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Investigating the impact of active PHBV films on the nutritional quality of minimally processed apples

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ABSTRACT

The aim of the study was to study the effect of an active film, a blend of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with a derivative of ferulic acid known as bis-O-dihydroferuloyl-1,4-butanediol (BDF), on the quality decay kinetic of minimally processed apples. Due to its properties, BDF is a promising candidate to serve both as a non-toxic biobased plasticizer and as an active additive, simplifying formulation of active packaging. Molecular modelling studies were conducted to explore the interaction between polyphenol oxidase (PPO) and BDF molecule, with the aim of understanding the mechanism of inhibition. The apple slices packed in different treatments exhibited an increase in PPO activity; however, the samples packed in active films showed a significantly slower rate of PPO activity ($P < 0.05$). Almost 50% of the ascorbic acid content degraded in the control samples (4.95–4.97 mg/100 g fruit weight (FW)) at d7 compared with the samples packaged in BDF-releasing films (5.81–6.1 mg/100 g FW) in which the degradation was only 30%. Using molecular modelling, it was observed that BDF (−6.3 kcal/mol) exhibited a stronger interaction with PPO than catechol (−4.5 kcal/mol) in the met and oxy states. BDF-releasing films showed superior preservative potential over the control as BDF competitively inhibits the PPO active site due to π–π stacking between the aromatic cycle of the BDF molecule and the PHE259 and hydrogen bonding with the active site.

1. Introduction

According to the World Health Organisation, eating plenty of fruits and vegetables (F&V) is one of the five keys to a healthy diet to provide humans with essential nutrients and bioactive compounds such as polyphenols, vitamins and minerals. However, over the last decade, there has been an increasing trend for the consumption of minimally processed F&V owing to the availability of a wide variety of minimally processed products in the market (Siroli et al., 2015). Although minimal processing operations such as cutting, peeling, washing and modified atmospheric packaging (MAP) have been reported to maintain the freshness of the food products, the shelf life of minimally processed F&V remains quite limited (5–10 days) compared with their fresh counterparts. The main reasons for the loss of nutritional quality are microbial contamination, inadequate packaging and improper handling, as reported in 2021 United Nations Environment Programme Report (UNEP Annual Report 2021). However, one area that has not been given much attention is the loss of quality due to oxidation reactions. Similarly, red delicious apples (*Malus domestica*) are among the most consumed fruits in the world. They are highly susceptible to browning when minimally processed, mainly due to the presence of polyphenol oxidases (PPOs), that activate the oxidation of phenolic compounds into quinones, leading to the formation of brown pigments (Ghinea & Leahu, 2022). This not only affects the visual appeal but also results in the loss of key nutrients, reducing the overall nutritional quality of the apples. Enzymatic oxidation can cause 5%–30% loss of ascorbic acid and polyphenolic content in minimally processed apples (Khan et al., 2021). Additionally, minimal processing disrupts the cellular structure of the apples, making them more vulnerable to microbial contamination and oxidation, which accelerates spoilage. Several strategies have been employed to maintain the nutritional quality of these apples, including the use of edible coatings (extensively in the past decade), anti-browning agents, drying

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methods and modified atmosphere packaging (MAP) (Joshi et al., 2011; Rocha et al., 2004; Volpe et al., 2019). However, these methods often have drawbacks, such as changes in colour, taste and texture of the food. Alternatively, antioxidants have been reported to preserve the quality of minimally processed F&V by interacting/binding with oxidative enzymes, although the exact mechanism remains to be fully understood (Khan et al., 2021). This opens the possibility of incorporating antioxidants in polymeric packaging to study their effect on the decay kinetics of food products, an area with limited research to the best of our knowledge.

Polymer packaging has revolutionized food preservation by providing several critical benefits that enhance the shelf life and quality of food products. These materials offer superior barrier properties against gases, moisture, and light, which are essential in slowing down oxidation, moisture loss, and microbial growth in food items. Nevertheless, researchers are shifting their attention towards bio-based polymers as alternatives to petrochemical-based plastics owing to their non-renewable nature (El Itawi et al., 2022; Fadlallah et al., 2021). However, it is not enough to be renewable; in this context, biopolymers of microbial origin, such as polyhydroxyalkanoates (PHAs), are an ideal option for food packaging applications as they are biocompatible and are carriers of bioactive compounds (Laycock et al., 2013). Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is one of the most promising PHA polymers as it has physical properties comparable to those of polyethylene and polypropylene (still it depends on the relative ratio of each monomer) (Laycock et al., 2013). However, its brittle nature limits its application in the food industry, specifically due to lower elongation at breaks. This drawback can be addressed by incorporating food additives such as essential oils and phenolic acids, which can improve the elasticity of the films depending on their nature (Ordoñez et al., 2022; Requena et al., 2019). But also helps to reduce enzymatic browning and nutrient loss, which are common challenges in the storage of minimally processed foods. Recently, the structure of phenolic acids has been modified to be utilised as active additives that can impart desired properties to polymeric materials (Khan et al., 2023), however, it suffers from delayed crystallization during long-term storage. For instance, ferulic acid is a natural antioxidant compound with a structure that can be modified to produce a potent antioxidant: bis-o-feruloyl-1, 4-butanediol (BDF). BDF exhibits sustained antioxidant release (up to 3% of the actual quantity added) in minimally processed F&V-based systems, unlike ferulic acid incorporated in PHA-based films where release is undetectable (Khan et al., 2023). These additives demonstrate a high effectiveness in plasticizing polylactic acid (PLA) or PHA (Gallos et al., 2021; Longé et al., 2022). This plasticization phenomenon doesn't involve transesterification or other chemical secondary reactions during the process. It was also demonstrated that BDF tends to partially migrate to the surface of the PLA (Raghuwanshi et al., 2022). BDF was also considered for toxicity testing where it was found to be not an endocrine active compound compared to bisphenol A (Maiorana et al., 2016). This means BDF is available in the material for other purposes and is likely to serve as a functional additive, as well as its plasticizing effect. This strategy would simplify the formulation of active packaging, reducing health hazards related to unexpected interactions between chemical compounds (Moeini et al., 2021). Furthermore, BDF has been shown to improve biodegradation (>40% at 100 h) of active PHB materials compared with control PHB with increasing concentrations (from 0 to 40 w/w%) in the blends (Carboué et al., 2022). Phenolic acids have been found to actively inhibit the activity of PPO by binding near the enzyme's active site, leading to the rearrangement of the secondary structure of the enzyme (Zhou et al., 2016). Ferulic acids act as inhibitors of PPO by binding to the enzyme's active site, competing with the natural substrates (like catechol) that PPO typically oxidizes. This competitive inhibition occurs because ferulic acid has structural similarities with the natural substrates of PPO, allowing it to interact effectively with the enzyme's active site through hydrogen bonding and π–π stacking interactions. These interactions can alter the enzyme's conformation and reduce its catalytic efficiency, leading to a decrease in the formation of brown pigments (Zhou et al., 2020). Thus, to gain a better understanding of the effect of BDF on the PPO activity, molecular docking simulations can be realised to identify binding modes, assess the stability of these interactions and highlight the mechanism of inhibition of the oxidative enzyme that can also be correlated with decay kinetics of the food product. Molecular dynamics simulations can then evaluate the stability of these binding poses. This type of modelling has notably been applied with benzoic acid and cinnamic acid on PPOs (Sun et al., 2022), as these molecules bind near the active site. Due to the structural similarity between BDF, polyphenols and the substrate catechol, BDF holds the potential as an active inhibitor of these oxidative enzymes to preserve the quality of minimally processed apples. The apple PPO (UniProt ID: P43309) is a protein composed of 593 residues. It possesses a signal peptide consisting of 89 first residues and becomes active only after losing its C-terminal end, which hinders the active site (Kampatsikas et al., 2019). The protein is cleaved within the sequence 455–459. PPO is also a metalloenzyme characterised by an active site with two copper ions, each coordinated by three histidine residues (Deeth & Diedrich, 2010). Different forms of active sites exist, depending on the catalytic cycle, namely, the deoxy, met or oxy state. The states used for modelling are the oxy and met states, which correspond to the states at which the catechol substrate interacts with PPO (Jiang & Lai, 2020). Thus, a molecular dynamics approach can be used since extremely limited information is available on product-specific PPO inhibition, which is still a new concept in the food packaging domain. Furthermore, the effect of release behaviour on the decay kinetics of the food product remains unexplored. While a previous study has shown the release kinetics of BDF molecules from different polymeric films, its effect on food decay remains to be investigated (Khan et al., 2023).

Thus, the objective of this study was to evaluate the effectiveness of antioxidant-releasing PHBV films in slowing down the decay kinetics of minimally processed apples. Various quality parameters were evaluated, including PPO activity as a marker of the decay kinetics. Furthermore, principal component analysis (PCA) was conducted to simultaneously compare the different treatments based on multiple quality parameters. Finally, a simulation study was conducted to uncover the underlying mechanism of PPO inhibition by the BDF molecule.

2. Materials and methods

2.1. Materials

Chloroform (67-66-3), gallic acid, ascorbic acid (91215), sodium carbonate (223530), 2,6-dichloroindophenol sodium salt hydrate (for vitamin C determination, D1878), meta-phosphoric acid (ACS reagent, chips, 33.5%–36.5%) and Folin's reagent (F9252) were purchased from Sigma-Aldrich (Milan, Italy). Polyvinylpolypyrrolidone (PVP-360), Triton™ X-100 and pyrocatechol (C9510) were acquired from Microtech s.r.l. (Napoli, Italy). Red delicious apples (*Malus domestica*) were purchased from a local market in Portici (NA, Italy). All the other reagents used in this study were of analytical grade. The PHBV was obtained from Biomer (Schwalbach, Germany). The BDF was synthesized according to the protocol described elsewhere (Raghuwanshi et al., 2022).

2.2. Methods

2.2.1. Film preparation

The control (PHBV) and active packaging films (PHBV.BDF 10%, PHBV.BDF 20%) were prepared from extruded polymeric blends using the solvent casting method, as previously described by (Khan et al., 2023). Briefly, PHBV/BDF blends (100:0, 90:10 and 80:20 wt ratios) were prepared via extrusion using a compounding twin screw extruder (HAAKE MiniLab II, Thermo Fisher Scientific, Waltham, USA) at 60 rpm and 170 °C by setting the screws in co-rotation. A HAAKE MiniLab Pro

Piston Injection Moulding System with a DMA test bar (60 × 10 × 1 mm) was used to mould the samples. Furthermore, the mould was maintained at 45 °C during the injection. These moulds were then used to prepare films using the casting method. The polymer/BDF blends (533 mg) were dissolved in 26.6-mL chloroform, and the solution was poured into stainless steel moulds (7.5 × 2.5 cm). The solvent was evaporated under a fume hood at room temperature to obtain uniform films.

2.2.2. Preparation of minimally processed apples

All the utensils used to prepare minimally processed apples were first sanitised with 70% ethanol. Apples (5 kg) without any apparent defect or injury were selected based on their uniform shape and size and then immediately transferred to the lab. Then, the selected apples were thoroughly and gently washed with tap water for 3 min. Subsequently, each apple was peeled and sliced into eight uniform slices (dimensions: 7 × 2.5 cm; weight: ~19 g) using a common household apple slicer (Volpe et al., 2019). The film samples were carefully tape-wrapped around individual apple slices (active sample) and placed in disposable aluminium trays (18.2 × 7.7 × 4.5 cm). The control samples involved apples slices without packaging. All the samples were stored at 4 °C ± 0.2 °C and 60% of relative humidity for 10 days in dark condition. The apple slices were taken out at regular intervals (0, 2, 4, 7 and 10 days) to evaluate their nutritional quality attributes with and without different treatments (active and control). For each treatment, five replicates were evaluated, for a total of 50 samples.

2.2.3. Weight loss

The apple slices wrapped in films were weighed after regular intervals for 10 days. The weight loss percentage (%) was calculated in triplicates from the difference between the initial and final weights of the sample.

2.2.4. Colour parameters

A hand-held colorimeter (Minolta CR-300, Osaka, Japan) with an 8-mm circular measurement area was used to measure the surface colour of the apple slices by placing them on blank foil. The standard chroma metre D65 illuminant was used as a light source (Tsikrika et al., 2021). The observation geometry used was d/0°. Briefly, five replicates of three apple slices were used for each storage interval. A uniform colour space CIE-$L^*a^*b^*$ (-Lab) was used to record the colour, where L^* indicates lightness; a^*, greenness to redness; and b^*, blueness to yellowness. The L^* value is a good indicator of browning during storage due to either oxidation reaction or accumulation of pigments; conversely, the a^* value is correlated with colour changes of fruit flesh; thus, both can be used as indicators of decay and oxidative browning.

2.2.5. Polyphenol oxidase activity

2.2.5.1. Enzyme extraction. PPO is the main indicator of oxidative browning and deterioration in the fresh and minimally processed F&V. Thus, it is important to measure the enzyme activity for the packed and unpacked apple slices to understand the difference between the decay kinetics of the food product (Khan et al., 2021). The PPO activity was measured using a wet chemistry method described by (Shrestha et al., 2020) with slight modifications. Initially, enzyme extraction was performed. Briefly, 5 g of the sample was homogenised (Ultra-Turrax T18, IKA Instruments Ltd., Milan, Italy) for 3 min (with 30-s intervals to avoid heating) at 12,000 rpm with phosphate buffered saline (PBS, pH 6.5, 0.1 M) containing Triton X_{100} (1% v/v) and PVP (1.5% w/v) under ice-cooling conditions. The homogenised samples were then centrifuged at 8000 rpm for 10 min at 4 °C. Next, the supernatant was filtered using Whatman filter paper no. 1.

2.2.5.2. Enzyme activity measurement. For the enzyme activity measurement, pyrocatechol was used as a substrate (the final reaction mixture contained 2.3-mL PBS, 1.5-mL pyrocatechol (40 mM) and 0.2-mL crude enzyme). The reaction mixture was left to stand for a few seconds, and then absorbance changes were recorded at 420 nm for 2 min every 10 s using a UV–Vis spectrophotometer (JASCO V550, Tokyo, Japan). One unit of enzyme activity (EAU) can be defined as an increase in absorbance of 0.001/min. A reference cuvette containing only the substrate solution was also used. For this analysis, three biological replicates were tested, which further included three technical replicates.

2.2.6. Total phenolic content

The total phenolic content (TPC) of the apple slices packed in control and active films was measured using the Folin–Ciocalteu method described by (Yavari & Abbasi, 2022) with modifications. Briefly, 1 g of the sample was homogenised with 10-mL sodium carbonate (6%), centrifuged at 8000 rpm for 10 min at 4 °C and filtered with bench paper. Subsequently, 0.5 mL of the filtrate was added into 2 mL of sodium carbonate (6%) and 2.5 ml of Folin's reagent (10%). The samples were incubated at 30 °C for 1 h and then at 4 °C for another 60 min. The absorbance was measured at 760 nm using a UV–Vis spectrophotometer (JASCO V550, Tokyo, Japan). A standard gallic acid calibration curve (0–100 mg/L; $y = 0.0136x - 0.0304$, $R^2 = 0.9975$) was used to represent the total phenols as mg gallic acid equivalent (mgGAE)/100 g of fresh FW (Equation (1)).

$$TPC(mg\ GAE\ /\ 100g\ FW) = \frac{C \times V}{Sample\ weight\ (g)} \times 100 \tag{1}$$

where C denotes the gallic acid concentration obtained from the standard curve and V denotes the extract volume in which the sample was dissolved.

2.2.7. Ascorbic acid degradation kinetics

Ascorbic acid content was determined using the colorimetric method described by (Thapa et al., 2022) with modifications. The sample (1 g) was extracted in 6% meta-phosphoric acid (10 mL) via homogenisation at 8000 rpm for 4 min. The homogenate was centrifuged for 7 min at 4 °C at 10,000 rpm. Subsequently, 1 ml of supernatant was mixed with 4-mL meta-phosphoric acid (6%) and 10-mL 2,6-dichlorophenol indophenol dye using a rapid delivery pipette, the reaction mixture was shaken well and the absorbance was read within 15–20 s at 518 nm using a UV–Vis spectrophotometer. The ascorbic acid concentration was determined using a standard ascorbic acid calibration curve between 40 and 200 μg/mL ($y = -0.0042x + 0.942$, $R^2 = 0.9879$) and expressed as mg/100g FW of the sample using the following equation:

$$\text{Ascorbic acid content}\ (mg\ /\ 100\ FW) = \frac{Ascorbic\ acid\ content \times extraction\ volume\ (ml) \times 100}{Extracted\ reaction\ volume\ (ml) \times sample\ weight\ (g)} \tag{2}$$

It is important to understand the degradation kinetics of ascorbic acid to predict quality losses during storage and the effect of release kinetics on the decay kinetics phenomenon. Thus, vitamin C degradation was described by the pseudo first-order model (Equation (3)) according to (Bai et al., 2013):

$$P = P_0 \, exp(-kt) \quad (3)$$

where P denotes the measured vitamin C content at time t; P_0, the initial ascorbic acid content; and k, the rate change constant. The quality of regression and fitted models was determined by the coefficient of correlation (R^2).

2.2.8. Molecular docking and simulation studies

PPO has an experimentally determined structure (PDB ID: 6ELS). However, 6ELS is missing residues 1–31, 349–359 and 454–456, and several residues miss some atoms (numbering starts after the signal peptide). For the modelling study, the AlphaFold model (ID: AF-P43309-F1) predicted for this protein was used. This model is almost identical to the 6ELS structure with the missing part modelled. The structure 6ELS presents an oxygen between the two copper atoms corresponding to a water molecule. Copper and oxygen were missing from the AlphaFold model, and the 6ELS structure has been used as a starting point to add these atoms to the AlphaFold model with PyMOL and model the met and oxy states. The models were subject to a minimisation step before docking.

For molecular dynamics simulations, the Amber99SB force field (Lindorff-Larsen et al., 2010) was used with GROMACS 2021.4 (Abraham et al., 2015). Parameters for the active site, in the met and oxy states, were calculated using the MCPB (a python-based metal centre parameter builder) software (Li & Merz, 2016). Parameters and charges were derived from the QM calculations at the B3LYP/631G(d) level of theory using Gaussian 16. The amber parameter and coordinate files were converted to the GROMACS file format using ParmEd (Shirts et al., 2017). A total of 1000 steps of the steepest descent were then carried out.

For molecular docking, the AutoDock 4.2 programme (Morris et al., 2009) was used to generate and rank the receptor–ligand complex conformations. The protein structure is kept rigid except for phenyl 259, which is known to hinder the active site and be displaced when the substrate interacts with the PPO protein (Kampatsikas et al., 2019). AutoDock explicitly considers ligand flexibility. The grid maps used for each protein conformation were 126 × 126 × 126-dimension points with a grid spacing of 0.2 Å and centred around the active site. The Lamarckian genetic algorithm was used to score the conformational space of the ligand. For each docking run, the individual population in GA was set to 150, and the maximum numbers of energy evaluations and generations were set to 25000000 and 27000, respectively. A total of 10 GA runs were performed for each protein–ligand system. This set of parameters is adequate to reach the convergence of the docking results. BDF and catechol were used as ligands with PPO in the met and oxy states. The parameters for copper were added to the AutoDock parameter file. The van der Waals and other needed parameters (Rii: 2.96; epsii: 0.005; vol: 12.00; solpar: −0.00110) were obtained from (Vazquez-Rodriguez et al., 2023). For the copper and oxygen atoms of the active site, the charges from MCPB were applied.

The four systems obtained after the docking were simulated by molecular dynamics with the AMBER ff99SB force field to test the stability of the binding poses. The active site parameters were those computed using the MCPB software. Missing hydrogens were added to the docking output structures. The systems were solvated (TIP3P) (Jorgensen et al., 1983) in a box with a 1-nm distance between the protein and the periodic boundaries. Antechamber was used for the parameterisation of BDF and catechol. Furthermore, the ParaMed tool was used to convert the topology files into GROMACS-compatible format. The system was neutralised by adding an equal number of counterions. Subsequently, it was subjected to energy minimisation using the steepest descent algorithm. The systems were equilibrated with position restraint simulations of 0.1 ns carried out under NVT and NPT conditions. The position restraint force was applied to the protein-heavy atoms. Finally, the systems were subjected to 25 ns of production dynamics. The molecular dynamics parameters were obtained from (Li & Merz, 2021). A time step of 2 fs was used with periodic boundary conditions. Short-range electrostatic and van der Waals interactions were calculated with a 1-nm cut-off. In addition, long-range electrostatics were calculated with PME in a 0.16-nm spaced grid. Temperature was controlled by a v–v-rescale thermostat to 300 K with a tau-T of 0.1 ps–1. The pressure was adjusted to 1 atm by a Parrinello–Rahman barostat with a 0.2-ps tau-P. The trajectories were visualised using VMD (Humphrey et al., 1996) and analysed using GROMACS tools and homemade scripts. XmGrace was used for the plots.

2.2.9. Statistical analysis

All the quantitative data obtained were expressed as mean ± standard deviation. After a normality test (Shapiro–Wilk) and one-way analysis of variance, the significant differences in mean values among the different treatments were examined using Tukey's HSD ($P < 0.05$). Statistical analysis was conducted using the SPSS software (version 27.0, SPSS Inc., Chicago, IL, USA). For the TPC and ascorbic acid analyses, five biological replicates were tested, which further included three technical replicates. PCA was conducted on the R programme (v 12.0) using the built-in function prcomp, and a PCA biplot was constructed using the function fviz_pca_biplot from the factoextra package to uncover the underlying trends on how different decay kinetics parameters are related to each other and how the different treatments compared when these parameters were considered simultaneously (Whitehead et al., 2023).

3. Results and discussion

3.1. Weight loss

For the data analysis, the Shapiro–Wilk test results indicated that the data met the assumption of normality ($p > 0.05$), justifying the use of parametric tests for subsequent analysis. A one-way ANOVA was conducted to evaluate the effect of the treatment on various quality parameters. When significant differences were found ($p < 0.05$), Tukey's Honest Significant Difference (HSD) post hoc test was performed to identify which specific treatments differed from each other on the same day. One of the most important factors directly influencing and reducing the shelf life of fresh and minimally processed F&V is weight loss. Thus, weight loss of a food product caused by moisture and nutrient loss when its surface is exposed to the external environment is an inevitable

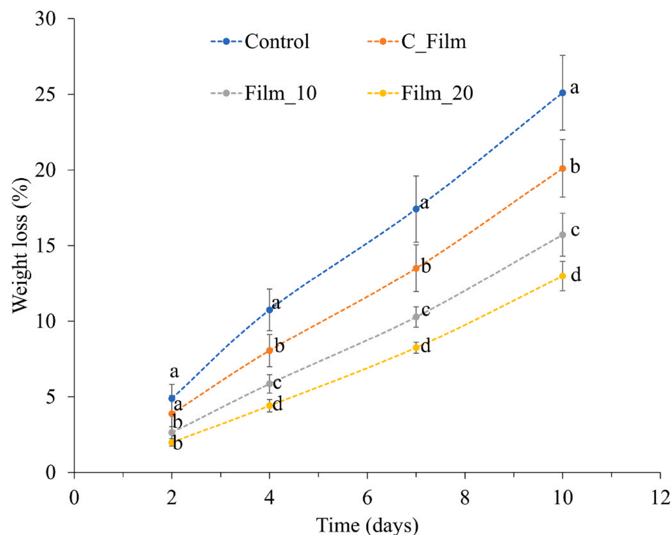

Fig. 1. Weight loss of apple slices packed in different treatments (without films = control, plain film = Film_C, PHBV.BDF 10% = Film_10 and PHBV.BDF 20% = Film_20). The different letters (a–d) above each point indicate significant differences among the mean observations ($P < 0.05$).

phenomenon that negatively impacts product quality (Cofelice et al., 2019). A significant difference was observed ($P < 0.05$) in the weight loss of the apple slices packed in active PHBV films compared with the control (product without packaging or packed in plain PHBV films) (Fig. 1). Initially, within the first 2 days of storage, the weight loss of the apple slices varied between 2% and 5%. The lowest weight loss was observed for apple slices packed in PHBV.BDF 20% films. However, the weight loss linearly increased for all samples, particularly for the unpacked apple samples, reaching 25% ± 2% after 10 days of storage, which can be attributed to increased microbial spoilage, fungal activities and loss of moisture from the product. However, this was not the case for the product packed due to the water vapour barrier properties of the PHBV (Eslami et al., 2022). Furthermore, the relative hydrophobic nature of the BDF molecule could have an impact on the water vapour transmission rate of the PHB films, leading to less weight loss during storage (Khan et al., 2023; Yavari & Abbasi, 2022). However, a strong correlation was observed between weight loss and ($R^2 \geq 0.996$) for the treatments (Rocha et al., 2004). The edible films/coatings have been reported to reduce weight loss in apple slices by preventing water loss by generating high relative humidity at the surface of the sliced apples (Cofelice et al., 2019). Furthermore, the ability of the film to reduce FW loss is also influenced by different permeabilities to the water vapours of the polymers used in the formulation. Nonetheless, lower weight loss in a coated food product can also be an indication of total soluble solid preservation over time due to π–π stacking of BDF molecule within PHBV (Vieira et al., 2016). Another possible reason for the reduced weight loss of the product packed in film samples could be the creation of a saturated atmosphere due to the hydrophobic films (Figs. S1 and S2), which minimised moisture loss even at high transpiration rates from peeled surfaces (Shrivastava et al., 2023). These findings can also be associated with the surface hydrophobicity of the films; however, it should be noted that other parameters such as oxidation reactions can contribute to the weight loss of the product by causing nutrient loss (Barbhuiya et al., 2021). Furthermore (Alves et al., 2017), found no significant difference in weight loss for apples wrapped in soy protein isolate films containing (1–4 g/L) ferulic acid at the end of the storage period due to the relative hydrophilic nature of ferulic acid as it can combine with water molecules and cause higher weight loss; however, this was not the case in our study as we used ferulic acid derivative, which is more hydrophobic.

3.2. Colour parameters

Table S1 presents the colour parameters (L^*, a^* and b^*) of samples stored with different treatments and at different times. The lowest L-values (65 ± 1) were observed for samples that were unpacked, whereas the highest L-value (69.7 ± 0.5) was observed for apple slices preserved in PHBV.BDF 20% on day 10. However, the rate of lightness decrease was different for each treatment. For instance, the samples preserved in active films had a significantly lower ($P < 0.05$) lightness compared with the control until day 4, which can be correlated with the consumption of substrates by PPO at different rates (Shemesh et al., 2016). The lightness (L-value) substantially decreased ($P < 0.05$) during the storage time for each treatment, which could be attributed to the generation of reactive oxygen species (ROS) caused by damage to cellular compartmentalisation response to physical stress, resulting in the contact between cellular PPOs and peroxidases with the polyphenolic substrates (Alves et al., 2017; Zhang et al., 2021). The active films might have released a sufficient amount of BDF into the food product to effectively bind with oxidative enzymes released during minimal processing operations that delayed browning. However, a sharp decrease in the L-value after day 4 could be due to the toxic effects of BDF on cellular contents on the fruit surface which might have caused organoleptic changes, as also reported by (Shemesh et al., 2016) for cherry tomatoes by carvacrol-loaded active packaging. Similarly, an increase in the a^*-value was observed, which can be directly correlated with a decrease in the L-value; both parameters contribute towards oxidative browning (Naqash et al., 2022). Initially, up to day 2, there was no significant difference in the a-values among all treatments; however, the effect of different film treatments was more pronounced after 4 days of storage. At the end of the experiment, the lowest a-value (−4.1 ± 0.1) was observed for apple slices packed in active films (PHBV.BDF 20%) due to the anti-enzymatic potential of BDF and the UV barrier properties. Contrarily, the control samples exhibited higher a-values (−3 to −3.45), indicating a more pronounced enzymatic browning, which can increase brown pigment concentrations (Gao et al., 2018). However, the b-value is not a reliable indicator to judge browning related to the enzymatic or non-enzymatic changes in fruits. Nonetheless, no significant difference was observed in the b-values of the samples packed in different packaging until day 7. The total colour difference (ΔE) increased during time ($P < 0.05$) up to a value of 14 ± 1 for the control sample. Samples packed with the active film showed a reduced increment of ΔE, which assumed the lowest value of 8.2 ± 0.2 for samples PHBV.BDF 20%. In agreement with (Volpe et al., 2019) ΔE of around 8 for minimally processed apples is still acceptable with no evidence of browning Similarly (Zhang et al., 2021), observed a protective effect of active tannic acid packaging on the browning index of apple wedges. This effect was attributed to the strong UV and oxygen barrier properties of the films, which protected fruit cells from peroxidation and maintained the integrity of cellular compartments of the cell membrane.

3.3. Polyphenol oxidase activity

One of the key changes associated with minimal processing of F&V is the release of oxidative enzymes such as PPOs. When these enzymes encounter phenolic compounds, they can initiate a degradation process. The abiotic stress caused by cutting and peeling can lead to the production of brown pigments (Olivas et al., 2007). Fig. 2 presents the PPO activity as a function of treatment and time. Although all the apple slices packed in different treatments exhibited an increase in PPO activity throughout the storage period, the rate of increase of the PPO activity was substantially faster ($P < 0.05$) in the unpacked apple slices or the ones packed in plain PHBV films. This was mainly because PPO quickly utilised the substrate on the apple surface, which led to the formation of quinones due to the oxidation of phenolic compounds. Therefore, no significant difference was observed in the PPO activity (90–95 EAU) of apple slices with control treatments ($P > 0.05$) (Fig. 2). Similarly (Zambrano-Zaragoza et al., 2014), observed an increasing trend in the PPO activity for control apple samples (37–140 U/min ml) until the 9th

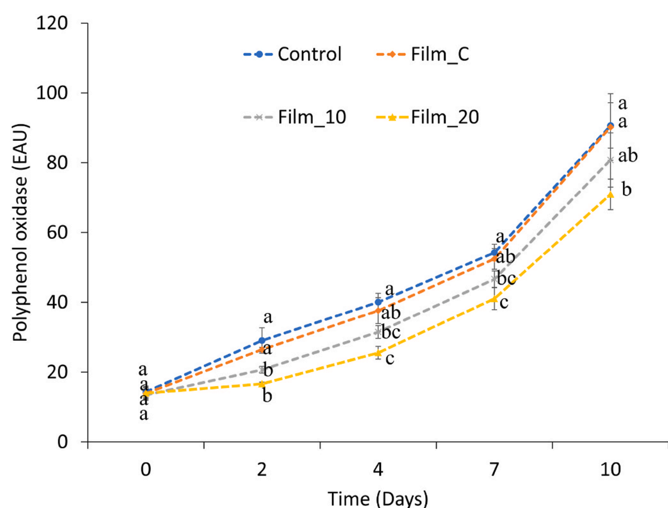

Fig. 2. PPO activity of apple slices packed in different treatments (without films = control, plain film = Film_C, PHBV.BDF 10% = Film_10 and PHBV.BDF 20% = Film_20). The different letters (a–d) above each point indicate significant differences among the mean observations ($P < 0.05$).

storage day. However, the PPO activity started to decline afterwards, possibly due to the covalent interaction of the generated quinones with the –NH_2 and –SH groups of the amino acids of the generated proteins, leading to alkylation and change in the secondary structure of the PPO enzyme. This resulted in a significant decrease in PPO activity (Wojdyło et al., 2008). However, we did not observe this decline in our study as the substrate was still available and the storage period was only 10 days, unlike the previous study that was conducted for 3 weeks. The PPO activity (absorbance/min/g) in this study remained below 0.4 until day 10, as observed by (Gardesh et al., 2016) for apples coated with nano-chitosan for 2 weeks.

Alternatively, apples packed with active PHBV films exhibited significantly lower PPO activity (70–80 EAU) due to the controlled and sustained release of BDF into the food systems, as confirmed by the back shift of the stretching vibrations of the C–O, O–H and C–H groups to their original wavenumbers after the release of BDF from PHBV films (Khan et al., 2023). Ferulic acid and its derivatives can effectively decrease the reaction quinones produced (by donating electrons to PPO from their hydroxyl groups) during the PPO-catalysed oxidation of polyphenols. They can also cross-link with PPO through hydrogen bonding and π–π stacking interactions, which can change the PPO polarity and reduce the formation of brown pigments. Another important factor is the structural similarity of the BDF additive to the phenolic substrates, which can allow BDF to act as a competitive inhibitor in a similar manner, as reported by (Nirmal & Benjakul, 2009) for ferulic acids.

It is noteworthy that the PPO activity exponentially increased after the 7th storage day, irrespective of the treatment, and there was no significant difference between the PPO activities of both controls and the PHBV.BDF 10% (80–90 EAU). This may be due to the limited BDF release, potentially reaching a plateau or interacting with other cellular components, which results in the limited availability of BDF towards the end of the storage period (Wang et al., 2015).

3.4. TPC

Phenolic compounds are secondary metabolites produced through phenylpropanoid metabolisation in many plants. These polyphenolic compounds can exhibit radical scavenging activity, chelate metal ions and modulate enzymatic activity (Peretto et al., 2017). All the treatments showed a decreasing TPC trend with an increasing storage time from d-0 to d-10; however, the rate of TPC degradation in the samples packed in active packaging was substantially slower ($P < 0.05$). The lowest and highest TPC values (545 ± 15 and 608 ± 8 mgGAE/100 g FW) were observed for apple slices unpacked and packed in 20% BDF-containing films (Fig. 3). These results can be directly correlated with the PPO activity as PPO enzymes are directly responsible for phenolic oxidation and degradation to produce brown pigments (Khaliq et al., 2019). However, a decrease in PPO activity due to interaction with BDF can slow down the decay kinetics of the phenolic content, as observed in the samples packed in active packaging. BDF possesses antioxidant properties that help protect phenolic compounds in the apples by scavenging free radicals and reducing oxidative stress. As the concentration of BDF increases, the availability of these antioxidant molecules on the apple surface also increases, enhancing the overall antioxidant effect. This leads to better preservation of TPC, as the phenolic compounds are less likely to undergo oxidation as also explained by (Deshmukh & Gaikwad, 2024) for phenolic compound concentration role in active packaging. An interesting phenomenon observed in the literature is the initial increase in phenolic content for two reasons. First, the stress caused by minimal processing operations can activate phenylalanine ammonia-lyase (PAL) which affects phenylpropane metabolism. Second, active coatings can stimulate changes in the non-enzymatic antioxidant components of F&V, such as polyphenols, by upregulating several key genes (Cao et al., 2023; Mendy et al., 2019). In our study, we only observed a non-significant increase in TPC for samples packed in control treatments at d-4, which could be attributed to the stress induced by the processing operations. However, a steady decline in the TPC of the samples packed in the active films was observed. This could be due to the interactions between the PAL enzyme and the released BDF molecule, which might have prevented the formation of more phenolic compounds. Similarly (Khaliq et al., 2019), observed a steady decline in the TPC of the product coated with *Aloe vera*-based coatings with the control showing faster degradation of total phenolics (from 137 to 85 mgGAE/100 g FW) than active coatings (from 139 to 132 mgGAE/100 g FW) due to higher PPO activity.

3.5. Ascorbic acid degradation kinetics

Ascorbic acid is a strong antioxidant that protects the F&V from oxidative deterioration caused by ROS (Blokhina et al., 2003). However, upon reacting with oxygen, ascorbic acid quickly degrades due to the activation of an oxidative enzyme, i.e. ascorbate oxidase. Thus, avoiding food contact with oxygen can delay the oxidative breakdown of ascorbic acid (Shah & Hashmi, 2020). The vitamin C degraded in all the apple slices, irrespective of the treatment applied throughout the storage

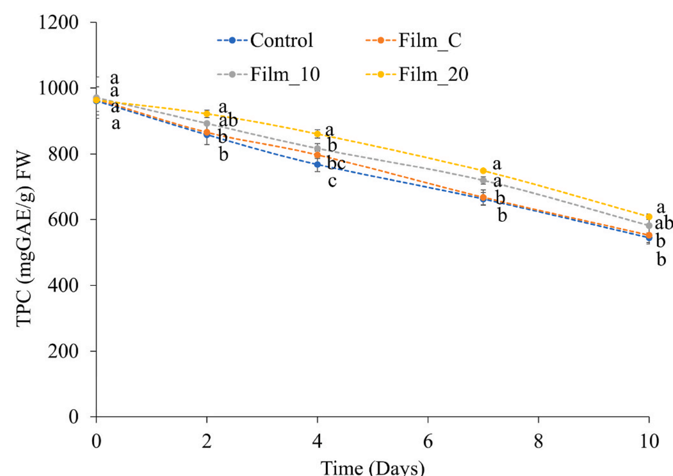

Fig. 3. TPC content of apple slices packed in different treatments (without films = control, plain film = Film_C, PHBV.BDF 10% = Film_10 and PHBV.BDF 20% = Film_20). The different letters (a–d) above each point indicate significant differences among the mean observations ($P < 0.05$).

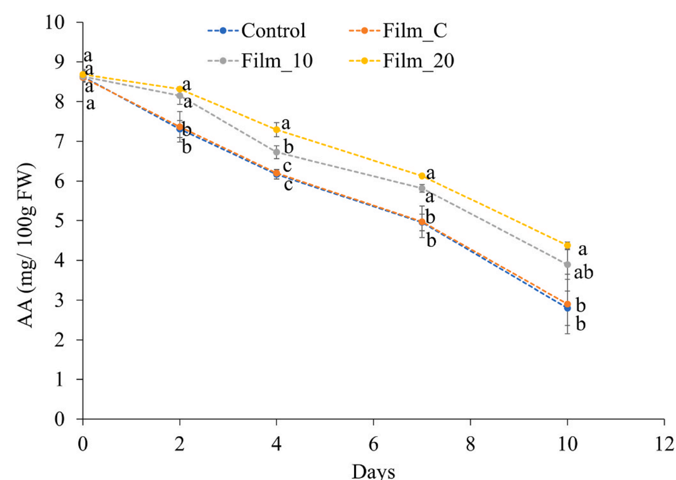

Fig. 4. Ascorbic acid degradation kinetics of apple slices packed in different treatments (without films = control, plain film = Film_C, PHBV.BDF 10% = Film_10 and PHBV.BDF 20% = Film_20). The different letters (a–d) above each point indicate significant differences among the mean observations ($P < 0.05$).

period. However, a significant difference was observed ($P < 0.05$) in the ascorbic acid contents of apple slices packed in active packaging and the control samples (Fig. 4). For instance, more than 50% of the ascorbic acid content degraded in the control samples (4.95–4.97 mg/100 g FW) at d-7 compared with the samples packed in BDF-releasing films (5.81–6.1 mg/100 g FW) in which the degradation was only 30%. This could be due to the release of small amounts of BDF onto the fruit surface, which could have interacted with the ascorbate oxidase enzyme to neutralise it after its activation during the cutting and peeling processes (Khan et al., 2022). However, at the end of the storage, no significant difference was observed between the ascorbic acid content of PHBV.BDF 10% films and the control, possibly due to the low release values or unavailability of the BDF owing to its higher affinity for other oxidative enzymes (Işık & Beydemir, 2021). Contrarily, some studies have reported an initial increase in vitamin C content with the application of edible gum coatings, followed by a steady decline possibly due to the nature of the coating composition (Daisy et al., 2020). Decay kinetics modelling was employed to describe ascorbic acid degradation over time. A critical quality index was established (at least 50% remaining ascorbic acid content at the end of the storage) to assess the influence of different treatments on vitamin C content. When the ascorbic acid concentration of apple slices was plotted against storage time, the coefficient of correlation (R^2) was found to be 0.98 for the pseudo first-order reaction (Fig. S3. Nonetheless, the models were a good fitting for the experimental data (Burdurlu et al., 2006). A significant decrease in k (from 0.067 to 0.043 day^{-1}) was found for the apple slices wrapped in active packaging films, indicating a much slower decay of vitamin C in such apple slices. Similarly (Maftoonazad & Ramaswamy, 2019), observed a higher k value (0.0074 day^{-1}) for the uncoated lime than those coated with pectin-based coatings (0.0066 day^{-1}). It has been reported that vitamin C is known to follow zero- or first-order reactions below 50 °C (Bosch et al., 2013). As can be seen from Fig. S3, active PHBV film containing 20% BDF was able to slow down vitamin C degradation by preserving at least 50% of its content at the end of storage due to the release of BDF onto the fruit surface.

3.6. Molecular modelling

Docking was performed using AutoDock with a 2.52-nm docking box centred around the active site. The binding energies computed from AutoDock (Table 1) exhibited a better interaction between BDF and PPO compared with catechol. For both molecules, the aromatic cycle was placed between PHE259 and the copper and oxygen ions (Fig. 5). A π–π stacking was observed between the aromatic cycle and the PHE259 and hydrogen bonding with the active site. This could lead to a change in the configuration of the secondary structure of the protein, which can inhibit the formation of oxidative products (Moon et al., 2020); however, this was not found in our study; nonetheless, the hydrogen bonding between PPO and BDF could lead to the formation of enzyme-inhibitor complex. Ferulic acid and its derivatives have also been reported to be placed well in the hydrophobic pocket of PPOs consisting of HIS61, HIS85, HIS 244, GLU256, PHE264, ALA286, and PHF292 due to strong hydrogen interactions, thus providing a mixed-type inhibitory effect (Yu & Fan, 2021). Similarly (Sun et al., 2022), observed a competitive inhibition of PPO upon the formation of a chelating structure between benzoic acid and PPO (copper atom), resulting in a decrease in the binding of the substrate at the PPO active site. Furthermore, hydrogen bonding can further facilitate the stabilisation of the enzyme–inhibitor complex. Molecular dynamics were conducted to evaluate the stability of the molecule at its interaction site. To compute contacts between molecules, a distance and angle cut-off of 3.5 Å and 30° were considered for hydrogen bonds and π-π interactions were computed as described by (Fu & Tian, 2011). The distance between the aromatics center of mass must be lower than 7.5 Å. For parallel configurations, the angle between aromatic planes must be lower than 40°. For perpendicular configurations, the angle between aromatic plans must be greater than 50° and one of the aromatics has to be above the plane of the other one within an angle lower than 40° according to the normal of the first aromatic plane (Fig. 6). Hydrogen bonds can be made between BDF and several amino acids. For the aromatic part of the BDF that is in the active site, these partners can be the PHE259 or the ASN258. As shown by (Deeth & Diedrich, 2010), hydrogen bonds can also be formed with the oxygen of the oxidised copper. RMSD analysis of the protein revealed that it remained stable throughout the simulation. Ligand RMSD analysis revealed that the aromatic group of the ligand molecule placed at the active site remained in that position during the whole simulation. However, the linker and the other aromatic groups of the BDF molecule exhibited greater mobility, resulting in higher RMSD (Fig. 7, dot lines). These results can also be correlated with the decrease in PPO activity observed in apple slices packed in active packaging systems compared with the control samples. While this study demonstrates the effectiveness of BDF-releasing PHBV films in inhibiting PPO activity and preserving the ascorbic acid content of minimally processed apples, there are several limitations that should be considered. First, the study was conducted exclusively on Red Delicious apples, which may limit the generalizability of the findings to other apple varieties or fruits with different PPO activity levels and antioxidant compositions. The controlled storage conditions (4 °C) may not fully represent real-world scenarios where temperature fluctuations could affect the BDF release kinetics and its effectiveness. Additionally, the study period was limited to 10 days, which does not capture longer-term storage effects, and further research could extend this period to better understand the long-term impacts. Moreover, while the study focuses on PPO inhibition and ascorbic acid preservation, potential interactions between BDF and other food components or enzymes were not explored not to mention the effects on microbial quality, which could influence overall food safety.

Table 1
Binding energies computed from AutoDock.

Binding energy (kcal/mol)	Met	Oxy
BDF	−6.39	−6.34
Catechol	−4.56	−4.51

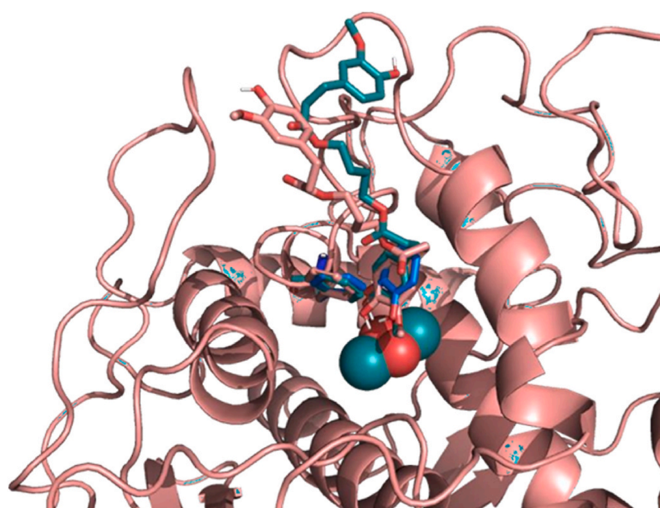

Fig. 5. Representation of catechol and BDF at the active site with the best energy of binding. PHE 259 is represented as sticks.

4. Conclusion

Nutritional losses due to oxidation are among the main and underrated contributors to food spoilage with oxidative enzymes, with PPOs being the primary culprits. In this context, this study was designed to produce BDF-releasing PHBV films using the casting method to

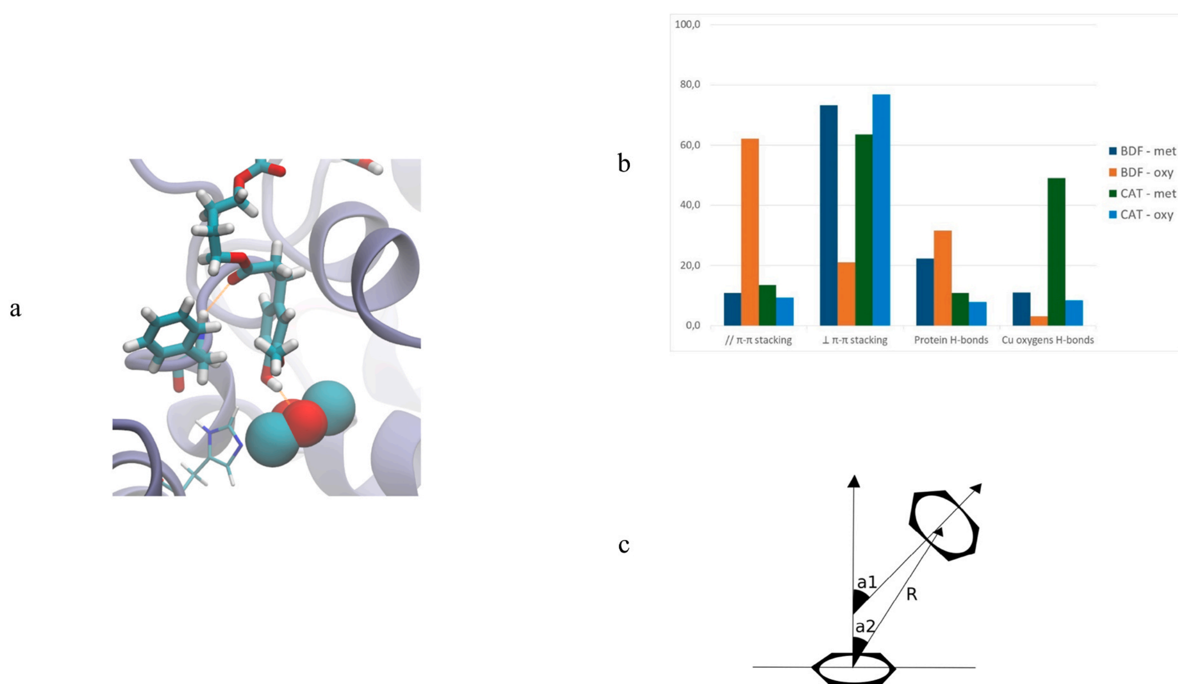

Fig. 6. a) Representation of the BDF at the active site with a visualization of its π- π stacking and hydrogen bonds with PPO. Hydrogen bonds are in yellow. b) Percentage of presence of the π- π stacking and hydrogen bonds during the PPO simulation between BDF or catechol and the protein in the met or oxy state. c) Spatial representation of the π-π interactions considered for the simulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

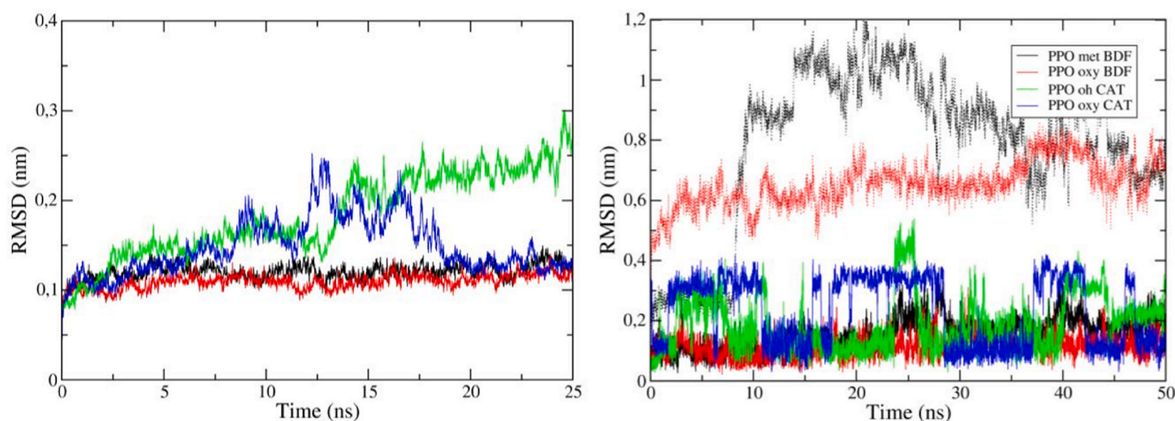

Fig. 7. RMSD on Cα after the least squares fit on Cα (left) and RMSD of catechol and BDF after the least squares fit of the protein on Cα (right). For BDF, the plain line corresponds to the aromatic part of the molecule, whereas the dot line corresponds to the whole molecule.

determine whether the BDF release has an influence on the decay kinetics of minimally processed products. Despite film brittleness upon/after application the surface hydrophobicity of the films led to a lower weight loss of the product compared with control samples without any packaging due to loss of moisture from the product as the relative humidity of the packaging headspace was higher than that of the surrounding environment. PPO quickly utilised the substrate on the apple surface, which led to the formation of quinones due to the oxidation of phenolic compounds. However, the oxidation rate was slower in samples packed in active packaging due to the donation of electrons to PPO from BDF hydroxyl groups, which restricted the oxidation of phenolics. This was also confirmed by molecular dynamics studies between PPO and BDF. PCA analysis revealed that PPO activity and TPC values are strongly correlated with each other (Fig. S4). This study confirmed that the release kinetics of bioactive compounds from packaging can be used as a tool to manage the decay of the food product. Furthermore, molecular modelling can be effectively used in the food packaging domain to understand the interactions between packaging and food components. The findings of this study have potential applications beyond minimally processed apples, but further investigation is needed to confirm their broader applicability. The mechanism by which BDF inhibits PPO activity may be relevant to other fruits prone to enzymatic browning, though differences in PPO chemical structure and fruit matrices should be accounted for. The use of BDF in other biodegradable polymers, such as polylactic acid (PLA) or starch-based films, could also be explored to assess its effectiveness across different packaging materials. Future research directions include testing the performance of BDF-releasing films under varying storage conditions, such as different temperatures and humidity levels, and investigating potential interactions of BDF with other food components.

CRediT authorship contribution statement

Muhammad Rehan Khan: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Jean-Marc Crowet:** Writing – original draft, Software, Data curation. **Sami Fadlallah:** Writing – review & editing, Visualization, Investigation. **Stefania Volpe:** Supervision. **Nicolas Belloy:** Validation, Software. **Florent Allais:** Writing – review & editing, Supervision. **Antoine Gallos:** Writing – review & editing, Supervision. **Elena Torrieri:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Muhammad Rehan Khan reports statistical analysis was provided by Reims Champagne-Ardenne University. Elena Torrieri reports financial support was provided by European Commission. If there are other authors, they 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116835.

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