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An insight into the mechanisms underpinning the anti-browning effect of *Codium tomentosum* on fresh-cut apples

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Abstract

This work focuses on understanding the action of a novel seaweed extract with anti-browning functionality in fresh-cut apples. Organic fresh-cut apples were coated by immersion in an aqueous *Codium tomentosum* seaweed extract (0.5 % w/v), packaged under ambient atmospheric conditions in plastic bags, and stored at 4 ˚C for 15 days. Browning-related enzymatic activities, as well as targeted gene expression related to superficial browning, were monitored immediately after coating and followed at five-day intervals, until a final storage period of 15 days. Gene expression was particularly affected one hour after coating application (day 0), with no expression registered for peroxidase (*mdPOD*) and phenylalanine ammonia-lyase (*mdPAL*) genes in the coated samples. A reduction in polyphenol oxidase expression levels was also observed. After 15 days of storage, the coated samples developed lower browning levels and presented distinctly lower activities of polyphenol oxidase and peroxidase - the oxidative enzymes predominantly involved in enzymatic browning. The observed post-coating suppression of *mdPAL* and *mdPOD* expression, and reduction in *mdPPO* expression, suggest that

the seaweed *C. tomentosum* extract delays the activation of these genes, and decreases enzymatic activity, which in turn accounts for the coating's anti-browning effect.

Keywords: Fruit, post-harvest, seaweed, coating, RNA extraction, gene expression, peroxidase activity.

1. Introduction

Apple (*Malus domestica*) is a fruit with a high economic value. Recently data suggests that 17 million tonnes of apples were produced in Europe in 2019 and the cost per tonne was 664 USD dollars (FAO, 2021). Apples are generally consumed as a whole fruit, but more recently there has been a rapid growth in the consumption of ready-to-eat fresh cut apples mainly due to the convenience it offers. Fresh-cut apples are made by washing the whole fruit followed by cutting, treating with a dip-solution and packaging (Tarancón et al., 2021; Yousuf et al., 2019). Fresh-cut processing demands a high standard of preparation and handling in order to prevent rapid deterioration due to microbial spoilage and browning (Yousuf et al., 2019).

In the production of fresh-cut apples, the mechanical damage induced by peeling and slicing operations triggers the onset of surface browning, which is a mechanistically complex process (Chen et al., 2021). Surface browning results in a loss of both nutritional and organoleptic quality, leading to a decline in consumer acceptance and commercial value (Rasouli & Koushesh Saba, 2018). Browning can be caused by enzymatic activity as well as by non-enzymatic reactions. The Maillard reaction, which is non enzymatic in nature and depends on the apple sugar content and ascorbic acid concentrations (Paravisini & Peterson, 2018), can potentially cause browning of fresh-cut apples but its contribution to browning in the case of fresh-cut apples is arguable (Paravisini & Peterson, 2018). On the other hand, enzymatic mechanisms which cause superficial browning in fresh-cut apples have been widely studied and reported in literature (Chen et al., 2021; Tang et al., 2020; Toivonen & Brummell, 2008). In whole apples, cellular organelles are compartmentalized, and metabolic pathways occur without external interference. However, cutting disrupts cellular membranes, causing enzymes like polyphenol oxidase (PPO, EC 1.10.3.1) and peroxidase (POD, EC 1.11.1.7) to come into contact with substrates thereby triggering browning (Toivonen & Brummell, 2008). More specifically, these enzymes catalyse the oxidation of phenolic compounds into quinones, which in turn undergo

condensation reactions which result in browning (Chen et al., 2021; Oliveira et al., 2021; Rasouli & Koushesh Saba, 2018; Toivonen & Brummell, 2008). The presence and concentration of phenolic compounds ranging between 60 and 220 mg per 100 g of apple are considered to be key factors for pro-oxidative enzyme activity (Zhu et al., 2020). It is therefore necessary to develop technological solutions to inhibit such enzyme mediated browning processes in fresh-cut apples.

Edible coatings formulated with active ingredients have been widely used as agents to prevent the development of enzyme-mediated browning (Maringgal et al., 2020). Active substances in the coatings may be chemically synthetic, like ascorbic acid (E300), calcium ascorbate (E302), and hydrogen sulphide or obtained from sources of natural origin, such as *Aloe vera*, lemongrass oil or edible seaweed extracts (Augusto et al., 2016; Carocho et al., 2018; Chen et al., 2021; Maringgal et al., 2020). Augusto et al. (2016) and Augusto et al. (2022a) reported on the efficacy of a green edible seaweed extract - *Codium tomentosum* - to reduce browning and microbial spoilage in fresh-cut apples and pears, without influencing the organoleptic quality of the fresh-cut products as evidenced through sensory triangular tests (Augusto et al., 2022b). The activities of PPO and POD in fresh-cut apples were assessed over a storage period of 20 days and a reduction of 36 % and 87 %, respectively, was observed (Augusto et al., 2016). In the case of fresh-cut pears, the samples treated with the seaweed extract solution were found to exhibit significantly lower rates of superficial browning than samples coated with an ascorbic acid-based synthetic formulation widely used in industry (Augusto et al., 2022a). Even though the efficacy of seaweed extracts have been conclusively established, the mechanism of their action is not clearly understood. This work aims to assess the possible mechanism underpinning anti-browning functionality of this extract, exploring the relation between gene expression regulation, oxidative-enzymes activities, and browning development in fresh-cut apples.

2. Materials and Methods

2.1. Seaweed extraction and sample coating

An aqueous seaweed extract was produced as the anti-browning coating for fresh-cut apples. Dry *Codium tomentosum* (particle size with an average of 1.5 mm) was extracted in a 1:15 ratio of biomass: deionized water. The extraction was conducted away from light for 3 hours under constant stirring (1500 rpm) at room temperature. Subsequently, the mixture was filtered, centrifuged and freeze-dried as described in Augusto et al. (2016.) The extract obtained was kept away from light until use.

Organically produced apples (*Malus domestica*, var. 'Fuji') without any post-harvest treatment were supplied from a Portuguese producer - Campotec S.A. Prior to the coating application, fruit with similar weight and maturation stage (total soluble solid content of 15.78 ± 0.40 %; and pH of 4.08 ± 0.14), were firstly disinfected by a 2-min immersion in a solution containing 2% of sodium hypochlorite, and then washed with deionized water and manually sliced. Each apple was cut into 6 to 8 slices, each slice weighing 16 g on average. In a preliminary study, four coating concentrations were evaluated: 0.25 %, 0.50 %, 0.75 % and 1 % (w/v). Following a storage for 9 days at 4°C, the concentration of 0.50 % (w/v) gave higher protection against browning development. Thus, in the present study, the slices were coated by immersion for 5 min in a solution containing 0.5 % (w/v) of the previously prepared seaweed extract dissolved in deionized water. The immersion time of 5 mins is consistent with the times followed in industrial practice. For comparison, a control treatment, comprising only immersion in deionized water, was adopted. After coating, the excess coating solution was drained, and samples were packaged in plastic bags (low density polyethylene – LDPE zip-seal bags) and sealed to avoid external contamination and to restrict air circulation during the storage period. Each sliced apple was considered to be a replicate, giving in a total 6 replicates per treatment. To promote the natural development of browning, treated and control samples were stored for 15 d at 4 ± 2 °C,

with analyses being conducted every 5 days (i.e. on 0, 5, 10, and 15 days). The time taken to slice, coat, package and store apples was around one hour. The aforementioned "0 day" corresponded to the time point just before the slices went into storage. Physicochemical parameters were evaluated in samples every 5 days. The analyses of browning compound absorbance, enzyme activities, and gene expression were undertaken on samples which were removed from storage at the stipulated time and frozen at -80 ˚C.

2.2. Browning compounds absorbance

Browning compounds absorbance was determined according to Paravisini and Peterson (2018) and Shao et al. (2018). In brief, 2 g of frozen apple slice was homogenised with 14 mL of deionized water (×10/25 Homogenizer, Ystral, Germany), followed by a 1-hour incubation period at room temperature. The mixture was centrifuged at 1000 × g for 5 min, after which 5 mL was collected from the supernatant, mixed with 96 % ethanol and re-centrifuged. The absorbance of the supernatant was measured at 440 nm in a microplate reader (4 wells per sample) (Epoch2 Microplate reader, Biotek, USA). Results are expressed in absorbance units. Six control slices and six coated samples were withdrawn for analysis at each point of storage time (n = 6).

2.3. Physicochemical analysis

To assess the treatment effects on fresh samples, the sample colour was measured with a focus on the browning index development (BI) and colour changes (ΔE*). Since colour measurement is non-destructive, the same samples were followed over the storage period. In order to avoid randomness in superficial colour development, 15 specific points were selected on the surface and the colour of these samples were followed during storage (Supplementary data, Figure S1). The average value of colour measured at each of the 15 points was considered for calculations. Browning Index (BI) and ΔE* were evaluated as described by Lante et al. (2016) and Augusto et al. (2016), respectively. The ΔE* was calculated by the difference between an individual sample and the gold standard defined as the colour parameters of a sliced apple analysed immediately

147 after cutting (L* = 79.81; a* = 0.90; b* = 24.46), and results are expressed as colour changes
148 (ΔE*). Browning index results are expressed as browning index. For each storage condition, six
149 different apple slices were analysed (n = 6).

150

151 *2.4. Gene expression*

152 *2.4.1. RNA Extraction, purification, and quality assessment*

153 Total RNA was isolated from each apple replicate using an adapted methodology from a CTAB-
154 based protocol, optimizing for maximum yields concomitant with higher qualities of the
155 extracted RNA (Gambino et al., 2008).

156 Firstly, 100 mg of frozen sample was ground to a powder with liquid nitrogen and transferred
157 immediately to a 2 mL microtube with 650 mg of 1.4 mm zirconium oxide beads. While avoiding
158 sample from thawing, a volume of 650 µL extraction buffer containing 2 % of PVP-40, 0.05 % of
159 spermidine, 2 % β-mercaepthanol, and 1 % IGEPAL® CA-630 (NP-40) in cetyltrimethylammonium
160 bromide extraction buffer (CTAB) was added, followed by homogenization in a bead beater using
161 2 cycles of 45 s at 6200 rpm with an interval of 5 s between cycles (Precellys Evolution, Bertin,
162 France). To promote protein degradation, 36 µL of 20 g L^{-1} of proteinase K was immediately
163 added, and the mixture was gently stirred by inversion, followed by a 10-min incubation at 70
164 ˚C and 1800 rpm (Thermomixer Comfort, Eppendorf, Germany). Afterwards, a centrifugation
165 step was performed for 5 min at 13000 × g (4 ˚C). The supernatant was then collected and mixed
166 with 530 µL of the mixture chloroform/isoamyl alcohol 24:1 (v/v) and stirred by inversion. To
167 promote phase separation, a new centrifugation step was performed at 12000 × g for 15 min (4
168 ˚C). The aqueous phase was then transferred, and the RNA was precipitated with 100 %
169 isopropyl alcohol (proportion of 1:1 to the aqueous phase) for 20 min at room temperature,
170 followed by a second centrifugation step. The resulting pellet was washed twice with 600 µL of

75 % ethanol, stirred by inversion and centrifuged for 5 min. After the complete removal of ethanol, the RNA was re-suspended in 10 µL of DNAse/ RNAse-free water.

Next, and according to the manufacturer's instructions, pooled RNA samples were purified using the RNA Clean & Concentrator™-5 Kit from Zymo Research, including the DNAse treatment to remove traces of gDNA. In the final step, RNA was eluted with 15 µL of DNAse/ RNAse-free water. Total RNA concentration, as well as DNA concentration for possible gDNA contamination, were determined with a fluorimeter, following the manufacturer instructions (Qubit® 2.0 Fluorometer, ThermoFisher Scientific, USA). Contamination with protein, polysaccharides, and other RNA contaminants was verified using a micro-spectrophotometer Nanodrop 2000 (Thermo Scientific, USA) by measuring the absorbance at 230 nm, 260 nm, and 280 nm and evaluating their ratios. Total RNA integrity was assessed after electrophoresis on a 1 % (w/v) agarose gel. Due to the still low RNA extraction yields obtained from the maximum biomass possible for each reaction (100 mg), five extractions of each apple replicate sample were performed and pooled, giving a final RNA amount of 666 ng in average for each pooled sample.

2.4.2. cDNA synthesis and qPCR amplification

iScript cDNA Synthesis Kit (Bio-Rad) was used for the first-strand cDNA synthesis, following the manufacturer's instructions. To this end, 150 ng of total RNA was reverse-transcribed in a total volume of 20 µL. Samples without reverse transcriptase were also amplified to assess possible interference of gDNA contamination on the RT-PCR amplification.

Next, to evaluate the effect of the seaweed extract on gene expression of browning-related enzymes (*mdPPO, mdPOD, mdPAL*), anti-oxidative enzymes (*mdSOD, mdCAT, mdDHAR*), and cell-wall related enzymes (*mdPME, mdα-Af*), the expression of those eight target genes and a reference/housekeeping gene (*mdH1*) was quantified. Oligo Explorer software (version 1.1.2, Gene Link™) was used to design primer sequences based on the available gene sequences in NCBI for this species (Table 1). Primer efficiency (E) was calculated according to the equation: $E= (10^{(-}$

196 $^{1/slope)}$ - 1) x 100, where the slope is obtained from the standard curve of sample serial dilution.
197 The specificity of each primer set in producing a single and specific amplification product was
198 assessed through melting curves analysis. To reject possible amplification resulting from gDNA
199 contamination, as well as primer dimers formation, different controls were performed: replicate
200 samples without the reverse transcriptase addition (-RT controls) and non-template control
201 replicates (NTC) (Taylor et al., 2019). For the qPCR amplification reactions, 2 µL of DNA template,
202 2 µL of each respective forward and reverse primer, 4 µL of DNAse/RNAse free water and 10 µL
203 of iTAQ™ Universal SYBR® Green Supermix were added (final volume of 20 µL). The thermal
204 cycling protocol comprised a first step of 30 s at 95 °C, followed by 60 cycles of a combined
205 denaturation (5 s at 95 °C) and annealing (30 s at 60 °C) steps. The presence of the desired
206 amplicon was verified by the melt curves which consisted in the measurement of fluorescence in
207 a range from 65 to 95 °C in each increase of 0.5 °C for 5 s. Amplification reactions were performed
208 in triplicates for all samples, using 96-well plates (Biorad, Multiplate® PCR Plates™), on a
209 thermocycling CFX Connect^{TM} Real-Time PCR System (Bio-Rad). The relative expression (ΔΔ CT)
210 of the target genes was normalized by the expression of the reference gene (*mdH1*) (Storch et
211 al., 2015), using the software CFX Connect™ Real-Time System (Biorad, USA) and following an
212 adaptation of the equation developed by Pfaffl (2007) as: ΔΔ CT = [TG $E^{(CTmin\ TG - CT\ value\ TG)}$ / RG E
213 $^{(CT\ min\ RG - CT\ value\ RG)}$], where E is the efficiency of each target (TG) and reference gene (RG). The
214 results are expressed as relative expression. Despite samples were kept in storage conditions for
215 15 days, gene expression analysis was performed only on 0, 5 and 10 days, since the molecular
216 effects that lead to the enzymatic alterations and visual effects at 15 days are expected to occur
217 sooner in time.
218
219
220

221 *2.5. Enzymatic activities*

222 The activity of four enzymes - polyphenol oxidase (PPO), peroxidase (POD), pectin
223 methylesterase (PME), and superoxide dismutase (SOD) was determined, and frozen samples
224 from coated and control groups were processed as described below.

225 To evaluate the efficacy of the seaweed extract to prevent browning development in coated and
226 control apple slices, enzymatic activities were evaluated, namely polyphenol oxidase (PPO) and
227 peroxidase (POD) - two enzymes which the extract appears to influence (Augusto et al., 2016),
228 and be mainly responsible for the enzymatic browning in fresh-cut apples (Toivonen &
229 Brummell, 2008). The evaluation of PPO and POD activities followed the procedure described in
230 Augusto et al. (2022a). Briefly, frozen samples were homogenised in 50 mmol L^{-1} sodium
231 phosphate buffer (pH 7.0) containing polyvinylpyrrolidone (PVP) (50 g L^{-1}) and the supernatant
232 collected for the enzyme's determination and protein quantification. For POD determination,
233 the reaction was followed at 470 nm and catalysed by mixing the enzymatic extract with a
234 solution containing 1 % of guaiacol and 0.3 % of hydrogen peroxide in 0.05 mol L^{-1} of sodium
235 phosphate buffer (pH 6.5). On the other hand, a solution of 20 mmol L^{-1} catechol in 5 mmol L^{-1}
236 sodium phosphate buffer (pH 7) was used as substrate mixture for the determination of PPO,
237 and the reaction absorbance read at 400 nm. The enzymatic activities were expressed as U per
238 kilogram total soluble protein, U kg^{-1}. Protein was quantified following the Bradford
239 methodology (Bradford, 1976).

240 The activity of the cell-wall related enzyme pectin methylesterase (PME) was measured
241 following the methodology described by Augusto et al. (2022a), and results were expressed as
242 U per kilogram protein, U kg^{-1}. Before PME determination, frozen apple slices were used for a
243 second extraction using an extraction buffer containing 1.5 mol L^{-1} of NaCl and 2.5 w/v of PVP
244 (pH 7.5) as described by Augusto et al. (2022a). The supernatant collected for the enzyme
245 determination and protein quantification. The enzymatic reaction was measured

spectrophotometrically at 610 nm, by mixing the enzyme extract with a substrate solution with 0.01 % of bromothymol blue and 5 g L^{-1} citrus pectin in 0.003 mol L^{-1} sodium phosphate buffer (pH 7.5).

The antioxidant enzyme superoxide dismutase (SOD) activity was also evaluated. A third enzymatic extraction was carried out for SOD determination. An adapted protocol from Collazo et al. (2018), Li et al. (2019), and Wei et al. (2019) was followed. Five g of frozen apple slice was homogenised with 10 mL of chilled 0.1 mol L^{-1} sodium phosphate buffer (pH 7.5) containing 20 g L^{-1} of polyvinylpyrrolidine (PVP), 2 mmol L^{-1} dithiothreitol (DTT), and 0.1 mmol L^{-1} ethylenediamine tetraacetic acid (EDTA). To remove larger particles, the mixture was filtrated through a cheesecloth, followed by a 30 min centrifugation at 14000 × g (4 °C), obtaining a clear supernatant ready to be used for SOD determination. A volume of 50 µL of enzyme extract was mixed with 150 µL of a subtract mixture composed of 50 mmol L^{-1} sodium phosphate buffer (pH 7.8) with 13 mmol L^{-1} methionine, 75 µmol L^{-1} NBT (nitrotetrazolium blue chloride), 10 µmol L^{-1} EDTA and 2 µmol L^{-1} riboflavin. The plate was incubated under fluorescent light for 10 min, and absorbance was read at 560 nm, before and after incubation. At the same time, and to discard possible interferences on the final absorbance determination, an identical plate was incubated in the dark (blank). SOD specific activity was expressed as U kg^{-1} of fresh weigh, U kg^{-1}, where one unit (U) of the enzyme activity was defined as the photoreduction inhibition of NBT by 50 %.

All enzymatic activities were performed in six biological replicates for each storage condition (n = 6), in triplicates, using an Epoch2 Microplate reader (Biotek, USA). In all the enzymatic determinations, a control assay was performed using the homogenization buffer instead of the enzymatic extract.

2.6. Statistical analysis

To assess the seaweed extract effects on the development of superficial browning and on gene expression of the sliced apples, generalized linear models (GLMs) with logarithm link functions were used. Based on the Akaike Information Criteria (AIC) and likelihood ratio tests (LRT), the best fitting models were chosen. For pairwise post hoc comparisons, the least significant difference (LSD) was run to test for individual differences between the factor levels. GLMs were performed in IBM SPSS Statistics version 27. CANOCO version 4.5 package was used for the Principal Component Analysis (PCA) design.

3. Results and Discussion

In fresh-cut apple processing, as an immediate response to the cutting process, the antioxidant system is activated by increasing the relevant enzyme activities like superoxide dismutase (SOD) and catalase (CAT). Consequently, there is an increase in the activity of browning-related enzymes like POD and PPO, which act on the phenolic substrates that have leaked from cell vacuole to result in superficial browning (Chen et al., 2021). Changes on the visual appearance of fresh-cut apples are mainly driven by the variation in enzyme activities that are preceded by metabolic responses occurring at a cellular level which regulate gene expression and protein levels (Lemos, 2021; Simões et al., 2019). The results here discussed are presented respecting the sequential events related to browning development (gene expression proceeded by the functional activity). These sequential events were then related between them and are also here discussed.

3.1. Colour development

The visual colour changes and reduction in browning are decisive factors establishing the efficacy of the seaweed extract. Browning development in fresh-cut apples can be determined by different methodologies, including spectrometric techniques and colour measurements.

In this study, the formation of browning compounds was determined as a function of storage time and the results, expressed as browning absorbance, browning index and colour (ΔE), can be seen in Figure 1 A-C. In general, in browning absorbance results (Figure 1A) comparing the data on days 0 and 15 there was no differences between the seaweed coated and the control group. The absorbance seems to decrease on day 5 but increase after 10 days, to reach similar values as day 0. Into the case of the seaweed extract coated group, an increase in absorbance was detected between days 0,10 and 15 days. Despite the observed increase in browning absorbance, the values for seaweed coated samples were lower than those for the control samples. These results clearly establish the efficacy of the seaweed extract coating on fresh-cut apples to inhibit browning, which has been associated with the presence of polysaccharides in the extract composition, mainly sulphated polysaccharides, which are already known to be present in green seaweeds, including *Codium* species (Fawzy, 2020). It is believed that these polysaccharides protect the cell wall membrane against external damages inflicted by the cutting process (Augusto et al., 2018; Augusto et al., 2022a). Preliminary studies based on FTIR analysis suggest amide, methyl, and sulphate as the main functional groups present in the seaweed extract. Additionally, FITR analysis also points to the presence of high carbohydrate content. A more detailed study, including chromatographic and spectroscopic techniques, is necessary to corroborate these findings and to better describe the extracts composition, which is on-going.

The results of colorimetric measurements in terms of browning index (BI) and total colour differences (ΔE) are presented in Figure 1 B-C. Lower browning index values were observed in samples coated with the seaweed extract, compared to control samples (Figure 1 B). As with the results of absorbance at 440 nm (Figure 1 A), the data on browning index also confirm the efficacy of seaweed coating treatment. The colour parameter ΔE increased between day 0 and 15 of storage, although the increase was much less pronounced in samples coated with the seaweed extract (ΔE_{15} control = 9.75 ± 4.29; seaweed extract = 6.22 ± 2.88). These changes are

consistent with the results of browning index (Figure 1B) and browning compounds absorbance (Figure 1A) since the development of browning colour has a strong bearing on the final colour of the fresh-cut apples. The differences between coated and uncoated samples are more pronounced on day 10 of storage, where control samples presented higher values of ΔE (ΔE_{10} = 8.77 ± 1.15) than samples coated with the seaweed extract (ΔE_{10} = 4.93 ± 1.15). Similar results were obtained by Augusto et al. (2016) in fresh-cut apple slices and puree treated with a solution of *C. tomentosum* extract. The same authors also observed a reduction in the development of superficial browning in fresh-cut pears treated with a coating solution containing 0.5 % w/v of *C. tomentosum* extract (Augusto et al., 2022a). In both studies, the mechanisms underlying the reduced browning in apple and pear coated with the seaweed extract were unknown, thus, the present study goes beyond the anti-browning effect of this seaweed-based coating, trying to understand the mechanism of action behind these effects.

3.2. Gene expression

Three of the studied target genes, namely *mdPAL*, *mdPOD*, and *mdPPO*, were initially selected as they are believed to be implicated in the mechanisms of browning in fresh-cut apples and linked between them through phenylpropanoid pathway (Chen et al., 2021). It is through this pathway that PPO and POD substrates, the phenolic acid compounds, are produced. Phenylpropanoids are secondary metabolites that derive from phenylalanine and tyrosine amino acids. Chemically, these compounds can be divided into five sub-groups: flavonoids, monolignols, stilbenes, coumarins, and phenolic acids, with the last sub-group representing the main substrates of PPO enzyme (Chen et al., 2021; Deng & Lu, 2017). Considered the main key factor for the phenylpropanoid biosynthesis, the enzyme phenylalanine ammonialyase (PAL) plays a primary role in the phenylpropanoid pathway, regulating indirectly the production of phenolic substrates (Chen et al., 2021; Dou et al., 2021; Liu et al., 2021b). Among phenolic compounds that can be found in apples, chlorogenic acid and procyanidin are the main phenolic substrates of PPO

346 enzyme (Treutter, 2001). Briefly, in the presence of phenolic substrates and oxygen, the activity
347 of PPO causes the generation of brown pigments. Firstly, the hydroxylation of monophenols to
348 *o*-diphenols is catalysed by PPO followed by the oxidation of *o*-diphenols, resulting in the
349 formation of *o*-quinones compounds which are responsible for the coloured brown spots in fresh-
350 cut apples (Chen et al., 2021; Rasouli & Koushesh Saba, 2018). Although being a controversial
351 subject, POD action in apple browning is believed to be a result of phenolic substrates oxidation,
352 a reaction catalysed by the presence of hydrogen peroxide, which provides the free radical
353 hydrogen essential for the enzyme activity (Chen et al., 2021; Oliveira et al., 2021).

354 In the present study, expression levels (*r*E) results of the genes encoding the mentioned
355 browning-related enzymes PAL, POD, and PPO, after 0, 5, and 10 days of storage time, can be
356 seen in Figure 2 A-C. Despite the lack of statistical differences between samples coated with the
357 seaweed extract and control from day 0, it was possible to observe a different response trend
358 in time between the control group and the seaweed extract group for these three genes. In the
359 control treatment, expression levels tended to increase between day 0 d and 5, followed by a
360 tendency for decreased or stabilized values at 10 days of storage (Figure 2). On the other hand,
361 in the samples coated with the seaweed extract it was not possible to detect any amplification
362 signal for *mdPAL* and *mdPOD* at day 0 (Figures 2 A and B), which expression only started to be
363 visible after 5 days and with a tendency to increase between day 5 and 10 of storage for both
364 genes. This same pattern of response was observed for *mdPPO,* although in this case some low
365 expression values could be detected at day 0 in the extract coated samples (Figure 2C). For the
366 three genes there was also an overall tendency pattern for higher expression of all of them in
367 control samples at days 0 and 5 in comparison to the seaweed extract coated samples, revealing
368 a possible seaweed extract early inhibition interference on the expression of those genes.
369 Although the non-detected *mdPAL* expression at day 0, in samples coated with the seaweed
370 extract (Figure 2A), after 5 days of storage, gene expression is identical in the two sets of
371 samples. But, at 10 days, samples coated with the seaweed extract seem to present increased

372 expression levels comparatively to control. As observed for *mdPPO*, the results suggest that
373 *mdPAL* reached its higher average expression levels earlier in control samples (*r*E = 0.89), on day
374 5, while the transcript level of treated samples reached its highest detected mean expression
375 levels on day 10 (*r*E = 0.98). Regarding *mdPOD*, similar results to those described for *mdPAL* were
376 observed, with even more pronounced evidence of the seaweed extract influence on gene
377 expression (Figure 2B). At day 5, the average transcript levels of control samples (*r*E = 1.15) were
378 considerably higher than those of treated samples (*r*E = 0.22).

379 In the present study, and although no statistical differences were identified between control
380 and treated samples for *mdPPO* expression within the same time-period, nor between control
381 treatments through time, the extract treatment significantly inhibited *mdPPO* expression at day
382 0 in comparison with the following days (Figure 2C). As previously mentioned, it is believed that
383 the expression of *mdPPO* is associated with browning development, and therefore the inhibition
384 of *mdPPO* gene expression may be directly related to browning reduction in fresh-cut apples
385 (Chen et al., 2021). After treating fresh-cut apples with hydrogen sulfide (H_2S), Chen et al. (2021)
386 observed a positive correlation between the significant reduction of *mdPPO* expression and
387 fresh-cut apples browning. Moreover, this inhibition was also observed in other food matrices,
388 namely in white button mushrooms treated with the amino acid ergothioneine (Qian et al.,
389 2021). White button mushrooms are prone to lose their original characteristics after harvesting,
390 and when submitted to fresh-cut practices, the intensity of tissue browning increase drastically.
391 The authors described a down-regulation in the expression of genes encoding browning-related
392 enzymes as PPO, thereby reducing the enzymatic activity, which resulted in samples with fewer
393 colour changes (Qian et al., 2021).

394 Overall, the seaweed extract addition seems to interfere with the transcription processes of
395 *mdPPO*, *mdPOD*, and *mdPAL*, with a greater influence on *mdPAL* and *mdPOD* transcripts. This
396 influence may lead to a delay in the transcription, resulting in lower values of gene amplification

397 at the beginning of the storage. These results also highlight the involvement and relevance of
398 *mdPOD* and *mdPAL* in the regulation of fresh-cut apple browning, instead of a single gene
399 regulation like *mdPPO*. Only recently, Chen et al. (2021), Qian et al. (2021), and Liu et al. (2021a),
400 relaunched the debate on the correlation between POD and PAL activities and browning
401 development of fresh-cut fruit and vegetables. The results discussed in the present study allow
402 to hypothesise that in the presence of the seaweed extract, the observed resistance to browning
403 development can also be highly mediated by PAL and POD, instead of a single PPO-browning
404 mediation. This is also, to the best knowledge, the first report where the possible involvement
405 of PAL on browning reduction of fresh-cut apples coated with the seaweed extract is
406 documented and discussed, fostering pertinence of further complementary studies to further
407 access the mechanisms underlying on fresh-cut apples browning. Additionally, in fruit and
408 vegetables, the induction of browning-related enzymes is a fast mechanism, and with the
409 injuries caused by cutting, this process can be almost instantaneous in fresh-cut fruit (Liu et al.,
410 2021a). This fact can support the observed differences in gene expression at day 0, with the
411 samples coated with the seaweed extract presenting overall lower expression levels, possibly
412 contributing to the protection conferred by the coating in the deteriorative processes, which is
413 not possible to obtain using water in the control treatment.

414 In fresh-cut processing, besides the changes in expression of browning-related genes (discussed
415 above), other protective processes may be occurring, such as the detoxification of superoxide
416 radicals that are converted into hydrogen peroxide by superoxidase dismutase (SOD). The
417 activity of SOD during induced stress prevents the accumulation of free radicals, as reactive
418 oxygen species (ROS), in the cell, contributing to cell protection (Rasouli & Koushesh Saba, 2018).
419 In apples, catalase (CAT) is also involved in the scavenging of ROS avoiding oxidative damages in
420 cells (Li et al., 2019), converting H_2O_2 into water (Abdelhai et al., 2019). Besides SOD and CAT,
421 dehydroascorbate reductase (DHAR) is also indirectly implicated in the antioxidant mechanisms,
422 particularly in the reduction of dehydroascorbate (DHA) into *L*-ascorbate (Davey et al., 2000; Do

et al., 2016), thus turning *L*-ascorbate re-available for the antioxidant system to mitigate excessive ROS levels.

The relative expression of the selected encoding genes for the antioxidant system-related enzymes *mdSOD* and m*dDHAR* can be observed in Figure 3 A-B (*mdCAT* was not possible to determine as further detailed below).

Considering the results of relative expression of *mdSOD* (Figure 3A), it is possible to observe that samples coated with the seaweed extract solution showed an overall trend for higher expression levels than control samples, through the 10 days of storage. In both control and seaweed extract coating samples, there was an increase in expression at day 5 (mean *r*E = 0.89 to 1.55 in control and mean *r*E = 1.12 to 1.66 in seaweed extract samples), followed by a decrease in control samples at day 10, which was not observed in seaweed extract samples. In the work by Liu et al. (2021a), the authors demonstrated that changes in the eggplant redox state, namely the delay of antioxidant genes transcription, could be associated with the development of fresh-cut eggplant browning. The observed differences in *mdSOD* transcript levels in the present study may thus suggest that the seaweed extract can induce the antioxidant system in fresh-cut apples, namely SOD activity, contributing to the browning resistance of samples coated with the seaweed extract (Liu et al., 2021a). Concerning *mdDHAR* relative expression (Figure 3B), overall, a tendency for lower expression levels in the seaweed extract group can be observed during the storage period, with a substantially lower relative expression (*r*E = 0.89) at day 5, when compared to control samples (*r*E = 1.22). These results suggest that the ascorbic acid recycling pathway is not activated at the same level in the seaweed extract samples as is in control samples. Although more studies are needed, increased expression of *mdDHAR* is expected to represent a protective response of the organism to ensure DHAR levels after apple slicing, which in the seaweed extract group may not be so relevant given the other protective actions in place against oxidative and deteriorating processes, as previously discussed.

Cell wall modifications involve multiple enzymes, and in fresh-cut apples, pectin methylesterase (PME) and α-arabinofuranosidase (*α*-Af) are two of the enzymes responsible for membrane integrity (Liu et al., 2021b). The study of these enzymes is important to understand their influence on browning development since, when active, these enzymes lead to cell membrane degradation, promoting the contact between phenolic substrates such as chlorogenic acid (stored in the vacuoles)and PPO stored in the plastid, leading to the production of quinones and melanin, and resulting in the dark areas on the fruit (Toivonen & Brummell, 2008; Wang et al., 2021). In this study (Figure 3C), while in the control group a sharp increase of *mdα-Af* expression was observed after 5 days of storage (average *r*E = 0.78 at day 0 to *r*E = 1.16 at day 5), followed by a decrease in expression at day 10 to a mean relative expression of 0.71, the presence of the seaweed extract in fresh-cut apples suggests an overall lower transcription of *mdα-Af.* In coated samples, no differences were observed in transcript values during the storage period, although it is possible to observe a slight incremental tendency in expression along the storage period, but never reaching the same maximum values as in the control group, even later in time (Figure 3C). Liu et al. (2021b) reported that the textural quality of fresh-cut apple during cold storage was maintained by the repression of *mdα-Af* transcript levels, which was associated with a treatment composed by 1.4 mg L^{-1} of aqueous ozone for 5 min. In the present study, the seaweed extract seems to inhibit the transcription of *mdα-Af*, contributing to the cell wall integrity maintenance.

Concerning *mdPME* and *mdCAT*, while there was an attempt to determine the expression of those genes, it was not possible to detect any amplification in both cases under the studied conditions, even with different sets of primers and performing a higher number of qPCR cycles (Table 1). As stated in the material and methods section, given the low yield of RNA retrieved from this type of samples, only 150 ng of total RNA were used as template for the first-strand cDNA synthesis, which may have contributed to the difficulty in detecting amplification of genes with very low transcription activities (Taylor et al., 2019).

3.3. Biochemical validation

With the fresh-cut processing, the first functional changes are observed at the biochemical level, where shifts in enzyme activities and protein levels can be directly linked to further visual effects as surface browning development. To proceed with the functional validation of effects through biochemical evaluations, and thus confirming the effect of the seaweed extract coating on the enzymatic activities of fresh-cut apples, the activities of PPO, POD, SOD, and PME enzymes were assessed (Figure 4 A-D).

The progress of PPO activity during the 15 days of storage period can be observed in Figure 4A. Overall, during the storage, samples coated with the seaweed extract solution had a lower PPO activity comparatively to control group. In more detail, control samples presented an increment in PPO activity between 20 % and 50 % in relation to the seaweed extract group, with this difference more evidenced at day 5. The observed difference in PPO activity at day 5, correspond to the same time-point where a higher expression of the corresponding gene *mdPPO* was detected (Figure 2C), thus indicating that the increased transcription resulted in higher translation and PPO activity. In turn, the lower activity detected in the seaweed extract group in relation to control could also be a direct effect of the overall lower *mdPPO* transcription values verified in the coated samples (Figure 2C). The seaweed extract effect on PPO activity observed in the present study can be corroborated by previous studies, where a reduction in PPO activity was described in fresh-cut apple (Augusto et al., 2016) and pear (Augusto et al., 2022a) coated with a solution containing 0.5 % w/v of *C. tomentosum* extract.

As expected, POD activity presented an increase between days 0 and 15 of storage in both control and seaweed extract groups (Figure 4B). Despite the observed POD increment in both sample groups, overall samples coated with the seaweed extract present a tendency of lower values of activity over the storage period. It is at day 10 that the difference between the two set

of samples is more denoted, where the enzyme activity was considerably lower in the seaweed extract group (19.10 $\times 10^3$ U kg^{-1} in contrast with 3.70 $\times 10^3$ U kg^{-1} in the seaweed extract group). These results strengthen those reported in previous studies conducted by Augusto et al. (2016) and Augusto et al. (2022a), documenting less POD activity in fresh-cut apples and pears coated with a seaweed extract solution and stored for 20 days and 15 days, respectively. As described for PPO, gene expression may also justify the lower values of POD activity in the seaweed extract group. In line with the observed transcription results (Figure 2B), the great disparity in POD values between the two sets of samples could be an effect of changes at the transcriptional level. A more detailed study comprising different phenolic substrates and the activity of PPO and POD will allow a better understanding of the mechanisms here proposed.

The effect of the seaweed extract on SOD activity was also evaluated (Figure 4C). To the best knowledge, this was the first report about the seaweed extract effect in SOD activity of fresh-cut apples. No differences were observed along sampling time-points between control group and samples coated with the seaweed extract. These results contrast with those presented by Chen et al. (2021), where the authors described a higher antioxidant capacity allegedly mediated by SOD activity, which resulted in a lower intensity of browning in fresh-cut apples. While in *mdSOD* gene expression (Figure 3A), differences between control and seaweed extract groups were detected, these did not affect the enzymatic level. The results suggest that at the biochemical level, the activity of SOD was not affected by adding a coating solution containing a seaweed extract. At the molecular level, the results may suggest a possible activation of the antioxidant mechanism, supported by the induction of CuZn SOD (Figure 3A), but this induction was not possible to verify at the enzymatic level considering that all types of SOD are present in the homogenate that is used for SOD determination. Additionally, a more detailed study linking the anti-oxidative and browning inducing enzyme activities, the production of compounds that resulted from oxidative stress in the presence of the seaweed extract will give more information about the extract mode of action.

The biochemical activity of PME was determined and results shown in Figure 4D. Both sampling groups presented a similar behaviour during the first 10 days of storage, including the sharp increase in PME activity of about 87 % between days 0 and 5. However, on day 10, control samples further increased their PME activity contrarily to the seaweed extract group that maintained the previous levels of activity. The efficacy of the seaweed extract to decrease PME activity of fresh-cut pears (Augusto et al., 2022a) and apples (Augusto et al., 2022b) was previously evaluated, and both studies suggested that the seaweed extract had influence in the reduction of PME activity. The results here presented may reinforce this positive influence on the activity PME, preventing the cell wall degradation and contributing to the cell integrity maintenance (Liu et al., 2021b).

3.4. Principal Component Analysis

A Principal Component Analysis (PCA) was performed considering the results obtained for the 13 parameters through assessments of gene expression, enzymatic activities, and colour evaluations (Figure 5). The first and second principal components (PC1 and PC2) are represented in the X and Y axis respectively and account for 88 % of data explanation (PC1: 59.7 % and PC2: 28.3 %), representing the largest fraction of variability. The proximity of the encoding genes for PPO and POD (*mdPPO* and *mdPOD*) vectors to control samples at day 5, reinforces what was previously seen that in this storage time and group of samples, the results were mostly characterized by the increase of *mdPPO* and *mdPOD* expression levels (Figure 2 B-C), with a negligible effect on seaweed extract samples group (observed by the 90 ° angle with *mdPPO* and *mdPOD* vectors). Only at day 10, it is possible to observe a greater influence of these genes in the seaweed extract samples, which further suggests a possible delay in gene transcription in samples coated with the seaweed extract solution, thus resulting in a lower browning rates and lower enzymatic activity, as previously reported in Figures 1 A-C and 4 A-B. This hypothesis - the delay in browning development in the seaweed extract samples group, can be strengthened by

550 the strong proximity between the vectors of colour evaluation and browning compounds with
551 the control group vector at day 10. Once again and considering the right angle between these
552 parameters to the seaweed extract group vector, results suggest a lower influence of browning
553 parameters on the coated samples results. With time, namely at day 15, the overall differences
554 between the two sets of samples are smaller, and mostly characterized by the activity of PPO
555 and POD. However, the lower angle between control samples and enzyme activities vectors may
556 suggest a stronger influence of PPO and POD on the results of these groups rather than in the
557 seaweed extract group, resulting in higher browning in non-treated samples, as observed in
558 Figure 1.

559 Thus, the mechanism of action of the seaweed extract involves the reduction in the expressions
560 of *mdPPO* and *mdPOD* as observed in the first five sampling days. Since these genes are known
561 to trigger the browning processes in fresh-cut fruits, the lower gene expression may explain the
562 significant decrease in the activities of PPO and POD, which results in lower rates of browning.
563 Additionally, the seaweed extract addition seems to have a greater influence on the activity of
564 POD than on PPO, which can be related to the apparent reduction in the *mdPOD* expression
565 observed at the beginning of the storage period.

566 **4. Conclusions**

567 This study elucidates the possible mechanisms by which a coating of seaweed extract *C.*
568 *tomentosum* acts to reduce browning in fresh-cut apples. At a molecular level, the coating
569 delayed the stimulation of the encoding genes for the main browning related enzymes namely
570 PPO, POD and PAL. The coating effect is visible since day 0, where *mdPPO* gene expression was
571 considerably lower in apple slices coated with the seaweed extract than in control samples. In
572 the same period, was not possible to detect any amplification signal for *mdPAL* and *mdPOD*.
573 Only after a period of 10 days of storage, the levels of transcripts in the seaweed extract samples
574 group reach identical values to control samples at day 5, suggesting a delay in gene expression.

The coating functionality was confirmed by the activity of PPO and POD that followed the same trend as the gene expression results, which lead to a lower browning development in fresh-cut apples coated with the seaweed extract.

Declarations of interest: none

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Appendix A. Supplementary data

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745 **Figure Captions**

746 **Figure 1.** Boxplots of the **(A)** browning compounds absorbance at 440 nm (calculated by
747 spectrophotometry), **(B)** browning index (BI, calculated by colour parameters), and **(C)** colour
748 changes (ΔE) between fresh-cut apples coated with seaweed extract vs control treatments.
749 Samples were stored for 15 days (d) at 4 ˚C, with periodic samplings on days 0, 5, 10 and 15.
750 Boxplots with different letters represent significantly different values (ANOVA, GLM, LSD test, p
751 < 0.05).

752 **Figure 2.** Relative expression of the browning-related genes (**A**) phenylalanine ammonialyase
753 (*mdPAL*), (**B**) peroxidase (*mdPOD*), and (**C**) polyphenol oxidase (*mdPPO*) in fresh-cut apples
754 coated with seaweed extract *vs* control treatments. Samples were stored for 10 days (d) at 4 ˚C,
755 with periodic samplings on days 0, 5, and 10. Boxplots with different letters represent
756 significantly different values (ANOVA, GLM, LSD test, $p < 0.05$).

757 **Figure 3.** Relative expression of the browning-related genes (**A**) superoxidase dismutase
758 (*mdSOD*), (**B**) dehydroascorbate reductase (*mdDHAR*), and (**C**) *α*-arabinofuranosidase (*mdα-Af*)
759 in fresh-cut apples coated with seaweed extract *vs* control treatments. Samples were stored for
760 10 days (d) at 4 ˚C, with periodic samplings on days 0, 5, and 10. Boxplots with different letters
761 represent significantly different values (ANOVA, GLM, LSD test, $p < 0.05$).

762 **Figure 4.** The activities of **(A)** polyphenol oxidase (PPO), **(B)** peroxidase (POD), **(C)** superoxide
763 dismutase (SOD), and **(D)** pectin methylesterase (PME) in fresh-cut apples coated with seaweed
764 extract *vs* control treatments. Samples were stored for 15 days (d) at 4 ˚C, with periodic

765 samplings on days 0, 5, 10, and 15. Boxplots with different letters represent significantly
766 different values (ANOVA, GLM, LSD test, $p < 0.05$).

767 **Figure 5.** Principal component analysis (PCA) of the different responses determined in fresh-cut
768 apples coated with seaweed extract *vs* control treatment, at the different storage times (0, 5,
769 10, and 15 days, d). *mdPPO* and PPO = polyphenol oxidase gene expression and enzyme activity,
770 respectively; *mdPOD* and POD = peroxidase gene expression and enzyme activity, respectively;
771 *mdPAL* = phenylalanine ammonialyase gene expression; *mdSOD* and SOD = superoxidase
772 dismutase gene expression and enzyme activity, respectively; *mdDHAR* = dehydroascorbate
773 reductase gene expression; *mdα-Af* = α-arabinofuranosidase gene expression; PME = Pectin
774 methylesterase activity; BC = browning compounds; ΔE = Colour changes; BI = Browning index.