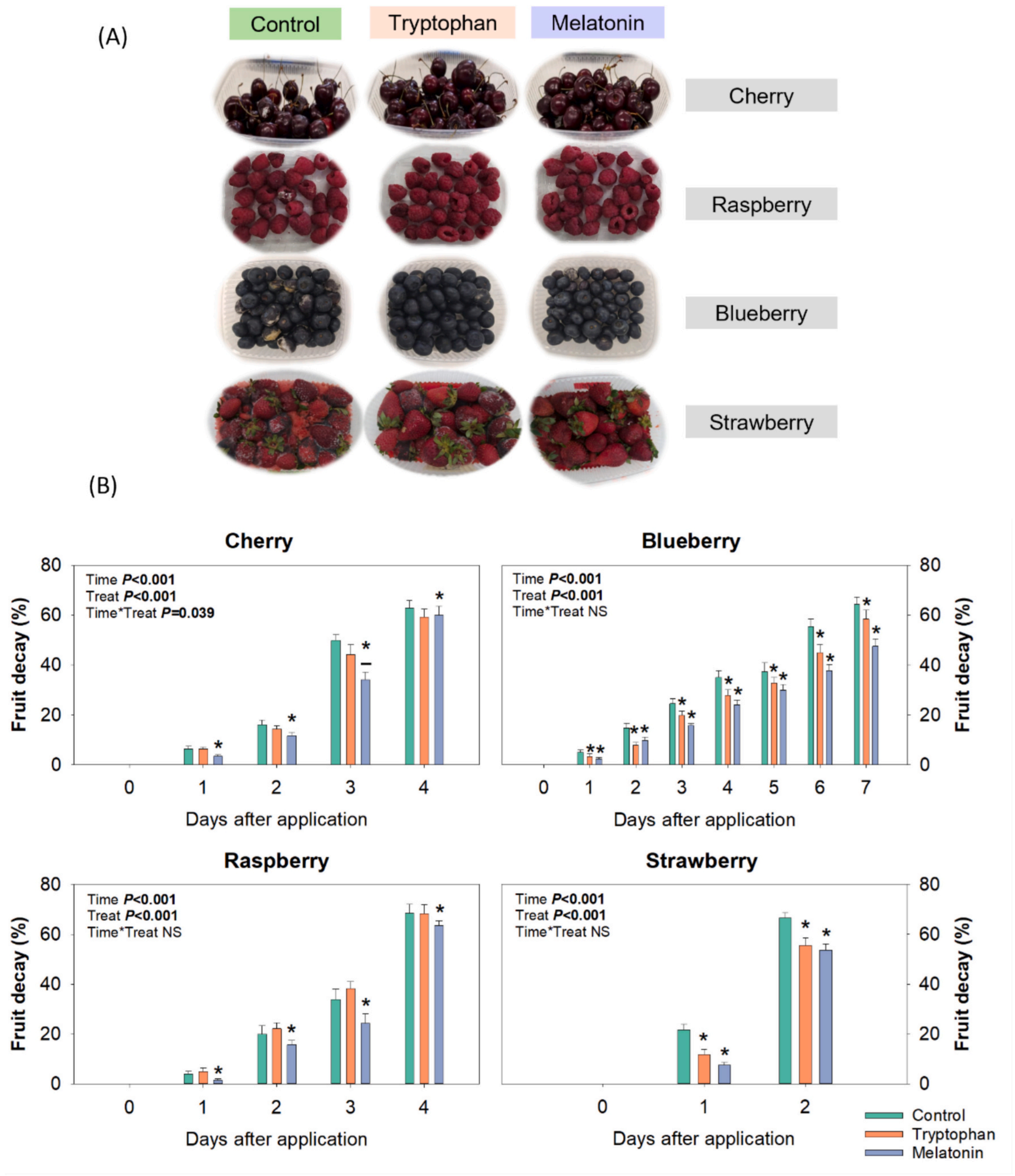


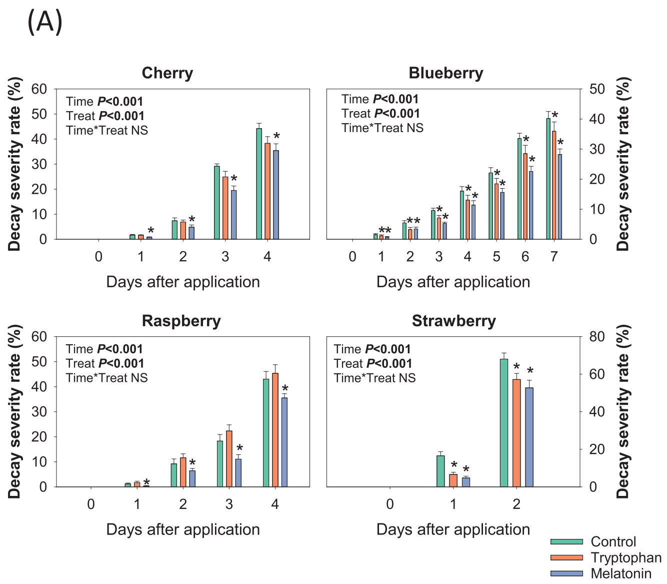
Fig. 1. (A) Appearance of cherries, raspberries, blueberries and strawberries at the end of the storage after non-treatment (control) or 100 μM melatonin and 100 μM tryptophan application. (B) Fruit decay (%) of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of $n = 10$ boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences between the control and that treatment during storage ($P < 0.05$). Underlined asterisk ($\underline{*}$) indicates significantly different values between the control and treated fruit on that day ($P < 0.05$).

2.4. Determination of total soluble solids, titratable acidity and pH

For estimating organoleptic fruit quality, total soluble solids (TSS), titratable acidity (TA) and pH were evaluated. For the analysis of TSS, 2 g of powdered frozen samples were homogenized with 10 ml of H_2O MilliQ and the obtained juice was used to determine °Brix in 1 mL using a refractometer (Hannah Instruments, Italy), as described by Boulton et al. (1999). Furthermore, pH was evaluated using a pH meter (Hanna instruments, Woonsocket, RI, USA). For TA measures, a 1:10 dilution of the juice with H_2O MilliQ was used with 0.1 M NaOH and 1 % phenolphthalein as an indicator, to estimate the quantity of major acid in each fruit following the method described by Latimer (2012).

2.5. Analysis of anthocyanins, total phenols, and total antioxidant capacity

To determine total anthocyanin, phenol content, and antioxidant capacity, methanolic extracts were used. In summary, 100 mg of each frozen sample were extracted in 750 μl 100 % (*v*/v) cold methanol. After vortexing, the extracts were ultrasonicated (Branson 3800 ultrasonic cleaner, Bransonic, United States) for 30 min in an ice bath at 4 °C and centrifuged (Labnet International Inc.) at 13000 rpm for 10 min and 4 °C. The supernatants were recovered in a new tube and the remaining pellet underwent one additional re-extraction with 750 μl of cold 100 % (v/v) methanol, repeating the previously described procedure. For



(C)

Melatonin application				
	Cherry	Raspberry	Blueberry	Strawberry
Fruit Decay	↓	↓	↓	↓
Decay severity rate	↓	↓	↓	↓
Fruit weight loss	↓	-	↓	↓
Hydration	-	-	-	-
Firmness	↑	↑	-	↑
L*	-	↑	-	-
a*	-	↓	↑	↑
b*	-	↓	-	↑
C*	-	-	-	↑
Hue	-	↑ Red	↑ Violet	-
TSS	-	-	-	-
TA	-	-	-	↓
TSS/TA	-	-	-	-
pH	-	-	-	-

Tryptophan application				
	Cherry	Raspberry	Blueberry	Strawberry
Fruit Decay	-	-	↓	↓
Decay severity rate	-	-	↓	↓
Fruit weight loss	↓	↑	↓	↓
Hydration	-	-	-	-
Firmness	↑	↑	-	↑
L*	-	-	-	↑
a*	-	-	↑	-
b*	-	↓	↓	-
C*	-	-	↑	-
Hue	-	↑ Red	↑ Violet	-
TSS	-	-	-	↓
TA	-	-	-	-
TSS/TA	-	-	-	↓
pH	-	-	-	-

Fig. 2. (A) Decay severity rate (%) and (B) firmness of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences between the control and that treatment during storage ($P < 0.05$). (C) Summary table on the changes in the different physicochemical quality parameters of the fruits (cherries, raspberries, blueberries and strawberries) after melatonin and tryptophan application. Up and down arrows indicate an increase or a decrease of that parameter, respectively. Dashes (-) indicate no change. L* = Lightness, a* = redness, b* = yellowness, C* = Chroma, Hue = Hue angle, TSS = Total Soluble Solids, TA = Titratable acidity.

anthocyanins, acidification was carried out with 10 N HCl (~37 %), and absorbance was measured at 530 nm using a spectrophotometer (CE Aquarius UV/Visible, Cecil Instruments Ltd., Cambridge, United Kingdom). The anthocyanin content was calculated using cyanidin-3-glucoside equivalents, following the method described by Siegelman and Hendricks (1958).

To estimate the total phenolic content (TPC), a volume of 100 μl of the prior extract was taken, and 1 ml of Folin-Ciocalteu reagent (diluted 1:10 with H_2O MilliQ) was added. This mixture was incubated for 4 min and 800 μl Na_2CO_3 5 % were added. Absorbances were measured at 765 nm spectrophotometrically (CE Aquarius UV/Visible, Cecil Instruments Ltd., Cambridge, United Kingdom). TPC was calculated using a gallic acid standard curve, as described by Singleton and Rossi (1965) and Li et al. (2007) expressed in mg gallic acid equivalents (GAE) / 100 g FW. The total antioxidant capacity (Radical Scavenging Activity, RSA) was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as was described in Barros et al. (2008) and Poojary et al. (2015). RSA was quantified with a gallic acid standard curve and indicated as mg GAE / 100 g FW.

2.6. Determination of melatonin, its precursors, 2-hydroxymelatonin and plant hormones content

Tryptophan, serotonin, melatonin, 2-hydroxymelatonin and ripening-related phytohormones including abscisic acid (ABA), jasmonates (12-*oxo*-phytodienoic acid (OPDA), jasmonic acid (JA) and the conjugated form JA-Ile), salicylic acid (SA), cytokinins (CKs) including trans-zeatin (t-Z), its riboside trans-zeatin (t-ZR), 2-isopentenyl adenine (2iP) and its riboside isopentenyl adenosine (IPA), indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) and gibberellic acid (GA_3) were extracted and quantified by ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UHPLC/ESI-MS/MS). Ground and frozen fruits (100 mg) were extracted with 200 μl cold 100 % (*v*/v) methanol containing deuterium-labeled hormones (Olchemim Ltd., Olomouc, Czech Republic), which were used as internal standards. The extracts were subjected to ultrasonication (Branson 3800 ultrasonic cleaner, Bransonic) in an ice bath at 4 °C for 30 min, followed by a 10 min centrifugation at 13000 rpm and 4 °C (Labnet International Inc.). The supernatant was collected, and the pellet was reextracted. Both supernatants were merged and filtered with hydrophobic PTFE filters of 0.22 μm (Phenomenex, Torrance, CA, United States) prior to UHPLC/ESI-MS/MS analysis. High-performance liquid chromatography was coupled to a triple quadrupole mass spectrometer (QTRAP 4000, AB Sciex, Concord, Ontario, Canada) for analyses as described by Müller and Munné-Bosch (2011) but with some modifications. Solvent A was acetonitrile with 0.05 % glacial acetic acid and solvent B was water with 0.05 % glacial acetic acid. The flow rate was set at 0.5 mL/min and a LUNA C18 column (Phenomenex Inc., United States [1,6 μm, 100 × 2,1 mm]) was used. In addition, tryptophan, serotonin, melatonin and 2-hydroxymelatonin were included in the method. To detect these compounds, positive mode (ESI+) was used. For tryptophan, the monitored product ion was 188 *m/z* and the collision energy (CE) 15 V, for serotonin it was 160 m/z and 13.5 V, for melatonin it was 174 m/z and 20.6 V and finally, for 2-hydroxymelatonin 189.3 m/z and 18 V. Quantification was made considering recovery rates for each sample by using the deuterium-labeled internal standards. For tryptophan, serotonin, melatonin and 2-hydroxymelatonin, d_4-Mel (178 m/z, 21.8 V CE) was used. Finally, calibration curves for each analyte were generated using MultiQuantTM 3.0.1 software.

2.7. Statistical analysis

For all analyzed parameters, a two-way analysis of variance (ANOVA) with interaction was performed considering time and treatment as factors. Multiple comparisons were conducted using a post-hoc Tukey test. Data were transformed for statistical analyses whenever necessary to achieve normal distribution and homoscedasticity of residuals; and if any transformation worked, individual nonparametric ArtAnova tests were performed. In all cases, differences were considered significant at a probability level of $p < 0.05$. All analyses were performed using RStudio (RStudio Team, 2020).

3. Results and discussion

3.1. Impact of melatonin and tryptophan post-harvest treatments on fruit decay and quality attributes

The storage of red fruits constitutes an important challenge, mainly because this type of fruits has a very thin epidermis that is sensitive to bruising and make them highly susceptible to deterioration and fungal infection. Consistently, the four fruits studied here had a very short, although still fruit-specific variable postharvest shelf life (Fig. 1). Blueberries had the greatest durability, up to 7 days at room temperature, and strawberries were the most perishable, only lasting 2 days after harvest. As indicated in Fig. S1, Duke blueberry is an early variety harvested at the beginning of the season, while Albion strawberry was already at the end of the season, which might have reduced its postharvest quality and greater fruit susceptibility (Yahia et al., 2019). As shown in Fig. 1A, strawberries became softer and lacked consistency, losing more than 50 % of water content (Fig. S2A) and promoting fungal appearance (Fig. 1A). Cherries and raspberries mean shelf life was up to four days after harvest, with their deterioration mainly marked by a decrease in firmness, hydration, and pathogen spoilage (Fig. 1, 2B, S2A).

Despite their short shelf life, melatonin treatment was effective in reducing the percentage of deterioration in all four studied fruits (Fig. 1B). For cherries and raspberries, melatonin was most effective in the first few days, reducing deterioration by up to 30 % compared with control fruits towards the third day of postharvest. Similarly, in the case of blueberries, melatonin reduced their deterioration by 30 % regarding control fruits throughout the entire storage period, with treated fruits showing less than 50 % decay on the last day. This positive effect was also very pronounced in strawberries, where deterioration was reduced by 65 % the first day after harvest and 20 % the second day in comparison to control fruits. Similar results were previously found in strawberries immersed in 100 μM melatonin but stored at 4 °C (Liu et al., 2018; Pang et al., 2020). In agreement with our results, Pang et al. (2023) also showed that soaked sweet cherries in 100 μM melatonin delayed their deterioration during storage at 20 °C.

Tryptophan, a cheaper and more cost-effective ingredient that was not investigated previously for the postharvest treatment of red fruits, also showed very positive results, but they were fruit specific (Fig. 1B). Tryptophan was effective for strawberries, reducing the percentage of deterioration by 45 % on the first day and by 16 % on the second day. Similarly, it achieved a 30 % reduction in blueberries decay during the first days and 15–20 % for the last few days. Nevertheless, it had no effect on the decay percentage in cherries and raspberries (Fig. 1B). Tryptophan is an aromatic amino acid that serves as a precursor of indolamines such as auxin, melatonin, phytoalexins, glucosinolates and both indole-and anthranilate-derived alkaloids. In this sense, it has been widely applied to plants in different ways to improve growth and productivity of various crops (Mosa et al., 2021; Mustafa et al., 2018), fruit quality through prebloom sprays in apples (Wójcik et al., 2019) and inhibit senescence in broccoli (Sohail et al., 2021), but to our knowledge, this is the first study showing positive effects for tryptophan during postharvest handling of fruits.

Melatonin also reduced the severity rate of fruit decay, particularly at the beginning of the postharvest period, showing reductions of 70 % in strawberries and raspberries, and 50 % in blueberries and cherries. Indeed, at the end of the storage, all melatonin-treated fruits had 20–30 % less severity of fruit deterioration (Fig. 2A). Melatonin also preserved fruit firmness during postharvest, with the most prominent effects in strawberries (Fig. 2B). This is consistent with a previous study in blueberries stored at 4 °C showing that 50 μM melatonin delayed pectin and cellulose degradation by inhibiting cell wall degrading enzymes, which is related to firmness maintenance (Liu, Shang, et al., 2023). Similarly to melatonin, tryptophan also attenuated the decay severity in blueberries and strawberries, as well as maintained fruit firmness in strawberries, raspberries, and cherries by more than 100 %, 30 %, and 20 % respectively (Fig. 2A, B, C). Both melatonin and tryptophan also led to less weight loss, with reductions of up to 20 % in blueberries, as well as positively affected surface color retention in blueberries, strawberries, and raspberries (Fig. S2B, S3, S4). These findings align with Miranda et al. (2020), who observed that the reduction in weight loss of sweet cherries after melatonin treatment was associated with the up-regulation of aquaporin and cuticle-related genes, and consequently to an improvement of water relations. Finally, no change in TSS/TA ratio was observed in red fruits after treatments, except in tryptophan-treated strawberries where a slight reduction in TSS occurred (Fig. S5). TSS and TA are important quality attributes for postharvest red fruits. El Sayed et al. (2014) reported an increase in TSS in pomegranate after preharvest tryptophan application, but there are no further reports on how tryptophan may influence sugar content during fruit overripening, which warrants further investigations.

Red fruits are renowned for their abundance of bioactive compounds, such as phenolic compounds which possess important biological activities and play an essential role in fruit protection and controlling senescence (Liu, Wei, et al., 2023; Valdés et al., 2015). Melatonin and tryptophan treatments did not result in an increase in total anthocyanin contents or total antioxidant capacity in any of the four fruits studied (Fig. S6, S7). However, exogenous applications of melatonin increased TPC in strawberries and blueberries by 36 % and 17 % at the end of storage, respectively (Fig. S6). Previous studies have demonstrated that melatonin can increase TPC during the postharvest period of various fruits such as plums, mulberries, peaches, and bananas alleviating the deterioration and extending the shelf life (Al-Qurashi et al., 2024; Arabia et al., 2022; Gao et al., 2018; Kakaei et al., 2024). Moreover, Magri and Petriccione (2022) found that melatonin increased polyphenols, flavonoids, and anthocyanins in blueberries after 2 weeks of cold storage, which mirrors our findings at room temperature. Similarly, Pang et al. (2020) reported melatonin up-regulating phenolic metabolism genes in

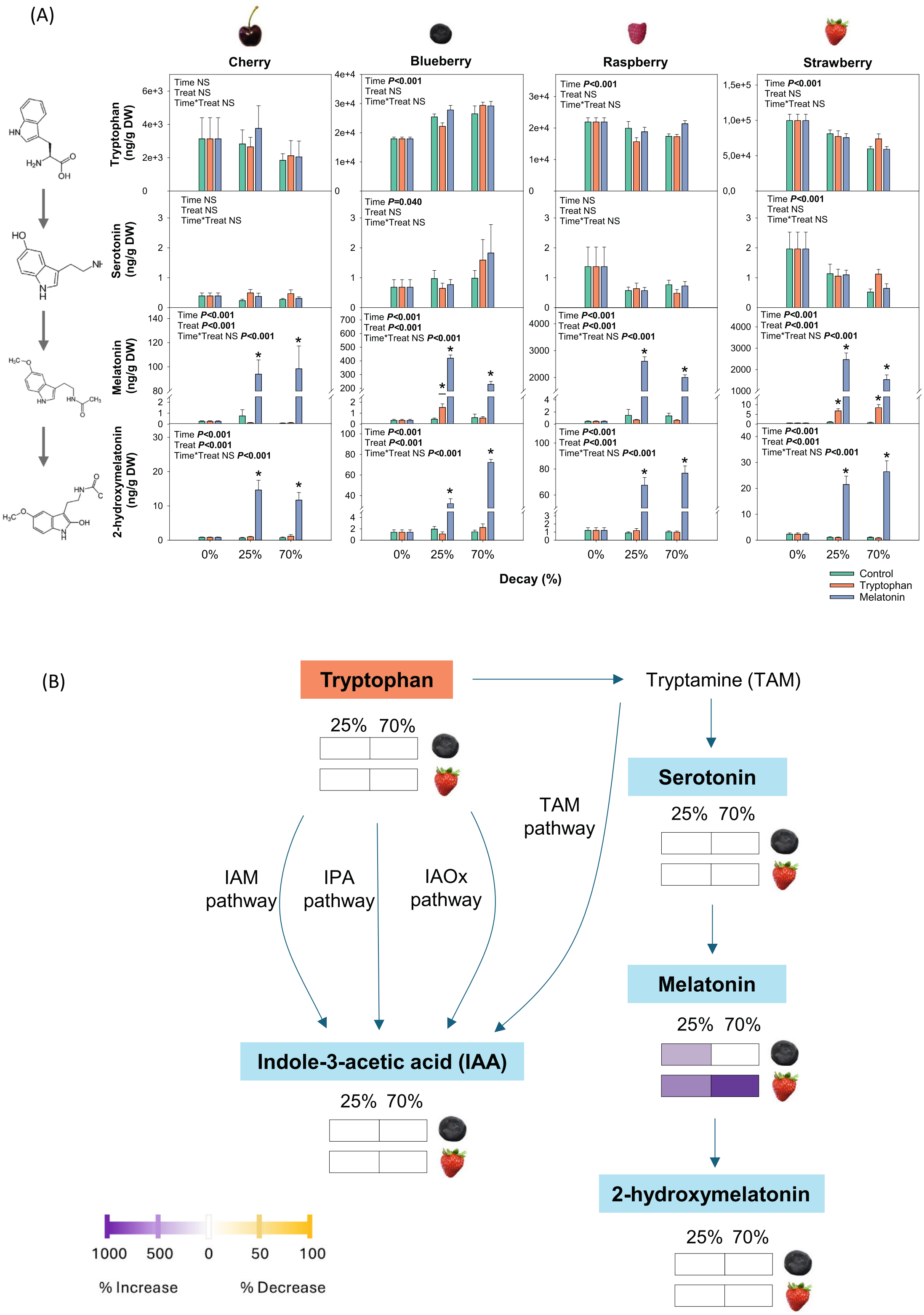


Fig. 3. (A) Endogenous content of tryptophan, serotonin, melatonin and 2-hydroxymelatonin of cherries, raspberries, blueberries and strawberries at 25 % decay and 70 % decay after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences between the control and that treatment during storage ($P < 0.05$). Underlined asterisk ($\underline{*}$) indicates significantly different values between the control and treated fruit on that decay percentage ($P < 0.05$). (B) Schematic representation of the changes in tryptophan, serotonin, melatonin, and indole-3-acetic acid contents after tryptophan application in blueberries and strawberries at 25 % and 70 % decay. An increase in their content is represented in purple colors, while a decrease in their levels is indicated in yellow colors. IAM = Indole-3- acetamide, IPA = Indole-3-pyruvic acid, IAOx = Indole-3-acetaldoxime, TAM = Tryptamine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

strawberries under cold storage, while Aghdam and Fard (2017) observed related effects in non-cold conditions. However, no differences were observed in cherries and raspberries in terms of TPC (Fig. S6) and the positive results on strawberries and blueberries occurred at the end of storage, too late to have a positive nutritional impact since deterioration was very advanced. Similarly, tryptophan treatment increased TPC at the end of storage in blueberries (Fig. S6). However, it slight decreased TPC at 25 % deterioration in the same fruit, which might reduce health benefits. It is interesting to note that in this fruit the dynamics in tryptophan-induced melatonin accumulation and the associated changes in TPC suggest that melatonin, rather than tryptophan, may be the substance directly modulating TPC in blueberries. Unfortunately, there is no previous evidence of other studies showing TPC variations upon tryptophan application in postharvest red fruits, thus further research is needed.

In summary, melatonin has proven to be effective in reducing decay and maintaining the quality of red fruits, even under challenging conditions for fruits, where extending shelf life is critical. Despite the botanical differences among the studied fruits, melatonin consistently showed positive results. Moreover, melatonin's efficacy was evident even when the postharvest life was already compromised, as seen with strawberries. Additionally, this study provides the first evidence that postharvest application of 100 μM tryptophan can reduce decay in red fruits, marking a promising new approach.

3.2. *Melatonin and tryptophan application alter derived-indolamines during postharvest of red fruits*

In the four fruits studied, endogenous melatonin was found at low concentrations in the range of 0.5–1.5 ng/g DW. After its exogenous application, its content increased significantly, reaching values of 100 ng/g DW in cherries, 400 ng/g DW in blueberries, and up to more than 2000 ng/g DW in strawberries and raspberries (Fig. 3A). However, despite the significant increase in melatonin in all the fruits, the extent of this increase varied depending on the fruit. This variation may be related to the activation capacity of its biosynthetic pathway as well as each fruit's ability to absorb the exogenous solution, which will be influenced by its cuticle. Nonetheless, in the case of strawberries, blueberries and raspberries, melatonin increments slightly diminished throughout the postharvest period. Furthermore, beyond increasing endogenous melatonin contents, which had already been described in other studies (Arabia et al., 2022; Liu et al., 2018; Liu, Shang, et al., 2023), the content of 2-HM also increased by more than 1000 % in all four red fruits (Fig. 3A). The endogenous contents of 2-HM found in control fruits were very similar to those of melatonin, around 1–2 ng/g DW in the different fruits. Here, exogenous melatonin increased 2-HM content to 26 ng/g DW in strawberries, 14 ng/g DW in cherries, and more than 70 ng/g DW in blueberries and raspberries. Melatonin can be metabolized to 2-HM through the enzyme M2H, which has a much higher catalytic activity compared to other melatonin biosynthetic enzymes (Byeon & Back, 2015). Moreover, Byeon et al. (2015) demonstrated that melatonin is metabolized rapidly to 2-HM and its accumulation predominates over that of melatonin, being the average 2-HM content 368-fold higher than the melatonin content. In this sense, other studies have also highlighted the role of 2-HM in plants. 2-HM can upregulate defense genes in plants, including *pathogenesis-related gene 1* (*PR1*) and *isochorismate synthase 1* (*ICS1*), suggesting it may play a partial role in defense against biotic stress (Back, 2021). Additionally, exogenous 2-HM treatment has been shown to alleviate oxidative stress in response to combined cold and drought stress (Lee & Back, 2019) and cadmium treatment (Shah et al., 2020). Recently, Korkmaz et al. (2023) also indicated that 2-HM confers multiple stress tolerance in pepper at seed germination stage having melatonin-like functions in enhancing abiotic stress tolerance. Moreover, Back (2021) suggested that the higher catalytic activity of melatonin catabolic enzymes amply demonstrates that melatonin's role (even in exogenous application) results from the combined effects of melatonin and its various metabolites, which possess unique features that differ from its parent molecule. Therefore, after melatonin application, part of the synthesized melatonin is rapidly metabolized into 2-HM, and it is possible that this compound also plays a role in fruit protection during postharvest. However, its specific physiological function in fruit overripening and senescence still needs to be clarified.

Regarding tryptophan treatment, it did not lead to an increase in the tryptophan content in any of the fruits studied, nor in serotonin levels (Fig. 3A). However, it raised the melatonin content throughout the postharvest period in strawberries, up to 11-fold compared to control fruits, rising from 1 ng/g DW in the control fruits to 8 ng/g DW in strawberries treated with tryptophan. Similarly, it also increased the endogenous melatonin content up to 3-fold in blueberries at the beginning of the postharvest period when they were at 25 % decay. Additionally, the application of tryptophan led to a reduction in the TRP/MEL ratio in blueberries and strawberries (Fig. S8), indicating an increase in melatonin levels relative to tryptophan. Nonetheless, there were no changes in the amount of melatonin in cherries and raspberries after tryptophan application. These results are consistent with the effectiveness of tryptophan in prolonging the shelf life of strawberries and blueberries, but not cherries and raspberries. Furthermore, in contrast to melatonin application, exogenous tryptophan did not lead to any changes in the 2-HM content. In plants, the relevance of tryptophan resides as a precursor of secondary metabolites such as IAA, serotonin and melatonin (Corpas et al., 2021). Generally, the signaling effect on plant growth and defense response following a tryptophan application has been associated with an actively metabolization to the phytohormone IAA (Jiang et al., 2022; Naveed et al., 2015). However, other studies also describe that tryptophan application in response to pathogenic infection in rice triggered defense responses via serotonin production (Ishihara et al., 2008). Similarly, a study on strawberry plants under short photoperiods and low temperatures showed that tryptophan application resulted in green foliage and fruit-setting flowers, linked to increased levels of serotonin and 2-HM in the flowers (Ayyanath et al., 2024). Contrary to these studies, in this case the beneficial effect of tryptophan in delaying overripening of strawberries and blueberries is not associated with an accumulation of serotonin or IAA (Fig. 3B). Therefore, these results indicate that tryptophan can extend the shelf life of red fruits when its metabolization to melatonin takes place. This finding aligns with previous research, such as the study by Xiang et al. (2024), indicating that applying tryptophan to grape berries before harvest enhances the melatonin content upon harvest.

Thus, in this study, we demonstrate that the positive effects of exogenous melatonin in prolonging fruit shelf life are mediated by the increase in its endogenous content and that of 2-HM, highlighting their potential physiological function. Indeed, melatonin application did not alter the levels of upstream molecules involved in its synthesis. Furthermore, we can also establish that tryptophan application leads to an increase in melatonin levels in fruits where it effectively delays overripening. Therefore, the beneficial effect of tryptophan in strawberries and blueberries is primarily associated with the accumulation of melatonin, rather than other indolamines such as IAA, serotonin or 2-HM.

3.3. *Variations in the contents of ripening-related phytohormones upon melatonin and tryptophan treatments during postharvest of red fruits*

The intricate signaling network between phytohormones is pivotal in regulating fruit senescence. This regulatory process encompasses both promoter and inhibitor signaling molecules, and their content throughout overripening plays an essential role in prolonging fruit shelf life (Xiang et al., 2021). In this study, the content of different plant hormones was analyzed to evaluate their relationship with the application of melatonin and tryptophan. In cherries and raspberries, no differences were found in the content of any of the studied plant

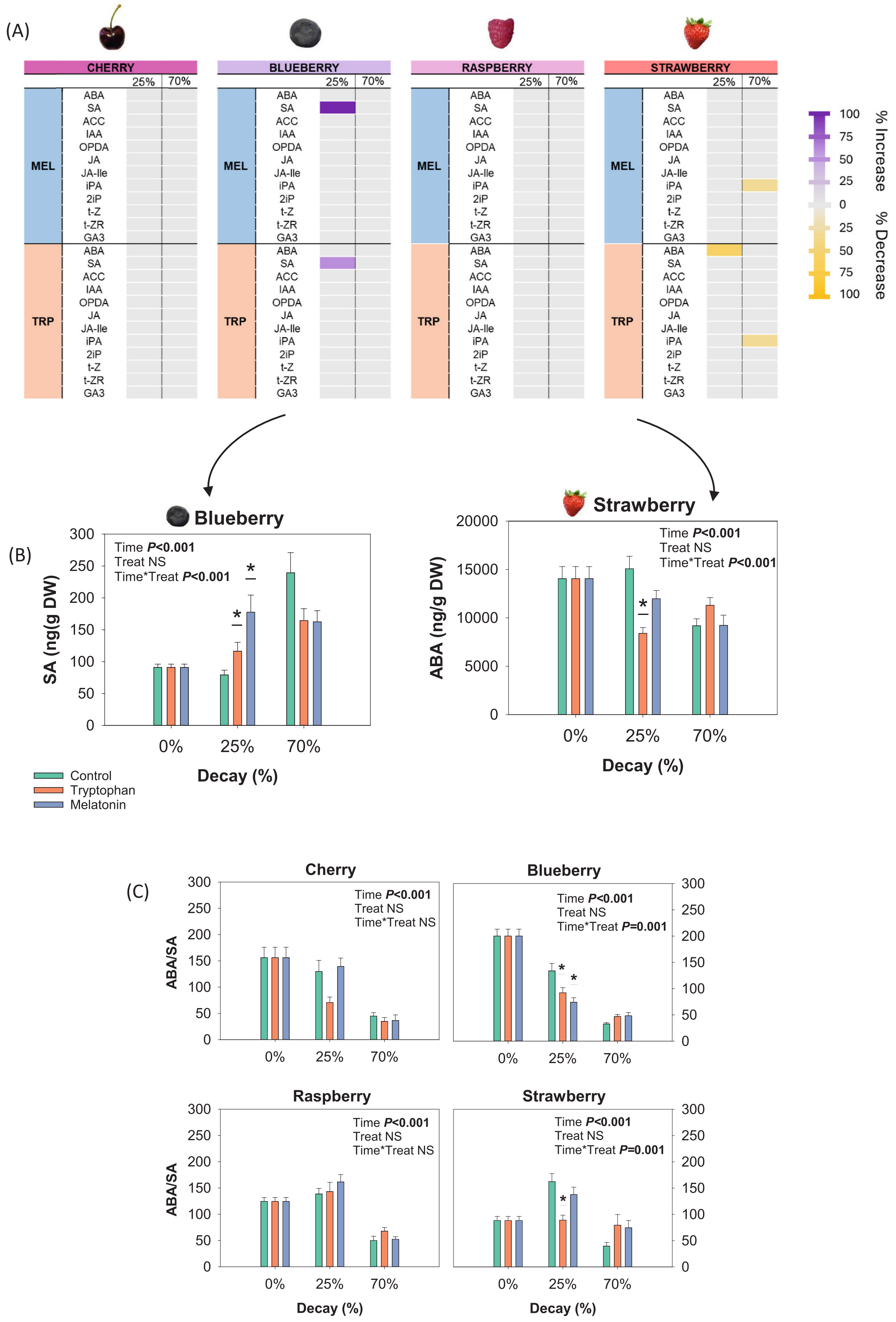


Fig. 4. (A) Changes in hormonal content due to melatonin (MEL) or tryptophan (TRP) treatment in cherries, raspberries, blueberries and strawberries. An increase in their content is represented in purple colors, while a decrease in their levels is indicated in yellow colors. (B) Endogenous content of salicylic acid in blueberries and abscisic acid in strawberries at 25 % decay and 70 % decay after melatonin and tryptophan applications. Control refers to untreated fruits. (C) ABA/SA ratio in cherries, raspberries, blueberries and strawberries at 25 % decay and 70 % decay after melatonin and tryptophan applications. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Underlined asterisk ($\underline{*}$) indicates significantly different values between the control and treated fruit on that decay percentage ($P < 0.05$). ABA = abscisic acid, SA = salicylic acid, ACC = 1-aminocyclopropane-1-carboxylate, IAA = indole −3-acetic acid, OPDA = 12-*oxo*-phytodienoic acid, JA = jasmonic acid, JA-Ile = jasmonoyl isoleucine, iPA = isopentenyl adenosine, 2iP = 2-isopentenyl adenine, t-ZR = trans-zeatine riboside, t-Z = trans-zeatin, GA3 = gibberellic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

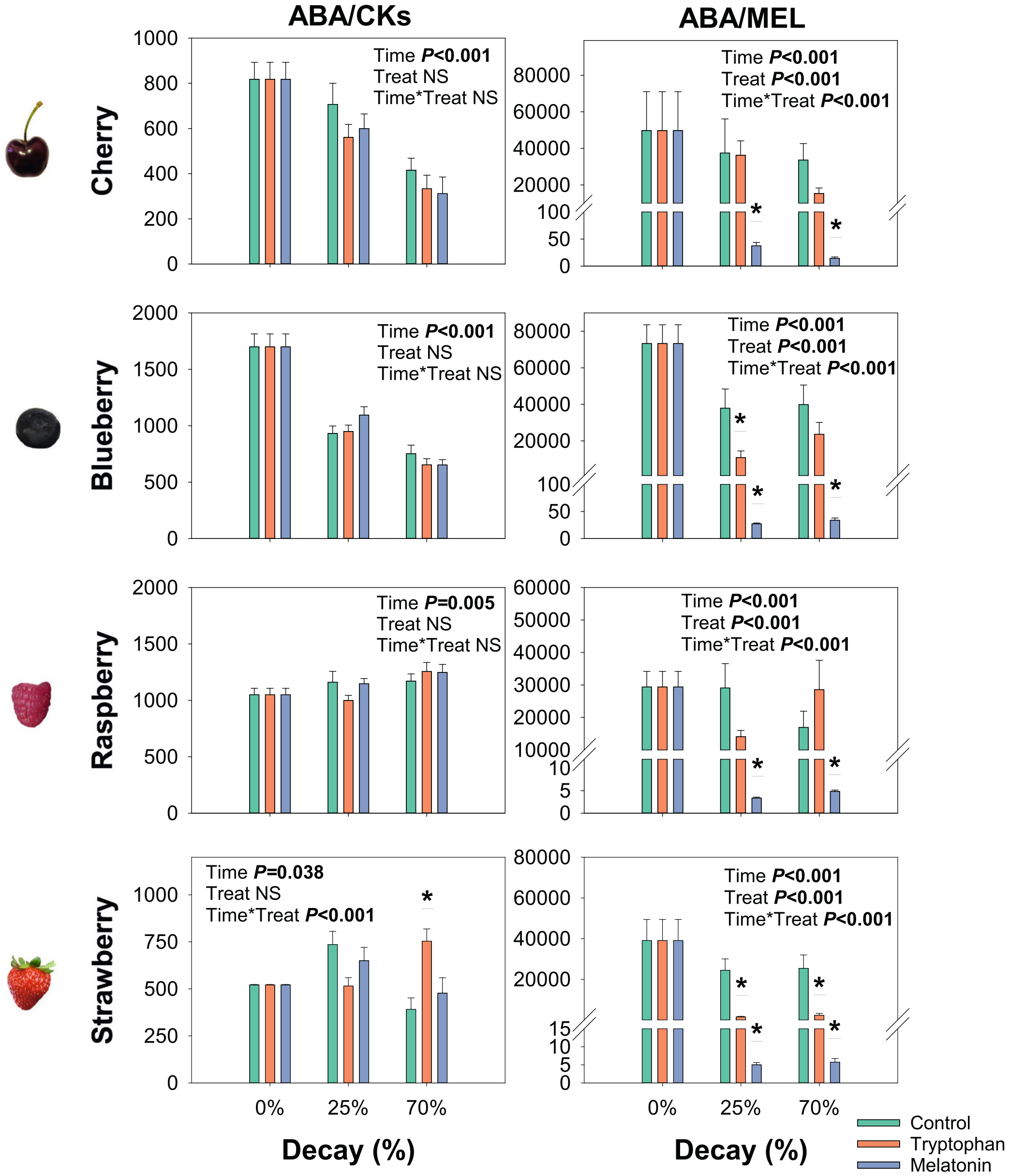


Fig. 5. ABA/CKs and ABA/MEL ratio in cherries, raspberries, blueberries and strawberries at 25 % decay and 70 % decay after melatonin and tryptophan applications. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Underlined asterisk ($\underline{*}$) indicates significantly different values between the control and treated fruit on that decay percentage ($P < 0.05$). ABA = abscisic acid; CKs = cytokinins (including the content of 2ip, iPA, Z and ZR); MEL = melatonin.

hormones (ABA, SA, ACC, JAs, CKs, and GA3) after melatonin and tryptophan application (Fig. 4A, S9, S10, S11, S12, S13, S14). Considering that both melatonin and IAA are derived from tryptophan, and the established relationship between the levels of IAA and melatonin treatment (Liu, Wei, et al., 2023; Wen et al., 2016), it was expected that melatonin or tryptophan application would interfere with the IAA content. However, neither application led to differences in terms of IAA levels (Fig.S9C).

Nevertheless, in the case of blueberries, an increase in the SA content was observed when fruits had 25 % deterioration after the application of both melatonin and tryptophan (Fig. 4B). The content of SA increased at the end of the postharvest period (70 % decay) in all untreated red fruits (Fig. 4B, S9B), but in blueberries the exogenous applications induced an increase of this phytohormone when fruits had 25 % decay, thereby accelerating SA boost. This increment in SA content was more notable with the application of melatonin, which resulted in a 125 % increase in SA content, while exogenous tryptophan led to a 46 % increase compared with untreated fruits. Moreover, it is known that SA alters the fruit physiology by increasing secondary metabolites in plant tissues which in turn increases the resistance level of plant organs (Prasad & Sharma, 2018). Molecular evidence also indicates that melatonin can up-regulate the expression genes of SA pathway decreasing pathogen susceptibility in Arabidopsis plants (Lee et al., 2014; Weeda et al., 2014). In fruits, Gao et al. (2018) reported that melatonin application enhances PAL activity stimulating the accumulation of TPC as well as delaying the loss of endogenous SA. Moreover, SA signaling pathway and phenylpropanoid metabolism were also involved in melatonin-induced disease resistance in tomato (Li et al., 2022). Therefore, it is suggested that the increase in SA may be related to the subsequent increase in phenols observed in blueberries, thus increasing the protective mechanisms of the fruit during postharvest. After tryptophan application in blueberries, SA content also increased. Zhang et al. (2024) showed that tryptophan

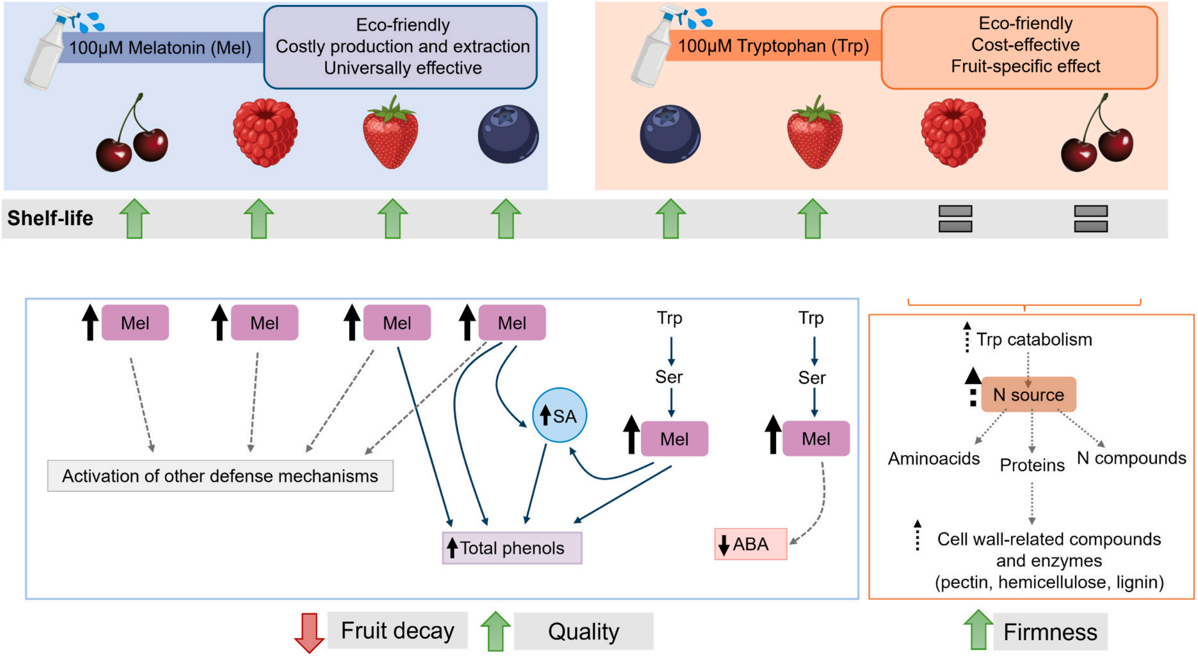


Fig. 6. Graphical representation of the effect and biochemical changes following exogenous melatonin and tryptophan application in different red fruits during postharvest. Trp = tryptophan, Ser = serotonin, Mel = melatonin, SA = salicylic acid, ABA = abscisic acid, N = nitrogen. Dashed lines indicate suggested mechanisms. This figure was created with BioRender.com. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

improved disease resistance in stored citrus fruits and slowed decay by upregulating genes involved in SA biosynthesis and transduction pathways. Here, it is probably that the SA increase in tryptophan-treated blueberries is mediated by the enhanced melatonin content, since the observed physiological response aligns with that observed in the exogenous melatonin application.

In strawberries, the tryptophan application also decreased ABA content by 45 % compared to control fruits at 25 % decay (Fig. 4B). ABA is widely known as one of the main plant hormones promoting fruit ripening, stimulating physiological changes such as softening or anthocyanins accumulation as well as inducing fruit senescence (Liu et al., 2020; Luo et al., 2014). For that, ABA levels decrease throughout postharvest fruit life (Tijero et al., 2019), which could also be observed in the red fruits of this study, where the lowest ABA levels are found at 70 % decay (Fig. 4, S9A). Therefore, considering its role in promoting ripening and senescence, a greater decrease in ABA content in strawberries at 25 % deterioration may be related to an overripening delay. In addition to the observed changes in content of SA and ABA, the ABA/SA ratio was also significantly affected (Fig. 4C). This ratio decreased in blueberries following treatments with melatonin and tryptophan, and it also showed a reduction in strawberries treated with tryptophan. However, considering the changes in individual hormone content, it is likely that in blueberries, the effect is primarily due to the increase in SA, whereas in strawberries, the reduction is more attributable to a decrease in ABA. The reduction in the ABA/SA ratio is particularly important in the postharvest context, as lower ABA/SA ratio may reflect an enhanced balance between stress response and senescence promotion. Beyond the hormonal changes already mentioned, melatonin did not lead to alterations in the balance between senescence-inhibiting hormones, such as cytokinins or IAA, and senescence-promoting hormones, like ABA and ACC. Therefore, the ABA/CKs, ABA/IAA and ACC/CKs ratios remained unchanged after the treatments (Fig. 5, S15, S16). However, melatonin application did reduce the ABA/MEL and ACC/MEL ratios in all fruits, as did the application of tryptophan in blueberries and strawberries (Fig. 5, S16). This suggests that the antisenescence effect of the postharvest treatments is not primarily due to changes in the content or balance of the major plant hormones, but rather to the increased melatonin levels, which may play a key role in delaying senescence and extending shelf life.

Therefore, these results indicate that the positive effects of the treatments are primarily driven by melatonin's antisenescence properties, showing that its effect is universal in red fruits. However, its mode of action varies depending on the specific fruit, either by regulating phytohormone content or through other defense mechanisms (Fig. 6). Moreover, tryptophan treatment followed the same pattern as exogenous melatonin, increasing SA content in blueberries. Tryptophan has only been effective in strawberries and blueberries, where part of exogenous tryptophan was converted into melatonin. Additionally, in cherries and raspberries, where tryptophan had no effect on shelf life extension but maintained firmness, no increase in melatonin, serotonin or the phytohormones analyzed was observed. Given that tryptophan, in addition to being a precursor of bioactive molecules, also serves as a N source through its catabolism (Shende et al., 2024; Tzin & Galili, 2010), it is plausible that these fruits have prioritized its use for increasing N availability. A greater source of N is essential for the synthesis of proteins, amino acids and nitrogenous compounds as well as metabolic regulation. Indeed, N is a fundamental component for the synthesis of enzymes and precursors necessary for the formation of compounds such as pectins, hemicelluloses, or lignin which enhance cell wall integrity and fruit firmness (Rivai et al., 2021; Vanholme et al., 2010; Wang et al., 2018). Thus, it may be that depending on the requirements and fruit needs at time of application, tryptophan is used for melatonin synthesis or not (Fig. 6). Nevertheless, it is suggested that it is the increase of endogenous melatonin that determines, in part, the success of its application.

4. Conclusions

In this study, melatonin application extended the shelf life at room temperature of all studied red fruits, including cherries, raspberries, blueberries, and strawberries, while tryptophan was effective in reducing post-harvest over-ripening in blueberries and strawberries.

These later effects were fruit-specific and appeared to be mediated, at least in part, by melatonin. Therefore, tryptophan treatment emerges both as an eco-friendly and highly cost-effective choice for the postharvest treatment of some red fruits. Additionally, we found that exogenous melatonin increased endogenous SA content in blueberries (an effect that was also observed upon tryptophan application), thus showing that melatonin may exert direct antisenescence effects in some fruits, but also indirect effects mediated by ripening-related hormones. Results also indicate that part of the beneficial effects of tryptophan delaying fruit decay are subject to its conversion to melatonin rather than to other indolamines. Overall, this study shows that melatonin treatment, and in some cases also its precursor tryptophan, is a sustainable and economical option to be used in the postharvest treatment of red fruits to extend their shelf life. This could contribute to reducing food waste, and minimize its impact on society, the economy, and the environment.

CRediT authorship contribution statement

Alba Arabia: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paula Muñoz:** Validation, Supervision, Methodology, Investigation, Conceptualization. **Sergi Munné-Bosch:** Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, 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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141487.

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