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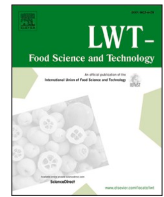

Influence of enzyme treatment approach on the phenolics content and antioxidant potential of sorghum grain samples

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A B S T R A C T

Enzyme-assisted extraction of phenolics was performed from commercial broom, and GK Emese and Farmsugro 180 hybrid sorghum grain residues with cellulolytic and esterolytic cocktails of *Rhizomucor miehei* NRRL 5282, *Gilbertella persicaria* SZMC 11086 and *Mucor corticolus* SZMC 12031 fungi. Results showed an increasing tendency for total phenolic content (TPC) during incubation that was in a positive relation with total flavonoid content (TFC) for most treatments. Best TPC and TFC yields were obtained on broom sorghum with maximal values of 256.9 ± 11.7 mg gallic acid equivalent (GAE)/100 g dry matter (DM) and 159.4 ± 6.8 mg catechin equivalent (CE)/100 g DM, respectively, achieved during *R. miehei* enzyme treatment. Reducing power and radical scavenging activity assays demonstrated improved antioxidant activity for most treated samples that was in positive correlation ($0.942 \geq r \geq 0.522$) with the TPC and TFC variables. Chromatography analysis revealed hydroxybenzoates, i.e., protocatechuic, 4-hydroxybenzoic and vanillic acids, as major phenolics with yields ranging from 5.40 ± 0.57 to 132.85 ± 14.21 μg/g DM, but the content of hydroxycinnamates increased to a greater extent during the treatments. The enzyme treatments can be reliable methods for enriching phenolic antioxidants in sorghum substances utilizable in gluten-free food products.

1. Introduction

Sorghum bicolor (L.) Moench is the fifth most produced cereal crop worldwide (Mabelebele, Siwela, Gous, & Iji, 2015). It is mainly cultivated in hot and arid regions (e.g., South Asia and Africa) due to its outstanding drought tolerance. In these regions, sorghum is considered as a staple food ensuring food security for millions of people (Cabrera-Ramírez et al., 2020; Dicko et al., 2005; Queiroz et al., 2015; Xu, Wang, & Zhao, 2021). Tons of waste sorghum grain and processing residues are generated annually due to the growing consumption of grain products (Salazar-López, Ovando-Martínez, & Domínguez-Avila, 2020). Sorghum contains some unique phenolics such as 3-deoxyanthocyanidins (e.g., apigeninidin and luteolinidin) (Awika, Rooney, & Waniska, 2004; Vanamala, Massey, Pinnamaneni, Reddivari, & Reardon, 2018; Wu, Wang, Liu, & Wang, 2023; Xiong, Zhang, Warner, & Fang, 2019). Phenolics are known to have a wide variety of health-promoting characteristics both *in vivo* and *in vitro* (Ed Nignpense, Francis, Blanchard, & Santhakumar, 2021) such as antioxidant, antimicrobial, anti-inflammatory, anti-diabetic, anti-atherogenic and anti-proliferative properties (Xiong et al., 2019; Xu et al., 2021). An imbalance between levels of free radicals and antioxidants can lead to oxidative stress associated unfavorable health conditions such as various neurodegenerative disorders (Olufunmilayo, Gerke-Duncan, & Holsinger, 2023). The grain phenolics are promising antioxidants, and the grain intake can prevent against the risk of certain cancer types, coronary heart diseases, and diabetes (Adom & Liu, 2002; Vanamala et al., 2018). The grain phenolics can bind metal ions, and absorb harmful UV radiation (Munteanu & Apetrei, 2021).

Majority of grain phenolics in the plant cell wall have been found in bounded form with non-starch polysaccharides, i.e., arabinoxylan, cellulose, hemicellulose, and lignin. These phenolic-saccharide complexes can be formulated via ester, ether, carbon-carbon (C–C) or glycosidic

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linkages, and thereby the bioavailability of the phenolic compounds can be reduced (de Lima et al., 2018; Schendel, 2019; Shahidi & Yeo, 2016). While free and soluble-conjugated phenolics can be isolated with solvents, bound phenolics require different methods to get liberated (Zhu et al., 2016). Acid and alkaline hydrolysis are the most common chemical methods to cleave bonds between phenolics and cell wall polymers (Shahidi & Yeo, 2016; Wang, Li, Ge, & Lin, 2020). However, these pre-treatments can result in a low-quality final product and have different toxicological effects (Puri, Sharma, & Barrow, 2012). Eco-friendly methods are the microwave-assisted and supercritical fluid extraction techniques, and the use of various hydrolytic enzymes (Soto, Acosta, Vaillant, & Pérez, 2016). In enzyme-assisted extraction, carbohydrate-cleaving enzymes are utilized as they can decrease the tissue rigidity in the plant cell wall leading to an increased efficiency for the phenolic release (Madeira Junior, Teixeira, & Macedo, 2015). These enzymes, including cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21), can cleave the bonds between the phenolics and the sugar residue contributing to the release of the phenolic compound from their glycoconjugates (Puri et al., 2012; Soto et al., 2016). Release of ester linked phenolics, e.g., the *p*-coumaric acid and ferulic acid (Adom & Liu, 2002; Awika & Rooney, 2004; Dilokpimol et al., 2016; Fardet, Rock, & Rémésy, 2008; Mastihuba, Kremnický, Mastihubová, Willett, & Côté, 2002; Schendel, 2019), can be efficiently supported by carboxylic-ester hydrolases, including feruloyl esterase (EC 3.1.1.73) and lipase (EC 3.1.1.3) activities. In fact, the shorter extraction time, high-yield production of phenolics and improved product quality are the main advantages of using enzymes to enrich phenolics from plant materials (Sowbhagya & Chitra, 2010).

Mucoromycota fungi (i.e., members of the former Zygomycota) are well known for being excellent producers of different exoenzymes, including those that are stable under harsh conditions (Papp et al., 2016). Lipases and β-glucosidases from *Rhizomucor miehei*, for instance, have been known to have high activity and stability at wide-ranges of temperature and pH conditions, and in organic solvent environments (Krisch, Bencsik, Papp, Vágvölgyi, & Takó, 2012; Takó et al., 2017). Cruz-Lachica et al. (2021) demonstrated that *Gilbertella persicaria* secretes a wide variety of hydrolytic enzymes during its growth on fruits. Results of Takó et al. (2015) revealed a high endoglucanase activity in the enzymatic cocktail of *G. persicaria* SZMC 11086 and *R. miehei* NRRL 5282 (SZMC 11005), and proved that *Mucor corticolus* SZMC 12031 is an excellent lipolytic enzyme producer. In addition, the *R. miehei* cellulase treatment was able to enrich the free phenolics yield in grape, apple and pitahaya samples (Zambrano et al., 2018).

Sorghum phenolic compounds and extracts with enhanced antioxidant activity can be used as food additives in different functional foods. The 3-deoxyanthocyanidins from sorghum was tested as a food preservative in meat products, and as a nutraceutical compound when encapsulated in microparticles (Links, Taylor, Kruger, & Taylor, 2015; Luckemeyer et al., 2015; Xiong et al., 2019). Various bakery products, e.g., bread, cookies, and expanded snacks, can be made using white or red sorghum grains without significant differences in texture and flavor compared to wheat products. Furthermore, sorghum grains are free from gluten, and the extrusion may facilitate the breakdown of high molecular weight grain tannins improving the nutraceutical value of the food. Products based on sorghum provide lower calorie intake and contain strong antioxidants, so they are beneficial for the human health. Pigmented sorghum bran is used for healthy and dark-colored bakery products, where the anthocyanins serve as natural food colorants (Awika, Dykes, Gu, Rooney, & Prior, 2003; Awika & Rooney, 2004; Salazar-López, González-Aguilar, Rouzaud-Sández, & Robles-Sánchez, 2018). Enzyme treatment operated by carbohydrase active cocktails from fungi may be a suitable method to produce free phenolics-enriched bioactive sorghum grain samples/extracts. Carbohydrases have been shown to support antioxidative phenolics mobilization in many cereals, e.g., rice, barley, millet (Liu et al., 2017a; Yadav, Singh, Bhattacharya, Yuvraj, & Banerjee, 2013; Zhu et al., 2016), and tannases and phytases were also tested against the tannin and phytate content, respectively, of sorghum samples (Schons, Battestin, & Macedo, 2012; Towo, Matuschek, & Svanberg, 2006). Carbohydrase treatment was applied to loosen the pericarp of the sorghum grain (Sruthi, Rao, Bennett, & Bhattarai, 2023), and these enzymes are important in saccharification of sorghum biomass for enhanced bioethanol production (Bakari et al., 2023). However, as we know, there was no investigation on carbohydrate active enzyme cocktails to improve the antioxidative phenolics content of sorghum grain samples to date.

In this study, an enzyme-assisted extraction approach was attempted to support the free antioxidative phenolics enrichment in grain residues of commercial broom sorghum, and GK Emese and Farmsugro 180 hybrid sorghum cultivars using cellulolytic and esterolytic cocktails from *G. persicaria*, *R. miehei* and *M. corticolus* fungi. Phenolic and flavonoid contents of grain residues were evaluated after the treatments; then, antioxidant properties, i.e., radical scavenging activity and reducing power of extracts obtained were also determined and compared. In addition, relationships between condensed tannin, phenolics content and antioxidant properties were analyzed, as well as the individual phenolic compounds before and after enzyme-assisted extraction were also monitored.

2. Materials and methods

2.1. Microorganisms and culture media

The *R. miehei* NRRL 5282 (SZMC 11005), *G. persicaria* SZMC 11086, and *M. corticolus* SZMC 12031 strains were obtained from the Szeged Microbiology Collection (SZMC, Szeged, Hungary). The microorganisms were subcultured regularly on malt extract agar and stored at 4 °C until use. To make a spore suspension, 72-h old cultures on plates were washed with 5 mL of sterilized distilled water. Then the spore number was enumerated in a Bürker-chamber under light microscope, and dilution with sterile distilled water was applied to set the required spore number. The malt extract agar was composed from 50 mL/L 20% (v/v) malt extract (VWR International, Radnor, PA, USA), 2.5 g/L yeast extract (VWR International, Radnor, PA, USA), 10 g/L glucose (Biolab, Budapest, Hungary) and 20 g/L agar-agar (Biolab, Budapest, Hungary).

2.2. Sorghum samples

Three types of *Sorghum bicolor* (L.) Moench grains were used for the experiments. Commercial broom sorghum was purchased in a local agricultural shop, Szeged, Hungary. The varieties GK Emese and Farmsugro 180 were obtained from the Cereal Research Non-Profit Ltd., Szeged, Hungary. GK Emese is a red sorghum variety, a hybrid domesticated to have a tannin content below 1%. Farmsugro 180 is a white variety and does not contain tannin. The protein content of the three sorghum samples was previously determined by our group, using the Kjeldahl method. The protein content of broom sorghum, GK Emese and Farmsugro 180 was 137.78 ± 3.58 g/kg, 99.02 ± 2.42 g/kg and 105.51 ± 3.53 g/kg, respectively.

2.3. Chemicals

Sodium acetate anhydrous, sodium nitrite and aluminum chloride hexahydrate were purchased from Reanal Laboratory Chemicals Ltd (Budapest, Hungary). Sodium dihydrogen phosphate anhydrous and disodium hydrogen phosphate dihydrate were purchased from Biolab (Budapest, Hungary). Dimethyl sulfoxide (DMSO), hydrochloric acid (37%) and sodium hydroxide were purchased from Molar Chemicals Ltd (Halásztelek, Hungary). Sodium carbonate anhydrous, methanol, ethanol (96%), iron(III) chloride hexahydrate, iron(II) sulfate, copper (II) chloride, ammonium acetate and acetic acid were purchased from VWR International (Radnor, PA, USA). Folin-Ciocalteu's phenol reagent, *p*-nitrophenol, *p*-nitrophenyl β-D-cellobioside, *p*-nitrophenyl β-D-

glucopyranoside, *p*-nitrophenyl palmitate, *p*-nitrophenyl acetate, gallic acid, vanillin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (+)-catechin hydrate, fluorescein disodium, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 2,9-dimethyl-1,10-phenanthroline (neocuproine), ethyl ferulate, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid and apigenin were purchased from Sigma-Aldrich (Munich, Germany).

2.4. Substrate preparation

The sorghum samples were handled separately during the experiments and were processed at the Department of Microbiology, University of Szeged. Grains were ground to coarse particles with an average diameter of 1–3 mm in a commercial mill and stored in glass flasks in dark and room temperature condition according to the recommendations of Oliveira et al. (2017) for storage parameters of sorghum samples. These samples were then used in the enzyme treatment experiments.

2.5. Preparation of the enzyme cocktails

The enzymatic cocktails were produced in a wheat bran-based solid-state fermentation (SSF) system. As the cultivation environment, a mass of 20 g of wheat bran and 20 mL of distilled water was prepared in 250-mL Erlenmeyer flasks. The flasks were then autoclaved at 121 °C for 25 min, and after cooling to room temperature, each flask was inoculated with 800 μL of 10^6 spore suspension. The inoculated flasks were incubated at 37 °C in case of *R. miehei*, and 28 °C in case of *G. persicaria* and *M. corticolus* for 6 days. At the end of the fermentation, the ferments were extracted with 120 mL of sodium acetate buffer (50 mmol/L, pH 6.0) and incubated at 4 °C for 12 h. After that, the extracts were filtered through gauze and centrifuged at 5040×*g* at 4 °C for 20 min. The supernatant was filtered through a Bio-Gel P-6 desalting cartridge (exclusion range 1–6 kDa; 50 mL; Bio-Rad, Hercules, CA, USA). The cartridge was previously equilibrated with 50 mmol/L of sodium acetate buffer (pH 6.0). Elution was performed with the same buffer at a flow rate of 9 mL/min, and the fraction containing cellulolytic and esterolytic enzyme activities were collected. The partially purified enzyme cocktail was sterilized by syringe filtration using 0.45 μm pore-size filter (Millex-HV, PVDF, Merck Millipore Ltd., Carrigtwohill, Ireland). The filtrates were stored at −20 °C until enzyme treatment experiments.

2.6. Cellobiohydrolase, β-glucosidase, lipase and esterase activity assays

The cellobiohydrolase and β-glucosidase activities of the enzyme cocktails were determined using 4.3 mmol/L *p*-nitrophenyl β-D-cellobioside and 6.6 mmol/L *p*-nitrophenyl β-D-glucopyranoside solutions prepared in sodium acetate buffer (50 mmol/L, pH 6.0), respectively. The reaction mixtures contained 180 μL of diluted enzyme cocktail and 20 μL of the appropriate substrate solution. Lipase and esterase activities of the enzyme cocktails were determined using *p*-nitrophenyl palmitate (3 mmol/L) and *p*-nitrophenyl acetate (5.5 mmol/L) substrate solutions, respectively, prepared freshly by DMSO. A volume of 1 mL of substrate solution was mixed with 1 mL of sodium phosphate buffer (100 mmol/L, pH 6.8). The reaction mixtures consisted of 50 μL of buffered substrate solution and 50 μL of enzyme cocktail. The mixtures were incubated at 50 °C (in case of *R. miehei*) or at 30 °C (in case of *G. persicaria* and *M. corticolus*) for 30 min. The reaction was stopped by adding either 50 μL (in case of the cellulase activities) or 25 μL (in case of the lipase activity) of 100 mmol/L sodium carbonate solution. Esterase activity was measured directly without addition of sodium carbonate. The *p*-nitrophenol release was measured at 405 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany). A standard curve was established using stock solution of 0.2 mmol/L *p*-nitrophenol in sodium acetate buffer (50 mmol/L, pH 6.0). One katal was defined as the amount of enzyme that releases 1 mol *p*-nitrophenol per sec under the conditions of the assay.

2.7. Determination of feruloyl esterase activity

Feruloyl esterase activity in the enzyme cocktails was assayed by using ethyl ferulate as the substrate. A 4.4 mmol/L stock solution from the ethyl ferulate was prepared in DMSO, and an equal volume of sodium phosphate buffer (100 mmol/L, pH 6.8) was added. A volume of 100 μL of buffered ethyl ferulate was added to 100 μL of enzyme cocktail, and the reaction mixtures were incubated for 24 h at 50 °C (in case of *R. miehei*) or 30 °C (in case of *G. persicaria* and *M. corticolus*). The reaction was stopped by boiling for 5 min; then, the samples were centrifuged at 17,000×*g* for 15 min and the ferulic acid release was monitored by HPLC (see 2.13). One katal was defined as the amount of enzyme that releases 1 mol ferulic acid per sec under the conditions of the assay.

2.8. Enzymatic treatment of grain samples

The enzyme cocktails produced in wheat bran-based SSF systems were used for the enzyme treatment experiments. A mass of 1 g of ground sorghum sample was mixed with 10 mL of purified enzyme cocktail in 50-mL Erlenmeyer flasks. The sorghum-enzyme extract mixtures were then incubated at 50 °C (in case of *R. miehei*) or 30 °C (in case of *G. persicaria* and *M. corticolus*) for 7 h under constant stirring at 130 rpm. Samples were taken at predefined intervals (0, 1, 3, 5 and 7 h), and the reaction was stopped in the samples by boiling for 3 min. Then, samples were centrifuged at 14,000×*g* for 10 min, and the supernatants were stored at −20 °C until further use. All enzymatic treatments were carried out in three independent experiments.

2.9. Determination of condensed tannin content

Extraction of condensed tannins of the three sorghum samples was performed by using the method of Sikwese & Duodu (2007). Condensed tannin content of the produced methanolic extracts was measured using the vanillin/hydrochloric acid method of Gaytán-Martínez et al. (2017). Catechin was used to establish a standard curve, and the results were expressed as mg catechin equivalent (CE)/g dry matter (DM).

2.10. Determination of total phenolics

The total phenolic content (TPC) of the extracts was determined using the Folin-Ciocalteu assay. The reaction mixture was prepared by mixing 30 μL of extract sample, 30 μL of ethanol (96%), 150 μL of distilled water and 15 μL of 50% Folin-Ciocalteu's phenol reagent. The mixture was incubated at room temperature for 5 min, then the reaction was initiated by adding 30 μL of sodium carbonate solution (5%). The mixtures were incubated in a dark place at room temperature for 60 min, then the absorbance was measured at 725 nm (SPECTROstar Nano, BMG Labtech, Offenburg, Germany). A standard curve was established using gallic acid solutions in the concentration range of 0–100 μg/mL. The TPC was expressed as mg of gallic acid equivalent (GAE)/100 g DM.

2.11. Determination of total flavonoids

The total flavonoid content (TFC) assay was carried out in 96-well microtiter plates, using the method reported by Herald, Gadgil, & Tilley (2012). The absorbance was measured at 510 nm via a SPECTROstar Nano (BMG Labtech, Offenburg, Germany) microplate reader. (+)-Catechin hydrate was used in the concentration range of 0–100 μg/mL to establish the standard curve. The TFC was expressed as mg of CE/100 g DM.

2.12. Antioxidant activity assays

2.12.1. Determination of DPPH scavenging capacity

The DPPH free radical scavenging activity of the extracts was determined according to the method of Varga, Jójárt, Fónad, Mihály, & Palágyi (2018) with slight modifications. The measurement was performed by mixing 10 μL of extract sample with 240 μL of 0.1 mmol/L DPPH solution prepared freshly in 80% (v/v) ethanol. These mixtures were incubated in a dark place at room temperature for 25 min, then the absorbance was determined at 517 nm (SPECTROstar Nano, BMG Labtech, Offenburg, Germany). The calibration curve was established using Trolox in a concentration range of 0–0.8 mmol/L. The free radical scavenging activity of extracts was expressed as μmol Trolox equivalent (TE)/100 g DM.

2.12.2. Determination of oxygen radical antioxidant capacity

The oxygen radical antioxidant capacity (ORAC) of the extracts was determined using the fluorogenic method of Huang, Ou, Hampsch-Woodill, Flanagan, & Prior (2002) with modifications according to the application note of BMG Labtech (2014). The ORAC values show the extent to which the sample is able to protect the fluorescein molecule against the oxidation by oxygen radicals generated during the reaction. For this measurement, the reaction mixtures were set up in a black 96-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany), and all solutions were prepared using sodium phosphate buffer (10 mmol/L, pH 7.4). A volume of 150 μL of fluorescein disodium solution (10 nmol/L) was mixed with 25 μL of extract, and the mixture was incubated at 37 °C for 30 min. Then, 25 μL of 240 mmol/L AAPH was pipetted into each well. After incubation in dark condition for 5 min, fluorescence measurements were performed (excitation: 485 nm, emission: 520 nm) using a FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany). Trolox in the concentration range of 0–40 μmol/L was used to establish the standard curve, and the results were expressed as μmol TE/100 g DM.

2.12.3. Determination of ferric reducing antioxidant power

The assay was performed using ferric reducing antioxidant power (FRAP) reagent contained 4 mL of 10 mmol/L TPTZ diluted in 40 mmol/L hydrochloric acid, 4 mL of 20 mmol/L iron(III) chloride solution, 20 mL of sodium acetate buffer (300 mmol/L, pH 3.6) and 2.2 mL of distilled water. The TPTZ solution was heated to 50 °C in a water bath before addition to FRAP reagent, and the prepared reagent was incubated at 37 °C until the measurement. For the reaction, a volume of 6 μL of extract was mixed with 200 μL of FRAP reagent. The reaction solution was then incubated at 37 °C for 30 min, and the absorbance was measured at 593 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany). The standard curve was established using 1 mmol/L iron(II) sulfate solution in the concentration range of 0.1–1.0 mmol/L. Results were expressed as μmol Fe(II)/100 g DM.

2.12.4. Determination of cupric ion reducing antioxidant capacity

The cupric ion reducing antioxidant capacity (CUPRAC) assay was performed following the method of Ribeiro, Magalhães, Reis, Lima, & Segundo (2011) with slight modifications. Briefly, a reaction mixture was prepared contained 50 μL of copper(II) chloride solution (10 mmol/L), 50 μL of neocuproine solution (7.5 mmol/L prepared in 96% ethanol) and 50 μL of ammonium acetate buffer (1 mol/L, pH 7.0). After an incubation at 37 °C for 15 min, a volume of 100 μL of extract was added to the mixture. The reaction solution was then incubated at 37 °C for 30 min, and the absorbance was measured at 450 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany). Trolox solution in the concentration range of 0–400 μmol/L was used to establish the standard curve. Results were expressed as μmol TE/100 g DM.

2.13. HPLC assay

HPLC analysis of the individual phenolic compounds was performed by the slightly modified method of Varga et al. (2018). A Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a membrane degasser, a binary pump, a standard autosampler, a thermostated column compartment and a variable wavelength detector was applied for the liquid chromatographic separation and quantification. Phenolic compounds were separated using a LUNA-PFP(2) (3 μm, 150 × 4.6 mm) column (Phenomenex, Torrance, CA, USA) thermostated at 40 °C. Mobile phase A consisted of water containing 0.1% (v/v) acetic acid, while methanol containing 0.1% (v/v) acetic acid served as mobile phase B. The linear gradient was performed as follows: 0.0 min, 5% B; 6.5 min, 25% B; 30.5 min, 37% B; 35.0 min, 55% B; 37.0 min, 95% B; 44.0 min, 95% B; 45.0 min, 5% B and 50.0 min, and 5% B for re-equilibration of the column. The flow rate was set to 0.7 mL/min and the injection volume was 10 μL. The phenolic compounds were monitored at 280 nm and 320 nm wavelengths.

For the measurement of feruloyl esterase activity, the separation of ethyl ferulate and ferulic acid was carried out using an isocratic elution with a flow rate of 0.7 mL/min, a column temperature of 25 °C, a mobile phase of water/methanol (45/55, v/v) containing 0.1% (v/v) acetic acid. The ultraviolet detection was set at 320 nm, while the injection volume was 10 μL.

Protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid and apigenin compounds were used to calibrate standard curves. Data were acquired using Trace Finder 4.0 software (Thermo Fisher Scientific, Waltham, MA, USA).

2.14. Statistical analysis

All measurements were performed in at least three independent experiments, and the data obtained were expressed as means ± standard deviation. Significance was calculated by multiple *t*-test with false discovery rate (FDR) ($Q = 10\%$), or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test in the GraphPad Prism 8.00 software (GraphPad Software Inc., San Diego, CA, USA). A P value less than 0.05 was considered as statistically significant. The relationship between TPC and antioxidant activity was investigated by applying the Pearson's correlation test calculated using GraphPad Prism 8.00 (GraphPad Software Inc., San Diego, CA, USA). The categories of very strong correlation ($0.999 \geq r \geq 0.800$), moderate correlation ($0.799 \geq r \geq 0.600$), fair correlation ($0.599 \geq r \geq 0.300$) and poor correlation ($0.299 \geq r \geq 0.100$) were defined to evaluate the correlation analysis (Akoglu, 2018).

3. Results and discussion

3.1. Enzyme cocktails used for treatments

For the preparation of cellulolytic and esterolytic cocktails to be used in enzyme-assisted extraction, the *R. miehei*, *M. corticolus*, and *G. persicaria* fungi were cultivated under SSF condition containing wheat bran as a growth and enzyme production support. The enzyme cocktail of *M. corticolus* showed the highest cellobiohydrolase and β-glucosidase activities (Table 1), which were about 1.5–5 times higher to those observed for *R. miehei* and *G. persicaria* cocktails ($P < 0.05$). A former study performed under SSF conditions also identified high cellobiohydrolase and β-glucosidase activities for the *M. corticolus* when the mixture of corn stalk and leaves and wheat bran was used as a growth substrate (Takó et al., 2015).

Among the fungal enzyme cocktails prepared, the *R. miehei* exhibited the highest esterolytic, i.e., lipase, esterase and feruloyl esterase activities (Table 1). Lipase activity of the *G. persicaria* enzyme cocktail was significantly lower ($P < 0.05$) than those presented by *R. miehei* and *M. corticolus*, that agrees with the previous experiment of Takó et al.

Table 1
Enzyme activities responsible for phenolics release in cocktails produced by Mucoromycota strains.

Fungal strains	Enzyme activity (nkat)				
	Cellobiohydrolase	β-Glucosidase	Lipase	Esterase	Feruloyl esterase
Rhizomucor miehei NRRL 5282	542.8 ± 27.1 a	5450.1 ± 22.1 a	11201.9 ± 160.3 a	4596.6 ± 115.6 a	2.2×10^{-4} ± 5.2×10^{-6} a
Gilbertella persicaria SZMC 11086	1106.7 ± 12.1 b	6576.9 ± 29.4 b	878.1 ± 27.3 b	1619.6 ± 60.8 b	6.7×10^{-5} ± 7.2×10^{-7} b
Mucor corticolus SZMC 12031	2946.7 ± 65.7 c	10431.6 ± 249.1 c	2911.4 ± 119.2 c	1028.6 ± 43.3 c	7.5×10^{-5} ± 6.7×10^{-7} b

Values are averages computed from three replicates ± standard deviation. Values within a column with different letters are significantly different according to the one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

(2015). The extent of esterase and feruloyl esterase activities were comparable in enzyme cocktails of *G. persicaria* and *M. corticolus* (Table 1). Esterase or feruloyl esterase activity has previously been identified for other *Rhizomucor* and *Mucor* fungi as well (Liu et al., 2013; Singh, Nigam, & Sachan, 2015). In a former study, remarkable endoglucanase, filter paper degrading and protease activities were measured in enzyme cocktails produced under wheat bran-based condition by the tested *R. miehei*, *M. corticolus* and *G. persicaria* fungi (Takó et al., 2015). Considering the cellulolytic and esterolytic activities achieved, the fungal enzyme cocktails prepared can be applicable for the hydrolysis of phenolic glycoside and phenolic ester conjugates.

3.2. Total phenolics and flavonoids in sorghum hydrolysates

TPC of the broom sorghum extract showed significant ($P < 0.05$) increase in all three enzymatic treatments (Fig. 1A). The *R. miehei* enzyme cocktail resulted in the highest TPC (256.9 ± 11.7 mg GAE/100 g DM), which was achieved at the 5th hour of incubation. For *G. persicaria* enzyme treatment, a 1.6-fold increase in the TPC was identified after a 7-h incubation, which was the highest degree of increase compared to the other treatments (Fig. 1A). All three enzyme treatments had also a positive effect on the TFC of broom sorghum extracts (Fig. 1A). In *R. miehei* enzyme treatment, the TFC values changed in parallel with the TPC ($r = 0.931$), indicating a considerable contribution of flavonoids to phenolic content. The *R. miehei* enzymatic cocktail resulted in the highest increase in the free flavonoid yield (1.93-fold) after 1 h of incubation. The obtained 159.4 ± 6.8 mg CE/100 g DM flavonoid yield is comparable to those obtained by Ofosu et al. (2021), where the TFC ranged from 114.0 to 138.5 mg CE/100 g of sorghum genotype varieties.

Concerning the GK Emese sample, a statistically significant ($P < 0.05$) increase (1.17-fold) in the TPC was observed after a 1-h incubation when the *R. miehei* enzyme extract was applied as a treatment solution (Fig. 1B). Then, a decrease was identified in the further stage of incubation (Fig. 1B). The highest TPC was achieved by using the *G. persicaria* enzyme extract after a 7 h incubation. A similar incubation time was effective for the *G. persicaria* extract to produce maximal TPC yield on broom sorghum sample as well (see Fig. 1A). The enzyme cocktail of *M. corticolus* did not significantly enhance the TPC of the GK Emese sample. In *R. miehei* extract treated samples, the TFC changed in accordance with the TPC results ($r = 0.943$), and the highest values (89.0 ± 6.4 mg GAE/100 g DM for TPC and 41.9 ± 2.4 mg CE/100 g DM for TFC) were measured at the beginning of extraction and after that a decrease was observed (Fig. 1B). A 1.42-fold increase in TFC was the highest that was achieved by *R. miehei* cocktail after a 3-h treatment. Results showed no significant changes in TFC when the GK Emese treatment was operated by *G. persicaria* enzyme cocktail. However, a significant decrease ($P < 0.05$) in TFC was identified in case of the *M. corticolus* enzyme treatment at the 7th incubation hour compared to the initial stage of reaction (Fig. 1B). Similarly, a decrease in flavonoid content was also reported for a *Lentinula edodes* carbohydrase treatment in rooibos samples after 6 h incubation (Pengilly, Joubert, van Zyl, Botha, & Bloom, 2008).

Both the *R. miehei* and the *M. corticolus* enzyme treatments could significantly increase the TPC for Farmsugro 180 sorghum ($P < 0.05$) (Fig. 1C). The highest, 1.32-fold increase was achieved with the *M. corticolus* cocktail after 5 h incubation. In *R. miehei* enzyme treated Farmsugro 180 sample, similar to as observed for broom sorghum, the TFC varied in relation to TPC data ($r = 0.975$) (Fig. 1C). The cocktails of *R. miehei* and *M. corticolus* were able to achieve significant ($P < 0.05$) TFC increases, with maximal yields of 37.6 ± 1.2 and 30.8 ± 1.3 mg CE/100 g DM, respectively (Fig. 1C). The 3-h treatment with the *R. miehei* enzyme cocktail resulted in the highest, i.e., 1.72-fold, increase in TFC.

The different sorghum cultivars may have different phenolic content (Bouargalne et al., 2022). For instance, TPC of African sorghums ranged between 8.1 and 29.6 mg GAE/g DM (Awika, Yang, Browning, & Faraj, 2009), which is higher than the overall data obtained for broom, GK Emese and Farmsugro 180 sorghum samples used. Some red and white sorghum varieties from Australia were characterized with a lower phenolic content of 0.24–0.88 mg GAE/g DM (Rao et al., 2018) to the sorghum grain residues investigated in this study.

Enzymatic treatment with carbohydrase active cocktails has been an effective approach for improving phenolic content in other applications, including cereal and fruit residue-based reaction systems. For instance, the cellulase and xylanase combined treatment had a positive effect on the free phenolic and flavonoid content of rice bran samples in the work

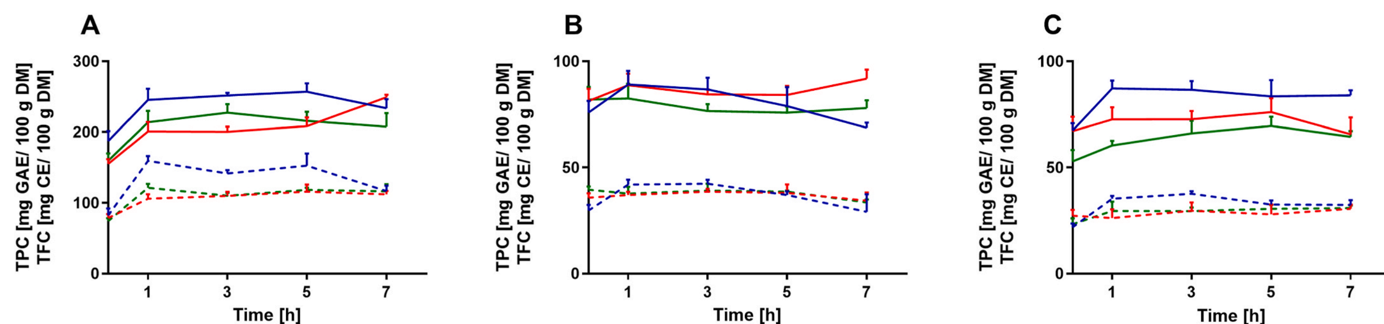

Fig. 1. Total phenolic content (TPC) and total flavonoid content (TFC) of broom sorghum (A), GK Emese (B) and Farmsugro 180 (C) sorghum extracts after direct enzyme treatment with enzymatic cocktail of *R. miehei* (blue line), *G. persicaria* (red line) and *M. corticolus* (green line). The continuous line represents the results of TPC measurements, while the TFC data have been indicated with dashed line. Results are presented as averages from three replicates; error bars represent standard deviation.

of Prabhu & Jayadeep (2015). A complex enzyme treatment, including glucoamylase, protease and cellulase activities, significantly increased the TPC and TFC yield also in fermented rice bran residues (Liu et al., 2017b). Wheat bran is also an effective substrate for phenolics yield developments. In the work of Arte et al. (2015), for instance, a combination of microbial fermentation and complex enzyme treatment with Depol 761 P and Viscoferm carbohydrases was able to improve the concentration of total phenols. In apple, pitahaya and grape residue samples, the cellulolytic extract of *R. miehei* could also be effective to improve the TPC after a 5-h treatment (Zambrano et al., 2018). Among the extracts tested here, treatment with *R. miehei* enzyme cocktail proved to be the most effective for TPC also in sorghum (Fig. 1) to which its high esterolytic activity (Table 1) may also have contributed. Carbohydrase cocktails of the zygomycete fungus *Rhizopus oryzae* obtained in liquid peptone-dextrose and wheat straw fermentations caused 5% and 9%, respectively, increase in the TPC of rooibos samples after 6 h incubation (Pengilly et al., 2008). The yield improvement was even greater for some flavonoids (Pengilly et al., 2008). Increase in soluble flavonoid content was observed by Chen, Xing, Huang, & Xu (2011) when *Ginkgo biloba* leaves were treated with *Penicillium decumbens* cellulase enzyme. Overall, there are a wide variety of factors, i.e., type of extraction solvent, duration and temperature of the extraction process, and DM/solvent ratio, that can influence the phenolics yield during enzyme treatments (Sharma et al., 2022).

3.3. Antioxidant ability before and after treatments

3.3.1. Evolution of reducing power

Reducing capacity of sorghum extracts was evaluated with the FRAP and CUPRAC methods. In broom sorghum samples, the highest FRAP, i.e., 2705.2 ± 40.1 μmol Fe(II)/100 g DM, was measured for the *G. persicaria* enzyme treatment after a 7-h incubation (Fig. 2A). This latter treatment showed a strong positive association between FRAP and TPC data (r = 0.846) during the reaction. The *M. corticolus* enzyme treatment resulted in the highest increase in FRAP as compared to the initial value (1.64-fold), and presented a maximal FRAP value of 2327.7 ± 228.6 μmol Fe(II)/100 g DM at the 7th day of incubation (Fig. 2A). CUPRAC improved significantly ($P < 0.05$) in all fungal enzyme treatments when broom sorghum was applied as a support (Fig. 2A). The highest degree of increase in CUPRAC was 1.47-fold obtained after 5 h incubation with the *R. miehei* enzyme cocktail (Fig. 2A), that was in a strong positive relation with TPC (r = 0.898) and TFC (r = 0.967) determined in the corresponding sample. Concerning broom sorghum, the highest CUPRAC data was 3884.6 ± 244.9 μmol TE/100 g DM, which was measured in the extract treated with *R. miehei* enzyme cocktail (Fig. 2A).

In GK Emese extracts, a significant increase was found in the FRAP values ($P < 0.05$) after treatments with both *R. miehei* and *M. corticolus* enzyme cocktails (Fig. 2B). The highest increase (1.48-fold) in FRAP was achieved with the cocktail of *R. miehei* after 5 h of incubation. This extract produced the highest FRAP, namely 1141.3 ± 63.3 μmol Fe(II)/100 g DM data, in GK Emese substance. A decrease in CUPRAC activity was observed for *R. miehei* and *M. corticolus* enzyme treatments, while there was an increased tendency in case of the *G. persicaria* enzyme treatment (Fig. 2B). The nature of individual phenolics found in samples determines the tendency of CUPRAC activity during the reaction (Apak, Güçlü, Özyürek, Bektaşoğlu, & Bener, 2008). There are certain phenolics, e.g., catechin and caffeic acid, to which the CUPRAC method responds faster and more sensitive than FRAP (Apak et al., 2008). The high-yield presence of such phenolic compounds can cause a difference between reducing power ability determined by the two approaches. The decrease in CUPRAC was significant ($P < 0.05$) for *R. miehei* enzyme treatment compared to initial sample, and this system exhibited the lowest value at the 7th incubation hour among the treatments. In line with this, important to note that both TPC and TFC showed a decreased tendency at the end of incubation in *R. miehei* enzyme treatment (see Fig. 1B). Treatment with *M. corticolus* enzyme cocktail did not affect the CUPRAC of GK Emese extract significantly ($P > 0.05$). In contrast, CUPRAC of GK Emese extract produced with enzymatic cocktail of *G. persicaria* increased significantly ($P < 0.05$) from the initial sampling up to the end of incubation (Fig. 2B). Here, a strong positive relationship (r = 0.912) between the TPC and CUPRAC was observed indicating the considerable role of CUPRAC sensitive phenolic compounds in the free phenolic yield obtained after the treatment.

In Farmsugro 180 sorghum, all three enzyme treatments resulted in an increase in FRAP (Fig. 2C). The overall FRAP of *R. miehei* enzyme treated sample improved most significantly ($P < 0.05$), presenting about a 1.9-time increment after 7 h incubation. This increase in FRAP was in moderate positive relation (r = 0.709) to TPC measured. The sample obtained after 3-h treatment with the *M. corticolus* enzyme showed the highest FRAP presenting a reducing power of 1232.2 ± 114.3 μmol Fe (II)/100 g DM. For CUPRAC analysis of Farmsugro 180 sample, a high increase in activity was observed up to the 1 h of incubation in all enzyme treatments, after that it was not changed considerably (Fig. 2C). Highest CUPRAC was measured after the *M. corticolus* enzyme treatment (1673 ± 257.01 μmol TE/100 g DM), while the highest degree of increase was achieved with the *R. miehei* cocktail. The obtained 1.48-fold CUPRAC increase, that was reached at the 3rd hour of incubation, was significant ($P < 0.05$) compared to the activity of initial sample (Fig. 2C). For *R. miehei* enzyme treatment, a strong positive correlation (r = 0.965) was found between the TPC and CUPRAC in Farmsugro 180 sample.

Literature has shown that the enzymatic treatment could improve the reducing power in cereal residues other than sorghum as well. In the works of Liu et al. (2017a, 2017b), for instance, the cellulase, protease, glucoamylase complex enzyme treatment significantly increased the

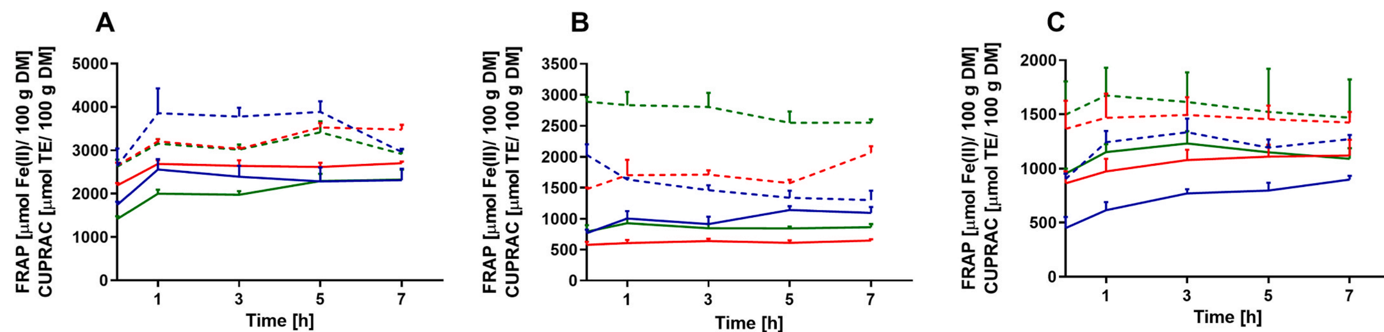

Fig. 2. Ferric reducing antioxidant power (FRAP) and cupric ion reducing antioxidant capacity (CUPRAC) of broom sorghum (A), GK Emese (B) and Farmsugro 180 (C) sorghum extracts after direct enzyme treatment with enzymatic cocktail of *R. miehei* (blue line), *G. persicaria* (red line) and *M. corticolus* (green line). The continuous line represents the results of FRAP measurements, while the dashed line represents results of CUPRAC measurements. Results are presented as averages from three replicates; error bars represent standard deviation.

FRAP values of fresh and lactic acid fermented rice bran residues compared to untreated samples. In other study, an increase in reducing power after tannase treatment was detected in rye flour samples (de Lima et al., 2018). The reducing power of sorghum samples has been improved by non-enzymatic extraction techniques as well. Roasting was effective for red sorghum samples (Irondi et al., 2019), while the solvent treatment of white sorghum resulted in higher reducing power to those described for colored sorghum varieties (Xiong et al., 2021).

3.3.2. Effect against free radicals

Free radical scavenging capacity of the sorghum extracts produced was evaluated using the DPPH radical scavenging activity and ORAC tests. After 1 h of incubation, the enzyme treatments affected positively both the DPPH and oxygen radical scavenging activities of broom sorghum samples (Fig. 3A). Although the increase in DPPH inhibition was not significant in case of the *R. miehei* and *G. persicaria* enzyme treatments, addition of the *M. corticolus* enzyme cocktail resulted in a considerable improvement ($P < 0.05$) up to the 5th incubation hour (Fig. 3A). *M. corticolus* enzyme treatment was the most effective, since it was able to increase the DPPH scavenging activity by a maximum of 2.28-fold compared to the initial sample. In addition, the correlation between TPC and DPPH inhibitory data was strongly positive ($r = 0.946$) for *M. corticolus* enzyme treatment. In broom sorghum sample, the highest inhibitory effect against DPPH was 799.2 ± 28.1 μmol TE/100 g DM identified in the *G. persicaria* enzyme treated system at the 3rd sampling hour (Fig. 3A). The highest ORAC data was measured at the first incubation hour of the treatment catalyzed by the *R. miehei* enzyme cocktail. The resulted 52.1 ± 1.5 μmol TE/100 g DM ORAC was about 1.3-fold higher to those detected in the initial sample ($P < 0.05$) (Fig. 3A). Up to the first hour of incubation, a marked increase ($P < 0.05$) in ORAC was identified during the *M. corticolus* enzyme treatment as well (Fig. 3A). However, incubation longer than 1 h did not result in a significant difference in ORAC activity (Fig. 3A).

In GK Emese sorghum, DPPH scavenging activity of *R. miehei* enzyme treated sample has improved for 1 h incubation (Fig. 3B). The measured 361.2 ± 41.9 μmol TE/100 g DM DPPH inhibitory effect was the highest among all enzyme treatments of GK Emese sample. DPPH inhibition of *G. persicaria* and *M. corticolus* enzyme treated GK Emese was slightly decreased during the first 3 h of incubation, but there was an increase at the 7th and 5th hours for the former and the latter treatments, respectively (Fig. 3B). There were fair positive correlations between TPC and DPPH inhibition in *R. miehei* ($r = 0.467$) and *M. corticolus* ($r = 0.535$) enzyme treated GK Emese, while a poor negative association ($r = -0.258$) was found by these variables for the *G. persicaria* enzyme treatment. In ORAC tests, none of the enzyme treatments of GK Emese sample was able to enhance the ability of the extracts to protect fluorescein (Fig. 3B). Moreover, a significant ($P < 0.05$) decrease of ORAC can be observed in case of the treatment with *R. miehei* cocktail (Fig. 3B).

Similarly, Connolly et al. (2019) described a decrease in ORAC values of brewer's spent grain samples after simulated gastrointestinal digestion treatment. In addition, there was no increase in ORAC of rice bran samples after liquefaction treatment in the work of Liu et al. (2017a). After an initial increment and a maximal antioxidant activity peak at the 2nd h of incubation, a decrease can be observed during the enzyme assisted treatment of algae samples as well (Sánchez-Camargo et al., 2016). In contrast, increased ORAC was documented by Ti, Zhang, Li, Wei, & Zhang (2015) for *in vitro* digested polished rice substrate compared to raw and cooked rice samples.

The overall DPPH radical scavenging capacity of Farmsugro 180 extracts was lower than that measured on the other two sorghum substances both before and after the enzyme treatments. However, all three enzyme treatments affected positively the DPPH radical scavenging activity of samples during the incubation ($P < 0.05$) (Fig. 3C). Maximal DPPH inhibition was achieved at different stages of incubation for each treatment. Namely, the *R. miehei* enzyme treatment resulted in maximal DPPH inhibition at the first hour of incubation presenting a 2.3-fold increase compared to the initial sample. The *G. persicaria* and *M. corticolus* enzyme catalyzed treatments reached a maximum DPPH inhibition with 2.1-fold and 3.2-fold increments at the 3rd and 5th sampling hours, respectively (Fig. 3C). In Farmsugro 180 sorghum, the relationship between TPC and DPPH scavenging activity was strong ($r = 0.951$) during *M. corticolus* enzyme treatment, while it was moderate and fair when *R. miehei* ($r = 0.655$) and *G. persicaria* ($r = 0.431$) enzyme assisted extractions, respectively, were applied. The initial ORAC of Farmsugro 180 sample did not change considerably during the incubations (Fig. 3C). A slight increase can be observed only for the *M. corticolus* enzyme treatment (Fig. 3C), that was in a positive correlation ($r = 0.779$) with the TPC of the corresponding extract. Overall, the Farmsugro 180 had low phenolic content (Fig. 1C) and moderate antioxidant activity (Fig. 2C and 3C) compared to the other sorghum substances studied. The white sorghum, however, may have many positive effects on the human health. For instance, the KARI-Mtama white sorghum induced the NAD(P)H:quinone oxidoreductase enzyme more efficiently compared to the red sorghum samples, despite the fact that it showed a lower antioxidant activity (Awika et al., 2009). In the study of Pontieri et al. (2016), antioxidant activity of food-grade white sorghum hybrids, however, was comparable to the values found in honey.

In most cereals, the DPPH scavenging activity is varied between 1200 and 3500 μmol TE/100 g DM (Fardet et al., 2008). Concerning sorghum extracts, the ability to inhibit DPPH after a solid-state fungal fermentation ranged between 126.67 and 133.67 mg TE/100 g DM (507 and 535 μmol TE/100 g DM) in the study of Espitia-Hernández et al. (2022).

3.3.3. Relationship between phenolic content and antioxidant activity

Association between total phenolic and flavonoid content and

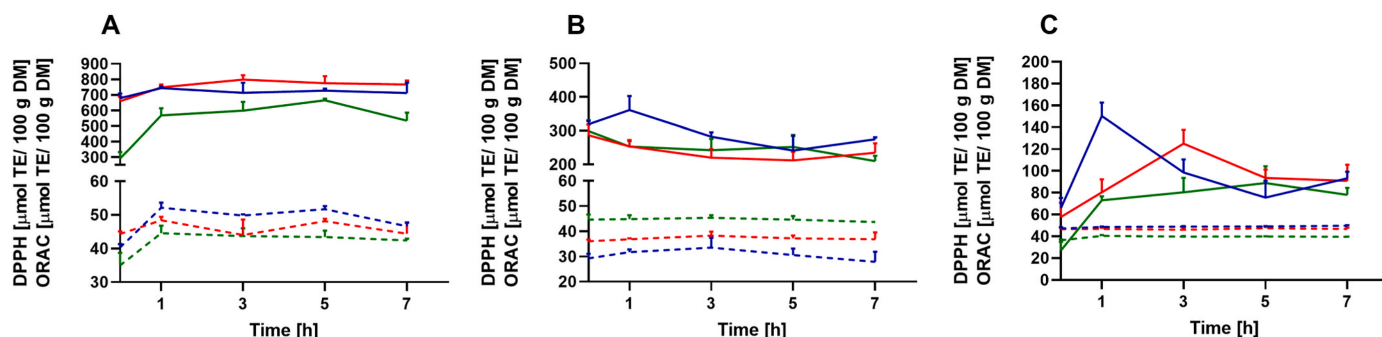

Fig. 3. DPPH radical scavenging capacity (DPPH) and oxygen radical absorbance capacity (ORAC) of broom sorghum (A), GK Emese (B) and Farmsugro 180 (C) sorghum extracts after direct enzyme treatment with enzymatic cocktail of *R. miehei* (blue line), *G. persicaria* (red line) and *M. corticolus* (green line). The continuous line represents the results of DPPH assay, while the dashed line represents results of ORAC measurements. Results are presented as averages from three replicates; error bars represent standard deviation.

antioxidant potential, i.e., FRAP, CUPRAC, DPPH and ORAC, was investigated. A large improvement in the bioactive properties of the sorghum substrates was mostly documented in the initial stage of incubation (see Figs. 1–3), therefore, data obtained after 1 h treatment was selected for the correlation analysis. After data analysis, a very strong positive correlation ($r = 0.989$) with a very high statistical significance ($P < 0.0001$) was found between the TPC and TFC of sorghum extracts (Table 2). Flavonoids have been shown to be as the predominant fraction of free phenolics in other sorghum substances as well (Ofosu et al., 2021). Antioxidant properties of extracts, except ORAC, associated strongly with the TPC and TFC showing a substantial role of phenolic compounds released in the improvement of antioxidative capacity of samples. Similarly, high positive correlations between phenolic content and antioxidant activity have been reported in recent studies for sorghum grain residues (Espitia-Hernández et al., 2022; Ghimire, Seo, Yu, Kim, & Chung, 2021; Seo et al., 2023). In fact, the polyphenols, as major antioxidants, may directly contribute to the antioxidant activity of cereal samples (Van Hung, 2016). Other plant substances investigated recently, such as sweet chestnut (Martínez, Fuentes, & Carballo, 2022), *Commiphora mollis* resin (Molole, Gure, & Abdissa, 2022), and tea (Dobrinas, Soceanu, Popescu, Carazeanu Popovici, & Jitariu, 2021) also showed positive associations between the TPC and antioxidant capacity.

The relationship between FRAP, CUPRAC, DPPH and ORAC antioxidant assays has also been studied. Very strong correlations with high significance were found when the FRAP data were subjected to analysis against CUPRAC ($P < 0.01$) and DPPH ($P < 0.001$) (Table 2). Similarly, the association was strong between CUPRAC and DPPH ($P < 0.01$) (Table 2). Both the FRAP, CUPRAC and DPPH found to be correlating with ORAC, however, it was just fair ($0.599 \geq r \geq 0.300$) in these relations (Table 2). In agreement with these results, positive correlations were identified between the different methods used for antioxidant measurements also in other sorghum grain samples (Seo et al., 2023).

3.3.4. Antioxidant properties in relation to condensed tannin content

Tannins are among the most important bioactive phenolic compounds in sorghum grains (Luthria & Liu, 2013). Majority of tannins in sorghum are condensed tannins. Although these compounds have been considered antinutritional factors, tannins are important antioxidants and may be active against the formation of some chronic diseases (Awika & Rooney, 2004; Dykes & Rooney, 2007; Gaytán-Martínez et al., 2017; Wang, Han, Li, & Zheng, 2020). In this assay, the broom, GK Emese and Farmsugro 180 sorghum samples used in enzyme treatment tests were subjected to condensed tannin content assay following the vanillin/hydrochloric acid colorimetric approach.

Condensed tannin content of colored sorghum types is generally higher to those found in white sorghum (Alfieri, Balconi, Cabassi, Habyarimana, & Redaelli, 2017; Semere, Tsehaye, Tareke, Westengen, & Fjellheim, 2023; Xiong et al., 2019). In line with this, no condensed tannin was detected by the method used for the analysis in Farmsugro 180 white type sorghum. In contrast, the colored broom and GK Emese substances have condensed tannins of 19.44 mg CE/g DM and 16.35 mg CE/g DM, respectively. These values are comparable with the concentrations of 3.68–28.36 mg CE/g DM (Alfieri et al., 2017), 20.54 mg CE/g DM (Gaytán-Martínez et al., 2017), 2.5–72.0 mg CE/g DM (Palacios, Nagai, Torres, Rodrigues, & Salatino, 2021) and 7.0–23.3 mg CE/g DM (Awika et al., 2009) described for condensed tannins in colored sorghum types using vanillin/acid solution method.

The higher the condensed tannins, the higher the phenolic content and antioxidant activity in sorghum samples (Awika et al., 2009; Dykes & Rooney, 2007; Xiong et al., 2021). In the work of Espitia-Hernández et al. (2022), for instance, the condensed tannin content correlated positively with the antioxidant potential of samples after fungal fermentation. Here, very strong correlation was found with DPPH scavenging activity ($r = 0.837$) and CUPRAC ($r = 0.828$), while it was moderately positive with TPC ($r = 0.684$) and TFC ($r = 0.682$). In addition, a fair positive association was identified in FRAP ($r = 0.593$).

3.4. Individual phenolics profile

The phenolic compounds in enzyme treated sorghum samples were determined by HPLC, and the obtained yields were compared to control data with no incubation. Concentration of most individual phenolics tested increased during the enzyme treatments, and the best yields were generally registered at the 7th hour of incubation (see Tables 3–5). For comparison, an increase in phenolic content was detected in the Folin test as well showing maximal values in the later stages, i.e., in the 5th or 7th hour of the treatments (Fig. 1). The Folin's phenol reagent used in TPC assay, however, can also react with nonphenolic substances such as amino acids, sugars, proteins and fatty acids found in extracts (Everette et al., 2010), which may overestimate the TPC yield obtained in each incubation hour.

In broom sorghum, the highest level of individual phenolics was mostly identified at the *R. miehei* enzyme treatment during the incubation (Table 3). This result agrees with those of TPC measurements, in which the *R. miehei* treatment also demonstrated the highest overall phenolic content and increases (Fig. 1A). Protocatechuic acid and vanillic acid were found in the largest concentration, but both 4-hydroxybenzoic acid and caffeic acid were also dominant in broom sorghum (Table 3). Compared to the control sample, the quantities of these compounds had increased about 1.8–5.4 times during *R. miehei* enzyme treatment ($P < 0.05$). Concerning caffeic acid, its marked presence in the *R. miehei* enzyme treated sample could be the one of the reasons of the high reducing power of the extract against cupric ion (Fig. 2A) (Apak et al., 2008). The apigenin content of the broom sorghum is also worth to note which, moreover, exhibited an increasing tendency during the enzymatic treatment (Table 3). For *G. persicaria* and *M. corticolus* enzyme treatments, the maximum individual phenolic yields were in a similar range showing about 1.3–3.3 times increments compared to initial control sample. In broom sorghum, the content of 4-hydroxybenzoic, *p*-coumaric and ferulic acids was highest at the earlier stages of the treatments (Table 3). The same trend was reported in the work of Faulds, Mandalari, LoCurto, Bisignano, & Waldron (2004), where majority of the *p*-coumaric and ferulic acids was released within 3 h incubation from the wheat bran samples treated with feruloyl esterase and xylanase enzymes. After maximal concentration, the amount of ferulic acid has decreased in the broom sorghum sample as the treatments progressed. The cellulase treatment of rice residues also caused a decrease in the amount of ferulic acid, but there has been an increasing tendency identified in the concentration of vanillic acid (Wanyo, Meeso, & Siriamornpun, 2014). A probable reason for this phenomenon can be the conversion of ferulic acid into vanillic and protocatechuic acids during the incubation (Wanyo et al., 2014). In fact, the yield of these latter two compounds increased significantly ($P < 0.05$) in the broom sorghum

Table 2
Correlation coefficients (Pearson r) between the total phenolic content (TPC), total flavonoid content (TFC), ferric reducing antioxidant power (FRAP), cupric ion reducing antioxidant capacity (CUPRAC), free radical scavenging activity (DPPH) and oxygen radical antioxidant capacity (ORAC) of sorghum extracts after a 1-h enzyme treatment.

Variables	TPC	TFC	FRAP	CUPRAC	DPPH	ORAC
TPC	1	0.989***	0.912**	0.879*	0.942***	0.528
TFC		1	0.905**	0.892*	0.921**	0.522
FRAP			1	0.862*	0.904**	0.521
CUPRAC				1	0.869*	0.513
DPPH					1	0.355
ORAC						1

Asterisks indicate significant associations between the variables according to a Pearson's correlation analysis performed by GraphPad Prism version 8.00, *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$. Categories: $0.999 \geq r \geq 0.800$, very strong correlation; $0.799 \geq r \geq 0.600$, moderate correlation; $0.599 \geq r \geq 0.300$, fair correlation; $0.299 \geq r \geq 0.100$, poor correlation (Akoglu, 2018).

Table 3
Comparative evaluation of best phenolic compound yields (μg/g DM) extracted from broom sorghum with *R. miehei*, *G. persicaria* and *M. corticolus* enzyme cocktails.

Compounds	Type of enzyme treatment								
	R. miehei			*G. persicaria*			*M. corticolus*		
	Control [a]	Maximum	*t*(incubation)/hour	Control	Maximum	*t*(incubation)/hour	Control	Maximum	*t*(incubation)/hour
Protocatechuic acid	37.35 ± 3.04	113.80 ± 17.82*	7	33.3 ± 2.69	71.50 ± 3.61**	7	34.40 ± 0.14	75.75 ± 0.35***	7
4-Hydroxybenzoic acid	55.75 ± 1.63	83.80 ± 5.11*	1	56.85 ± 8.84	73.70 ± 0.57	1	37.15 ± 3.18	68.95 ± 0.35**	3
Vanillic acid	75.15 ± 6.58	132.85 ± 14.21*	7	73.70 ± 3.65	97.95 ± 2.47*	7	73.40 ± 0.42	100.40 ± 1.98**	7
Caffeic acid	19.25 ± 0.03	104.10 ± 8.49**	7	19.65 ± 2.05	60.10 ± 2.83**	7	18.20 ± 0.28	60.50 ± 1.98**	7
p-Coumaric acid	41.50 ± 0.71	54.00 ± 1.25**	1	34.60 ± 0.42	44.55 ± 0.49**	3	31.65 ± 1.34	49.60 ± 6.51	3
Ferulic acid	33.50 ± 4.95	33.50 ± 4.95	0	22.15 ± 1.63	39.45 ± 0.21**	3	14.85 ± 5.44	33.40 ± 5.37	3
Apigenin	11.40 ± 0.71	48.40 ± 5.66*	5	n. d. [b]	26.50 ± 1.41**	7	13.00 ± 2.40	29.45 ± 0.35*	7

Data presented are averages of concentration values determined in three replicates ± standard deviation. Asterisks indicate significant differences between the respective control and enzyme treated samples according to a multiple *t*-test performed by GraphPad Prism version 8.00, FDR (Q = 10%), *P < 0.05, **P < 0.01, ***P < 0.0001.
[a] Concentration of the phenolic compound measured in the presence of the respective enzyme extract without incubation (0th incubation hour).
[b] Not detected.

Table 4
Comparative evaluation of best phenolic compound yields (μg/g DM) extracted from GK Emese sorghum with *R. miehei*, *G. persicaria* and *M. corticolus* enzyme cocktails.

Compounds	Type of enzyme treatment								
	R. miehei			*G. persicaria*			*M. corticolus*		
	Control [a]	Maximum	*t*(incubation)/hour	Control	Maximum	*t*(incubation)/hour	Control	Maximum	*t*(incubation)/hour
Protocatechuic acid	46.43 ± 4.27	98.13 ± 26.99	3	56.09 ± 1.28	98.15 ± 4.28**	7	44.38 ± 5.01	82.41 ± 1.93**	1
4-Hydroxybenzoic acid	34.70 ± 0.64	125.86 ± 18.52*	7	44.07 ± 1.14	94.15 ± 3.98**	7	42.75 ± 8.02	87.84 ± 2.93*	5
Vanillic acid	22.91 ± 5.44	68.59 ± 18.93	3	9.01 ± 0.80	41.85 ± 1.25**	7	14.38 ± 1.36	28.41 ± 1.86*	1
Caffeic acid	n. d. [b]	23.44 ± 1.49**	7	14.51 ± 0.47	19.86 ± 1.06*	7	n. d.	9.42 ± 1.11**	3
p-Coumaric acid	n. d.	9.45 ± 0.74**	7	2.74 ± 0.27	7.60 ± 0.35**	7	2.51 ± 0.57	6.84 ± 0.96*	5
Ferulic acid	n. d.	11.12 ± 2.18*	7	4.27 ± 1.29	14.57 ± 1.14*	7	2.67 ± 1.17	7.53 ± 0.51*	1
Apigenin	n. d.	n. d.	–	n. d.	n. d.	–	n. d.	n. d.	–

Data presented are averages of concentration values determined in three replicates ± standard deviation. Asterisks indicate significant differences between the respective control and enzyme treated samples according to a multiple *t*-test performed by GraphPad Prism version 8.00, FDR (Q = 10%), *P < 0.05, **P < 0.01, ***P < 0.0001.
[a] Concentration of the phenolic compound measured in the presence of the respective enzyme extract without incubation (0th incubation hour).
[b] Not detected.

samples (Table 3).

Although GK Emese is also a red sorghum variety, its phenolic content was quite different compared to broom sorghum. After enzyme treatments, for example, level of vanillic, caffeic, *p*-coumaric and ferulic acids was lower, but the protocatechuic acid content was comparable to broom sorghum (Table 4). Anyway, all enzyme treatments applied were able to improve the bioavailability of these compounds on GK Emese substance. Among phenolics, vanillic acid content of the *G. persicaria* enzyme treated extract exhibited the largest increase, i.e., 4.6 times (P < 0.01), during incubation. Caffeic acid content of GK Emese after the *G. persicaria* enzyme treatment was also considerable (Table 4), which may be responsible to the more sensitivity of this sample towards CUPRAC assay than FRAP (Fig. 2B) (Apak et al., 2008). In addition, there was a notable, i.e., about 2.1–3.6 times (P < 0.05), increase also in the 4-hydroxybenzoic acid yield because of the enzyme extractions (Table 4). It is worth to note, however, that no apigenin was detected in GK Emese irrespectively of the enzyme treatments. Except for ferulic acid, the highest individual phenolic yields were observed in the *R. miehei* enzyme cocktail surrounding. Concentration of most phenolics reached their maximum at the 7th h of incubation for *R. miehei* and *G. persicaria* enzyme treatments, while largest yields were identified at earlier stages of incubation in the case of *M. corticolus* cocktail (Table 4). A decrease in the phenolic content was then detected during *M. corticolus* treatment. This may be attributed to the action of certain enzymes, e.g., phenolic acid decarboxylases or phenolic acid reductases (Svensson, Sekwati-Monang, Lutz, Schieber, & Gänzle, 2010) that can cause the polymerization or degradation of plant phenolics. However, such enzyme activities have not yet investigated in the partially purified enzyme cocktails used for the treatments.

Concentration of many individual phenolics was lower in Farmsugro 180 residue to that detected for broom sorghum (Tables 3 and 5). But the resulted compound yields were comparable to those reached in GK Emese, where only the hydroxybenzoates (protocatechuic acid, 4-hydroxybenzoic acid and vanillic acid) demonstrated a higher content

Table 5
Comparative evaluation of best phenolic compound yields (μg/g DM) extracted from Farmsugro 180 sorghum with *R. miehei*, *G. persicaria* and *M. corticolus* enzyme cocktails.

Compounds	Type of enzyme treatment								
	R. miehei			*G. persicaria*			*M. corticolus*		
	Control [a]	Maximum	*t*(incubation)/hour	Control	Maximum	*t*(incubation)/hour	Control	Maximum	*t*(incubation)/hour
Protocatechuic acid	8.50 ± 0.14	16.55 ± 0.78**	7	6.85 ± 0.21	10.95 ± 0.21**	7	8.70 ± 0.85	13.05 ± 0.07*	7
4-Hydroxybenzoic acid	43.95 ± 0.92	101.45 ± 2.19**	7	33.45 ± 0.92	63.65 ± 2.33**	7	42.25 ± 3.04	74.65 ± 5.44*	7
Vanillic acid	34.85 ± 1.20	44.70 ± 0.42**	7	5.40 ± 0.57	8.25 ± 0.49*	7	7.60 ± 0.69	12.60 ± 1.27*	7
Caffeic acid	9.15 ± 0.78	29.80 ± 0.14**	3	13.65 ± 0.92	24.70 ± 1.13**	3	14.00 ± 1.15	29.00 ± 0.85**	3
p-Coumaric acid	4.45 ± 0.21	18.45 ± 0.64**	7	4.55 ± 0.78	10.45 ± 1.20*	7	5.05 ± 0.49	12.30 ± 0.85**	7
Ferulic acid	4.63 ± 0.06	26.84 ± 0.45**	7	9.25 ± 1.34	22.90 ± 1.41*	7	12.50 ± 0.28	27.65 ± 2.47*	7
Apigenin	n. d. [b]	n. d.	–	n. d.	n. d.	–	n. d.	n. d.	–

Data presented are averages of concentration values determined in three replicates ± standard deviation. Asterisks indicate significant differences between the respective control and enzyme treated samples according to a multiple *t*-test performed by GraphPad Prism version 8.00, FDR (Q = 10%), *P < 0.05, **P < 0.01, ***P < 0.0001.

[a] Concentration of the phenolic compound measured in the presence of the respective enzyme extract without incubation (0th incubation hour).

[b] Not detected.

after enzymatic treatments (Tables 4 and 5). In keeping with TPC/TFC and antioxidant activity results (see Figs. 1–3), data were generally the lowest in case of the white variety Farmsugro 180 sorghum compared to pigmented types tested. Similarly, Wu et al. (2016) reported fewer types of individual phenolics and a less phenolic concentration in white sorghum compared to the pigmented ones. Nevertheless, all three enzyme treatments were positively affected ($P < 0.05$) the liberation of the studied individual phenolics from their glycosides or esters in the Farmsugro 180 substance as well (Table 5). And maximal yields for each phenolics were generally achieved after a 7-h incubation with the enzyme cocktails. In particular, the content of hydroxycinnamates, i.e., caffeic acid, *p*-coumaric acid and ferulic acid, improved significantly ($P < 0.05$) as a result of the enzyme treatments compared to the GK Emese substrate. The free *p*-coumaric acid and ferulic acid contents, for instance, increased to 4.1 and 5.8 times ($P < 0.01$), respectively, after *R. miehei* enzyme-assisted extraction of Farmsugro 180. The *G. persicaria* and *M. corticolus* enzyme treatments also resulted in at least two times improvement in the yield of these phenolic compounds (Table 5). A 5.7-fold accumulation in the free ferulic acid content was registered in fermented wheat bran samples during the work of Manini et al. (2014), in which the ferulic acid release was considered to the action of esterases and other fiber degrading and solubilizing enzymes. Ferulic acid bound fraction can mainly be found cross-linked to macromolecules in the plant cell wall, thus the higher the bound ferulic acid content, the harder the sorghum grain (Li et al., 2021). It is also important to mention that the improved DPPH radical scavenging activity of Farmsugro 180 extracts (Fig. 3C) could ascribe to the increased ferulic acid content during incubation (Table 5), similar to the findings described by Huang et al. (2013) for an esterase treated wheat bran substrate.

Comparing the overall phenolic compound yields, the total concentration of hydroxybenzoic acids was superior to that of hydroxycinnamic acids on all sorghum substrates (Tables 3–5). However, highest degree of increase during enzyme treatments was mostly documented for the hydroxycinnamic acids indicating the presence of a considerable mass of these phenolic acids in conjugated forms. The flavonoid compound apigenin was found only in broom sorghum by the chromatography technique of the current study (Table 3). Similarly, no apigenin was detected in white and some red sorghum varieties in the work of Wu et al. (2016), in which a relationship between the phenolic profile and genotype and growth temperature conditions of sorghum samples has also indicated. Anyway, the yields achieved for each individual phenolics by the enzymatic treatments performed here were noticeable and were comparable to those documented for other sorghum varieties. For instance, the caffeic acid, *p*-coumaric acid, ferulic acid, protocatechuic acid and apigenin levels measured in treated broom sorghum was superior to those detected recently in an extract from SSG59-3 red sorghum cultivar (Punia, Tokas, Malik, Satpal, & Sangwan, 2021). In addition, the caffeic, *p*-coumaric and ferulic acids in enzyme-treated Farmsugro 180 showed higher yields compared to S-713 and PC5 white sorghum cultivars (Punia et al., 2021).

Overall, treatment with *R. miehei* enzyme cocktail proved to be most effective extraction method to release individual phenolics in sorghum samples, that agrees with the results documented for TPC and antioxidant activity. This indicates the contribution of high esterolytic activity of *R. miehei* cocktail to the yield of free phenolics during reaction. In addition, the high incubation temperature (50 °C) applied as optimal working condition for the *R. miehei* enzymes can also support the phenolics enhancement and solubility (Hou et al., 2016).

4. Conclusions

In this work, cellulolytic and esterolytic cocktails from *R. miehei*, *G. persicaria* and *M. corticolus* were produced on wheat bran-based SSF. These enzyme cocktails were then used to improve the free phenolics content and antioxidant activity of grain residues of commercial broom sorghum and GK Emese and Farmsugro 180 hybrid sorghum varieties. Results revealed a positive effect by the enzyme treatments on most bioactive parameters studied. A large development of TPC and some antioxidative properties was detected even after 1 h of treatment, indicating the applicability of a shorter incubation time for the extraction. For most enzyme treatments, the TPC associated well with TFC and antioxidant capacity of sorghum samples during incubation. Strong positive association was also found between FRAP, CUPRAC and DPPH data, coinciding with a fair to strong positive relationship with condensed tannin content. Chromatography showed more hydroxybenzoate content in sorghum residues than hydroxycinnamate, but the yield of the latter compounds increased to a greater extent during the enzyme treatments.

The broom sorghum exhibited the highest yield for both soluble and insoluble bound phenolics. Among the enzyme extracts, the highly esterolytic *R. miehei* cocktail proved to be the most effective in the mobilization of phenolics from the sorghum samples, indicating a major

role for the esterase enzymes in the phenolics extraction. In conclusion, the enzymatic treatment using cellulolytic/esterolytic enzyme mixtures may be a suitable approach to develop phenolic enriched antioxidative extracts from sorghum substances. As far as we know, our findings are the first that show the characterization of the free phenolic content and antioxidant activity of sorghum residues after a cellulase/esterase treatment. The results can also contribute to the economic utilization of by-product samples generated during sorghum grain processing. The obtained sorghum samples/extracts with enhanced bioactive properties can be utilized as additives in functional food developments, which may be even more important considering the role of sorghum in the future production of gluten-free products.

CRediT authorship contribution statement

Tamás Kovács: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Dóra Anna Papp:** Investigation, Methodology. **Mónika Varga:** Investigation, Methodology, Validation. **András Szekeres:** Formal analysis, Methodology, Resources. **Andrea Palágyi:** Methodology, Resources. **Csaba Vágvölgyi:** Conceptualization, Project administration, Resources. **Tamás Papp:** Conceptualization, Funding acquisition, Resources. **Judit Krisch:** Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Miklós Takó:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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