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Interaction of bread and berry polyphenols affects starch digestibility and polyphenols bio-accessibility

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ABSTRACT

In this study, the effect of berry polyphenols on starch digestion was tested *in vitro* both by co-digestion of berry extract with bread or by fortifying bread with berry extract. Results show that the co-digestion of bread with berry extracts significantly reduce the rate and extent of starch digestion. Sixty one percent of starch digestion is inhibited by co-digesting 1 g of raspberry extract with 4 g of the bread. The inhibition obtained by co-digesting berry extracts and bread is much higher than the inhibition obtained by digesting berry-fortified bread. Interactions of polyphenols with matrix reduce polyphenols bio-accessibility, thus reducing the amount of polyphenols available for α-amylase inhibition. The interaction of polyphenols and starch seems also a crucial mechanism for the inhibition of starch digestion. This study shows that the co-ingestion of berry polyphenols with bread is a promising strategy to reduce glycaemic index of starchy food.

1. Introduction

Energy-dense, nutrient-poor diets containing high amounts of carbohydrates combined with sedentary lifestyles are the major drivers of the global obesity epidemic with high prevalence of type-2 diabetes (Medina-Remón, Kirwan, Lamuela-Raventós, & Estruch, 2018). Dietary carbohydrates, mainly occurring as starch in the human diet, can be hydrolysed by enzymes present in the upper gastrointestinal tract and absorbed as monosaccharides. Therefore, reducing the rate of starch digestion through a dietary intervention is a promising strategy for a better glycaemia control and this can be achieved by inhibiting the enzymes responsible for starch digestion (α-amylase and/or α-glucosidase) (Lim, Kim, Shin, Hamaker, & Lee, 2019; Takahama & Hirota, 2018).

Polyphenols have been shown to inhibit α-amylase and/or α-glucosidase, thus modulating the glycaemic response to carbohydrates (Barrett, Farhadi, & Smith, 2018; Di Stefano, Oliviero, & Udenigwe, 2018; Figueiredo-González et al., 2018; Silva, Sampaio, Freitas, & Torres, 2018). The mechanism of the inhibition depends on the type and concentration of polyphenols. Monomeric polyphenols can inactivate the two primary digestive enzymes by blocking the catalytic sites (Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). Polymeric polyphenols can precipitate with the digestive enzymes to form a non-digestible complex (Barrett et al., 2018). If the concentration of the polyphenols is high enough, they can also interact with food nutrients (like protein and starch) to form a polyphenol-coated particle or even large complexes (Amoako & Awika, 2016a,b). All these mechanisms can slow digestion of carbohydrates and reduce the rate of glucose uptake in the blood stream. Therefore, the use of polyphenols as a more natural substitute for anti-diabetic drugs such as acarbose was proposed for effective glycaemic control (Boath, Stewart, & McDougall, 2012; Lin, Teo, Leong, & Zhou, 2019).

Among the various polyphenols-rich food blueberry and raspberry are becoming popular in human diet, not only because of their appealing taste, but also for their health benefits (Garcia et al., 2017; Louis et al., 2014). Polyphenol-rich extracts from a range of berries containing anthocyanins and proanthocyanidins can inhibit α-amylase and α-glucosidase *in vitro* (Grussu, Stewart, & McDougall, 2011). *In vitro* studies and *silico* molecular docking studies confirmed the inhibitory effect of anthocyanins (like cyanidin-3- glucoside, cyanidin-3,5-glucoside, cyanidin-3-rutinoside, and peonidin-3-glucoside) on pancreatic α-amylase (Sui, Zhang, & Zhou, 2016). Proanthocyanidins also showed inhibitory effect on α-amylase (Mullen et al., 2002).

The inhibition of polyphenols on α-amylase and α-glucosidase has been often investigated in simple model systems but rarely in a real food matrix (Grussu et al., 2011; McDougall et al., 2005; Yuan et al., 2018). The influence on starch digestive enzymes of the actual availability of the polyphenols under digestive-physiological conditions, as well as the influence of the interactions between polyphenols and food components during digestion are still unknown.

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To investigate how the food matrix influences the role of polyphenols on starch digestibility, the berry extracts was either co-digested with bread or used to fortify a bread by mixing it to the dough. The co-digestion of the control bread plus different concentrations of berry polyphenols extracts was performed to simulate a meal in which bread is consumed along with berries; while by preparing a berry-fortified bread the effect of baking and bread matrix was investigated. In this paper, we aim at investigating the inhibition of starch degradation during *in vitro* digestion of white bread (1) fortified with raspberry or blueberry extracts and (2) co-digested with the same raspberry or blueberry extracts. The kinetics of starch digestion and polyphenols bioaccessibility were measured and compared in the two sets of samples.

2. Methodology

2.1. Materials

Blueberry (*Vaccinium* spp.), raspberry (*Rubus idaeus*), wheat flour (carbohydrate 73%, fat 1.6%, gluten 11%) and dry yeast were purchased from a local supermarket.

Cyanidin-3-glucoside, procyanidin B-2, pepsin (800–2500 units/mg), pancreatin (P1750; 4X USP specifications), amyloglucosidase (129 U/mg), ferric ammonium sulphate, butanol, bovine serum albumin (BSA), sodium dodecyl sulphate (SDS), triethanolamine (TEA) were purchased from Sigma–Aldrich (St Louis, MO, USA). Acetonitrile, methanol and absolute ethanol were HPLC grade. All other chemicals were of analytical grade.

2.2. Polyphenol extract preparation

The preparation of crude polyphenol extracts was carried out according to a previously published method with a slight modification (Kan, Nie, Hu, Liu, & Xie, 2016). The fresh blueberry and raspberry were dried firstly in a freeze dryer (Alpha 2–4 LDplus, Christ). Then, 200 g of dry powder of the fruits were extracted three times with 2 L of methanol. The extraction was carried out through an ultrasound equipment (Sonication, China) for 30 min. Ice was added to the ultrasound equipment to keep the temperature at 0 °C. After each extraction, extracts were centrifuged at 4000*g* for 15 min. The supernatants were combined and concentrated on a rotary evaporator to remove the methanol. Finally, the extracts were freeze-dried. The berries polyphenol extract powder was stored at −20 °C. For the preparation of the heated extracts, 5 g of berry extract was placed in a boiling water bath for 45 min to simulate the baking process. Finally, the heated berry extract was tested for the α-amylase inhibition assay and *in vitro* digestion assay (see Sections 2.4 and 2.6).

2.3. Polyphenol composition

2.3.1. Anthocyanins

Anthocyanin analysis was performed on a HPLC system equipped with a diode array detector based on a previous method with some modification (Kan et al., 2017). The separation was carried out on a Varian Polaris 5 C18-A (4.6 × 150 mm) column. The mobile phase consisted of water (10% of formic acid, eluent A) and methanol (eluent B). The flow rate was 1 mL/min. A multi-step programme was as follows: 5–60% B (20 min), 60–100% B (5 min), 100% B (5 min), 100–5% B (1 min). The run time was 31 min. The injection volume was 10 μL. The monitoring was performed at 520 nm. The total anthocyanin content was expressed as mg/g berry extract (cyanidin-3-glucoside equivalents).

2.3.2. Proanthocyanidins/condensed tannins

The acid butanol method was used for condensed tannins quantification (Han et al., 2015). Briefly, 0.5 mL of suitable diluted extract was mixed with 3 mL of butanol-acid reagent (95:5, *v/v*) and 0.1 mL of ferric reagent (2% ferric ammonium sulphate in 2 M HCl). Then, the mixture was boiled for 30 min. After cooling, the absorbance at 550 nm was measured on a spectrophotometer (Agilent Cary 60 UV.VIS). Procyanidin B-2 was used to make a calibration curve. The results were expressed as mg of procyanidin B-2 equivalents per gram of berry extract.

2.3.3. Protein precipitation capacity

BSA precipitation assay was used for measuring the protein precipitation capacity of berry tannins (Kyraleou et al., 2015). Briefly, 0.5 mL of dissolved extract was added to 1 mL of buffer 1 (200 mM acetic acid; 170 mM NaCl; pH = 4.9, containing BSA (1 mg/mL). Then the mixture was shaken slightly for 15 min. After the shaking, the samples were centrifuged to pellet the protein-tannin precipitate. The supernatant was discarded. The protein-tannin pellet was dissolved in a buffer containing 5% TEA (v/v) and 5% SDS (w/v). The dissolved tannins solution was mixed with 125 μL of ferric chloride reagent (10 mM $FeCl_3$ in 10 mM HCl). The mixture was incubated at room temperature for 10 min, and the reading at 510 nm was observed. Catechin was used as a standard, and the results expressed as mg catechin equivalents per gram of berry extract.

2.4. α-amylase inhibition assay

The inhibition of α-amylase was performed according to a previous method (Zhang et al., 2010). Briefly, 1% starch solution was prepared by dissolving 1 g starch in 100 mL of 0.1 M phosphate buffer (pH 6.9 containing 6.7 mM sodium chloride). Porcine pancreatin (α-amylase activity is 40 U/mg) was dissolved in the same buffer to give a final concentration of 20 mg/mL. Different concentrations of berry extract was dissolved in methanol. Then, 400 μL of methanol or berry extract solution or gastric supernatant (see Section 2.7) was mixed with 200 μL of starch and incubated at 25 °C for 10 min, and finally 200 μL of pancreatin was added to start the reaction. The final concentration of the berry extracts in the mixture was 5, 10, 20, 30, 40 and 50 mg/mL. The final concentration of the gastric supernatant in the mixture was the actual concentration for α-amylase inhibition during *in vitro* digestion. The α-amylase activity of the final mixture is 200 U/mL. After 3 min at 25 °C, 400 μL of 96 mM dinitrosalicylic acid reagent was added and the mixture was put in boiling water bath for 5 min. After cooling down, 4 mL of water was added before measured at 540 nm on a microplate reader. The α-amylase inhibition was calculated according to the following equation:

$$\text{Inhibition } (\%) = (1 - (A_{sample} - A_{blank})/(A_{test} - A_{control})) \times 100 \quad (1)$$

where A_{sample} is the absorbance of the mixture of phenolic samples, starch, enzyme and DNS reagent; A_{blank} is the absorbance of the mixture of phenolic samples, starch and DNS reagent without enzyme; A_{test} is the absorbance of the mixture of buffer (instead of phenolic sample), starch, enzyme and DNS reagent; $A_{control}$ is the absorbance of the mixture of buffer, starch and DNS reagent without enzyme.

Finally, the α-amylase inhibitory effect of the berry extract was expressed as IC_{50}, which was defined as the concentration of extract required to inhibit 50% of the enzyme activity, and expressed as milligram berry extract per millilitre solvent (mg/mL). The α-amylase inhibitory effect of the gastric supernatant was calculated according to Eq. (1).

2.5. Preparation of berry polyphenol-fortified bread

Baked bread fortified with 0% (control), 2.5% and 5% berry polyphenol extract were prepared using a bread-baking machine. The content of berry extract is based on fresh bread. The bread recipe was shown in Table 1. The ingredients of baked bread included wheat flour, water and yeast (Goh et al., 2015). All the ingredients were put in the bread-baking machine (Philips, HD 9020) and the bread was made using a standard program (Table 1).

Table 1
The recipe of berry polyphenol-fortified bread.

Ingredients	Control	1%	2.5%	5%
Water/mL	220	220	220	220
Wheat flour/g	350	345	337	324
Berry extract/g	0	5	13	26
Yeast/g	7	7	7	7
Fresh weight/g	516	516	516	516

Standard program of white bread: baking time is 45 min and baking temperature is 120 °C.

2.6. In vitro digestion study

2.6.1. In vitro digestion model

A standard protocol was used for the *in vitro* digestion study (Minekus et al., 2014) which was modified for the amount of α-amylase and the absent of the salivary amylase. The fresh bread samples were sieved through a 2 mm sieve. Five grams of sieved bread is mixed with 4 mL of simulated salivary fluid, 25 μL of 0.3 M $CaCl_2$ and 975 μL of water. The mixture was mixed thoroughly. Then two digestion phases (gastric and intestinal phase) were performed. For the gastric phase, 10 mL of the mixed sample were combined with 7.5 mL of simulated gastric fluids and 1.6 mL of pepsin (5.86 mg/mL, 4268 U/mg). The pH was adjusted to 3 by 1 M HCl. All the samples were put in the shaking water-bath (37 °C) for 2 h. For the intestinal phase, the samples from gastric digestion were combined with simulated intestinal fluids and pancreatin (40 mg/mL; α-amylase activity of the pancreatin is 40 U/mg.) to give an α-amylase activity of 200 U/mL in the final solution and the pH was adjusted to 7. All the samples were put in the shaking water-bath (37 °C) for 2 h. Individual sample tubes were prepared for each digestion time point of intestinal phase. Totally 8 time points (0, 10, 20, 40, 60, 80, 100 and 120 min) was chosen for the intestinal phase. Then all the samples from each time point was centrifuged immediately at 4 °C (4000*g*, 10 min). Finally 1 mL of the supernatant was mixed with 4 mL of ethanol to stop the reaction and this mixture was used for further analysis of glucose.

To understand the matrix effect of bread on the efficacy of berry polyphenol, a co-digestion study was carried out, aiming to simulate a meal in which bread is consumed along with berry. Then 0, 0.125, 0.25, 0.5 and 1 g of berry extract were mixed with 5, 4.875, 4.75, 4.5 and 4 g of bread and marked as 0% (control), 2.5%, 5%, 10% and 20% of berry extracts co-digestion.

2.6.2. Determination of the percentage of the digested starch

For the glucose measurement, amyloglucosidase was added to complete starch digestion (Rovalino-Córdova, Fogliano, & Capuano, 2018): one millilitre of supernatant was combined with 5 mL of amyloglucosidase solution (27.16 U/mL) in acetate buffer (0.1 M, pH 4.8) and incubated at 37 °C for 1 h. The tubes were boiled for 5 min to inactivate the enzyme activity. The samples were centrifuged at 4000*g* for 15 min. The supernatant was collected for glucose measurement.

Preliminary experiments have indicated that colorimetric enzymatic methods for glucose measurements are poorly accurate when coloured extracts are used or when the presence of polyphenols may inhibit the enzymes used in the assay. HPLC-ELSD was used to quantify the glucose from starch digestion (Ma, Sun, Chen, Zhang, & Zhu, 2014). The separation was carried out on a Grace prevail carbohydrate ES (5 μm, 250 × 4.6 mm) column. The mobile phase consisted of water (eluent A) and acetonitrile (eluent B). The flow rate was 0.6 mL/min. The programme was 75% B for 25 min. The injection volume was 20 μL. For the ELSD settings, evaporating and nebulizer temperature were 90 °C and 50 °C, and the carrier gas flow was 1.6 slm (standard litre per minute). The released glucose from bread was quantified based on the peak area from HPLC (The digested starch = released glucose/0.9). The initial amount of total starch in bread was measured by Total Starch Assay kit (amyloglucosidase/α-amylase method), Megazyme Inc. (Bray, Ireland). The results were expressed as the percentage of digested starch (% of digested starch = digested starch/initial amount of starch).

The digested starch data for this study were fitted to a first order model (Eq. (2)) as previously proposed (Goñi, Garcia-Alonso, & Saura-Calixto, 1997):

$$C_t - C_0 = C_\infty(1 - e^{-kt}) \quad (2)$$

where C_t, C_0 and C_∞ are the percentage of digested starch at time t, time 0 and at infinite time, respectively, and k is a pseudo-first order rate constant. Solver from Excel was used for estimating k and C_∞ values by minimizing the residual sum of square values. In this study, the change of the two parameters (k and C_∞) caused by polyphenols will be discussed.

In this study, the initial reaction rate was calculated by Eq. (3).

$$Initial\ \ reaction\ \ rate = (C_{10} - C_0)/10 \quad (3)$$

where C_{10} and C_0 are the percentage of digested starch at time 10 and 0 (min), respectively, and 10 is the time that was chosen for the calculation of initial reaction rate.

The inhibition of the berry polyphenols on starch digestion could be calculated by Eq. (4):

$$\text{Inhibition}\ \ (\%) = ((C_{t\,(control)} - C_{t\,(sample)})/C_{t\,(control)}) \times 100 \quad (4)$$

where $C_{t\ (control)}$ is the percentage of digested starch of control bread at time t, and $C_{t\ (sample)}$ is the percentage of digested starch of berry-fortified or co-digested bread at time t.

2.7. Bio-accessibility of polyphenols

During the *in vitro* digestion process, the digested samples at the end of gastric (time point, 0 min) and intestinal phase (time point, 120 min) were collected and centrifuged for 10 min at 4000*g*. The both supernatants were collected for measuring anthocyanins and proanthocyanidins directly as described in 2.3. The supernatant from gastric phase was also used for measuring the α-amylase inhibition as described in 2.4. Then the bio-accessibility of polyphenols was expressed as percentage of polyphenols available in the supernatant compared to the initial amount of polyphenols in the crude extract.

2.8. The interaction between polyphenols, digestive enzymes and bread matrix

To investigate the interactions between polyphenols, digestive enzymes and bread matrix in co-digestion samples, 250 mg of berry extract were mixed with gastric and intestinal digestive fluids as a control. Then the digestive enzymes (pepsin and pancreatin) or 5 g of bread were added separately. All the mixtures were allowed to stand at room temperature for 2 min. Then the mixture was centrifuged for 15 min at 4000*g*. The supernatant was collected and the percentage of polyphenols in supernatant was determined as described in 2.3. The results were expressed as the percentage of polyphenols distributed in supernatant compared to the initial content in berry extracts.

To study the interaction preference between berry polyphenols and bread components (starch and gluten) in co-digestion samples, starch and gluten were mixed with berry extract separately. Briefly, starch or gluten were added to some water. The mixture was boiled for 5 min and then cooled down to room temperature to allow for starch gelatinization. Then 2.5 g of starch or 0.4 g of gluten (the corresponding amount in 5 g of bread) were mixed with 250 mg of berry extract separately. Then gastric and intestinal fluids were added. The mixture was allowed to stand at room temperature for 2 min. Quantification of polyphenols in the supernatant was carried out as described in 2.3.

To explain the interaction of polyphenols and food matrix in fortified bread, 5 g of 5% berry-fortified bread was mixed with gastric and intestinal digestive fluids. The mixtures were allowed to stand at room

temperature for 2 min. Then the mixture was centrifuged for 15 min at 4000*g*. The supernatant was collected and the percentage of polyphenols in supernatant was determined as described in 2.3. The results were expressed as the percentage of polyphenols distributed in supernatant compared to the amount of polyphenols in berry-fortified bread. Moreover, 5% berry-fortified dough (before baking) was also prepared to ascertain the influence of baking on polyphenol stability. Then the dough was mixed with gastric and intestinal fluids and the following steps was the same as fortified bread.

2.9. Statistical analysis

All experiments were performed in triplicate. The results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by the Duncan's multiple range test was used to compare the means among different groups by the SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. α-amylase inhibition and protein precipitation capacity of berry extracts

Table 2 shows the polyphenol composition of berry extracts. Anthocyanins (10.0 mg/g) and proanthocyanidins (44.5 mg/g) are detected in blueberry extract. Whereas in raspberry extract, less anthocyanins (4.4 mg/g) and proanthocyanidins (13.5 mg/g) were detected. Raspberry showed some protein precipitation capacity of 9.6 mg/g. No precipitation capacity was detected in blueberry extract. Blueberry and raspberry extract also contained 70.2% and 66.9% of sugars respectively (data not shown). After heating at 100 °C, almost half of the anthocyanins and proanthocyanidins were degraded for both berry extracts. There was no significant change of protein precipitation capacity after heating.

The inhibition of berry extracts on α-amylase is also shown in Table 2. The blueberry extract had a smaller IC_{50} value (17.3 mg/mL) than raspberry extract (25.5 mg/mL). The heating also had some influence on the inhibitory effect of both berry extracts. Although half of the anthocyanins and proanthocyanidins were degraded, the direct inhibition on α-amylase was increased (Table 2).

3.2. Starch digestibility in co-digestion and fortification samples

The starch digestion curves for bread co-digested with different concentrations of berry extracts and for fortified bread are shown in Fig. 1. A dose-dependent reduction in the rate and extent of starch digestibility was observed for both extracts that were co-digested with bread. The starch digestion curves are fit to a fractional conversion model (Dona, Pages, Gilbert, & Kuchel, 2010) and the estimated parameters are shown in Table 3. In our study the C_∞ value of control white bread was 86.9%. A significant decrease in C_∞ values of 4.9%, 14.7%, 38.2% and 56.5% was observed when the white bread was co-digested with different levels of blueberry extracts and an even larger decrease of 12.9%, 23.7%, 51.1% and 61.2% is found with raspberry extracts. Higher inhibition was found in both heated berry extracts that were co-digested with control bread compared with non-heated extract (16.7% vs 14.7% for blueberry and 28.2% vs 23.7% for raspberry). The initial rate also decreased significantly when co-digested with berry extract. The starch was digested at a similar rate (k value) as control bread when co-digested at low concentration of polyphenols (2.5%). When co-digested with higher polyphenols concentration (above 5%), the k value slightly increased.

The results about the fortified bread revealed that the inhibitory effect of polyphenols on starch digestion in berry-fortified bread was lower compared with the co-digestion. Regarding the raspberry bread, the 2.5% and 5% of raspberry extracts fortification led to a significantly inhibitory effect that was confirmed by the decrease of the C_∞ values from 86.9% (control bread) to 80.8% (2.5% fortification) and 74.0% (5% fortification) (Table 3). Regarding the blueberry bread, it can be noticed that the addition of extract did not produce any significant effect on starch digestion (Fig. 1). The C_∞ values from the kinetics model confirmed this trend (Table 3).

Table 2
The amount of polyphenols in the berry extract or heated berry extract and their IC_{50} values for α-amylase inhibition.

Extract	Anthocyanin[a]	Condensed tannins[b]	PPC[c]	IC_{50} [d]
BPE	10.0 ± 0.0 a	44.5 ± 1.5 a	nd	17.3 ± 0.4 c
RPE	4.4 ± 0.1 b	13.5 ± 1.0 b	9.6 ± 0.5 a	25.5 ± 0.3 a
Heated BPE	5.5 ± 0.1 c	26.7 ± 0.9 c	nd	15.8 ± 0.5 d
Heated RPE	2.1 ± 0.0 d	7.4 ± 0.6 d	9.5 ± 0.6 a	22.0 ± 0.3 b

BPE: blueberry polyphenol extract; RPE: raspberry polyphenol extract. nd: not detected, Values followed by the different letter in the same column are significantly different ($p < 0.05$).

[a] Results expressed as mg cyanidin-3-glucoside equivalents per gram of extract or heated extract.

[b] Results expressed as mg procyanidin B-2 equivalents per gram of extract or heated extract.

[c] PPC, Protein precipitation capacity. The Results expressed as mg catechin equivalents per gram of extract or heated extract.

[d] Results expressed as IC_{50} values which means the concentration of extract (mg/mL) required to inhibit 50% of the α-amylase activity.

3.3. Polyphenols bio-accessibility and α-amylase inhibition of gastric supernatant

Polyphenols bio-accessibility after gastric phase and intestinal phase are shown in Fig. 2. For both berry samples, the polyphenols bio-accessibility of co-digestion samples is higher than that of fortification samples. Regarding the blueberry co-digestion samples, almost 50~60% of anthocyanins was bio-accessible after gastric digestion and no clear increase after intestinal digestion. Less proanthocyanidins was bio-accessible after gastric digestion compared to anthocyanins. Regarding the blueberry fortification samples, less than 30% of anthocyanins and 5% of proanthocyanidins was bio-accessible after gastric digestion. The raspberry polyphenols bio-accessibility showed the similar trend with blueberry polyphenols, but no protein precipitation capacity was detected after gastric digestion or intestinal digestion of all the samples. The α-amylase inhibition of the bio-accessible polyphenols after gastric phase was also investigated (Table 4). No enzyme inhibition was detected in all the fortification samples. Regarding bio-accessible blueberry polyphenols after gastric phase, 5.2%, 37.3% and 65.2% of α-amylase inhibition was found in the bio-accessible polyphenols from 5%, 10% and 20% co-digestion. But less α-amylase inhibition was found in bio-accessible raspberry polyphenols from co-digestion experiments.

3.4. Interaction among polyphenols, digestive enzymes and bread matrix

The interaction among polyphenols, digestive enzymes, bread, gluten and starch was measured and shown in Fig. 3. Regarding the blueberry, 61% of anthocyanins and 52% of the proanthocyanidins were detected in the supernatant after diffusion in the digestive fluids. Less anthocyanins (56%) and proanthocyanidins (50%) were detected when pepsin and pancreatin were added to digestive fluids. The raspberry showed the same trend as blueberry. When both berry extracts were mixed with control bread, much less polyphenols were detected in the supernatant. The interaction of polyphenols with starch and gluten is also shown in Fig. 3. When both berry extracts were separately mixed with starch and gluten (at the corresponding amount present in the

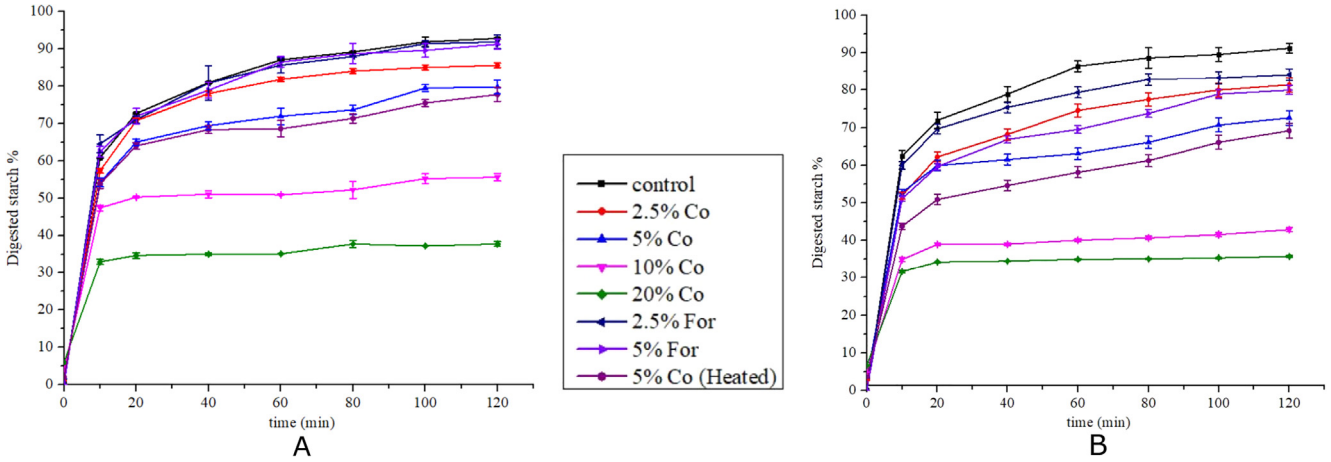

Fig. 1. *In vitro* starch hydrolysis profiles of control bread co-digested or fortified with different concentrations of blueberry polyphenol extract (A) and raspberry polyphenol extract (B). Co: Co-digestion. For: Fortification.

Table 3
Estimated kinetic parameters for starch digestion obtained from *in vitro* digestion of wheat bread co-digested or fortified with different levels of berry extracts.

	Co-digestion						Fortification	
blueberry	Control	2.5% Co BPE	5% Co BPE	5% Co heated BPE	10% Co BPE	20% Co BPE	2.5% Fortification	5% Fortification
k (min^{-1})	0.11 ± 0.00 cd	0.11 ± 0.00 cd	0.15 ± 0.01 b	0.12 ± 0.01 c	0.21 ± 0.00 a	0.21 ± 0.03 a	0.11 ± 0.00 cd	0.11 ± 0.00 d
C_∞ (%)	86.9 ± 1.2 a	82.0 ± 0.6 b	72.2 ± 0.2 c	70.2 ± 0.2 d	48.7 ± 0.4 e	30.4 ± 0.1 f	87.2 ± 0.8 a	87.6 ± 0.1 a
Initial rate (%*min^{-1})	5.8 ± 0.2 ab	5.6 ± 0.2 b	5.4 ± 0.1 c	5.0 ± 0.2 d	4.3 ± 0.1 e	2.7 ± 0.1 f	5.9 ± 0.2 a	5.6 ± 0.0 bc
Residual sum of squares	136.2 ± 5.5	40.3 ± 0.6	27.9 ± 1.4	86.0 ± 46.7	32.2 ± 4.2	11.4 ± 1.7	180.2 ± 25.9	105.5 ± 9.6
raspberry	Control	2.5% Co RPE	5% Co RPE	5% Co heated RPE	10% Co RPE	20% Co RPE	2.5% Fortification Fortification	5% Fortification
k (min^{-1})	0.11 ± 0.00 e	0.09 ± 0.00 g	0.14 ± 0.00 c	0.10 ± 0.00 f	0.18 ± 0.00 f	0.21 ± 0.01 b	0.12 ± 0.00 a	0.10 ± 0.00 d
C_∞ (%)	86. 9 ± 1.2 a	74.0 ± 1.8 c	63.2 ± 1.6 d	58.8 ± 1.7 e	35.8 ± 0.4 f	25.7 ± 2.8 g	80.8 ± 1.5 b	74.0 ± 1.0 c
Initial rate (%*min^{-1})	5.8 ± 0.2 a	4.8 ± 0.1 b	4.5 ± 0.1 c	3.7 ± 0.1 d	3.0 ± 0.1 e	2.5 ± 0.0 f	5.7 ± 0.1 a	4.7 ± 0.1 b
Residual sum of squares	136.3 ± 5.7	110.5 ± 5.9	99.8 ± 8.1	143.9 ± 8.1	8.9 ± 1.0	1.1 ± 0.3	72.8 ± 3.1	99.8 ± 8.1

Values expressed as mean ± sd. Values followed by the different letter in the same row are significantly different ($p < 0.05$).

bread), more polyphenols were detected in the supernatant of gluten-polyphenol mixture than starch-polyphenol mixture.

Blueberry-fortified bread and dough were mixed with digestive fluids as well, and few anthocyanins (6.0 and 3.6%) and proanthocyanidins (1.8 and 1.1%) were found in bread and dough, respectively. A similar behaviour was observed in raspberry-fortified bread.

4. Discussion

Given the relevance of post-prandial glycaemia on the incidence of chronic diseases, non-communicable strategies to reduce the GI of staple starch-based foods are intensively explored. A promising strategy is the addition of polyphenols which has been reported to reduce the rate of starch digestion. However, the vast majority of the scientific reports have been produced in simple model systems (*i.e.*, just containing enzymes, polyphenols and a simple substrate like p-nitrophenyl-α-D-glucopyranoside (pNPG) or starch), but rarely in a real food matrix (McDougall et al., 2005). We have selected white bread as a model starchy food, berries as sources of polyphenols and used the INFOGEST *in vitro* digestion method to simulate the digestion of bread. The extent of starch digestion we observed was in line with other reports where 87% of starch was hydrolysed in white bread using the same *in vitro* digestion model (Bustos, Vignola, Pérez, & León, 2017). The C_∞ values were markedly reduced when increasing the polyphenol concentration from blueberry and raspberry in the co-digestion and the fortification experiments (Fig. 1A & B). However, the rate constant k became larger when co-digested with higher amount of berry extract, even though the rate of starch digestion during the first minutes was clearly lower as visually judged by the slope of the starch digestion curves during the initial stage of digestion. This counterintuitive finding is rather difficult to explain but might partly be related to the smaller amount of starch available for digestion at very high polyphenols content and thus, to the shorter time needed to reach C_∞. In such situations, the calculation of the initial rate of digestion would give a more accurate depiction of the digestion kinetics compared to k (Table 3).

Taken together our results suggest that the effect of polyphenols on starch digestion is modulated by the presence of the food matrix and of other digestive enzymes. In particular, when the IC_{50} values reported in Table 2 are compared to the kinetics reported in Table 3, it is clear that the behaviour of the berry extracts in the starch digestion of bread cannot be accurately predicted by the IC_{50} values calculated in the simple model system containing just starch, polyphenols and α-amylase. For example, in the 20% co-digestion experiment (corresponding to a concentration of raspberry extract in the intestinal digestion of 25 mg/mL) an inhibition of 57.4% (Table 4) was found based on the starch digestion kinetics of the first 10 min which is higher than 50% inhibition reported in Table 2, and much higher inhibition (70.4%) was found based on the starch digestion kinetics of the infinite time

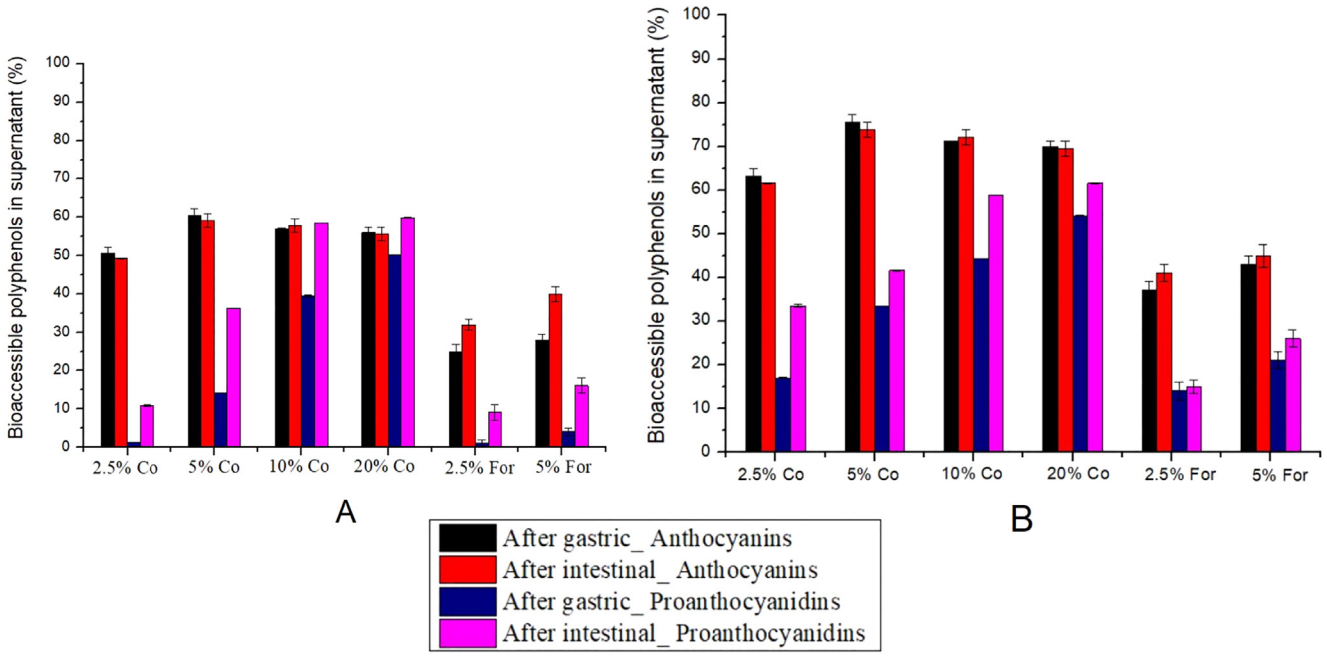

Fig. 2. Bioaccessibility of berry polyphenols after gastric and intestinal digestion. Results expressed as percentage of bioaccessible polyphenols at the end of gastric and intestinal digestion compared to the initial amount of polyphenols in crude extract. A: Blueberry; B: Raspberry; Co: Co-digestion; For: Fortification. No protein precipitation capacity was detected after gastric and intestinal digestion.

(Table 4). What's more, the order of inhibitory potential of berries that we observed in the experiment with bread (Table 3) is the opposite of what observed with the inhibition experiments (Table 2). Therefore the IC_{50} value can not accurately predict the inhibition of starch digestion in food matrix.

When results in Fig. 1A and B are considered, it is clear that co-digestion of the berry extract with bread is more effective in reducing the rate of starch digestion than incorporating berry extract into the bread matrix. We first hypothesized that this effect may be partially due to the degradation of the phenolic compounds during baking. Indeed, a substantial reduction in the content of extractable polyphenols was observed after baking (Table 2). However, heated berry extracts showed better inhibition than non-heated extracts in both the simple inhibition assay (Table 2) and in the *in vitro* simulated digestion (Table 3 & Fig. 1). This could mean that the degradation products from anthocyanins or proanthocyanidins can exert an inhibitory effect on α-amylase at least of the same magnitude as the parent compounds. A recent study has showed that the thermal degradation products from anthocyanins, like chalcone, could inhibit α-amylase through non-competition inhibition (Zhang et al., 2019).

Interactions with food matrix modulate the amount of polyphenols available to inhibit α-amylase. To consider how interactions with food matrix influence the role of polyphenols on α-amylase, we investigated the bio-accessibility of polyphenols in a variety of systems (Figs. 2 & 3).

We firstly investigated the bio-accessibility of polyphenols after gastric phase digestion which is an indication of the actual concentration for α-amylase inhibition. As reported in Fig. 2, the bio-accessibility of polyphenols in fortification samples was lower than that of co-digestion samples but the loss of phenolics due to the baking step must be considered as well. Since a direct comparison with fortified bread was difficult given this loss, we further compared the bio-accessibility of polyphenols after mixing with bread to the bio-accessibility of polyphenols in a dough matrix. The results in Fig. 3 shows that the bio-accessibility of polyphenols was higher in the co-digestion experiment compared to the dough. We therefore hypothesize that, regardless the exact type of enzyme inhibition (*i.e.* uncompetitive, non-competitive, *etc.*) (Barrett et al., 2018; Grussu et al., 2011), the intensity of the inhibition on digestive enzymes is proportional to the amount of solubilized polyphenols that can interact with α-amylase, which represented the bio-accessible fraction, not blocked by other interactions, *e.g.*, with gluten, or starch. This is in line with previous reports discussing the relevance of interactions within the food matrix on the digestive enzyme activities (Capuano, Oliviero, Fogliano, & Pellegrini, 2018; Sun, Gidley, & Warren, 2018).

Although interactions with matrix reduce the amount of polyphenols available for α-amylase inhibition, the direct interaction with starch could also be a crucial mechanism to inhibit starch digestion. Another possible mechanism of the inhibition of the starch digestion is

Table 4
α-amylase inhibition of digested samples after gastric digestion (%).

		Co-digestion				Fortification	
		2.5%	5%	10%	20%	2.5%	5%
Blueberry	Amylase inhibition	nd	4.8 ± 0.2 c	33.2 ± 4.2 b	60.6 ± 4.6 a	nd	nd
	Inhibition calculated from C_∞	5.7 ± 0.7 d	16.9 ± 0.2 c	44.0 ± 0.5 b	65.1 ± 0.1 a	0	0
	Inhibition calculated from C_{10}	2.5 ± 1.7 d	6.9 ± 1.01 c	25.9 ± 1.1 b	54.1 ± 1.5 a	0	0
Raspberry	Amylase inhibition	nd	nd	4.7 ± 0.4 b	33.5 ± 2.6 a	nd	nd
	Inhibition calculated from C_∞	14.8 ± 2.0 d	27.3 ± 1.9 c	58.8 ± 0.5 b	70.4 ± 3.2 a	7.0 ± 1.8 e	14.9 ± 1.3 d
	Inhibition calculated from C_{10}	17.7 ± 1.1 d	22.3 ± 1.3 c	48.7 ± 1.0 b	57.4 ± 0.0 a	1.5 ± 1.6 e	19.5 ± 0.9 d

The results were expressed as the mean ± sd; The different letters in the same row mean significant difference.

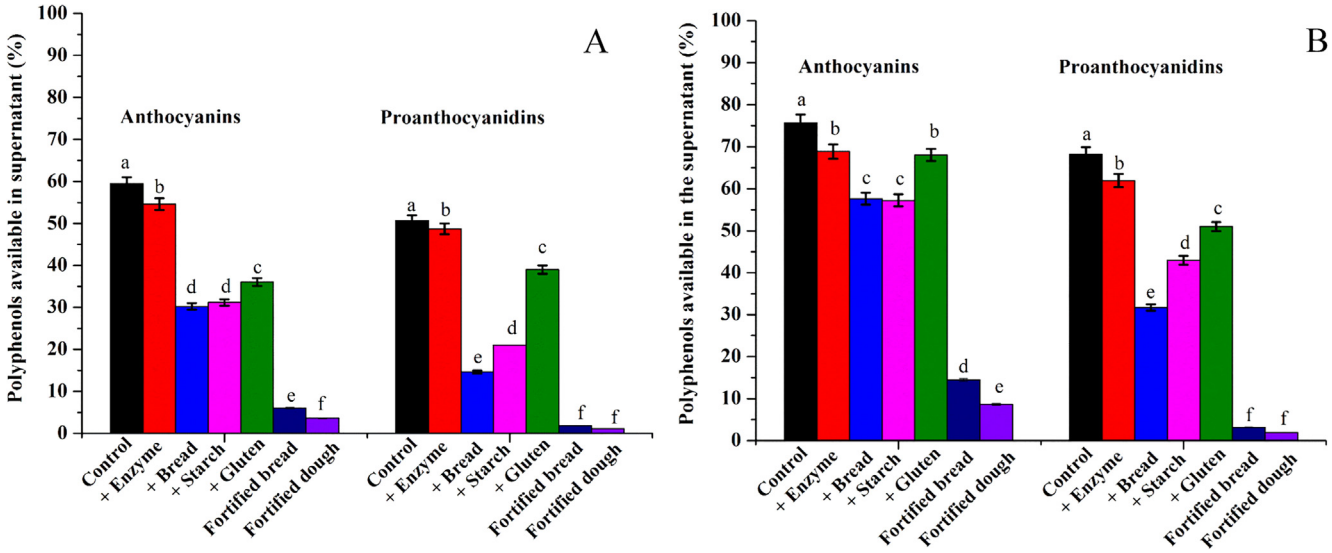

Fig. 3. Relative percentage of polyphenols in supernatant upon addition of digestive enzymes and bread components (gluten and starch) compared to the initial content in berry extracts. The berry extracts were mixed with digestive fluids as a control. Then the mixture was mixed either with digestive enzyme, or with bread, or with starch, or with gluten (Gluten and starch were added at the corresponding amount as they are present in the bread). In addition, the berry-fortified bread or dough was mixed with digestive fluids and the percentage of polyphenols in supernatant was also measured as well. The different letters mean significant difference. A: blueberry extract, B: raspberry extract. (No protein precipitation capacity was detected in all the samples.)

the absorption of polyphenols on the surface of starch granules, possibly shielding the amylase from its substrates. The accessibility of available binding sites on the starch granules for amylase would be reduced in presence of a large amount of polyphenols. In this case, the inhibitory effect would be proportional to the extent of starch coverage by polyphenols. In fact, the drastic decrease in C_∞ at very high concentration of berry extract (Table 3), suggests that the interaction between starch and polyphenols is producing a fraction of starch that is hardly, if ever, digestible *i.e.*, resistant starch. To further confirm to what extent the direct interaction of polyphenols with starch could influence the starch digestion, we investigated the α-amylase inhibition of the intestinal supernatant after the gastric phase and compared it with the inhibition calculated by the bread digestion experiments (C_{10} and C_∞). Data of Table 4 highlights that the inhibition calculated by C_{10} and C_∞ are higher than the α-amylase inhibition in most of the cases. Even more importantly, for some of the co-digestion or fortification experiments with 2.5% and 5% extracts, no α-amylase inhibition was found even if some inhibition was calculated from the C_{10} and C_∞. The bio-accessibility of polyphenols at the end of intestinal phase (Fig. 2) also confirmed that substantial amount of polyphenols stably interacted with food matrix. Therefore, α-amylase inhibition is not the only way to inhibit starch digestion, interaction with starch is also a crucial mechanism for inhibition of starch digestion.

Whatever mechanism of the inhibition on starch digestion is, the polyphenols bio-accessibility is modulated by the food matrix. To further explore the factors modulating bio-accessibility of polyphenols during digestion of a bread matrix, we showed that this bio-accessible fraction depends on polyphenols solubility and stability and is further reduced by the addition of pancreatic secretions and by the food matrix (Fig. 3). The addition of digestive enzymes reduces bio-accessibility of polyphenols. This was expected given the protein nature of digestive enzymes but the net effect was rather modest. It must be noted however, this effect depends on the amount of enzymes present in the digestive fluids used *i.e.*, varies depending on the *in vitro* digestion model selected. The effect of adding bread instead was substantial, both for anthocyanins and proanthocyanidins. Further insights were obtained when gluten and starch were separately added to the mixture of digestive fluids and polyphenols. Whereas an interaction of polyphenols with the gluten network was expected, given the high affinity of polyphenols with proteins, we observed a surprising contribution of starch on polyphenols bio-accessibility. Polyphenols have been already reported to bind directly with starch through hydrophobic forces and hydrogen bonding (Zhu, 2015). Here we show that despite the stronger interactions of polyphenols with proteins, in a starch-rich matrix such as bread, the contribution of starch in binding polyphenols is greater than that of gluten (Fig. 3). Incidentally, the interaction with digestive enzymes and food matrix will also modify the way polyphenols are delivered to the gut microbiota (bound to starch or proteins versus free) even though the effect these interactions can have on polyphenols utilization by gut microbiota is still unknown.

The bio-accessibility of polyphenols is not the only factors determining the extent of the inhibition on starch digestion, the type of polyphenols being equally important. This is shown by the fact that inhibition is better after addition of raspberry extracts compared to blueberry extracts despite the higher IC_{50} value of raspberry extract from the results of the simple α-amylase inhibition assay (Table 2). Raspberry is different from other berries for its substantial amount of high molecular weight tannins, *i.e.* ellagitannins. High molecular tannins are reported to show better protein precipitation capacity compared with condensed tannins (McDougall et al., 2005). This was in line with our results that blueberry extract did not show any protein precipitation capacity though it had substantial amount of condensed tannins (Table 2). However, we did not detect any protein precipitation capacity after gastric and intestinal phase as shown in Fig. 2. That means the high molecular weight tannins from raspberry are likely to interact with starch, thus reducing the accessibility of the starch for the α-amylase as we discussed before. This is also further confirmed by results in Table 4, *i.e.*, raspberry bio-accessible polyphenols have lower α-amylase inhibition compared to blueberry bio-accessible polyphenols, but higher inhibition of starch digestion calculated by C_{10} and C_∞. That means some raspberry polyphenols, most likely the high molecular weight tannins, interacted with starch, thus inhibiting the starch digestion. This is in line with the others reports that high molecular weight tannin-starch complexes can block digestibility of starch (Amoako & Awika, 2016a,b).

Our study shows that it is possible to slow down starch digestion in a starchy food like bread providing there is a sufficient amount of polyphenols in the gastrointestinal tract. However, such an amount is hardly achievable through fresh berries. The yield of the extract from fresh berries was around 5%. Based on the extract yield from berries, co-

digestion with 5% berry extract for 100 g of bread would require 5 g of berry extract, *i.e.* about 100 g of fresh berries, which is an amount that can be realistically achieved in a meal (Furlan et al., 2019). However, co-digestion with higher amount of berry extract would require unrealistic amounts of berries. For example, 200 g and 400 g of fresh berries are needed for 100 g of bread at a level of 10% and 20% of berry extract. On top of that, bio-accessibility of polyphenols from a fruit matrix is limited by the fruit matrix itself which may result in even milder effects compared to berry polyphenols pre-extracted from the fruit matrix (Capuano et al., 2018).

5. Conclusion

In summary, this study investigated the effects of berry polyphenols on the *in vitro* digestibility of white bread either when they are co-digested or incorporated in bread. A significant reduction was observed in starch digestion kinetics from the co-digestion of bread with berry extract. The fortification of bread with berry extracts was less effective in inhibiting the starch digestion. The effect of polyphenols on starch digestion is modulated by the presence of the food matrix. On one hand, the interactions between polyphenols and the food matrix reduces the bio-accessibility of the polyphenols, thereby reducing the amount of polyphenols available for α-amylase inhibition. On the other hand, the interaction between starch and polyphenols is also a crucial way to inhibit starch digestion by reducing the accessibility of the starch for α-amylase. Finally, polyphenols type also influences their way for the inhibition of starch digestion. This study shows that the co-ingestion of berry polyphenols with bread is a promising strategy to reduce glycaemic index, however, the lower bio-accessibility due to the interaction with food matrix must be taken into account.

6. Ethics statements

This paper did not include any human subjects and animal experiments.

CRediT authorship contribution statement

Lijiao Kan: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Visualization, Writing - original draft. **Teresa Oliviero:** Conceptualization, Writing - review & editing, Validation, Supervision. **Ruud Verkerk:** Conceptualization, Writing - review & editing, Validation, Supervision. **Vincenzo Fogliano:** Supervision, Writing - review & editing. **Edoardo Capuano:** Conceptualization, Writing - review & editing, Validation, Supervision.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests.

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