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# Inhibition of starch hydrolysis during *in vitro* co-digestion of pasta with phenolic compound-rich vegetable foods

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ABSTRACT

The ability of phenolic compounds to inhibit amylolytic enzymes activities has been investigated, suggesting their possible role in type-2 diabetes management. However, these studies have been carried out with purified enzymes and synthetic substrates and are distant from simulating a real physiological situation. The objective of the present research was to evaluate the ability of phenolic-rich vegetable foods to inhibit starch hydrolysis during *in vitro* co-digestion with pasta resulting in a potential anti-diabetic effect and mimicking as closely as possible a real scenario. Some tested vegetable foods, such as capers, red-skinned onion, red radish, and olives, determined a decrease in starch hydrolysis by 21.5%–31.7% during *in vitro* co-digestion with pasta. The quali-quantitative phenolic profiles of *in vitro* co-digested samples were elucidated and selected standard compounds were tested for their ability to inhibit porcine pancreatic α-amylase and mammalian α-glucosidase. The inhibitory potential of these compounds, especially against α-glucosidase, explained the effect observed during co-digestion experiments. The most active phenolic compounds against α-glucosidase were quercetin-4'-*O*-glucoside, quercetin-3-*O*-rutinoside, luteolin-7-*O*-glucoside and quercetin-3-*O*-glucoside-4'-*O*-glucoside with $IC_{50}$ values of 20.67, 52.23, 68.84 and 87.58 μmol/L, respectively. This is the first report suggesting that these compounds are potent inhibitors of mammalian α-glucosidase. This study indicates that consuming starchy foods (i.e., pasta) with phenolic-rich vegetable foods may result in an inhibition of starch digestion possibly reducing the post-prandial glucose levels.

## 1. Introduction

Diabetes mellitus is a metabolic chronic status characterized by high blood glucose levels and is considered one of the most important reasons for death worldwide. Diabetes morbidity is continuously increasing, with an actual prevalence of 10.5% in populations of high-income countries, which is expected to rise to 12.2% in 2045 (Kaur et al., 2023). Of the two distinct types of diabetes, i.e., type-1 and type-2, the second one is the most prevalent, representing more than 90% of diagnosed cases. This chronic disease is considered a major health concern, negatively affecting the quality of life and the health care system. The increasing prevalence of type-2 diabetes mellitus in the global population is mainly driven by changes in lifestyle (increasing sedentary lifestyle) and eating habits (increasing Western diet spread), which results in a condition of overweight and obesity strongly related to an increased likelihood of developing diabetes (Klein et al., 2022). The main hallmark of diabetes is a constant hyperglycaemic condition, which can evolve towards the onset of cardiovascular, renal, and nervous system complications (Kaur et al., 2023; Schmidt, 2019).

To date, the most employed pharmacological therapy for type-2 diabetes aims to reduce plasma glucose levels while keeping hyperglycaemia under control, particularly in the post-prandial phase (Tarigopula & Davies, 2014). Inhibitors of amylolytic enzymes, such as acarbose and metformin, are among the most used drugs for type-2 diabetes treatment (Usman et al., 2019). Their mechanism of action lies in their ability to inhibit the activity of the enzymes α-amylase and α-glucosidase, which are involved in carbohydrate metabolism. The gastro-intestinal hydrolysis of starch and other complex carbohydrates begins with the action of the enzyme α-amylase. This enzyme, secreted by the pancreas in the intestinal fluid, can hydrolyse starch and other glucose-based polysaccharides liberating shorter oligosaccharides (such as dextrins, maltotriose, and maltose), which are further cleaved into free glucose by the action of the brush-border enzyme α-glucosidase. However, these drugs may have serious side effects, especially at the gastro-intestinal tract level (Usman et al., 2019). In this context, attention has been paid in the last years to studying natural α-glucosidase

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inhibitors, especially from food samples (Di Stefano et al., 2018).

Among the food-derived α-glucosidase inhibitors, special consideration has been given to phenolic compounds (Giuberti et al., 2020; Proença et al., 2022). Phenolic compounds are plant secondary metabolites ubiquitously present in vegetable foods with several pharmacological properties and health effects (Cao et al., 2019; Carregosa et al., 2021; Micek et al., 2021). Most physiological effects of dietary phenolic compounds are mediated by their ability to interact non-covalently with proteins, including the active site of enzymes (Shahidi & Dissanayaka, 2023). Several studies have demonstrated the ability of phenolic compounds to inhibit the amylolytic enzymes α-amylase and α-glucosidase (Cao et al., 2019; Giuberti et al., 2020; Proença et al., 2022). The inhibitory activity has been documented both with pure compounds and with food extracts by using specific enzymatic assays (Cattivelli et al., 2022; Giuberti et al., 2020; Proença et al., 2017, 2019). However, these studies are far from the physiological conditions occurring during food consumption and digestion. First of all, in a real scenario, starch is embedded in the food structure (such as in pasta or bread) and has to be released during digestion before its hydrolysis by gastro-intestinal α-amylase and α-glucosidase. Moreover, starchy foods contain different classes of starch, such as resistant starch, slowly digestible starch, and rapidly digestible starch (Dodi et al., 2023). Another crucial point is that phenolic compounds have to be released from the food matrix and stable during digestion (i.e., have to be bioaccessible) to exert their inhibitory activity against starch digestive enzymes (Cattivelli et al., 2021). Furthermore, most vegetable foods are consumed only or mainly after cooking, which may have a massive impact on phenolic compounds stability, bioaccessibility, and bioactivity (Cattivelli et al., 2022).

Despite these considerations, to the best of our knowledge, no studies have explored the potential inhibitory activity of phenolic compounds in vegetable foods during *in vitro* co-digestion with pasta.

Therefore, the present study was designed to investigate the ability of phenolic compounds present in vegetable foods (capers, cherry tomatoes, dark purple eggplant, olives Kalamata, red radish, and red-skinned onion Tropea), commonly consumed in the context of the Mediterranean diet in combination with pasta, to inhibit starch hydrolysis during *in vitro* gastro-intestinal co-digestion with pasta. For some selected vegetable foods (dark purple eggplant, red radish, and red-skinned onion Tropea), the typical homemade thermal treatments have been applied since they are included in pasta dishes after cooking. The aim was to give a more physiological and realistic picture of what may happen during gastro-intestinal digestion of a real meal by assessing the anti-diabetic properties of selected vegetable foods (i.e., inhibition of starch digestion) and differentiating their effect in function of their phenolic compounds content. To this purpose, the phenolic profiles of the intestinal samples from *in vitro* digested pasta and *in vitro* co-digested pasta and vegetables were assessed by high-resolution mass spectrometry. Finally, the most relevant compounds were tested for their *in vitro* inhibitory activity against porcine pancreatic α-amylase and rat α-glucosidase.

## 2. Materials and methods

### 2.1. Materials

Chemicals and enzymes for the analytical determinations and *in vitro* gastro-intestinal digestion as well as rat intestinal acetone powder were purchased from Sigma (Milan, Italy). Solvents for extraction and mass spectrometry analysis were obtained from BioRad (Hercules, CA, USA). The glucose assay kit was purchased from Megazyme (Auchincruive, UK). Pasta (spaghetti type) and vegetable foods (capers, cherry tomatoes, dark purple eggplant, olives Kalamata, red radish, and red-skinned onion Tropea) were bought in a local supermarket (Reggio Emilia, Italy). The list of phenolic compounds used as standards for mass spectrometry quantification is reported in Supplementary Table S1. Selected pure phenolic compounds used for the *in vitro* inhibitory assays against α-amylase and α-glucosidase were 3-*O*-caffeoylquinic acid (purity ≥99%), 4-*O*-caffeoylquinic acid (purity ≥99%), hydroxytyrosol (purity ≥98%), luteolin-7-*O*-glucoside (purity ≥98%), luteolin-7-*O*-glucuronide (purity ≥98%), luteolin-7-*O*-rutinoside (purity ≥95%), quercetin-4'-*O*-glucoside (purity ≥99%), quercetin-3-*O*-rutinoside (purity ≥99%), quercetin-3-*O*-glucoside-4'-*O*-glucoside (purity ≥99%) and kaempferol-3-*O*-rutinoside (purity ≥98%). All the tested standard phenolic compounds were obtained from Extrasynthese (Genay, France) with the exception of luteolin-7-*O*-glucuronide and luteolin-7-*O*-rutinoside that were purchased from Cayman Chemical (Ann Arbor, MI, USA).

### 2.2. Preparation of vegetable foods and pasta

Vegetable foods were prepared following the typical domestic procedures used to prepare pasta and vegetable dishes. Capers, olives, and cherry tomatoes are usually added raw to pasta dishes. These foods were carefully washed with distilled water, dried on absorbent paper, and stored at −80 °C until use. Red-skinned onion, dark purple eggplant and red radish are instead used for the preparation of pasta-based recipes after being fried. Red-skinned onion was peeled and cut into slices about 0.3 cm thick. Then the slices were fried as reported in Cattivelli et al. (2021). Briefly, red-skinned onion slices were fried in a domestic deep fryer for 8 min at 140 °C in sunflower oil. Dark purple eggplant was washed with distilled water, cut into small cubes with a volume of approximately 1 $cm^3$, and then fried for 10 min at 170 °C in sunflower oil by using a domestic deep fryer (Martini et al., 2021). Red radish was washed with distilled water, sliced in portion of 5 cm length and 0.5 cm width, and then fried in sunflower oil at 140 °C for 8 min in a domestic deep fryer. Finally pasta was cooked for 10 min in unsalted boiling water (140 g of pasta in 2 L of boiling water). All the vegetables and pasta were stored at −80 °C for a maximum of 2 days.

### 2.3. Rat α-glucosidase extraction from rat intestinal acetone powder and determination of α-glucosidase activity in the extract

Rat α-glucosidase was extracted from rat intestinal acetone powder following the procedure reported in Oki et al. (1999) with minor modifications. Briefly, 200 mg of rat intestinal acetone powder were mixed with 6 mL of a 0.9% NaCl solution and homogenized with an ultra-turrax. The homogenization procedure consisted of 4 cycles of 30 s each at a speed of 6000*g*. The mixture was constantly kept on ice during the homogenization procedure and, after each cycle, the sample was left to rest on ice for 30 s. Next, the homogenized sample was subjected to 4 cycles of 30 s of sonication interspersed by 30 s of rest on ice. The extract was then centrifuged at 8,000*g* for 30 min at 4 °C.

The α-glucosidase activity was assayed as reported in Cattivelli et al. (2022). Briefly, 20 μL of rat intestinal α-glucosidase extract were mixed with 71.7 μL of potassium phosphate buffer (0.1 mol/L, pH 6.8), 3.3 μL of reduced glutathione (3.3 mmol/L) and 5 μL of *p*-nitrophenyl-glucose 5 mmol/L. Next the reaction mixture was incubated at 37 °C for 20 min after that the reaction was stopped by adding 150 μL of sodium carbonate 1 mol/L. The amount of released *p*-nitrophenol was determined by measuring the absorbance at 405 nm using a microplate reader. One unit of α-glucosidase activity was defined as the amount of enzyme able to release 1 μmol of *p*-nitrophenol per min.

### 2.4. In vitro digestion of pasta, vegetable foods and co-digestion experiments

*In vitro* gastro-intestinal digestion was carried out by following the INFOGEST 2.0 protocol (Brodkorb et al., 2019) modified by addition of the α-glucosidase extract during the intestinal phase of the digestion as reported in Bellesia et al. (2015). Pasta and vegetable foods were homogenized before the digestion with a pestle and mortar to simulate the

mastication of the foods. Next, 10 g of sample were added to 10 mL of salivary fluids containing salivary α-amylase (150 U/mL of final concentration in the digestive system). To mimic the oral phase of the digestion the mixture was incubated at 37 °C for 2 min in a rotating wheel (10 rpm). The gastric phase of the digestion was simulated by mixing 20 mL of gastric fluid with the bolus. The pH was then brought to 3 with 6 mol/L HCl before the addition of pepsin at 2000 U/mL (final concentration in the digestive system). After incubation at 37 °C in a rotating wheel (10 rpm) for 120 min, 40 mL of intestinal fluid were mixed with the bolus and the pH corrected to 7.5 with concentrated NaOH. After 30 min of incubation in a rotating wheel (10 rpm) at 37 °C, pancreatin (final concentration in the digestive system based on trypsin activity of 200 U/mL) and 0.5 mL of rat α-glucosidase extract (final concentration of α-glucosidase in the digestive system 0.1 mU/mL) were added to the chyme. Finally, the chyme was incubated in a rotating wheel (10 rpm) at 37 °C for 120 min.

Pasta was digested alone and in combination with single vegetable foods or a mixture of the vegetable foods in a proportion that mimics a real Italian recipe. The composition of the digested samples with the proportion of pasta and vegetable foods is depicted in Table 1.

Control digestions were carried out by omitting pasta and replacing it with water, to consider the possible interferences due to the digestive system and the presence of glucose in vegetable foods.

All the digestions were carried out in triplicate.

### 2.5. Glucose quantification in in vitro digested samples

The amount of glucose released at the end of the digestions of both vegetables alone and in combination with pasta was quantified using a hexokinase and glucose-6-phosphate dehydrogenase assay kit and following the manufacturer instructions. The data from co-digestions were corrected for those from digestions of vegetables alone.

### 2.6. Identification and quantification of phenolic compounds by high-performance mass spectrometry in chemical extracts and in vitro digested samples

High-resolution mass spectrometry identification and quantitative analysis of phenolic compounds in *in vitro* digested samples was performed by using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled to a UHPLC Ultimate 3000 module (Thermo Fisher Scientific, San Jose, CA, USA). Separation of phenolic compounds was achieved with a C18 column (Acquity UPLC HSS C18 Reversed phase, 2.1 × 100 mm, 1.8 μm particle size, Waters, Milan, Italy) with a gradient of acetonitrile/formic acid (mobile phase B; 99.9:0.1 v/v) and water/formic acid (mobile phase A; 99.9:0.1 v/v) and a flow rate was 0.3 mL/min. The gradient began with 1% B to then increase linearly to 40% B in 20 min. Column was then washed by increasing the concentration of B to 99% in 6 min to then came back to the initial conditions. The chromatographic and mass spectrometry parameters are reported in Martini et al. (2020). Quantification was carried out by building external calibration curves with the available standard compounds as depicted in Supplementary Table S1. Data are expressed as μmol/L of digesta.

**Table 1**
Composition of the samples submitted to *in vitro* gastro-intestinal digestion. In the corresponding control digestions pasta was replaced with the same amount of water.

| Digested sample code | P | P–C | P-CT | P-DPE | P–O | P-RR | P-RSO | P-Recipe |
|---|---|---|---|---|---|---|---|---|
| Pasta | 3.2 g | 3.2 g | 3.2 g | 3.2 g | 3.2 g | 3.2 g | 3.2 g | 3.2 g |
| Capers | / | 6.8 g | / | / | / | / | / | 0.7 g |
| Cherry tomatoes | / | / | 6.8 g | / | / | / | / | / |
| Dark purple eggplant | / | / | / | 6.8 g | / | / | / | / |
| Olives | / | / | / | / | 6.8 g | / | / | 1.4 g |
| Red radish | / | / | / | / | | 6.8 g | / | 2.8 g |
| Red skinned onion | / | / | / | / | / | / | 6.8 g | 1.7 g |
| Water | 6.8 g | / | / | / | / | / | / | 0.2 g |
| Total weight | 10 g | 10 g | 10 g | 10 g | 10 g | 10 g | 10 g | 10 g |

### 2.7. Enzyme inhibition assays with authentic standard phenolic compounds

Standard phenolic compounds stock solutions were prepared at 50 mmol/L concentration in dimethyl sulfoxide (DMSO). Working standard phenolic compound solutions were prepared daily by diluting the stock solutions in the reaction buffer so as that the DMSO concentration was always ≤1% in the reaction mixture.

The inhibitory activity of standard phenolic compounds versus the enzyme α-amylase was assayed as described by Cattivelli et al. (2022). Briefly, 70 μL of sodium phosphate buffer (0.1 mol/L; pH 6.9; NaCl 6.7 mmol/L) were added of 20 μL of standard phenolic compound at a concentration of 500 μmol/L and 10 μL of a 2 U/mL solution of porcine pancreatic α-amylase. The mixture was incubated 20 min at 37 °C before the addition of 100 μL of 1% starch solution dissolved in the same sodium phosphate buffer. Then the reaction mixture was further incubated for 10 min at 37 °C. Starch hydrolysis was assessed by adding 100 μL of dinitrosalicylic acid solution to the reaction mixture than followed by boiling for 15 min in water. After addition of 900 μL of distilled water, 200 μL of reaction mixture were transferred to a 96-well plate and read at 540 nm using a microplate reader.

The α-glucosidase inhibitory activity of standard phenolic compounds was determined by using the same protocol as reported in paragraph 2.3 with slight modifications. Briefly, 71.7 μL of potassium phosphate buffer (0.1 mol/L, pH 6.8) were added of 10 μL of rat α-glucosidase extract (final concentration in the assay of 0.1 mU/mL), 10 μL of standard phenolic compound at concentrations ranging from 5 to 500 μmol/L and 3.3 μL of reduced glutathione (3.3 mmol/L). The mixture was pre-incubated at 37 °C for 20 min before the addition of 5 μL of *p*-nitrophenyl-glucose 5 mmol/L. After 40 min of further incubation at 37 °C the reaction was stopped by adding 150 μL of sodium carbonate 1 mol/L and the amount of released *p*-nitrophenol was determined by measuring the absorbance at 405 nm using a microplate reader.

For the most active compounds the $IC_{50}$ values, defined as the concentration of phenolic compound able to inhibit the enzyme activity of 50%, was calculated. The $IC_{50}$ values were calculated by non-linear regression analysis by correlating the base-10 logarithm of the phenolic compounds concentration with the percentage of enzyme inhibition.

### 2.8. Statistics

Data are presented as mean ± SD for three analytical replicates for each prepared sample. Each experiment was performed in triplicate. Differences among samples were determined by one-way ANOVA (univariate analysis of variance) with Tukey's post-hoc test by using Graph Pad prism 6.0 (GraphPad Software, San Diego, CA, U.S.A.). The differences were considered significant with $P < 0.05$. The $IC_{50}$ values were calculated through Graph Pad prism 6.0.

## 3. Results and discussion

### 3.1. Vegetable foods inhibit starch hydrolysis during in vitro co-digestion with pasta

*In vitro* gastro-intestinal digestion of pasta resulted in the release of 5.45 ± 0.16 g of glucose/100 g of pasta, roughly corresponding to 4.91 g of starch (Fig. 1). When the pasta was co-digested with the different tested vegetables, an inhibition in starch hydrolysis was observed, resulting in a decrease in the release of glucose (Fig. 1). The highest inhibition (31.7%) was observed after co-digestion of pasta and capers, whereas cherry tomatoes determined the lowest inhibitory effect (12.2%) when co-digested with pasta. Olives and red radish displayed similar starch hydrolysis inhibitory activity (25.4% and 24.8%), followed by red-skinned onion (21.5%) and dark purple eggplant (18.0%).

The four most active vegetable foods (i.e., capers, olives, red radish, and red-skinned onion) were combined in a real Italian recipe in the proportions reported in Tables 1 and *in vitro* co-digested with pasta. As reported in Fig. 1, the *in vitro* co-digestion of pasta with the vegetable mix resulted in a 28.3% lower glucose release from starch compared to pasta digestion.

Whereas several studies have investigated the inhibitory effect of phenolic compounds after *in vitro* gastro-intestinal digestion of vegetable foods on α-amylase and α-glucosidase (Cattivelli et al., 2022; Dou et al., 2022; Wu et al., 2023; Zahid et al., 2023) only a few studies have explored the inhibitory activity of phenolic compounds or foods and beverages during *in vitro* digestion. Bellesia et al. (2015) *in vitro* co-digested potatoes with pomegranate juice by finding a decrease in the released glucose at the end of the digestion due to the inhibitory ability of pomegranate ellagitannins against α-glucosidase. In another study, Pacheco et al. (2023) *in vitro* co-digested pasta with a phenolic-rich ethanol extract of *Durvillaea incurvate* by observing a reduction in starch hydrolysis due to the capability of phenolic compounds in the extract to inhibit α-glucosidase activity. However, this is the first study that measured starch hydrolysis during *in vitro* co-digestion of pasta with vegetable foods individually or in combination in the proportions mimicking a real recipe.

While no *in vivo* studies demonstrate that the simultaneous consumption of pasta and vegetable foods affects the post-prandial glycaemic peak, other studies carried out with pasta or bread enriched with high phenolic content products went in this direction. For example, Turco et al. (2016) demonstrated that the enrichment of pasta with *Faba bean* flour increased the phenolic content of the product and resulted in a lower glycaemic index and load in healthy human volunteers. The authors linked the *in vivo* effect on glucose metabolism with the higher α-amylase and α-glucosidase inhibitory activity of enriched pasta compared to the one not enriched. Similarly, clinical studies performed with functional bread enriched with legume or hazelnut flours showed a decrease in post-prandial glucose levels in humans (Amoah et al., 2022).

### 3.2. Identification of phenolic compounds in in vitro digested pasta and in vitro co-digested pasta and vegetables foods

The quali-quantitative profile of the *in vitro* digested samples (pasta and pasta in combination with the vegetable foods) was determined by high-resolution mass spectrometry. The mass spectrometry and the quantitative data of the individual phenolic compounds identified after *in vitro* digestion of pasta and pasta in combination with the vegetables are reported in Supplementary Tables S1 and S2, respectively. Fig. 2 instead displays data about the total amount of bioaccessible phenolic compounds (sum of the individual phenolic compounds) calculated for each digested sample. High variability in the phenolic content among the digested samples was noted. *In vitro* digested pasta contained the lowest amount of total phenolic compounds (7.56 ± 0.16 μmol/L), followed by *in vitro* digested pasta and cherry tomatoes (19.17 ± 0.26 μmol/L). A higher amount of phenolic compounds was detected after *in vitro* digestion of pasta and red-skinned onion (120.52 ± 1.30 μmol/L) and pasta and capers (314.91 ± 10.62 μmol/L). No significant differences were found between the total phenolic content of *in vitro* digested pasta and olives and *in vitro* digested pasta and dark purple eggplant (1075.84 ± 16.77 μmol/L and 1011.87 ± 9.88 μmol/L, respectively). However, the highest amount of total phenolic compounds was found after *in vitro* digested pasta and red radish (8293.52 ± 69.93 μmol/L). The amount of total phenolic compounds in the digested recipe (7154.99 ± 49.35 μmol/L) was similar to that found after the *in vitro* digestion of pasta and red radish, this last vegetable being the predominant one in the recipe.

No clear relationship was found between the total amount of phenolic compounds and the inhibitory activity against starch hydrolysis during *in vitro* co-digestion with pasta, suggesting that the inhibitory potential is related to the type of phenolic compound in the specific vegetable food rather than the total amount of phenolic compounds.

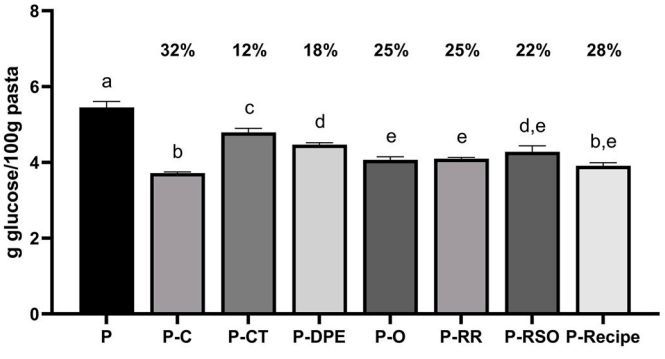


**Fig. 1. Effect of *in vitro* co-digestion of vegetable foods and pasta on starch hydrolysis.** Starch hydrolysis was assessed by measuring the amount of free glucose released at the end of the *in vitro* gastro-intestinal digestion. The percentage of inhibition is also reported. P: digestion of pasta alone; P–C: co-digestion of pasta and capers; P-CT: co-digestion of pasta and cherry tomatoes; P-DPE: co-digestion of pasta and dark purple eggplant; P–O: co-digestion of pasta and olives; P-RR: co-digestion of pasta and red radish; P-RSO: co-digestion of pasta and red-skinned onion. Values are means of three assay replications ± standard deviation (SD). Different letters among samples denote significant differences ($P < 0.05$).

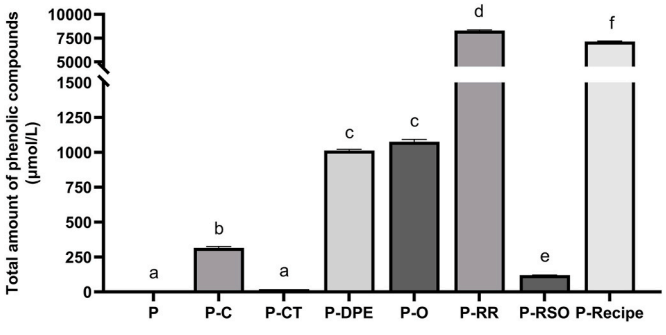


**Fig. 2. Total amount of phenolic compounds determined by mass spectrometry analysis in the various digested samples.** The total amount of phenolic compounds was calculated by summing the amount of each individual quantified phenolic compound in the digested samples. P: digestion of pasta alone; P–C: co-digestion of pasta and capers; P-CT: co-digestion of pasta and cherry tomatoes; P-DPE: co-digestion of pasta and dark purple eggplant; P–O: co-digestion of pasta and olives; P-RR: co-digestion of pasta and red radish; P-RSO: co-digestion of pasta and red-skinned onion. Data about the concentration of individual phenolic compounds in each sample is reported in Supplementary Table S2. Values are means of three assay replications ± standard deviation (SD). Different letters among samples denote significant differences ($P < 0.05$).

### 3.2.1. Identification and quantification of individual phenolic compounds

Fig. 3 shows the percentage of incidence of the different classes of phenolic compounds in the samples. Only four phenolic compounds were identified in *in vitro* digested pasta, with the two isomers of the flavone apigenin-*C*-hexoside-*C*-pentoside being the predominant one (Supplementary Table S2). The other two identified phenolic compounds were hydroxybenzoic and di-hydro-coumaric acids.

Among the 28 phenolic compounds identified after *in vitro* digestion of pasta and capers, the majority of them belong to the class of flavonols (18 phenolic compounds) (Supplementary Table S2). Flavonols represented 89.73% of total phenolic compounds, followed by hydroxycinnamic acids (8.46% of total phenolic compounds) (Fig. 3A). Among flavonols, the 3-*O*-rutinoside-derivatives of kaempferol and quercetin were present at the highest concentration of 165.20 ± 6.48 and 97.92 ± 8.30 μmol/L, respectively, and represented together the 83.56% of total phenolic compounds identified in *in vitro* digested pasta and capers samples (Supplementary Table S2). Regarding the *in vitro* digested pasta and cherry tomatoes samples, mass spectrometry allowed the identification of 22 phenolic compounds, predominantly hydroxycinnamic acids (13 compounds) and hydroxybenzoic acids (6 compounds) (Supplementary Table S2). From a quantitative point of view, hydroxycinnamic acids were the most abundant phenolic compounds class, representing 71.7% of total phenolic compounds (Fig. 3B).

Hydroxycinnamic acids were also the most abundant phenolic compounds class identified in *in vitro* digested pasta and dark purple eggplant samples, representing 99.64% of total phenolic compounds (Fig. 3C and Supplementary Table S2). The most representative compounds were the *trans* isomers of 3-*O*-caffeoylquinic acid (758.74 ± 9.69 μmol/L), 4-*O*-caffeoylquinic acid (141.59 ± 0.60 μmol/L), and 5-*O*-caffeoylquinic acid (55.93 ± 1.70 μmol/L) (Table S2). These three compounds accounted for 94.50% of total phenolic compounds in *in vitro* digested pasta and dark purple eggplant samples (Supplementary Table S2).

Flavones and phenylethanoids dominated the phenolic profile of *in vitro* digested pasta and olives samples (Fig. 3D and Supplementary Table S2). The compound present in the highest amount was hydroxytyrosol (353.45 ± 15.21 μmol/L) followed by luteolin-7-*O*-rutinoside isomer 2 (159.89 ± 0.55 μmol/L), luteolin-7-*O*-glucoside (137.88 ± 1.99 μmol/L) and luteolin-7-*O*-rutinoside isomer 3 (119.71 ± 5.15 μmol/L) (Supplementary Table S2).

In the *in vitro* digested pasta and red radish samples, hydroxycinnamic acids were the predominant phenolic compounds class from a qualitative point-of-view, with 39 out of 92 identified compounds (Supplementary Table S2). However, flavones represented the most abundant phenolic compounds class from a quantitative point-of-view, accounting for 87.76% of total phenolic compounds (Fig. 3E and Supplementary Table S2). Luteolin-7-*O*-glucoside was the predominant phenolic compound in the *in vitro* digested pasta and red radish samples (2124.83 ± 25.56 μmol/L) together with luteolin-7-*O*-glucuronide (1955.96 ± 17.77 μmol/L) and luteolin-7-*O*-rutinoside (1217.30 ± 5.88 μmol/L) (Supplementary Table S2). Indeed, 3-*O*-caffeoylquinic acid *trans* was also detected in a high amount (514.89 ± 56.47 μmol/L) after digestion of pasta and red radish (Supplementary Table S2).

As expected, *in vitro* digested pasta and red-skinned onion samples resulted in the accumulation of flavonols, representing 84.78% of total phenolic compounds (Fig. 3F and Supplementary Table S2). Among flavonols, the most abundant were quercetin-4'-*O*-glucoside and quercetin-3-*O*-glucoside-4'-*O*-glucoside detected in the amount of 37.18 ± 0.61 and 32.10 ± 0.97 μmol/L, respectively.

Finally, as reported in Supplementary Table S2, the phenolic profile of *in vitro* digested pasta and vegetable mix (recipe) strongly resembled that of *in vitro* digested pasta and red radish samples.

### 3.3. Inhibition of α-amylase and α-glucosidase by selected standard phenolic compounds

Based on the high-resolution mass spectrometry data on *in vitro* co-digested samples, ten phenolic compounds that dominated the phenolic profiles of digested samples were selected for further studies regarding their ability to inhibit porcine pancreatic α-amylase and rat intestinal α-glucosidase. The selected standard compounds were: two hydroxycinnamic acids, 3-*O*-caffeoylquinic and 4-*O*-caffeoylquinic acids that were present in high concentrations in digested dark purple eggplant and red radish; one phenylethanoid, hydroxytyrosol, which was the major phenolic compound detected in *in vitro* digested olives; three flavones, luteolin-7-*O*-glucoside, luteolin-7-*O*-rutinoside and luteolin-7-*O*-glucuronide which dominated the phenolic profile of digested red radish and were also present in a high amount in digested olives; and four flavonols, quercetin-4'-*O*-glucoside and quercetin-3-*O*-glucoside-4'-*O*-glucoside found in a high amount in *in vitro* digested red-

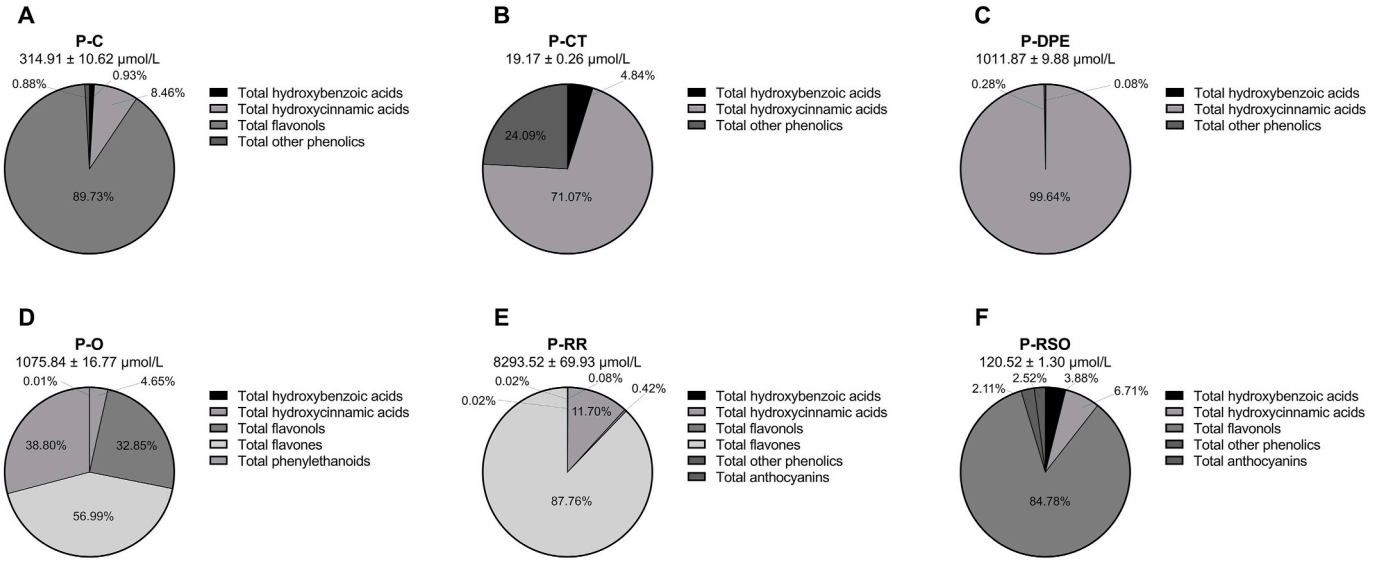


**Fig. 3. Percentage of incidence of phenolic compounds divided by classes in the various digested samples.** The total amount of phenolic compounds is also shown for each sample. P–C: co-digestion of pasta and capers; P-CT: co-digestion of pasta and cherry tomatoes; P-DPE: co-digestion of pasta and dark purple eggplant; P–O: co-digestion of pasta and olives; P-RR: co-digestion of pasta and red radish; P-RSO: co-digestion of pasta and red-skinned onion. Data about the concentration of individual phenolic compounds in each sample is reported in Supplementary Table S2. Values are means of three assay replications ± standard deviation (SD).

skinned onion and quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside that were the most important phenolic compounds identified in *in vitro* digested capers.

The selected standard phenolic compounds were first screened at a concentration of 500 μmol/L (final concentration in the assay) to establish their inhibitory effect on porcine pancreatic α-amylase and rat intestinal α-glucosidase.

### 3.3.1. Inhibition of α-amylase by selected standard phenolic compounds

As displayed in Fig. 4, all the tested compounds were weak inhibitors of porcine pancreatic α-amylase. 3-*O*-Caffeoylquinic acid showed the highest inhibitory activity with a 40.92% ± 1.12% enzymatic activity inhibition. Furthermore, the positional isomer 4-*O*-caffeoylquinic acid displayed about a 4-time lower inhibitory activity (10.61% ± 0.88% inhibition). Previous studies with both porcine pancreatic and human α-amylase and using starch as a substrate demonstrated that caffeoylquinic acids were weak inhibitors of α-amylase with $IC_{50}$ values in the magnitude order of mmol/L (Nyambe-Silavwe & Williamson, 2018; Wang et al., 2022; Zheng et al., 2020).

The second most active compound against α-amylase was the flavonol quercetin-4'-*O*-glucoside, with a percentage inhibition of 39.63% ± 1.10%. The addition of a glucose moiety in position 3 (as in quercetin-3-*O*-glucoside-4'-*O*-glucoside) hampered the α-amylase inhibitory activity (Fig. 4). The other two tested flavonols (quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside) were only weak inhibitors of α-amylase, as already suggested (Proença et al., 2019, 2022; Swilam et al., 2022).

Among the tested flavones, luteolin-7-*O*-glucoside exhibited the highest α-amylase inhibitory activity (24.89% ± 1.20%), followed by luteolin-7-*O*-rutinoside (20.23% ± 1.10%) and luteolin-7-*O*-glucuronide (12.44% ± 0.95%) (Fig. 4). Previous studies found these compounds as only weak inhibitors of α-amylase (Asghari et al., 2015; Lam et al., 2024; Proença et al., 2022). Finally, no inhibitory activity was observed when hydroxytyrosol was tested at 500 μmol/L concentration (Fig. 4).

### 3.3.2. Inhibition of α-glucosidase by selected standard phenolic compounds

Otherwise, most of the tested phenolic compounds were potent inhibitors of α-glucosidase (Fig. 5). The flavonols quercetin-4'-*O*-glucoside, quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside as well as the flavone luteolin-7-*O*-glucoside totally inhibited α-glucosidase activity at 500 μmol/L concentration (Fig. 5). A slightly lower inhibitory activity was observed for quercetin-3-*O*-glucoside-4'-*O*-glucoside and luteolin-7-*O*-rutinoside (88.46 % ± 2.70% and 87.93% ± 3.20%, respectively). Luteolin-7-*O*-glucuronide was a weaker inhibitor of α-glucosidase (50.57% ± 2.70%), whereas the two caffeoylquinic acids and hydroxytyrosol did not inhibit α-glucosidase activity at 500 μmol/L concentration (Fig. 5). In contrast, previous studies identified hydroxycinnamic acids as potent inhibitors of α-glucosidase using yeast as the source of the enzyme (Ćorković et al., 2022; Iwai et al., 2006; Kamitani et al., 2009). It has been demonstrated that the application of diverse enzyme sources for the enzymatic assays (i.e., yeast α-glucosidase vs mammalian α-glucosidase) may give different results due to the structural divergences between the two enzymes (Barber et al., 2021; Farazi et al., 2023). Enzymatic assays carried out with mammalian α-glucosidase demonstrated that hydroxycinnamic acids (including caffeoylquinic acids) were only weak inhibitors of α-glucosidase (Nyambe-Silavwe et al., 2018; Wu et al., 2023).

The $IC_{50}$ values against α-glucosidase were calculated for the most active compounds, as shown in Fig. 6. The lowest $IC_{50}$ value, meaning the highest inhibitory potency, was found for quercetin-4'-*O*-glucoside (20.67 ± 1.12 μmol/L). No studies on the inhibitory activity of quercetin-4'-*O*-glucoside are present in the literature. However, a previous study demonstrated the ability of *in vitro* digested red-skinned onion to inhibit α-glucosidase, and through chemometric analysis, the authors identified quercetin mono-hexosides as the compounds responsible for this inhibitory activity (Cattivelli et al., 2022). In agreement with the reported data, in the cited work quercetin-4'-*O*-glucoside was the predominant quercetin mono-hexoside identified in digested red-skinned onion (Cattivelli et al., 2022). Quercetin-4'-*O*-glucoside exhibited an $IC_{50}$ value against mammalian α-glucosidase lower than that of quercetin, quercetin-3-*O*-glucoside, and others 3-*O*-glycosylated

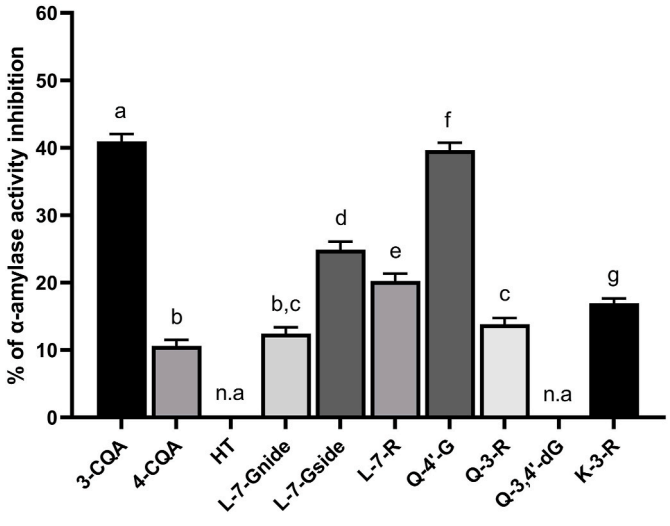


**Fig. 4. Percentage of inhibition of porcine pancreatic α-amylase activity in presence of selected phenolic compounds.** Phenolic compounds were tested at the concentration of 500 μmol/L (final concentration in the assay). Selected standard were: 3-CQA: 3-*O*-caffeoylquinic acid; 4-CQA: 4-*O*-caffeoylquinic acid; HT: hydroxytyrosol; L-7-Gnide: luteolin-7-*O*-glucuronide; L-7-Gside: luteolin-7-*O*-glucoside; L-7-R: luteolin-7-*O*-rutinoside; Q-4'-G: quercetin-4'-*O*-glucoside; Q-3-R: quercetin-3-*O*-rutinoside; Q-3,4'-dG: quercetin-3-*O*-glucoside-4'-*O*-glucoside; K-3-R: kaempferol-3-*O*-rutinoside. N.a. means non active compound. Values are means of three assay replications ± standard deviation (SD). Different letters among samples denote significant differences ($P < 0.05$).

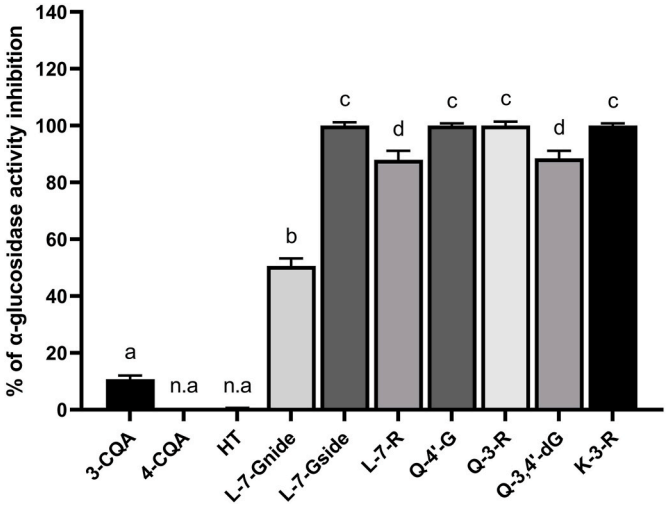


**Fig. 5. Percentage of inhibition of mammalian α-glucosidase activity in presence of selected phenolic compounds.** Phenolic compounds were tested at the concentration of 500 μmol/L (final concentration in the assay). Selected standards were: 3-CQA: 3-*O*-caffeoylquinic acid; 4-CQA: 4-*O*-caffeoylquinic acid; HT: hydroxytyrosol; L-7-Gnide: luteolin-7-*O*-glucuronide; L-7-Gside: luteolin-7-*O*-glucoside; L-7-R: luteolin-7-*O*-rutinoside; Q-4'-G: quercetin-4'-*O*-glucoside; Q-3-R: quercetin-3-*O*-rutinoside; Q-3,4'-dG: quercetin-3-*O*-glucoside-4'-*O*-glucoside; K-3-R: kaempferol-3-*O*-rutinoside. N.a. means non active compound. Values are means of three assay replications ± standard deviation (SD). Different letters among samples denote significant differences ($P < 0.05$).

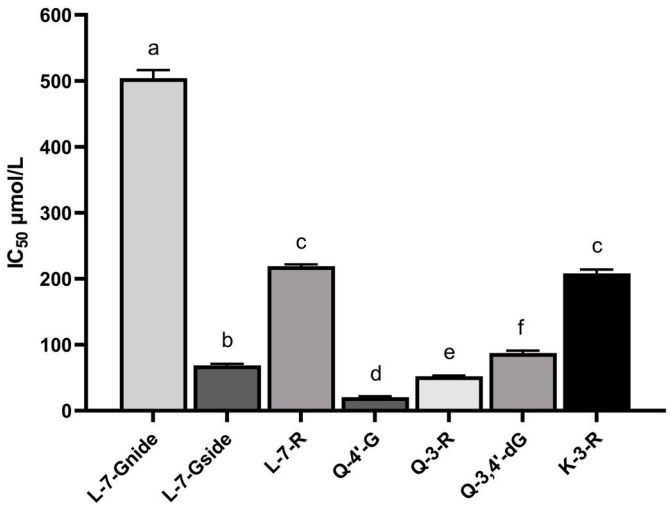


**Fig. 6. $IC_{50}$ values against α-glucosidase of the most active phenolic compounds.** Selected standards were L-7-Gnide: luteolin-7-*O*-glucuronide; L-7-Gside: luteolin-7-*O*-glucoside; L-7-R: luteolin-7-*O*-rutinoside; Q-4'-G: quercetin-4'-*O*-glucoside; Q-3-R: quercetin-3-*O*-rutinoside; Q-3,4'-dG: quercetin-3-*O*-glucoside-4'-*O*-glucoside; K-3-R: kaempferol-3-*O*-rutinoside. Values are means of three assay replications ± standard deviation (SD). Different letters among samples denote significant differences ($P < 0.05$).

quercetin-derivatives (Barber et al., 2021; Flores-Bocanegra et al., 2015). In contrast, quercetagetin displayed higher inhibitory potency than quercetin-4'-*O*-glucoside against human α-glucosidase (Barber et al., 2021). Two additional flavonols, quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside-4'-*O*-glucoside, showed $IC_{50}$ values against α-glucosidase of 52.23 ± 0.89 µmol/L and 87.58 ± 3.60 µmol/L (Fig. 6). Whereas no previous studies investigated the inhibitory activity of quercetin-3-*O*-glucoside-4'-*O*-glucoside against α-glucosidase, some data have been reported on quercetin-3-*O*-rutinoside. These reports described a lower inhibitory potency of quercetin-3-*O*-rutinoside against yeast α-glucosidase once again indicating the high variability in the inhibitory profile among the enzymes from different species (Jia et al., 2019; Ma et al., 2015). The last tested flavonols, kaempferol-3-*O*-rutinoside, displayed an $IC_{50}$ value about 4 times higher than quercetin-3-*O*-rutinoside (Fig. 6). The only difference between these two compounds is the lack of one OH group in the B ring of kaempferol-3-*O*-rutinoside compared to quercetin-3-*O*-rutinoside. Therefore, the hydroxylation pattern of the B ring of flavonols was pivotal in determining their α-glucosidase inhibitory activity, as already suggested (Barber et al., 2021; Proença et al., 2022). Among the three tested flavones, luteolin-7-*O*-glucoside displayed the lowest $IC_{50}$ value (68.84 ± 2.20 µmol/L), followed by luteolin-7-*O*-rutinoside (219.10 ± 2.50 µmol/L) and luteolin-7-*O*-glucuronide (504.00 ± 12.30 µmol/L). Previous studies have shown a strengthened inhibition of luteolin-7-*O*-glucoside and luteolin-7-*O*-glucuronide against yeast α-glucosidase (Asghari et al., 2015).

### *3.3.3. Potential contribution of individual phenolic compounds on the inhibition of starch hydrolysis in co-digested samples*

The high inhibitory potency of quercetin-3-*O*-glucoside-4'-*O*-glucoside and especially quercetin-4-*O*-glucoside against α-glucosidase may explain the effect of red-skinned onion on starch hydrolysis during the co-digestion with pasta. This is especially true for quercetin-4'-*O*-glucoside, present in the digested pasta and red-skinned onion samples at a concentration almost double its $IC_{50}$ value against α-glucosidase. Similarly, in the digested pasta and caper samples, the concentration of quercetin-3-*O*-rutinoside was approximately double its $IC_{50}$ value against α-glucosidase, whereas the concentration of kaempferol-3-*O*-rutinoside was near to the $IC_{50}$ value. These two compounds may account for the inhibitory effect versus starch digestion observed during caper and pasta digestion. Likewise, luteolin-7-*O*-glucoside, luteolin-7-*O*-rutinoside, and luteolin-7-*O*-glucuronide were present in digested pasta and red radish samples in concentrations much higher than their $IC_{50}$ values against α-glucosidase. Besides, at the concentrations found in digested pasta and red radish samples it is conceivable that these compounds, and also 3-*O*-caffeoylquinic acid, may exert some inhibitory effect against α-amylase. Furthermore, luteolin-7-*O*-glucoside and luteolin-7-*O*-rutinoside may also be responsible for the inhibition of starch hydrolysis observed during co-digestion of pasta and olives. Finally, in the case of dark purple eggplant, the slight inhibitory effect observed during co-digestion with pasta may be due to an inhibition of α-amylase activity by 3-*O*-caffeoylquinic acid, which was present at a concentration near the $IC_{50}$ value.

## 4. Conclusion

This study demonstrates that phenolic-rich vegetable foods slow down starch hydrolysis during *in vitro* co-digestion with pasta, mimicking a real-life scenario as closely as possible. Results underlined that the simultaneous consumption of starchy-rich foods, such as pasta, with vegetable foods may inhibit digestion, possibly reducing the post-prandial glycaemic peak.

The most active foods were flavonoid-rich vegetables rather than phenolic acid-rich vegetables such as cherry tomatoes and eggplant. There was no clear relationship between the total amount of phenolic compound determined by mass spectrometry and the extent of the inhibitory activity. Rather, the observed reduction in starch hydrolysis may be supported by the inhibitory activity of specific phenolic compounds, especially against the enzyme α-glucosidase. Given the concentration of these compounds detected in the intestinal fluid, it is possible to hypothesize an effect *in vivo* on starch digestion and post-prandial glucose concentrations. However, any contribution to the inhibition of starch hydrolysis of other unidentified phenolic compounds, in particular the high molecular weight ones, cannot be excluded.

Anyway, the present results highlighted the importance of the timing of consumption of phenolic-rich food to limit starch hydrolysis and possibly post-prandial glucose levels. The main limitation of this study is related to the use of an *in vitro* model that, although designed to reproduce in the most faithful possible realistic physiological conditions, did not consider the complexity of the human organism. Nonetheless, this study lays the foundations for designing future *in vivo* studies, which will have to reflect the type of phenolic-rich food as well as methods and times of consumption to get the maximum effect.

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This research did not receive any specific fund.

## CRediT authorship contribution statement

**Alice Cattivelli:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Melissa Zannini:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Angela Conte:** Writing – review & editing, Validation, Supervision, Data curation, Conceptualization. **Davide Tagliazucchi:** Writing – original draft, Validation, Supervision, Project administration, Data curation, Conceptualization.

## Declaration of competing interest

We wish to confirm that there are no known conflicts of interest: Associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the

criteria for authorship but are not listed.

We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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## 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.fbio.2024.104586.

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