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Assessing the impact of food-derived bioactives on digestive proteases by *in vitro* and *in silico* approaches

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The interactions between food components and digestive tract enzymes can affect nutrient absorption and impact an individual's health. Certain components, particularly polyphenols, are reported to inhibit digestive enzymes and are commonly referred to as anti-nutritional factors. Reports on this subject often contradict each other, highlighting the need for consistent methodologies to assess the potential impact of bioactive compounds. This study evaluated the "*in vitro*" activity of pepsin, trypsin, and chymotrypsin using ovalbumin, gluten, and haemoglobin as substrates in the presence or absence of twenty-five bioactive natural compounds belonging to different chemical classes at gastro-intestinal physiological concentrations (0.1 mM). The results indicate that bioactives may have opposite effects on proteolytic activity depending on the substrate/enzyme combination and bioactives structure. With ovalbumin as substrate, piceid and resveratrol were described as strong chymotrypsin activators (+1.46- and 1.17-fold change, respectively), phloridzin dihydrate as a weaker activator (+0.41-fold change), while phloretin was a strong inhibitor (−0.65-fold change). A computational approach based on molecular docking and dynamics simulations was used to investigate the interactions between selected bioactives, chymotrypsin and ovalbumin. The "*in silico*" study included piceid and phloridzin dihydrate, as well as their respective aglycones (resveratrol and phloretin). The results obtained through computational modelling indicate that all four bioactives can interact with chymotrypsin. However, only those bioactives that enhance *in vitro* proteolytic activity induce a partial unfolding of ovalbumin's structure. This suggests that the effect of bioactive compounds on protein digestion may be substrate-dependent, and may vary depending on the specific protein being digested.

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1. Introduction

Natural antioxidants, such as polyphenols and terpenoids, are recognized as key determinants of the health advantages associated with a diet rich in fruits and vegetables.^{1,2} However, the beneficial effects of polyphenol consumption on human health have been associated with a negative impact on the breakdown of macromolecules during digestion.³ Indeed, due to the generally low absorption of this family of bioactive compounds, most of them remain in the gastrointestinal tract, ranging from 0.4 to 3 mM,⁴ with reported inhibitory effects on

enzymes involved in the breakdown of macromolecular nutrients, such as starch and oligo- or polysaccharides.⁵ In particular, the inhibitory activity of a polyphenol against α -amylase is determined by molecular binding interactions such as hydrogen bonding and hydrophobic interactions. The effective inhibition of the enzyme is dependent on the presence of specific characteristics, including hydroxyl groups, galloyl substituents and conjugated systems, which are inherent to bioactive compounds.⁶

The inhibitory effects of phenolics on the digestion of saccharides and lipids may be considered one of the mechanisms, together with the modulation of neurohormones/peptides involved in food intake,⁶ in the prevention of obesity and other metabolic diseases.⁷ However, inhibitory effects on the digestion of proteins are not desirable, as it has been also demonstrated that fermentation of non-hydrolysed proteins by colonic flora increases the risk of colon cancer.⁸ Furthermore, several reports have indicated the formation of complexes between proteins and polyphenols in food, which may impair

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the enzymatic activities associated with the involved proteins and affect digestibility.^{9,10}

The picture is complicated also by conflicting reports on the effect of polyphenols on the activity of proteolytic enzymes in the digestive tract. For instance, He *et al.*¹¹ reported an inhibitory effect of green tea polyphenols on pepsin activity using haemoglobin as a substrate, whereas Donmez *et al.*¹² reported an increase of pepsin activity on β -lactoglobulin in the presence of epigallocatechin gallate. Aside from methodological issues related to the choice of a limited set of proteases and substrates, most of the available studies were limited to a quite narrow set of polyphenols.

To address at least some of these limitations, this study undertook a comprehensive investigation into the potential influence of phenolics on protein breakdown. Standardized approaches have been used to evaluate the impact of 25 bioactives belonging to different chemical classes (stilbenoids, dihydrochalcones, phenolic alcohols, phenolic acids, flavonoids, anthraquinone, and terpenoids) on the activity of key digestive proteolytic enzymes (pepsin, trypsin, and chymotrypsin). Stilbenoids are widely present in grapes, red wine and various berries,¹³ phenolic alcohols in olive oil,¹⁴ phenolic acids in red berries and citrus fruits,¹⁵ while flavonoids represent the main class in green tea, and legumes.¹⁶ Furthermore, the respective glycosylated forms were included to assess the effect of the glycoside in comparison to the aglycone alone. Each enzyme was tested on three different protein substrates (bovine haemoglobin, chicken egg albumin, and wheat gluten). Haemoglobin was selected as reference protein as one of the most common substrates for the assessment of pepsin activity,^{11,17–19} which inclusion may facilitate comparison of the results obtained in this study. Ovalbumin and gluten were tested as they are commonly used in real food or meals and can be considered as good food models for two extreme cases: (i) solutions of native globular proteins²⁰ and (ii) low digestible²¹ supramolecular assemblies of water-insoluble intrinsically disordered proteins,²² respectively. The study also included representative species of seldom studied glycosidic derivatives of representative phenolics,^{23,24} as well as terpenoids and anthraquinones.^{25,26}

To rationalise the results obtained from enzyme activity measurements, the structure/function relationship in selected polyphenol/enzyme/substrate combinations was addressed through the performance of molecular docking studies and molecular dynamics simulations. *In silico* analyses considered the most blatant effects observed for specific bioactive/enzyme/substrate combinations, regardless of whether the added bioactive enhanced or inhibited the proteolytic activity.

The final aim of this study is to define some of the molecular basis of the structure–activity relationship (SAR) in these systems, pointing out structural features (3D structure, substitution pattern, binding affinity) relevant to specific effects. This could represent a significant advancement in our comprehension of the digestive-modulating properties of food bioactives, with potential implications for the rational design and development of functional foods and food supplements.

2. Materials and methods

2.1 Materials

Bovine haemoglobin (H2625), chicken ovalbumin (A5503), wheat gluten (G5004), porcine pepsin (P6887; ≥ 3200 units per mg protein), bovine trypsin (T1426; $\geq 10\,000$ BAEE units per mg protein) and α -chymotrypsin (C4129; ≥ 40 units per mg protein), piceide (15721), phloretin (PHL82624), phloridzin dihydrate (274313), gallic acid (842649), ferulic acid (128708), caffeic acid (8.22029), oleuropein (12247), (+)-catechin hydrate (22110), procyanidin B1 (PHL89764), naringenin (N5893), quercetin (PHR1488), rutin (PHL89270) were from Sigma-Aldrich (St. Louis, MO, USA). Resveratrol (BD4672) and epigallocatechin gallate (BD42886) were from BLD Pharm (Reinbek, Germany). Pterostilbene (F093412) and tyrosol (F077935) were from Fluorochem (Hadfield, United Kingdom). Quercetin 3- β -O-glucoside (482-35-9) and 4-hydroxy benzyl alcohol (H20806) were from HWI Group (Appenweier, Germany) and Alfa Aesar (Haverhill, MA, USA), respectively.

2.2 Bioactives synthesis

δ -Viniferin, ϵ -viniferin, and a pterostilbene dimer were prepared according to Mattio *et al.*²⁷ Aloe-emodin was obtained through the oxidation of aloin as described by Dallavalle *et al.*²⁸ Safranin was prepared from picrocrocin (from saffron residues) as reported by Catinella *et al.*²⁹ Naringin and limonin were extracted from Citrus paradisi seeds as reported by Magurano *et al.*³⁰ Stock 10 mM solutions of each compound were prepared in ethanol. Fig. 1 reports the chemical structure of the selected bioactive compounds included in the study.

2.3 Pepsin, trypsin, and chymotrypsin activity

Pepsin, trypsin, and chymotrypsin activities were determined according to Borgonovi *et al.*, with slight modification.³¹ A very small volume (0.006 ml) of a stock 10 mM ethanolic solution of the investigated compound (or of ethanol alone, in control runs) was added to 0.494 ml of 3% solutions of haemoglobin, gluten, or ovalbumin, in water at pH 2 (for pepsin), or at pH 7 (for trypsin and chymotrypsin). The pH value was adjusted – when required – with 200 mM HCl. The reaction was started by adding 0.1 ml of a solution of the appropriate enzyme (30 mg L⁻¹ in 0.15 M NaCl, 0.0115 M CaCl₂, adjusted to pH 2 for pepsin, and pH 7 for trypsin and chymotrypsin). The final bioactives, substrates and enzymes concentration were 0.1 mM, 2.47%, and 5 mg L⁻¹, respectively. After 10, 15, 20, and 30 min, the reaction was stopped by adding 1 mL of 20% (w/v) trichloroacetic acid (TCA). After centrifugation (12 000g for 10 min), TCA-soluble peptides in the supernatant were detected at 280 nm. For this study, one proteolytic unit corresponds – regardless of the enzyme being used – to a 0.001 increase in A280 per minute. Activity was normalized for enzymes concentration and expressed as U mg⁻¹ enzyme. The effect of a given bioactive was calculated as the change (positive or negative) with respect to control. The final ethanol concentration was 1%, which did not result in a significant alteration to enzyme activity in comparison to the absence of ethanol.



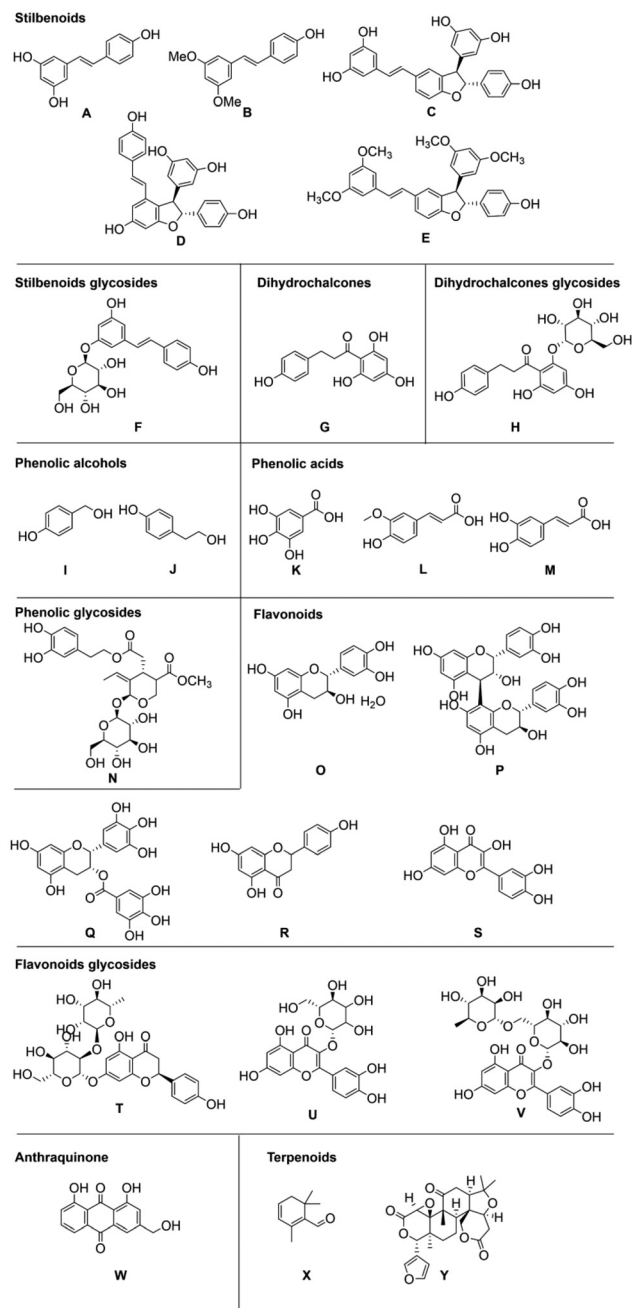


Fig. 1 Chemical structure of selected bioactive compounds. Resveratrol (A), pterostilbene (B), δ -viniferin (C), ϵ -viniferin (D), pterostilbene dimer (E), piceide (F), phloretin (G), phloridzin dihydrate (H), 4-hydroxybenzyl alcohol (I), tyrosol (J), gallic acid (K), ferulic acid (L), caffeic acid (M), oleuropein (N), (+)-catechin hydrate (O), procyanidin B1 (P), epigallocatechin gallate (Q), naringenin (R), quercetin (S), naringin (T), quercetin 3- β -O-glucoside (U), rutin (V), aloe-emodin (W), safranal (X), limonin (Y).

2.4 Proteins and polyphenols model design

A complete, good-resolution (2.00 Å) structure of bovine chymotrypsin was used (PDB code 1ACB; <https://www.rcsb.org>; last accessed September 2023).³² The UCSF Chimera software (version 1.16)³³ was used to process further the structure

removing water and co-crystallized ligand, and adding hydrogens before running further analysis. The 3D structure of ovalbumin was derived from the AlphaFold (<https://alphafold.ebi.ac.uk/>) predicted structure retrieved in .pdb format from Uniprot (code: P01012), since the crystallographic structure in the RCSB database is not complete. AlphaFold is a deep learning-based system commonly used for the prediction/analysis of the structure of proteins.³⁴ The AlphaFold structure was compared to the best resolution structure from PDB (ID 1OVA; 1.95 Å; last database access September 2023)³⁵ recording an RMSD of 0.49 Å, pointing to high comparability of the two structures and supporting reliability of AlphaFold structure as a valid model.

The 3D structures of the polyphenols used for *in silico* studies (resveratrol, CID: 445154; piceid, CID: 5281718; phloretin, CID: 4788; phloridzin dihydrate, CID: (4789), were taken from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>; last database access September 2023)³⁶ in the Structured Data File format (.sdf). The molecules were converted into Tripos Mol2 format using Open Babel³⁷ for further analysis.

2.5 Molecular docking

Molecular docking was performed to assess the interaction between the selected polyphenols and chymotrypsin and ovalbumin. The simulations were carried out using GOLD software (version 2021).³⁸ The ligands were kept fully flexible and the proteins semi-flexible, allowing polar hydrogens to rotate freely. Local docking was performed defining the chymotrypsin binding site within a 10 Å radius sphere around the centroid of the co-crystallized ligand of the 1AFQ structure. Four water molecules (#507, #539, #595, and #596, according to PDB structure 1AFQ) were kept during docking simulations as found buried within the ligand binding site and thought important to shape the space available to arrange ligands. Additionally, the arrangement of each ligand with the protein pocket was facilitated using the aromatic 'pharmacophore' constraint (150 units weight), set in a 0.7 Å radius sphere centred in correspondence to the centroid of aromatic ring of 1AFQ ligand. This promoted the arrangement of the aromatic rings in accordance with the crystallographic data. The ovalbumin binding site was defined based on previous studies^{39,40} as a 5 Å radius sphere around the pocket's centroid. Ten poses were generated for each ligand, and only the best-scoring pose, according to the internal scoring function PLP Score was used in the analysis.

2.6 Molecular dynamics simulations

Molecular dynamics simulations were carried out to verify the stability of proteins-polyphenols and monitor their conformational changes over time.⁴¹ They were performed by using GROMACS (version 2019.4) with CHARMM27 all-atom force field parameters support.⁴² Each ligand was processed and parametrized with the SwissParam tool (<https://www.swissparam.ch>).⁴³ To investigate how these molecules may interact in a real-time context, an environment as similar as possible to a real biological system was obtained solvating the



input structures with SPCE water in a cubic periodic boundary condition and neutralizing the complexes by adding Na^+ and Cl^- as counter ions. Each system was then energetically minimized to avoid steric clashes and correct improper geometries using the steepest descent algorithm with a maximum of 5000 steps. Subsequently, each system underwent 100 ps simulations under isothermal (300 K, coupling time 2 ps) and isobaric (1 bar, coupling time 2 ps) conditions. Finally, molecular dynamics simulations were run for chymotrypsin-polyphenols and ovalbumin-polyphenols systems. The simulations were 80 ns long and were conducted at 300 K with a coupling time of 0.1 ps and 1 bar with a coupling time of 2 ps, in agreement with previous studies that successfully investigated systems like those under analysis.^{44,45}

2.7 Statistical analysis

Enzyme activity in the absence of selected polyphenols was compared using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for statistical analysis. The effect of bioactives on enzyme activity was evaluated using the ANOVA followed by Dunnett's post-test. All the statistical analyses were

performed using GraphPad Prism 10 (GraphPad Software Inc.; San Diego, CA, USA).

3. Results and discussion

3.1 Pepsin, trypsin, and chymotrypsin activity

3.1.1 The nature of substrate affects the enzymes' activity. The activity of pepsin, trypsin, and chymotrypsin on the different substrates in the absence of the selected dietary bioactive compounds is reported in Table 1. Pepsin exhibited the highest apparent proteolytic activity, which is likely attributable to the loss of structural integrity of the substrate proteins at low pH.⁴⁶ The three proteases demonstrated a clear preference for haemoglobin as a substrate. Conversely, trypsin exhibited limited activity on wheat gluten, which is thought to possess a relatively low abundance of the positively charged amino acid sidechains that are a specific target for trypsin.⁴⁷

3.1.2 Bioactives modulate the enzymes' activity. Modulation of pepsin activity by selected dietary bioactives using haemoglobin, ovalbumin, and gluten as substrates are reported in Fig. 2A–C, respectively. In the presence of haemoglobin and gluten (Fig. 2A and C), naringin was found to be one of the most potent promoters of pepsin activity, while its counterpart aglycone naringenin showed no activation. Conversely, in the presence of ovalbumin, the naringenin aglycone demonstrated the most potent effect, while the glycosylated molecule appeared to be irrelevant (Fig. 2B). The effect of sugar on the small molecule's activation capacity was observed in relation to the substrate type for both phloridzin dihydrate and its aglycone phloretin. The activation capacity was potent for both forms in the presence of gluten (Fig. 2C), and null in the presence of haemoglobin and ovalbumin (Fig. 2A and B).

Table 1 Digestive enzymes activity with the different substrates. Data are means \pm SD of at least four replicates and are expressed as U mg^{-1} enzyme. Statistical analysis was carried out by one-way ANOVA (all: $p < 0.05$) with Tukey's *post hoc* test. Different capital letters in the same row and lowercase letters in the same column indicate significant differences (at least $p < 0.05$)

	Haemoglobin	Ovalbumin	Gluten
Pepsin	1183.6 \pm 164.7 ^{A,a}	222.4 \pm 22.7 ^{B,a}	142.5 \pm 1.4 ^{B,a}
Trypsin	98.2 \pm 25.3 ^{A,b}	58.7 \pm 9.3 ^{B,b}	24.9 \pm 4.4 ^{C,c}
Chymotrypsin	98.8 \pm 10 ^{A,b}	47.09 \pm 11.4 ^{B,b}	56.8 \pm 10.6 ^{B,b}

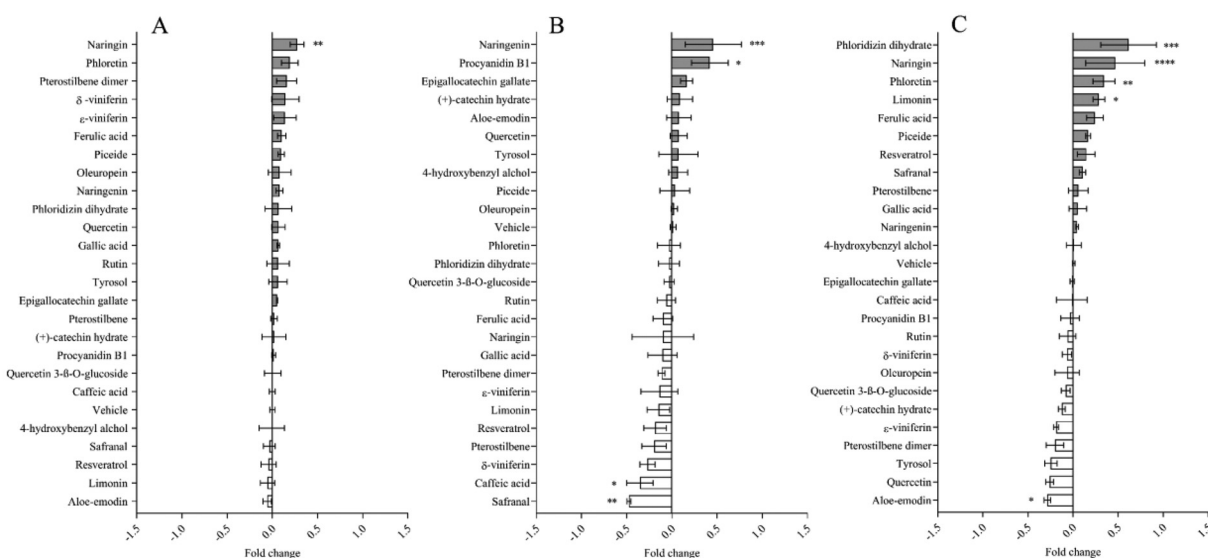


Fig. 2 Modulation of pepsin activity by dietary bioactives with haemoglobin (A), ovalbumin (B), or gluten (C) as substrates. Data are means \pm SD of at least four replicates and are expressed as fold change with respect to control. Statistical analysis was the ANOVA (all: $p < 0.05$) followed by Dunnett's post-test with respect to the vehicle (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).



In contrast, no difference was found between quercetin 3- β -O-glucoside and quercetin. Both compounds have no effect in the presence of haemoglobin (Fig. 2A), ovalbumin (Fig. 2B), and gluten (Fig. 2C). The substrate plays a key role in modulating proteolytic activity in the presence of terpenoids. Safranal and limonin had varying effects on pepsin activity depending on the substrate: safranal decreased, limonin increased, and they had no effect on activity in the presence of ovalbumin (Fig. 2B), gluten (Fig. 2C), and haemoglobin (Fig. 2A), respectively.

Fig. 3A–C show similar experiments carried out with trypsin as the enzyme. The impact of glycoside presence on substrate type is particularly noticeable when trypsin is present as an enzyme. Naringin, phloridzin dihydrate, and piceide, which are the glycosylated molecules, are some of the most effective activators of trypsin activity when haemoglobin (Fig. 3A) and ovalbumin (Fig. 3B) are present as substrates. In contrast, the presence of gluten as substrate eliminates the proteolysis-promoting activity of naringin, phloridzin dihydrate, and piceide (Fig. 3C). Furthermore, both quercetin 3- β -O-glucoside and its aglycone demonstrated no effect in the presence of ovalbumin and gluten (Fig. 3B and C), and contrasting effects in the presence of haemoglobin (Fig. 3B). Regardless of the glycoside, the substrate-dependent effect was confirmed. Tyrosol was found to promote trypsin activity in the presence of gluten (Fig. 3C), but no effect in the presence of haemoglobin and ovalbumin (Fig. 3A and B).

Modulation of chymotrypsin activity by selected dietary bioactives using haemoglobin, ovalbumin, and gluten as substrates are reported in Fig. 4A–C, respectively. In the case of chymotrypsin, the presence of the glycoside has been shown to modulate enzyme activity.

Specifically, the glycoside increases the catalytic activity of phloretin in haemoglobin and gluten (Fig. 4A and C) and of quercetin in ovalbumin and gluten (Fig. 4B and C) compared

to the corresponding aglycone (Fig. 4A and C). However, there were no differences in the modulation of enzyme activity between resveratrol and its glycosylated form, regardless of substrate type (Fig. 4A–C). In contrast, the effect of the glycoside was more complex when bound to naringenin. Naringin positively modulated the enzyme's activity in the presence of haemoglobin and gluten (Fig. 4A and C), but had no effect in the presence of ovalbumin (Fig. 4B). The corresponding aglycone, naringenin, increased proteolysis in the presence of haemoglobin (Fig. 4A), but not in the presence of ovalbumin or gluten (Fig. 4B and C). Despite the presence of glycoside, bioactive compounds still had a substrate effect on modulation. Epigallocatechin gallate was found to be a potent promoter or inhibitor of enzyme activity in the presence of haemoglobin (Fig. 4A), respectively, but had no effect when ovalbumin and gluten were present (Fig. 4B and C). It is worth noting that bioactive compounds can have similar or opposing effects on enzyme activity, depending on the type of enzyme and regardless of the substrate. For instance, in the presence of haemoglobin, naringin has been shown to promote all enzymes activity (Fig. 2–4A). On the contrary, in the presence of ovalbumin, resveratrol has been shown to promote chymotrypsin (Fig. 4B) but have no effect on pepsin and trypsin activity (Fig. 2–3B). Regioisomerism had an opposite effect on chymotrypsin activity on haemoglobin in the presence of δ - and ϵ -viniferin, causing a decrease and a null effect, respectively (Fig. 4A).

A 2-dimensional coloured dataset visualization representing the magnitude of individual values inherent effect of glycosylation and regioisomerism is reported in Fig. 5.

The current literature lacks agreement about the effect of polyphenols and polyphenols-rich food products on the activity of proteolytic enzymes in the digestive tract.^{17,19,48–51}

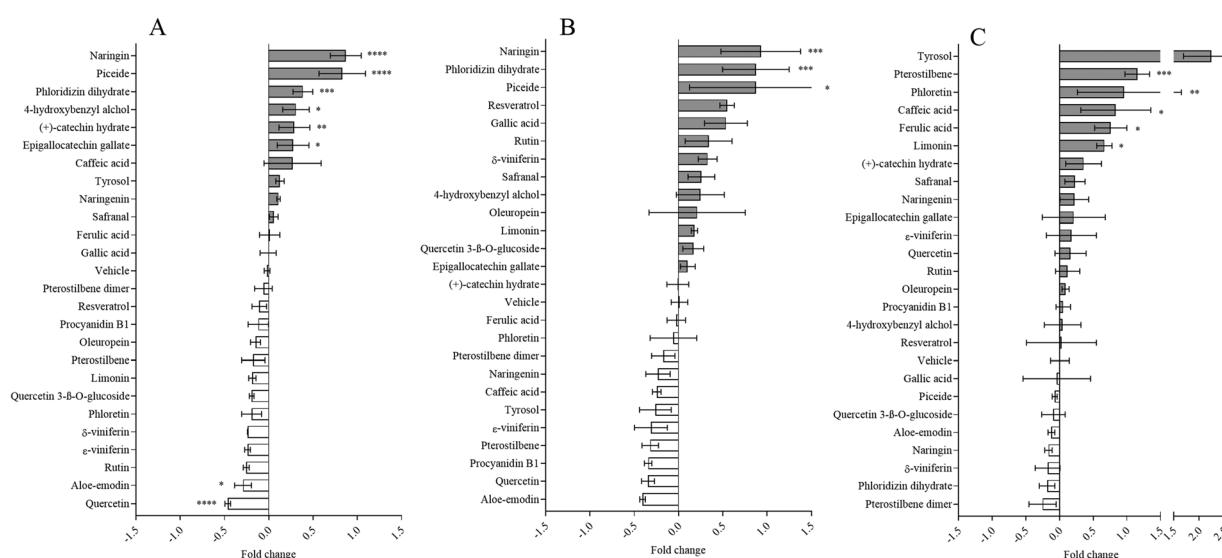


Fig. 3 Modulation of trypsin activity by dietary bioactives with haemoglobin (A), ovalbumin (B), or gluten (C) as substrate. Data are means \pm SD of at least four replicates and are expressed as fold change with respect to control. Statistical analysis was the ANOVA (all: $p < 0.05$) followed by Dunnet's post-test with respect to the vehicle (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).



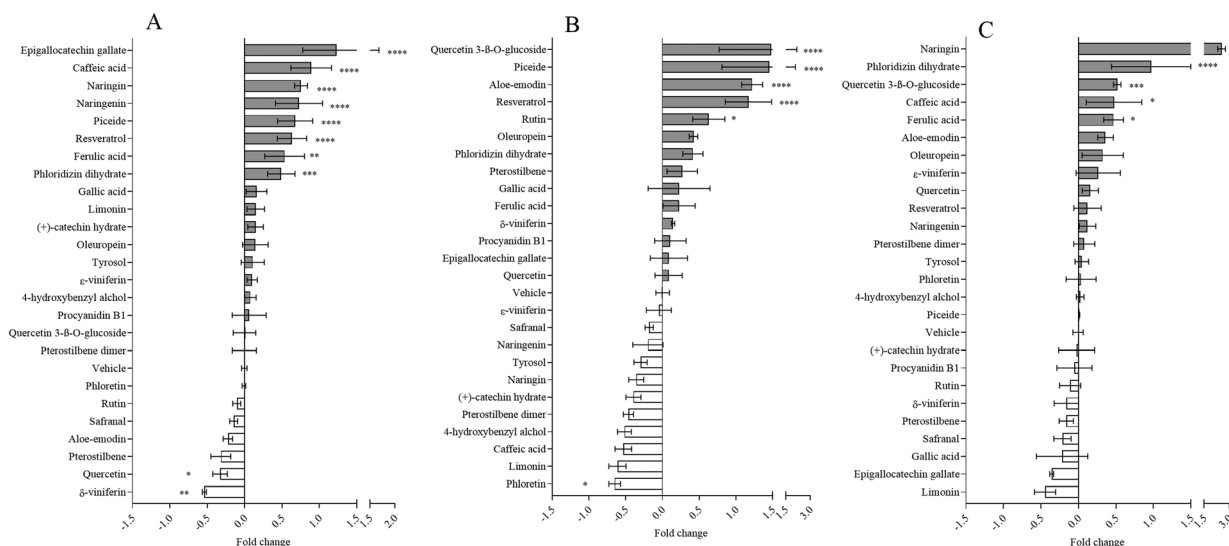


Fig. 4 Modulation of chymotrypsin activity by dietary bioactives with haemoglobin (A), ovalbumin (B), or gluten (C) as substrates. Data are means \pm SD of at least four replicates and are expressed as fold change with respect to control. Statistical analysis was the ANOVA (all: $p < 0.05$) followed by Dunnet's post-test with respect to the vehicle (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

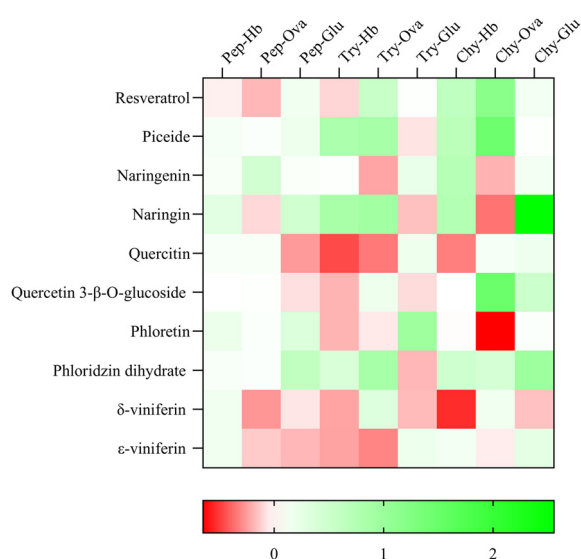


Fig. 5 Heatmap representation of the effect of glycosidation and regioisomerism on digestive proteases activity with different substrates. Pep: pepsin; Try: trypsin; Chy: chymotrypsin; Hb: haemoglobin; Ova: ovalbumin; Glu: gluten.

Recently, Urbinati *et al.* indicated that the polyphenol-rich balsamic vinegar was able to decrease pepsin, but not trypsin, activity using haemoglobin and *p*-toluene-sulfonyl-L-arginine methyl ester as substrate.¹⁹ On the contrary, Myint *et al.*⁴⁹ and Huang *et al.*⁴⁸ evidenced that polyphenols from stevia and tea leaves were able to inhibit trypsin activity using the synthetic peptide benzoyl-DL arginine-*p*-nitroanilide, respectively. Similarly, Naz *et al.* reported an inhibitory effect of trypsin and chymotrypsin activity by epigallocatechin-3-gallate using *N*-

α -benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester, respectively.¹⁷ On the contrary, Tantoush *et al.* reported that pepsin-resistant proteins, such as β -lactoglobulin and Ara h 1 and Ara h 2, degrade faster in the presence of catechin-enriched green tea polyphenols.⁵¹ These effects were also confirmed by Tagliazucchi *et al.* who reported that resveratrol > quercetin > epigallocatechin-3-gallate > catechin enhanced the proteolytic activity of pepsin using haemoglobin as protein substrate by increasing the V_{\max} of the reaction.⁵⁰ The conflicting results can be partially explained by the different experimental conditions adopted in these studies, such as bioactives concentration, substrate type and concentration, bioactives/enzyme ratio, bioactives/substrate ratio, and the way activity was assessed (SDS-PAGE, OPA assay, absorbance at 280 nm).⁵²

Also, it is worth noting that many studies have used experimental settings that are far from normal physiological conditions, such as using different substrate types (proteins *vs.* synthetic peptides) and varying bioactives and substrate concentrations.⁵²

The choice of protein substrates seems to be crucial, especially considering their resistance to digestion and frequency in the diet. Although in the present study the chosen substrates may appear less resistant to digestion compared to legume proteins, a recent article has compared the protein digestibility of cereals and legumes.²¹ The main findings indicate that the protein digestibility of legumes was generally higher than that of cereals, with wheat having a protein digestibility comparable to that of most of the legumes studied (adzuki bean, pea, lentil and soya bean). Furthermore, although gluten is not the primary dietary source of protein, cereals and cereal products are a fundamental part of the human diet, providing a significant proportion of dietary energy and nutrients in both developed and developing countries.⁵³



Also the selection of bioactives concentration represents a critical factor in achieving a result that can be extrapolated to reflect the *in vivo* scenario, particularly considering their well-established concentration-dependent effect on the modulation of protease activity.^{49,50} In a recent study, Vyas *et al.* reported that curcumin, diosmin, morin, and 2',3',4'-trihydroxychalcone were able to inhibit pepsin, trypsin, and chymotrypsin activity, with the degree of inhibition being concentration-dependent.⁵⁴

To circumvent these limitations, the enzyme's activity was appraised through a standardised approach at a single concentration that exclusively comprised native proteins and bioactives. The substrate concentration (2.47%) and bioactive concentration (0.1 mM) were selected in accordance with the standardised static *in vitro* digestion INFOGEST protocol⁵⁵ and predicted gut polyphenols concentration,⁴ respectively. In a recent study, Tamargo *et al.* demonstrated that the total concentration of hydroxybenzoic acids, hydroxycinnamic acids, flavonols and flavan-3-ols in red wine after *in vitro* gastrointestinal digestion was approximately 0.18 mM.⁵⁶ Indeed, the concentration of polyphenols within the intestine can fluctuate between 0.4 and 3 mM, contingent upon the quantity of these compounds present in the ingested food, the portion size consumed, and the extent to which they are released into the digestive tract (bioaccessibility). Consequently, it is challenging to establish a universally applicable value.

For this reason, the present study focused exclusively on a single concentration of bioactives and protein substrates, as previously reported.^{11,57} The addition of other concentrations would have resulted in an excess of experimental conditions, rendering it unfeasible to comprehensively explicate and discuss all the results within the confines of a single study. However, further studies are required to ascertain the impact of bioactive concentration on the diet.

The varying effects found among different bioactive compounds cannot be solely attributed to the different experimental conditions adopted in the studies. The effects on protein digestibility may be due to the binding of the bioactives compounds to digestive proteases and protein substrates, which is dependent on their chemical structure, affecting protease activity and accessibility of protein substrates. Enzyme activity may vary because of phenolic chemical binding, which generally can relax or stabilize enzyme structure. Besides competitive inhibitors targeting the substrate binding site, enzymes can be inhibited when their structure is altered to the point of disrupting the correct positioning and orientation of substrate and/or catalytic residues. For this reason, it is important to predict protein conformational changes, particularly for phenolics with bulky structures such as tannins or glycosides of phenolic compounds.⁵⁸

Furthermore, the binding of phenolic compounds can hinder the catalytic or substrate binding sites, reducing the proteolytic activity⁷ or causing enzyme inhibition through allosteric effects. On the contrary, polyphenols may act as allosteric effectors⁵⁹ or modify enzyme conformation (stabilization) for a better fitting of protein/substrate.⁶⁰ Like proteases, polyphenolic compounds can either stabilize or destabilize the

structure of protein substrates, making them less or more susceptible to proteolytic attack, respectively. Phenols may specifically reduce the accessibility of proteases that prefer hydrophobic residues⁶¹ by binding to proteins and primarily interacting with their hydrophobic sites.⁶² On the contrary, the binding of phenolic compounds can destabilize the protein structure by causing partial unfolding, which increases the protein's accessibility to enzymes. Generally, phenolic compounds may influence protein hydrolysis through their interactions with both the protein substrate and the protease itself, making it difficult to determine the net effect of phenolic compounds on protease activity when using protein substrate. In this complicated but intriguing scenario, *in silico* investigations can help to study the behaviour of small molecules, within the binding site of a target protein and understand the fundamental molecular mechanisms underlying their action.⁶³

3.2 *In silico* bioactives interaction with chymotrypsin and ovalbumin

3.2.1 Molecular docking. The interactions between digestive enzymes and polyphenolic compounds modulating their activity still need further analysis.⁷ In this respect, we applied a molecular docking approach that previously succeeded in estimating the geometries of binding of ligands.⁶⁴ These theoretical methods may estimate the binding affinity and the identification of crucial residues for ligand recognition.⁶⁵ In addition, molecular dynamic simulations can predict the protein–ligand complex movement over time based on a general physics model that governs interatomic interactions.⁶⁶ These simulations can capture critical biomolecular processes, such as conformational changes for protein and ligand as well as ligand binding/unbinding phenomena. Herein, a 3D *in silico* molecular modelling study made of docking and molecular dynamics simulations was conducted on a selection of polyphenols, chosen as case studies, to investigate the mechanisms possibly underlying their effects on enzyme activity from a molecular perspective. Specifically, ovalbumin was chosen among the substrates considered in this study, being an ideal model system that could be more proficiently investigated *in silico* compared to gluten and haemoglobin. Concerning the selection of polyphenols and enzymes, resveratrol, piceid, phloretin, and phloridzin dihydrate were selected among those tested *in vitro* as they presented an interesting chemical landscape to study because: (i) piceid and phloridzin dihydrate are the glycosides of resveratrol and phloretin, respectively; and (ii) the conjugation had either a huge and opposite (as for phloretin and phloridzin dihydrate) or a similar (as for resveratrol and piceid) impact on the activity of respective aglycones on chymotrypsin activity. Indeed, according to the fold changes observed (Fig. 4B), piceid and resveratrol were described as strong chymotrypsin activators (+1.46- and 1.17-fold change, respectively), phloridzin dihydrate as weak activator (+0.41-fold change), while phloretin was an inhibitor (−0.65-fold change).

Based on docking analysis and the positive scores recorded (Table 2; the higher the score, the better the interaction, as per



Table 2 Docking PLP scores of the selected polyphenols within chymotrypsin and ovalbumin

	Chymotrypsin	Ovalbumin (binding site)
Resveratrol	201.11	44.95
Piceid	214.93	44.73
Phloretin	211.75	52.27
Phloridzin dihydrate	212.62	53.77

manufacturer declaration), the selected polyphenols (*i.e.* resveratrol, piceid, phloretin, and phloridzin dihydrate) seemed to favourably interact with both chymotrypsin and ovalbumin.

Specifically, as per manufacturer declaration (<https://www.ccdc.cam.ac.uk>), positive scores indicate the capability of ligands to satisfy the physicochemical requirements of the pocket and can be used to estimate the likeliness of protein-ligand complex formation. Germane to the case study, all the ligands recorded positive scores pointing to their theoretical capability to favourably interact with both proteins. Of note, the scores for chymotrypsin were around four times higher than those recorded for ovalbumin due to the constraint used to dock the ligands within the former which enhances the score when satisfied. This prevented the inter-system comparison of scores, which were instead comparable intra-system, describing the ligands considered similarly able to satisfy the requirements of each pocket.

The analysis of the docking poses revealed the capability of all the tested polyphenols to display polar interactions (hydrogen bonds) with both residues of the binding pocket and water molecules. Specifically, resveratrol (Fig. 6B) and phloretin (Fig. 6C) docked the binding site in accordance with the pharmacophore constraint. Conversely, piceid (Fig. 6D) and phloridzin dihydrate (Fig. 6E) placed the sugar moiety in the inner part of the pocket. In either case, polyphenols could theoretically interact close to the enzyme's catalytic triad (Ser195, His57, Asp102),⁶⁷ suggesting their capability to compete with enzyme's substrates (Fig. 6A–D). In more detail, as reported in Fig. 6, the hydrogen bond distances between the polyphenols under investigation and the residues and water molecules were calculated at the catalytic site. The analysis revealed that the whole set of polyphenols could interact with at least one of the water molecules, which may act as a bridge to the protein. Moreover, three out of four of the considered polyphenols (*i.e.* phloretin, piceid and phloridzin dihydrate) also interact with at least one of the residues of the catalytic triad. Interestingly, all of them recorded an interaction with Ser195 characterized by a distance lower than that observed for the known non-covalent synthetic inhibitor co-crystallized in the 1AFQ structure⁶⁸ (Fig. 6A). Conversely, resveratrol showed a slightly different mode of interaction with no direct polar contacts with the catalytic triad, though it was found interacting close to the active pocket contacting Ser217 (data not shown). As a general comment, different interaction networks were observed for the considered polyphenols, though all in line

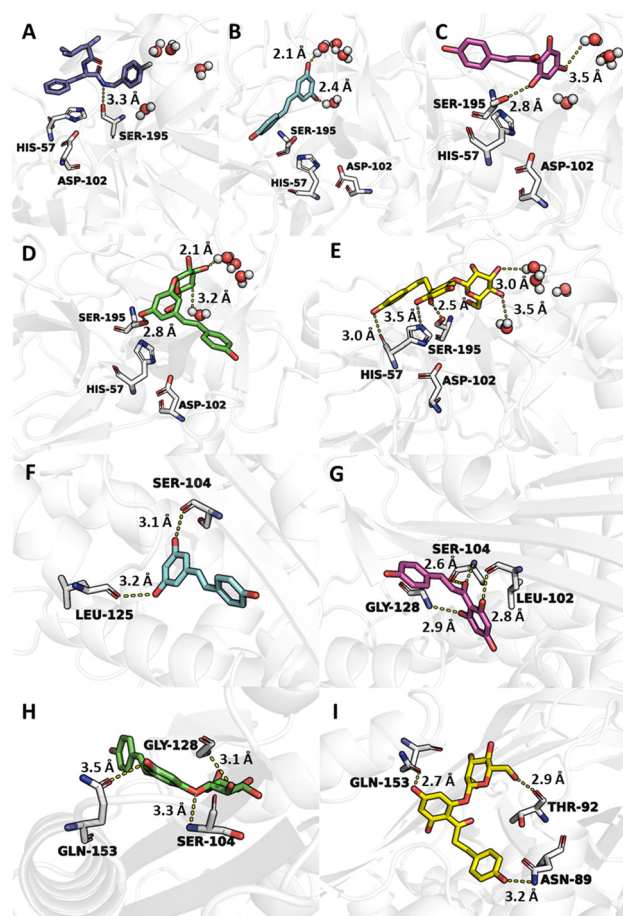


Fig. 6 Docking poses of the selected polyphenols within chymotrypsin and ovalbumin. Proteins are represented in white cartoons, while polyphenols and the amino acids of the proteins involved in polar interactions are represented in sticks. The water molecules at the chymotrypsin binding site are shown in balls-and-sticks. Yellow dashed lines indicate hydrogen bonds (interatomic distances in Å). Interaction of a known synthetic inhibitor with chymotrypsin binding site (as per PDB structure 1AFQ) (A). Interaction of resveratrol (cyan sticks) within chymotrypsin binding site (B). Interaction of phloretin (pink sticks) within chymotrypsin binding site (C). Interaction of piceid (green sticks) within chymotrypsin binding site (D). Interaction of phloridzin dihydrate (green sticks) within chymotrypsin binding site (E). Interaction of resveratrol (cyan sticks; F), phloretin (pink sticks; G), piceid (green sticks; H) and phloridzin dihydrate (yellow sticks; I) within ovalbumin binding site (F–I).

with the architecture of binding previously reported for other proteases inhibitors either in terms of polar bond length or residues involved.^{45,69}

Therefore, all the tested polyphenols were found to be potential chymotrypsin inhibitors. This result is consistent with previous evidence that generally describes polyphenols as inhibitors of peptidases, including chymotrypsin.⁷ For instance, it was demonstrated that resveratrol was capable of decreasing the activity of trypsin.⁷⁰ This finding suggests a possible inhibitory effect against chymotrypsin as well, based on the high similarity between the two enzymes, which enables many inhibitors to act on both. This hypothesis has



been widely and convincingly demonstrated for other inhibitors.⁷¹ With respect to ovalbumin, similarly to what observed for chymotrypsin and based on the positive docking scores recorded (Table 2), piceid, resveratrol, phloretin, and phloridzin dihydrate were predicted to interact positively with the designated binding pocket (Fig. 6F–I). Of note, the pocket was chosen based on previous evidence attesting the capability to bind a variety of ligands, including polyphenols.^{39,40}

Taken together, the results of docking simulations could not provide a plausible explanation for the diverse impact of the selected polyphenols on the chymotrypsin activity over ovalbumin as all of them could theoretically interact with both. In particular, the interaction with chymotrypsin should have been reasonably resulted in a reduction of ovalbumin degradation, laying unexplained the enhanced degradation observed for resveratrol, piceid and phloridzin dihydrate. Therefore, the best scored docking pose of each compound when in complex with both chymotrypsin and ovalbumin was used as input for molecular dynamics simulations to check the evolution of each complex overtime. This may allow investigating further the molecular mechanisms underpinning the diverse outcome the considered polyphenols had on chymotrypsin *in vitro*. It arose the hypothesis that the considered polyphenols may induce a diverse dynamic of ovalbumin, making it a better substrate for hydrolysis when in complex with resveratrol, piceid and eventually with phloridzin dihydrate than when in complex with phloretin.

3.2.2 Molecular dynamics. To test the hypothesis mentioned above, each complex underwent molecular dynamics simulations to investigate protein–ligand complex motions over time. Such an approach allowed the analysis of the stability of protein–ligand complex over time, monitoring proteins and ligands root-mean-square deviation (RMSD). This was a fundamental criterion to calculate the capability of the molecules under analysis to interact with chymotrypsin and ovalbumin, as previously reported.^{44,45} In addition, molecular dynamic simulations enabled also to study the dynamic evolution of a specific portion of ovalbumin, as a rational to explain the increased hydrolysis described for resveratrol, piceid and phloridzin (see below).

With respect to the complex with chymotrypsin, the outcome of molecular dynamics revealed that all the polyphenols under investigation were able to stably interact with the protease, aligning with the steady state RMSD trend of ligands and protein (Fig. 7A and B). Of note, this is a measurement able to determine whether the ligands were stably interacting at the designated binding site.⁷² Regardless of the ligand bound, the protein experienced a geometrical settling and reached stability from around 45 ns onward, suggesting that after an initial phase of adjustment, the systems remained stable over time. With respect to ligands, they showed a steady state interaction from around 25 ns onward, except for piceid which showed the highest mobility though within the range observed for the other ligands. These results confirmed the theoretical capability of the four polyphenols considered to have a certain degree of inhibitory activity against chymotryp-

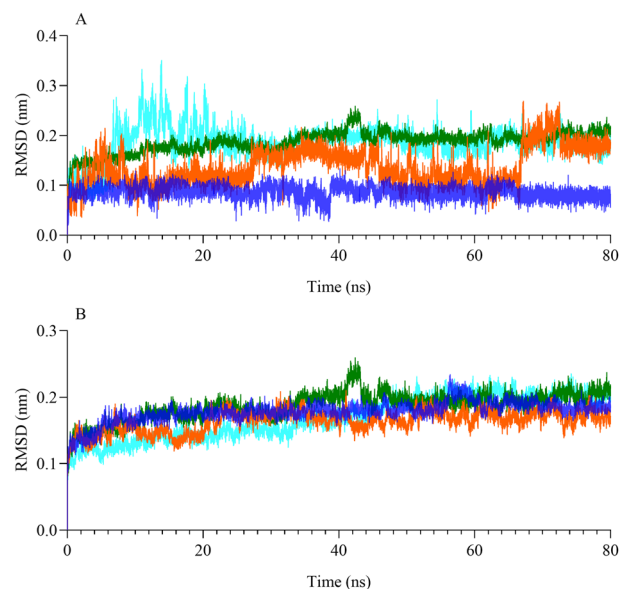


Fig. 7 Ligands and chymotrypsin RMSD variations. Resveratrol (blue), piceid (orange), phloretin (green), and phloridzin dihydrate (light blue) RMSD variations within chymotrypsin over time (A). Chymotrypsin C-alpha RMSD variations when in complex with resveratrol (blue), piceid (orange), phloretin (green), and phloridzin dihydrate (light blue) (B).

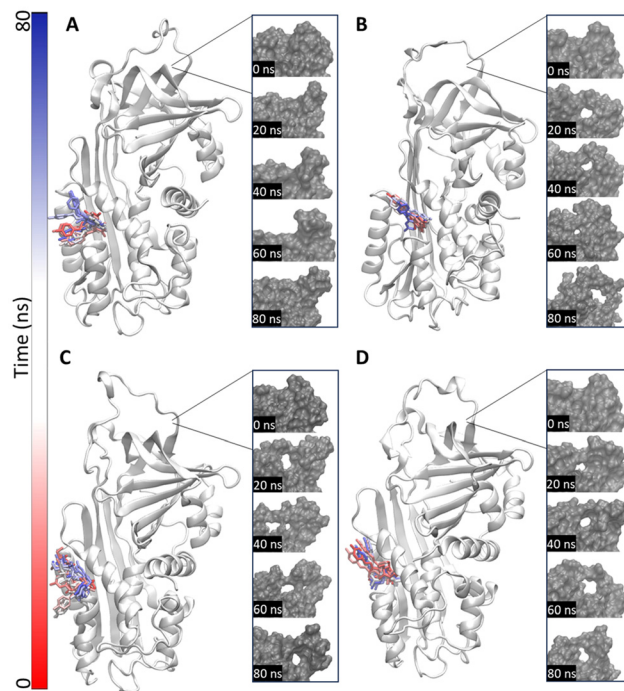


Fig. 8 Molecular dynamics results of the selected polyphenols within ovalbumin and close-up on the newly formed pockets. Time step representation of phloretin (A), resveratrol (B), phloridzin dihydrate (C), and piceid (D) within ovalbumin and close-up of the new pocket at different times (0 ns, 20 ns, 40 ns, 60 ns, 80 ns). Protein is represented in white cartoons and in grey surface; polyphenols are represented in sticks. The red-to-blue colour switch indicates the stepwise changes of coordinates during the simulations.



sin, as discussed above and in line with previous evidence.⁷⁰ This corroborated the hypothesis that they might induce differential dynamics when in complex with ovalbumin.

Therefore, an 80 ns molecular dynamics simulation was performed for each polyphenol within the ovalbumin binding site (Fig. 8).

The results indicate that phloretin had a different effect on ovalbumin compared to other polyphenols. Indeed, during the entire simulation, ovalbumin appeared well compacted when in complex with the digestion inhibitor phloretin (Fig. 8A). In contrast, when complexed with resveratrol, piceid, or phloridzin dihydrate, ovalbumin exhibited the opening of an additional surface cleft (Fig. 8B–D), which might prelude conformational changes allowing an increased accessibility to proteases and a subsequent enhanced susceptibility to proteolytic cleavage.

This phenomenon could provide a plausible though partial explanation to the diverse outcome the selected polyphenols showed *in vitro*.

4. Conclusion

This study demonstrates how food-bioactive compounds can affect the proteolytic activity of digestive proteases in an enzyme- and substrate-specific way. Additionally, it has been observed that glycosylation in small molecules can significantly alter reaction kinetics. These differences may be due to non-covalent interactions – mainly hydrogen bonds – at the substrate or enzyme level. In this scenario, the assessment of enzymatic activity in an assay alone is insufficient for predicting interactions among bioactives, enzymes, and substrates. To gain a deeper understanding of the mechanistic basis of *in vitro* outcomes from a molecular standpoint, it is necessary to conduct *in silico* analysis. The *in silico* studies reported here demonstrate that all the chosen polyphenols can interact with chymotrypsin in a way that likely impairs enzyme activity. However, the polyphenols that enhance *in vitro* proteolytic activity can diversely impact on the substrate structure, reasonably making it more susceptible to undergo the proteolytic cleavage. This suggests a combined substrate- and ligand-dependent relationship that may explain why polyphenols can have opposite effects on protein digestion.

To conclude, this study proves the feasibility and the significance of defining – on a molecular basis – the role of phenolics in modulating the activity of proteolytic enzymes in the digestive tract. Furthermore, this study highlights the importance of specific structural characteristics associated with the observed effects of these bioactive compounds. These characteristics include their three-dimensional structure, binding affinity, orientation, and geometry. Thus, this report is expected to contribute to further evaluation of this family of bioactive compounds as “digestive modulating agents” in the formulation of functional foods targeting specific consumers’ needs. In this frame, it is worth underscoring that isolated food components may exhibit a different behaviour if compared to those occurring when they are part of a food matrix,

and this ‘matrix effect’ must be duly considered when evaluating any property of the system and all the resulting nutritional and health implications. In this intricate scenario, *in vitro* digestion assays can be regarded as a valuable research method for investigating and comprehending alterations, interactions, and the bioaccessibility of nutrients and non-nutritive substances in food.⁷³ Specifically, it can be utilised to evaluate digestion endpoints or the kinetics of digestion phases, such as gastric and small intestinal hydrolysis.^{74,75} Notwithstanding the limitations of *in vitro* methods in fully reflecting the intricacies of *in vivo* digestion, they are regarded as valuable screening tools for various diet-related concerns, including digestibility, the release of bioactive compounds, structural changes in food, and the interaction between nutrients and bioactive food components. In any case, further investigations are required, as there is a paucity of *in vivo* studies in animal models and human, in contrast with the promising *in vitro* and *in silico* data.

Author contributions

Conceptualization: A.P., S.D., S.I., M.D.N. Methodology: L.D., M.D.N. Software: F. P., L.D., L. P. Validation: L.D., M.D.N. Formal analysis: S.M.B., F.P., L.D., L.P., M.D.N. Investigation: S.M.B., F.P., L.P., F.A. Resources: G.G., A.P., S.D., S.I., M.D.N. Data curation: S.M.B., F.P., L.P., F.A. Writing – original draft: S. M.B., F.P., L.D., M.D.N. Writing – Review & editing: G.G., A.P., S.D., S.I., M.D.N. Visualization: S.M.B., F.P. Supervision: L.D., M.D.N. Project administration: L.D., M.D.N. Funding acquisition: G.G., A.P., S.D., S.I., M.D.N.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Conflicts of interest

There are no conflicts to declare.

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