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Functional potential of crackers formulated with an innovative fermented cooked chickpea flour

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Incorporating legume ingredients into staple foods offers a sustainable strategy to promote legume intake while improving the nutritional quality. Fermentation can further enhance the nutritional and functional properties of chickpea flour. This study evaluated the use of cooked chickpea flour (CF) and *Lentilactobacillus diolivorans* 13-4A-fermented cooked chickpea flour (FF) as functional ingredients in crackers, replacing refined wheat flour at 10% or 25% levels. Four enriched formulations (C10CF, C25CF, C10FF, C25FF) were compared to a conventional wheat cracker (CW). All products underwent *in vitro* digestion to obtain bioaccessible fractions (BFs) and digestion residues (RFs). BFs were analyzed for polyphenol bioaccessibility and bioavailability (using a Caco-2 cell model), total antioxidant capacity (TAC), and inhibitory activity against α-amylase, α-glucosidase, and dipeptidyl peptidase-IV (DPP-IV), while RFs were assessed for residual TAC. Fermentation tripled CF polyphenol content, with FF-enriched crackers, particularly C25FF, showing the highest polyphenol levels. Pyrogallol was detected only in FF and related crackers. FF exhibited the highest pre-digestion TAC, while post-digestion, C25FF and C10CF had the highest bioaccessible and residue TAC, respectively. CF or FF enrichment (10%) most effectively inhibited α-amylase (~50%), whereas FF and C25FF showed the strongest α-glucosidase inhibition (~8%). CF demonstrated the strongest DPP-IV inhibition. All BFs were non-cytotoxic; FF-derived BF enhanced the transcellular permeability and polyphenol transport in Caco-2 cells. Pyrogallol and sinapic/protocatechuic acids glucosides were the most bioavailable (40–100%). Overall, *Lent. diolivorans* 13-4A-fermented chickpea flour demonstrates strong potential to improve the nutritional and functional properties of crackers, contributing to the development of healthier cereal-based snacks.

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1. Introduction

In recent decades, there has been a growing interest in dietary strategies that support healthier lifestyles, including the development of functional foods rich in fiber and plant-based proteins.[1,2] Legumes have gained considerable attention due to their association with improved metabolic health, positive modulation of the gut microbiota, and their potential as sustainable alternatives to anima-based proteins.[3] Despite their nutritional and environmental advantages, legume consumption remains well below the recommended 50–100 g per day intake established by the Food and Agriculture Organization (FAO).[4] One promising approach to promote legume intake is the incorporation of legume-derived ingredients, such as chickpea flour, into widely consumed products like snacks and baked goods. Chickpeas (*Cicer arietinum* L.) are particularly valued for their high-quality protein content, slowly digestible carbohydrates, dietary fiber, and naturally gluten-free composition.[5–7] Their inclusion in cereal-based products has been shown to enhance nutritional profiles by improving amino acid balance, increasing antioxidant activity, and contributing bioactive phytochemicals of products such as bread,[8–10] snacks,[2] muffins,[11] and pasta.[12] However, the incorporation of chickpea flour in food products poses several technological and sensory challenges, including the presence of anti-nutritional factors (*e.g.*, phytic acid, fermentable oligosaccharides), off-flavors (*e.g.*, earthy, beany), and undesirable textural changes.[3,13] Fermentation has emerged as an effective, non-invasive and biotechnological process to overcome these limitations by modifying the food matrix. It can improve the organoleptic qualities of legumes while also enhancing the bioavailability of functional compounds such as bioactive peptides (BAPs), phenolic compounds, and antioxidant

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molecules.[14,15] Notably, the functionality of these bioactives is influenced not only by their concentration but also by their release from the food matrix and bioaccessibility during digestion.[16] Polyphenols, for example, are often bound within complex structures of dietary fiber and proteins, which can limit their release and subsequent absorption.[17] Moreover, the interactions between bioactive compounds and the food matrix during processing may alter their biological properties, underscoring the importance of evaluating both the ingredient and the final product in functional food development.[18] Crackers represent a convenient, widely accepted, versatile, food product, making them an ideal model-system for functional ingredient incorporation. Previous studies have shown that legume flour enrichment can enhance the nutritional value of crackers without negatively affecting sensory attributes at moderate inclusion levels (<30%).[19] Moreover, fermentation of legume-enriched products has demonstrated improvements in protein digestibility, antioxidant capacity, and the release of phenolics and essential amino acids *in vitro*.[14,15] We recently screened 14 lactic acid bacteria strains for their growth performance in chickpea-based purees and their ability to modulate key metabolites, including polyphenols, bioactive peptides, and phytic acid.[20] Each strain generated distinct chemical and functional profiles, enabling the identification of candidates with multifunctional potential. Metabolomic analysis of the resulting lyophilized powders showed that fermentation with *Lentilactobacillus diolivorans* 13-4A produced a flour characterized with a well-balanced combination of phenolic and proteolytic features. Principal component analysis positioned this fermented flour within an intermediate cluster, highlighting its multifunctional properties. Moreover, *Lent. diolivorans* 13-4A exhibited robust growth and adaptability in the chickpea matrix, supporting its suitability as a starter culture. Beyond its balanced metabolomic profile, this strain is also known to produce exopolysaccharides, which may contribute to improved texture and stability in food formulations.[21] Based on these findings, we hypothesized that chickpea powder fermented with *Lent. diolivorans* 13-4A could function as a multifunctional ingredient in baked products, enhancing nutritional and functional properties without compromising overall product quality. The aim of this study was therefore to evaluate the functional potential of *Lent. diolivorans* 13-4A-fermented chickpea powder as a novel ingredient in cracker. To this end, crackers were formulated by partially replacing wheat flour at 10% and 25% (w/w) with either fermented or non-fermented chickpea powder and compared with a conventional wheat-based control. The ingredients and resulting crackers were evaluated for antioxidant capacity, polyphenol bioaccessibility and intestinal bioavailability using a Caco-2 cell model, as well as for their *in vitro* inhibitory activity against digestive enzymes involved in glycemic control. Overall, this study provides a step forward in translating fermented chickpea-based ingredients, optimized through targeted microbial fermentation, into practical food formulations with validated functional and nutritional properties.

2. Materials and methods

2.1 Chemicals and reagents

All the solvents used were high-pressure liquid chromatography (HPLC) and mass spectrometry (MS) grade or analytical grade and were purchased from Sigma-Aldrich (Milan, Italy) as were water, acetonitrile, methanol, formic acid (99–100%), acetic acid glacial. The standard 4-hydroxyphenyl acetic acid (≥95%), *p*-hydroxybenzoic acid (≥99%), pyrogallol (≥98%) and 2,2-difenil-1-picrylidrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Trizma, starch azure, Gly-Pro *p*-nitroanilide hydrochloride, *para*-nitrophenyl-α-D-glucopyranoside and sodium phosphate were purchased from Sigma Aldrich (Milan, Italy). Potassium chloride, sodium chloride, magnesium chloride, ammonium bicarbonate, and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). The enzymes α-amylase from porcine pancreas (13 U mg^{-1} solid), α-glucosidase from *Saccharomyces cerevisiae* (≥10 U mg^{-1} solid), pepsin from porcine gastric mucosa (599 U mg^{-1} solid), pancreatin from porcine pancreas (100 U mg^{-1} solid), human dipeptidyl peptidase 4 (≥10 U mg^{-1} solid), and the bile salts were purchased from Sigma Aldrich (Milan, Italy). Cellulose powder from spruce was obtained from Fluka (Taufkirchen, Germany). For Caco-2 cell culture, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAA), *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES) buffer, antibiotic solution (penicillin–streptomycin), antimycotic solution (fungizone), phosphate buffered solution (PBS) and trypsin-EDTA were purchased from Gibco Invitrogen (Carlsbad, USA). While for the assays 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), dimethyl-sulfoxide (DMSO) were purchased from Sigma Aldrich (St Louis, MO, USA).

2.2 Chickpea flour preparation

Chickpeas (*Cicer arietinum* L.) of the Cicerale cultivar, a traditional variety from the Campania region (Southern Italy) and recognized as a Slow Food Presidium, were used in this study. Chickpea seeds (harvest 2023) were obtained from a local producer (Azienda Agricola Iuorio Luigia, Villamina, Campania region, Italy). The seeds, cultivated throughout the Campania region, were processed to prepare cooked and freeze-dried fermented and unfermented chickpea powders. Since both ingredients were incorporated as wheat flour substitutes in the formulation of innovative crackers (section 2.3), they are hereafter referred to as cooked chickpea flour (CF) and fermented cooked chickpea flour (FF), respectively. The preparation has been previously described.[20] Briefly, 500 g of chickpeas were cleaned with 500 mL tap water for 2 min at room temperature and then cooked at 121 °C for 20 min (Vapour Line 80B, VWR International PBI srl) in deionized water (4 : 1 water-to-seed ratio). Cooked chickpeas were blended to obtain a 20% w/v puree and cooled to 30 °C. The chickpea-puree concentration (20%, w/w) was selected based on a previous screening study showing that this level was sufficient to support microbial

growth, adaptation, and stable fermentation performance.[20] For CF, the puree was directly lyophilized. For FF, the cooled puree was inoculated with *Lent. diolivorans* 13-4A (previously isolated from Sardinian fermented milk) at a concentration of ~10^7 CFU mL^{-1} and incubated at 30 °C for 48 h prior to lyophilization. Both CF and FF were sieved through a 2 mm mesh and stored until use.

Prior to fermentation, the strain was precultured in MRS broth (Oxoid, Thermo Fisher Scientific) at 30 °C for 48 h. The pH and microbial loads were determined at the beginning of fermentation (after inoculum) and at the end of the fermentation period by pH measurement and standard plate counting on MRS agar (Oxoid), respectively. In addition, High-Performance Liquid Chromatography (HPLC) analyses were performed as previously described,[20] to assess the availability of fermentable sugars (glucose and fructose) for LAB growth and to quantify the main metabolites produced during fermentation.

2.3 Cracker design and development

The detailed formulations of all crackers are provided in Table 1. Wheat flour (type 00′, SELEX S.p.A, Italy), sodium bicarbonate (CRASTAN S.p.A), and fine salt (Italkali, S.p.A) were used to produce the crackers. All these ingredients were purchased by a local supermarket (Sole 365 S.p.a). CF and FF produced as described above (section 2.2), were used to formulate four novel cracker prototypes, which were evaluated for their functional properties in comparison with a conventional wheat-based cracker (CW). The CW was prepared by adapting the recipe reported by Gangola *et al.*[22] All the ingredients were mixed for 10 min, and the resulting dough was rolled to a thickness of ~0.5 mm. Baking was carried out in a forced-air convection oven (fan speed level 2 on a 1–5 scale) to ensure uniform heat distribution. Baking conditions were optimized through preliminary trials by testing different temperature (170, 180, and 190 °C) and time (10, 15, and 20 min) combinations to obtain crackers with colour and crispness comparable to those of commercially available products (data not shown). Based on these trials, the final baking conditions were set at 180 °C for 15 min. After cooling, crackers were freeze-dried (Thermo scientific Heto, Denmark), ground using a GRINDOMIX 2000 (Retsch Italia, Verdere Scientific S.r.l, Bergamo, Italy) and sieved through a 2 mm mesh prior to analysis.

Previous studies on bakery products containing chickpea flour have shown that enrichment levels below 30–50% can significantly increase the protein and fiber content while maintaining acceptable sensory quality and desirable rheological and textural properties of the final product.[19,23,24] The nutritional composition of the crackers is reported in Table S1. It was calculated from the dough formulations (Table 1) using nutrient data for the individual ingredients obtained from the CREA food composition database (dry-matter basis). Total nutrient contents were adjusted to account for water loss during baking (33% for CW; 35% for C10CF and C10FF; 36% for C25CF and C25FF). Nutritional values were expressed per 100 g of finished product, based on the experimentally determined moisture content (lyophilization in triplicate), as reported in Table S1.

In the novel formulations, replacement of wheat flour with FF or CF at 10% and 25% (w/w) allowed the products to meet the "source of protein" and "source of fiber" nutrition claims as defined by Regulation (EC) no. 1924/2006, as amended by Regulation (EU) no. 1047/2012.[25] At substitution levels of 10% and 25%, proteins contributed approximately 14% and 16% of the total energy value, respectively, while fiber content reached 3.5 g and 5.2 g per 100 g of product. Moreover, these substitution levels ensured satisfactory dough-handling properties, enabling evaluation of the effects of different replacement levels on the functional characteristics of the final products.

2.4 *In vitro* digestion

To investigate the bioaccessibility of polyphenols, *in vitro* gastrointestinal digestion was performed on CF, FF, and the corresponding crackers using the standardized INFOGEST method.[26] Simulant salivary (SSF), gastric (SGF), and intestinal fluids (SIF) were prepared accordingly. For the oral phase, 2.5 g of each freeze-dried sample were mixed 1 : 1 with water to form a paste-like bolus, to which SSF (pH 7.0), amylase (75 U mL^{-1}), and $CaCl_2$ (1.5 Mm in SSF) were added. The mixtures were incubated for 2 min at 37 °C and 160 rpm before proceeding to the simulation of gastric phase. After 2 min, SGF (pH 3.0) and $CaCl_2$ (0.15 mM in SGF), were added and the pH was adjusted to 3 with HCl 1 M prior adding pepsin (2000 U mL^{-1}). The samples were then incubated for 2 h at 37 °C at 130 rpm. For the intestinal phase, SIF (pH 7.0), pancreatin (800 U mL^{-1}), bile salts (10 mM), and $CaCl_2$ were added. The pH was adjusted to 7.0 with NaOH 1 M, and the samples were incubated for 2 h at 37 °C and 100 rpm. At the end of intestinal phase, samples were centrifuged at 4200*g* for 15 min at 4 °C to separate the supernatant (bioaccessible fraction) from the pellet (residual fraction). The pellet was freeze-dried and gently ground to obtain a homogeneous powder, which was subsequently used to determine the residual antioxidant activity retained by the undigested fraction, as described in section 2.5.3. Aliquots (1 mL) of each digested sample were freeze-dried and used for

Table 1 Formulation of different prototypes of crackers. The ingredients are reported in grams

Ingredient	CW	C10CF	C25CF	C10FF	C25FF
Wheat flour (WF)	102.2	92.0	76.7	92.0	76.7
Cooked chickpea flour (CF)	—	10.2	25.6	—	—
Fermented cooked chickpea flour (FF)	—	—	—	10.2	25.6
Baking soda	1.3	1.3	1.3	1.3	1.3
Salt	0.5	0.5	0.5	0.5	0.5
Water	46.0	46.0	46.0	46.0	46.0
Total	150.0	150.0	150.0	150.0	150.0

CW: 100% wheat-based cracker; C10CF: 10% cooked chickpea flour-enriched cracker; C25CF: 25% cooked chickpea flour-enriched cracker; C10FF: 10% fermented cooked chickpea flour-enriched cracker; C25FF: 25% fermented cooked chickpea flour-enriched cracker.

the analyses reported below. For the *in vitro* bioavailability study, 5 mL of each supernatant were collected, freeze-dried, and subsequently resuspended in 2.5 mL of DMSO prior to the experiments described in section 2.8.

2.5 Total antioxidant capacity (TAC)

2.5.1 Extraction of samples. The determination of soluble antioxidant activity and total polyphenols in the products (flours and crackers) was carried out on extracts produced according to the method reported by Chiacchio *et al.*[20] Briefly, 100 mg of each sample were added with 1 mL of a methanol/water solution (70 : 30 v/v), vortexed for 30 s and centrifuged at 4 °C for 10 min at 21 100*g*. The resulting supernatant was separated from the pellet and filtered using 0.45 μm PTFE filters prior analysis. For the extraction of bioaccessible fractions, the aliquots (1 mL) of supernatant collected at the end of digestion were freeze-dried, resuspended in 1 mL of a methanol/water solution (70 : 30 v/v), and extracted as described in this section for the undigested products.

2.5.2 Soluble TAC. The antioxidant activity was measured on the products and their bioaccessible fractions through the DPPH as described by Hamzalioglu *et al.*[27] This assay allows the evaluation of the radical scavenging activity of the antioxidants present in the extracts. A DPPH radical solution was prepared by diluting DPPH powder in methanol to reach a final absorbance of 0.9 ± 0.02 at 517 nm. The reaction was started by adding 200 μL of extracted sample to 1 mL of DPPH radical solution. After 5 min, the absorbance was measured at 517 nm with a UV-VIS spectrophotometer (PG Instruments, UK). The percentage of inhibition was calculated against blank and compared to a Trolox standard curve, and the results expressed as μmol of Trolox equivalent (TE) per gram of dry pellet.

2.5.3 Direct TAC. The TAC was measured on the insoluble pellet obtained at the end of digestion using the QUENCHER method described by Hamzalioglu *et al.*[27] The radical solution was prepared as described above for soluble TAC determination. The dried pellet was incubated with DPPH radical solution for 30 min. Thereafter, the mixture was centrifuged at 4200*g* for 5 min at 4 °C and the absorbance of the supernatant was measured at a wavelength of 517 nm with a UV-VIS spectrophotometer (PG Instruments, UK). The percentage of inhibition was calculated against control and compared to a Trolox standard curve and the results expressed as μmol of Trolox equivalent (TE) per gram of dry pellet.

2.6 Determination of polyphenols by HPLC-UV/VIS analysis

Phenolic compounds were characterized in the extracted samples and bioaccessible fractions as reported by Chiacchio *et al.*[20] Chromatographic analysis was performed using an HPLC SHIMADZU equipped with a detector UV/VIS SPD 20° set at wavelength of 280 nm and a Prodigy ODS3 100 Å column (250 mm × 4.6 mm, particle size 5 μm) (Phenomenex, CA, USA). The eluents were HPLC-grade water with 0.2% (v/v) formic acid (solvent A) and a mixture of acetonitrile/methanol (60 : 40 v/v) (solvent B). The gradient was set as follows: 20% B (2 min), 30% B (8 min), 40% B (18 min), 50% B (26 min), 90% B (34 min), 90% B (37 min), 20% B (39 min), and 20% B (43 min). The injection volume was 20 μL and the flow rate of 1 mL min^{-1}. The analysis was performed in triplicate for each sample, and the results were expressed as μg g^{-1} of dry weight using a calibration curve built with the corresponding standard compound. This analysis allowed for the identification of *p*-hydroxybenzoic acid, 4-hydroxyphenyl acetic acid, and pyrogallol. The limits of detection (LOD), quantification (LOQ), and the retention times of the identified polyphenols are reported in Table S2. Protocatechuic-4-*O*-glucoside, sinapoyl D-glucoside, and kaempferol 3-*O*-glucoside were identified through a high-resolution mass spectrometry (LC-HMRS) analysis as we previously reported.[20] Molecular formula, *m*/*z*, and retention time of polyphenols identified by LC-HRMS are reported in Table S3.

2.7 Potential anti-diabetic activity: inhibition of digestive enzymes

2.7.1 α-Amylase inhibition. The inhibitory activity of α-amylase was assessed following the method reported by Llorens *et al.*[28] Aliquots (1 mL) collected at the end of the intestinal phase of digestion, corresponding to the bioaccessible fraction, were lyophilized and subsequently resuspended in 1 mL of distilled water. The substrate solution was prepared by adding 0.4 mL of a Tris-HCl 0.1 M buffer (pH 6.9) containing calcium chloride (0.01 M) to 4 mg of starch azure. The mixture was boiled for 5 min and incubated at 37 °C for 5 min. The reaction was initiated by adding 0.2 mL of a Tris-HCl 0.1 M buffer solution (pH 6.9) containing the enzyme α-amylase from porcine pancreas (2 U mL^{-1}) to 0.2 mL of the sample and 0.2 mL of substrate solution. The mixture was incubated at 37 °C for 10 min in a thermostatic bath. Thereafter, the reaction was stopped by adding 0.5 mL of a 50% acetic acid/water solution (v/v) and ultracentrifuging at 4 °C, 21 100*g* for 10 min. To evaluate the maximum activity of the enzyme (100%), the same reaction was reproduced by replacing the sample with the buffer Tris-HCl 0.1 M. Similarly, to evaluate the minimum activity of the enzyme (0%), the sample and the enzyme were replaced with Tris-HCl 0.1 M. For each sample, a blank was prepared, in which the buffer Tris-HCl 0.1 M was added instead of the enzyme. The absorbance of the resulting supernatants was measured at 595 nm with a UV-VIS spectrophotometer (PG Instruments, UK). Each determination was conducted in triplicate, and the results were expressed as %inhibition using the following formula:

$$\%\text{Inhibition} = \frac{[\text{Abs}_{100\%} - \text{Abs}_{0\%}] - [\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}]}{[\text{Abs}_{100\%} - \text{Abs}_{0\%}]} \times 100$$

where $\text{Abs}_{100\%}$ is the absorbance of the sample containing the TRIS-HCl buffer instead of the digests; $\text{Abs}_{0\%}$ is the absorbance of the samples containing TRIS-HCl buffer instead of the digests and the enzyme; $\text{Abs}_{\text{blank}}$ is the absorbance of the samples containing TRIS-HCl buffer instead the enzyme.

2.7.2 α-Glucosidase inhibition. The α-glucosidase inhibition assay was carried out as reported by Llorens *et al.*[28]

Freeze-dried soluble intestinal fractions were resuspended in 1 mL of distilled water. 125 μL of the sample was added to 870 μL of 0.1 M phosphate buffer (pH 6.8). The reaction was initiated by adding 125 μL of a 0.1 M phosphate buffer solution (pH 6.8) containing the enzyme α-glucosidase from *Saccharomyces cerevisiae* (1 U mL^{-1}) and 125 μL of a 0.1 M phosphate buffer solution containing *para*-nitrophenyl-α-D-glucopyranoside (3 mM) as the substrate for the enzyme. The samples were incubated at 37 °C for 20 min in a thermostatic bath and then ultracentrifuged at 21 100*g* for 10 min at 4 °C. After this period, the absorbance was measured at 410 nm with a UV-VIS spectrophotometer (PG Instruments, UK). To evaluate the maximum activity of the enzyme (100%), the same reaction was reproduced by replacing the sample with the phosphate buffer solution 0.1 M. Similarly, to evaluate the minimum activity of the enzyme (0%), the sample and the enzyme were replaced with phosphate buffer solution 0.1 M. For each sample, a blank was prepared, in which the phosphate buffer solution 0.1 M was added instead of the enzyme. Each determination was conducted in triplicate, and the results were expressed as %inhibition using the following formula:

$$\%\text{Inhibition} = \frac{[\text{Abs}_{100\%} - \text{Abs}_{0\%}] - [\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}]}{[\text{Abs}_{100\%} - \text{Abs}_{0\%}]} \times 100$$

where $\text{Abs}_{100\%}$ is the absorbance of the sample containing the phosphate buffer solution 0.1 M instead of the digests; $\text{Abs}_{0\%}$ is the absorbance of the samples containing phosphate buffer solution 0.1 M instead of the digests and the enzyme; $\text{Abs}_{\text{blank}}$ is the absorbance of the samples containing phosphate buffer solution 0.1 M instead the enzyme.

2.7.3 Dipeptidyl peptidase-IV (DPP-IV) inhibition. The bioaccessible fractions collected at the end of the gastrointestinal digestion were tested for the ability to inhibit the enzyme DPP-IV activity according to You *et al.*[29] Briefly, 25 μL sample were add in a 96-well plate and 25 μL Gly-Pro *p*-nitroanilide hydrochloride (1.59 mM) were added and incubated for 10 min at 37 °C. The reaction was started by adding 50 μL of human DPP-IV (≥10 U mg^{-1} protein) and stopped after 1 h of incubation at 37 °C by adding 100 μL acetate buffer 1M (pH 4.0). All reagents were diluted in 100 mM Tris-HCl buffer pH 8.0. Absorbance was measured at a wavelength of 405 nm with SpectraMax Mini plate reader (Molecular Devices). Each determination was conducted in triplicate, and the results were expressed as %inhibition using the following formula:

$$\%\text{Inhibition} = \frac{(\text{Abs}_1 - \text{Abs}_2)}{(\text{Abs}_1 - \text{Abs}_3)} \times 100$$

where Abs_1 is the absorbance of the sample containing TRIS-HCl buffer instead of the digests; Abs_2 is the absorbance of the digested sample; Abs_3 is absorbance of the sample containing Tris-HCl buffer instead of the enzyme solution and the potential inhibitor.

2.8 Bioavailability of polyphenols *in vitro*

2.8.1 Cell culture. To investigate the bioavailability of polyphenols released upon *in vitro* digestion, a model of human colon adenocarcinoma (Caco-2) was used. According to Juan-García *et al.*[30] cells were grown in Dulbecco's Modified Eagle's medium (DMEM), enriched with 10% fetal bovine serum (FBS), 1% HEPES buffer (v/v), 1% NAA (v/v), 100 U mL^{-1} of both penicillin–streptomycin, and 0.1% fungizone. The cells were kept in an incubator at 37 °C with environmental conditions set at as follows: 5% CO_2 and 95% air. The cells were sub cultivated after trypsinization (trypsin-EDTA) once or twice per week and were subculture routinely with only a small number of sub-passages (<70 subcultures) to maintain the genetic homogeneity.

2.8.2 Cell viability of digested extracts on Caco-2 cells. Prior to the bioavailability study, the Caco-2 cells were exposed to digested extracts to test their potential cytotoxicity through the yellow soluble tetrazolium salt (MTT) test.[30] Briefly, 200 μL per well of a suspension of Caco-2 cells (2×10^6 cells per mL) was seeded in a 96-well plate. After the confluency of cells was >90%, the culture medium was refreshed with 200 μL of the same medium and cells were exposed to the digested samples and their serial dilutions from 1 : 0 to 1 : 256 (1 : 2 dilution). The plates were incubated for 24 h and 48 h at 37 °C. After 24 h and 48 h, the media containing the extracts was removed and 200 μL of fresh medium along with 50 μL of MTT solution were added. This assay allows to measure the viability of cells that are metabolically active by determining the reduction of the yellow soluble tetrazolium salt to an insoluble purple formazan crystal *via* a mitochondrial reaction which reflects the viability of cells.[30] Therefore, after 4 h of incubations at 37 °C, formazan crystals were dissolved in 200 μL of DMSO and 25 μL of Sorensen's solution (glycine 0.4 μmol L^{-1} + 0.1 μmol L^{-1} NaCl, pH 10.5) were added. The amount of converted dye was determined by measuring absorbance at 570 nm wavelength with the ELISA plate reader Multiskan EX (Thermo Scientific, MA, USA). Cell viability was expressed in percent relative to control cells which were treated with 1% DMSO.

2.8.3 Differentiation of Caco-2 cell monolayer and trans epithelial resistance (TEER) measurement. Differentiated Caco-2 cells were used as an *in vitro* model to study the intestinal absorption and transport of polyphenols.[30] For the differentiation, the cells were seeded at 2×10^6 cells per cm^2 into a 6-well Transwell Permeable Supports with a diameter of 4.5 mm and a pore size of 0.4 mm (Corning, NY, USA). The cells were considered suitable for the study of polyphenol bioavailability once they reached TEER values ≥400 Ω cm^2 indicative of the formation of a mature and intact monolayer (Fig. S1).[28] The apical (AP) and basolateral (BL) compartments were filled with 1.5 mL of 10%-DMEM medium and refreshed 3 times a week. During this period, the TEER was measured to ensure membrane integrity with a Millicell ERS voltammeter equipped with electrodes (Millicell-ERS2, Bedford, MA). The Caco-2 cells reached complete differentiation within 2 weeks with TEER values in the range 360–580 Ω. Resistance values

were obtained considering the resistance values and the filter surface area using the following formula:

$$\text{TEER } (\Omega \text{ cm}^2) = (R_1 - R_0) \times A$$

where R_1 is the resistance value of the Caco-2 cell compartment, Ω; R_0 is the resistance value of the unseeded cell compartment, Ω; and A is the membrane area of the compartment, cm^2.

2.8.4 Transport of polyphenols and effect of barrier integrity. Polyphenol transport was studied as reported by Juan-García *et al.*[30] by exposing differentiated Caco-2 cells to undiluted extracts of digested samples, as no cytotoxic effect was found. By the 14th day, the medium from the AP (upper) and BL (lower) compartments was removed and the digests or Hank's Balanced Salt Solution (HBSS) buffer (control) were inoculated on the AP side, while fresh HBSS was replaced in the BL compartment. The permeates were collected from the BL side at 1 h, 2 h, 3 h and 4 h, followed by replacement with fresh HBSS buffer. TEER was regularly registered for 4 h (0, 2, and 4 h) of treatment and calculated as reported by Bocsik *et al.*[31] by subtracting the resistance of the cell-free insert from the measured values. This measurement was performed to evaluate the effect of transport on the permeability of the cell monolayer, as TEER is associated with the integrity of tight junctions (TJs) and, consequently, with barrier integrity. The aliquots collected at each time point were lyophilized, extracted, and analyzed as described above for the samples and their corresponding bioaccessible fractions for the polyphenols content (section 2.5.1 and section 2.6). The results were expressed as concentration (ng mL^{-1}) and as bioavailability (%) according to the following formula:

$$\text{Bioavailability } (\%) = \frac{[\text{concentration in BL}]}{[\text{concentration in AP}]}$$

where concentration in BL is the phenolic concentration in the basolateral compartment after the time of incubation at 1 h, 2 h, 3 h, and 4 h; concentration in AP is the phenolic concentration in the apical compartment.

2.9 Statistical analysis

All the analysis were performed in three replicates and the results were expressed as mean ± SD. The results of cell viability (MTT test) were expressed as mean ± SD of eight independent replicates. Statistical analysis was performed using statistical software SPSS (version 20.0, SPSS, Inc., Chicago, IL, USA). The differences between samples were assessed by *T*-test and one-way ANOVA and Tukey's *post hoc* tests ($p < 0.05$). The visualization of polyphenols abundance with heatmap was performed using R version 4.0.3 (https://www.r-project.org) ComplexHeatmap package.[32]

3. Results and discussion

3.1 Antioxidant capacity of the flours and crackers

Lent. diolivorans 13-4A showed a very good growth performances during chickpea puree fermentation, reaching 9.00 ± 0.13 log CFU mL^{-1} and lowing the pH from 6.23 to 4.33 (Table S4). The chickpea puree before fermentation (t0 h) contained about 6 g L^{-1} of monosaccharides (glucose and fructose) (Table S4). Fermentation depleted about half of the initial monosaccharides and produced lactic acid (6.22 ± 0.17 g L^{-1}), acetic acid (0.76 ± 0.11 g L^{-1}) and ethanol (1.32 ± 0.09 g L^{-1}) as e consequence of obligate hetero-fermentative metabolism of *Lent. diolivorans*.[33] The high residual sugar, in relation to the high lactic acid produced, suggests the fermentation of some oligosaccharides. In fact, *Lent. diolivorans* strains able to ferment raffinose were described in literature.[33,34] In addition, the *Lent. diolivorans* 13-4A was originally isolated from Sardinian fermented milk as raffinose fermenting (data not shown). The TAC of fermented chickpea flour (FF) was compared with that of unfermented chickpea flour (CF) and results are reported in the Fig. 1A (green bars). Fermentation resulted in a substantial increase in TAC, with FF exhibiting approximately 11-fold higher than CF. This finding aligns with previous studies reporting enhanced antioxidant capacity, up to 3-fold, in chickpea-based products fermented with LAB strains including *Lactiplantibacillus plantarum* CRL2211, *Weissella paramesenteroides* CRL2182, *Lactobacillus plantarum* 299v.[24,35–37] This enhancement is likely due to microbial enzymatic activity during fermentation, which promotes structural modification of the matrix and the release of antioxidant compounds such as free phenols, bioactive peptides, and amino acids.[38,39] Following the *in vitro* digestion, soluble antioxidant activity decreased in both CF and FF bioaccessible fractions (Fig. 1A, blue bars), by approximately 7-fold and 2.8-fold, respectively, compared to the products. However, FF retained markedly higher TAC in the pellet (non-solubilized residual) fraction, (Fig. 1B) which corresponds to the material that could reach the colon and interact with the gut microbiota *in vivo*.

Among the five cracker formulations, C10CF (10% unfermented chickpea flour) showed the highest TAC, followed by the conventional product (CW) (Fig. 1, green bars). In contrast, Sáez *et al.*[24] observed an improved antioxidant activity in crackers produced with chickpea flours, in which the fermented one accounted for 75% of the total flour used in the formulation, compared to the control. Interestingly, in our study, C10CF displayed a 7-fold increase in TAC compared to its ingredient (CF), suggesting that baking played a major role in the release and/or the formation of antioxidant compounds such as Maillard reaction products (*i.e.*, Amadori compounds, hydroxymethylfurfural, furfural, aldehydes, aldols and melanoidins).[40,41] Specifically, thermal treatment above 150 °C are well known to promote the formation of high-molecular-weight melanoidins which exhibit radical-scavenging activity, thereby contributing to overall antioxidant capacity of bakery products.[42] In addition, Maillard reaction products have also

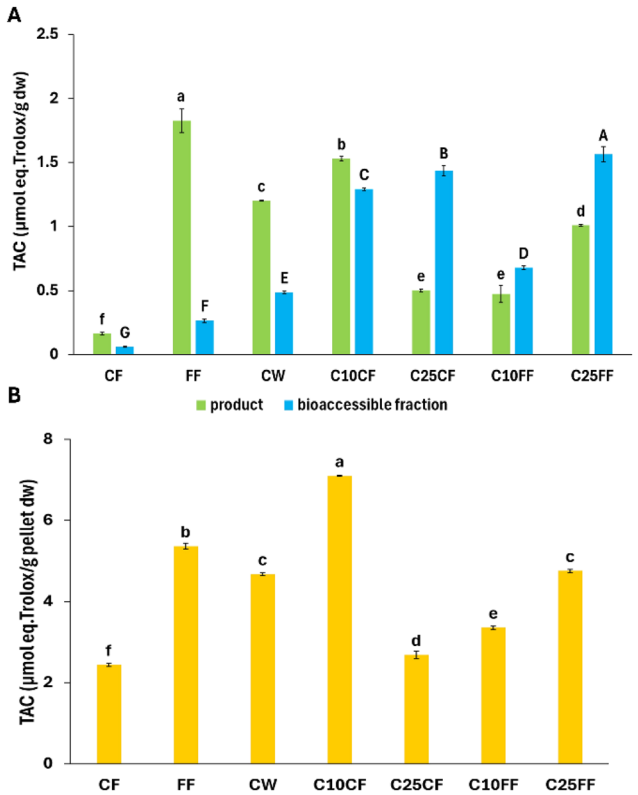

Fig. 1 (A) Soluble antioxidant activity of product (green bar) and bioaccessible fraction (blue bar) of cooked chickpea flour (CF), fermented cooked chickpea flour (FF), 100% wheat-based cracker (CW), 10% cooked chickpea flour-enriched cracker (C10CF), 25% cooked chickpea flour-enriched cracker (C25CF), 10% fermented cooked chickpea flour-enriched cracker (C10FF), and 25% fermented cooked chickpea flour-enriched cracker (C25FF) expressed as µmol eq. Trolox per g of dry weight sample (mean ± SD). Different lowercase letters indicate differences among the products whereas different uppercase letters indicate differences among the bioaccessible fractions (One-way ANOVA and Tukey's *post hoc*, $p < 0.05$). (B) Antioxidant activity of residue fraction from the digestion expressed as µmol eq. Trolox per g of dry weight pellet (mean ± SD). Different letters indicate differences between the samples assessed by One-way ANOVA and Tukey's *post hoc* tests ($p < 0.05$).

been reported to interact with dietary fiber and cereal-derived polyphenols (*e.g.*, ferulic acid), forming so-called "Maillardized dietary fiber", which may further enhance the antioxidant activity in the final product.[43] In FF-enriched crackers, C25FF showed approximately twice the TAC of C10FF, indicating a dose-dependent effect of fermented flour addition. Conversely, in CF-enriched crackers, C10CF exhibited a higher TAC than C25CF. This discrepancy may not be related directly to total polyphenol content, which was higher in both C10FF and C25FF compared to CF crackers, but rather to differences in polyphenol profile and their interactions with each other and with free radicals and/or to the release of bioactive peptides with antioxidant potential.[44,45] Moreover, in protein-rich matrices, such as crackers containing 25% chickpea flour, high-molecular-weight melanoidins may form and interact with chickpea-derived polyphenols, potentially limiting their contribution to the measured antioxidant activity compared to less enriched formulations, such as C10FF.[42,46] As with the flours, *in vitro* digestion led to a reduction in the soluble antioxidant activity of CW and C10CF bioaccessible fractions, by 2.5- and 1.2-fold, respectively, compared to their undigested forms (Fig. 1A, blue bars). In contrast, the TAC of C25CF, C10FF, and C25FF increased post-digestion by 2.9-, 1.4-, and 1.5-fold, respectively. In CW, *in vitro* digestion appeared to induce a reduction and/or transformation of antioxidant compounds associated with the cereal matrix, likely driven by digestive factors such as pH variations and interactions with other matrix components, including dietary fiber, as previously reported for cereal-based products.[16,43] This result correlates with the modest antioxidant activity retained in the undigested residue fraction of CW.

In contrast, in chickpea-enriched and fermented formulations, digestion is consistent with an enhanced release of polyphenols from the legume–cereal matrix, leading to increased antioxidant activity in the bioaccessible fraction. These differences indicate that matrix structure and composition together with changes in formulations involving more complex matrices (chickpea) and the impact of thermal treatments, are key factors influencing the release of antioxidant compounds during digestion.[18] Consistently, the residual fractions from digested crackers exhibited a promising radical scavenging activity (Fig. 1B). Notably, C10CF retained the highest TAC in the residual fraction, approximately 1.8 times greater than the other formulations, followed by C25FF and CW, which showed ~1.2-fold higher values compared to the remaining samples. The high antioxidant activity in the residual fraction may result from the stabilization of polyphenols during digestion, potentially through interactions with proteins and peptides that entrap them within the insoluble matrix. Although not absorbed in the upper gastrointestinal tract, these compounds may reach the colon, where they can exert health benefits through interactions with the gut microbiota.[27] In comparison with other fermented legume-based systems, the antioxidant activity of FF-enriched crackers was within the range of DPPH radical-scavenging activities reported for fermented legume beverages and non-baked matrices, such as legume-based sourdoughs and fermented soybean, lupin, pea, and bean products.[41,47,48] For instance, Vurro *et al.*[41] reported that focaccia bread enriched with pea-based sourdough at 40% exhibited DPPH radical-scavenging activity comparable in magnitude to that observed in the present FF-enriched cracker formulations with value below 1 µmol eq. Trolox per g samples. Taken together, these results indicate that fermented chickpea flour can contribute to measurable antioxidant activity in complex baked matrices, even at relatively low levels of inclusion.

3.2 Polyphenol bioaccessibility

In the present study, six phenolic compounds were identified in the flours, whereas four and nine of them were detected in the CW and cracker formulated with the innovative ingredients, respectively (Table 2).

Table 2 Polyphenol content in flours and enriched cracker expressed as μg g^{-1} of dry weight sample

Compound	CF	FF	CW	C10CF	C25CF	C10FF	C25FF
p-Hydroxybenzoic acid	6.8 ± 0.32^{b}	11.1 ± 0.6^{a}	$2.8 \pm 0.02^{d,e}$	$3.1 \pm 0.1^{d,e}$	3.4 ± 0.1^{d}	2.4 ± 0.03^{e}	4.8 ± 0.2^{c}
Protocatechuic 4-*O*-glucoside	27.3 ± 0.5^{c}	46.0 ± 0.4^{a}	—	9.7 ± 0.01^{f}	21.6 ± 0.3^{d}	10.9 ± 0.2^{e}	33.7 ± 0.61^{b}
4-Hydroxyphenyl acetic acid	9.8 ± 0.2^{b}	21.1 ± 0.2^{a}	—	2.0 ± 0.2^{d}	2.9 ± 0.02^{c}	1.7 ± 0.1^{d}	2.8 ± 0.20^{c}
Sinapoyl D-glucoside	43.4 ± 0.8^{a}	41.2 ± 1^{b}	—	6.3 ± 0.1^{d}	8.4 ± 0.1^{c}	1.2 ± 0.03^{e}	5.44 ± 0.02^{d}
Kampferol 3-*O*-glucoside	3.9 ± 0.01^{b}	5.7 ± 0.05^{a}	—	1.9 ± 0.1^{e}	$2.2 \pm 0.1^{d,e}$	2.1 ± 0.01^{d}	2.82 ± 0.01^{c}
Pyrogallol	—	123.5 ± 1.1^{a}	—	—	—	6.5 ± 0.04^{c}	11.6 ± 0.04^{b}
Sinapic acid	—	—	2.4 ± 0.1^{a}	1.6 ± 0.01^{b}	1.0 ± 0.1^{c}	0.7 ± 0.1^{d}	0.6 ± 0.05^{d}
Ferulic acid	—	—	5.8 ± 0.4^{a}	3.0 ± 0.04^{b}	2.4 ± 0.1^{c}	2.4 ± 0.02^{c}	1.6 ± 0.00^{d}
Vanillic acid	—	—	1.2 ± 0.1^{a}	—	—	—	—
Total	91.2 ± 0.8^{b}	248.6 ± 0.9^{a}	12.2 ± 0.2^{f}	27.7 ± 0.5^{e}	41.8 ± 0.3^{d}	27.8 ± 0.3^{e}	63.3 ± 0.6^{c}

CF: cooked chickpea flour; FF: fermented cooked chickpea flour; CW: 100% wheat-based cracker; C10CF: 10% cooked chickpea flour-enriched cracker; C25CF: 25% cooked chickpea flour-enriched cracker; C10FF: 10% fermented cooked chickpea flour-enriched cracker; C25FF: 25% fermented cooked chickpea flour-enriched cracker. Data are shown as mean ± SD. Different letters indicate differences among the samples before digestion within the same row for each compound, as assessed by One-way ANOVA and Tukey's *post hoc* ($p < 0.05$).

The fermentation of chickpea flour increased the polyphenol content by approximately 2.7 times. Similarly, Sáez *et al.*[24] and De Pasquale *et al.*[36] reported that the solid-state fermentation of chickpea flours increased the polyphenol content by 2-fold compared to the control flours. In chickpea seeds, phenolic compounds are mainly in bound forms; therefore, the enzymes expressed by LABs (*i.e.*, esterases, phenolic acid decarboxylases, and glycosidases) may have caused the breakdown and their release from the plant cell walls.[20,49] Compared to the non-fermented flour, FF contained double the amount of *p*-hydroxybenzoic acid, 4-hydroxyphenyl acetic acid, kaempferol 3-*O*-glucoside, and protocatechuic 4-*O*-glucoside. Xu *et al.*[50] reported that the glucoside of protocatechuic acid is one of the main phenolic compounds released from the cell wall matrix during chickpea germination and contributed to their higher antioxidant activity compared to non-germinated chickpeas. Conversely, synapoyl D-glucoside was found more abundant in CF flour and its derived-products. During fermentation new bioactive molecules can be produced by LABs. Indeed, pyrogallol was the predominant phenolic in FF but was absent in CF. This is likely due to the bioconversion of gallic acid, following tannin hydrolysis and subsequent decarboxylation mediated by LABs as previously reported.[20]

Consistent with the flour composition, the predominant phenolic compounds identified in the crackers were glucosides of sinapic acid and protocatechuic acid. The novel prototypes showed, on average, a 3-fold higher total polyphenol content compared to the conventional cracker (CW), with C25FF and C25CF exhibiting the highest concentrations, 1.8-fold and 1.2-fold greater than the other formulations, respectively. These findings suggest that the percentage of chickpea flour enrichment, particularly with FF, plays a critical role in enhancing the functional properties of the final product. As expected, crackers formulated with a higher inclusion level of chickpea flour (25%), both fermented and non-fermented, contained significantly more polyphenols than those enriched at 10%. However, in the undigested samples, the increase in total polyphenol content did not mirror the respective measured antioxidant activity. This observation aligns with previous reports indicating that the antioxidant capacity of plant-based foods cannot be attributed solely to total phenolic content, as synergistic interactions among polyphenols, and with other bioactive compounds, may influence the overall activity through different modes of interaction with the DPPH radical.[51,52] Additionally, interactions between phenolic compounds and the food matrix can modulate their bioactivity. Within complex food systems, polyphenols may bind with dietary fibers, proteins, or carbohydrates, potentially enhancing or diminishing their antioxidant potential depending on the nature and strength of these interactions.[53] The influence of matrix effects is also compound-specific, as shown by Sęczyk *et al.*,[54] who reported a reduction in antioxidant activity in white bean–fortified pasta due to interactions between polyphenols and macromolecules in the pasta matrix. In the present study, no significant difference in total polyphenol content was observed between C10FF and C10CF ($p > 0.05$), indicating that fermentation at this lower inclusion level did not markedly affect overall phenolic release from the matrix. At lower inclusion levels, interactions between polyphenols and the wheat-based matrix may have limited phenolic release, thereby attenuating the differences between fermented and non-fermented flours.[43,55]

As shown in Table 3, *in vitro* digestion resulted in 1.3- to 4.1-fold increase in total polyphenols compared with the undigested products. The greatest release was observed for C10FF, followed by C25FF and FF, with increases of approximately 4.0-, 2.4-, and 1.9-fold, respectively. After digestion, protocatechuic and sinapic acid glucosides remained the predominant phenolic compounds, along with pyrogallol, whose bioaccessibility in FF-enriched crackers exceeded 100%. This effect may be explained by the release of additional phenolic compounds previously entrapped within the food matrix during digestion, driven by the physicochemical conditions of the gastrointestinal process, including pH changes and digestive enzyme activity.[56] Inclusion of FF at both 10% and 25% substitution levels resulted in up to a twofold increase in polyphenol release during digestion compared with the corresponding unfermented formulations. This enhanced bioaccessibility

Table 3 Polyphenol content of bioaccessible fractions of chickpea flours and enriched cracker expressed as μg g^{-1} of dry weight sample

Compound	CF	FF	CW	C10CF	C25CF	C10FF	C25FF
p-Hydroxybenzoic acid	7.4 ± 0.4^{c}	30.8 ± 0.5^{a}	4.4 ± 0.1^{e}	7.6 ± 0.6^{c}	9.0 ± 0.1^{b}	6 ± 0.2^{d}	7.5 ± 0.06^{c}
Protocatechuic 4-*O*-glucoside	42.5 ± 0.3^{c}	200.2 ± 2^{a}	—	11.7 ± 0.1^{f}	30.1 ± 0.2^{d}	15.9 ± 0.2^{e}	53.1 ± 0.08^{b}
4-Hydroxyphenyl acetic acid	21.5 ± 0.4^{b}	33.5 ± 0.42^{a}	—	2.7 ± 0.2^{e}	6 ± 0.04^{d}	2.6 ± 0.2^{e}	10.6 ± 0.3^{c}
Sinapoyl D-glucoside	61.1 ± 0.6^{b}	64.0 ± 0.2^{a}	—	7.2 ± 0.3^{e}	17.8 ± 0.03^{c}	6.5 ± 0.4^{e}	14.8 ± 0.9^{d}
Kampferol 3-*O*-glucoside	4.6 ± 0.04^{b}	7.5 ± 0.2^{a}	—	2.9 ± 0.2^{c}	3.2 ± 0.14^{c}	2.4 ± 0.2^{d}	4.4 ± 0.3^{b}
Pyrogallol	—	144.0 ± 0.04^{a}	—	—	—	76.9 ± 2.0^{b}	61.4 ± 4.4^{c}
Sinapic acid	—	—	1.6 ± 0.04^{a}	0.6 ± 0.02^{d}	0.5 ± 0.01^{d}	1.1 ± 0.00^{b}	0.9 ± 0.04^{c}
Ferulic acid	—	—	10.3 ± 0.2^{a}	5 ± 0.1^{b}	3.6 ± 0.00^{d}	4.2 ± 0.10^{c}	2.1 ± 0.02^{e}
Vanillic acid	—	—	1.1 ± 0.02^{a}	—	—	—	—
Total	137.2 ± 0.5^{c}	480.0 ± 2.5^{a}	17.4 ± 0.2^{f}	37.8 ± 0.2e	70.1 ± 0.3^{d}	115.5 ± 2.6^{c}	154.0 ± 4^{b}

CF: cooked chickpea flour; FF: fermented cooked chickpea flour; CW: 100% wheat-based cracker; C10CF: 10% cooked chickpea flour-enriched cracker; C25CF: 25% cooked chickpea flour-enriched cracker; C10FF: 10% fermented cooked chickpea flour-enriched cracker; C25FF: 25% fermented cooked chickpea flour-enriched cracker. Data are shown as mean ± SD. Different letters indicate differences among the bioaccessible fractions after digestion within the same row for each compound, as assessed by One-way ANOVA and Tukey's *post hoc* ($p < 0.05$).

may be attributed to the lower pH of the fermented matrix, resulting from acidification during fermentation which can promote disruption of complex matrix structures and facilitate the release and solubilization of bound polyphenols.[57] Consistent with pre-digestion results, the conventional wheat-based cracker exhibited the lowest total polyphenol content in the bioaccessible fraction, further highlighting the functional advantage conferred by FF enrichment.

3.3 Potential antidiabetic effect *in vitro*: enzyme inhibition

The bioaccessible fractions were tested for the inhibitory activity towards the digestive enzymes α-amylase, α-glucosidase, and DPP-IV (Table 4).

All bioaccessible fractions from innovative crackers showed higher enzyme inhibition than control cracker, which was not effective in reducing the activity of α-amylase and α-glucosidase. The inhibition rate of α-amylase ranged from 30% to 50%. Similarly, Arnal *et al.*[58] reported an inhibition rate of around 60% for the bioaccessible fractions of cooked chickpeas, whereas values of up to 30% were obtained for fermented chickpea-based beverage by Zhu *et al.*[59] Interestingly, the 25% enrichment with both FF and CF led to lower α-amylase inhibitory activity compared with 10% enrichment, with the overall ranking being C10FF = C10CF > C25FF > C25CF. At higher levels of chickpea flour incorporation, the increased contents of starch, protein, polysaccharide, and dietary fiber may limit the release or accessibility of inhibitory compounds during digestion or promote non-specific interactions that reduce effective enzyme inhibition. Indeed, polyphenols are known to interact with proteins and soluble polysaccharides both before and after reaching the small intestine, thereby reducing their availability to interact with digestive enzymes and attenuating α-amylase inhibition.[60] In addition, processing-induced compounds such as melanoidins may contribute to enzyme inhibition in a non-linear manner, partially masking dose-dependent effects attributable to polyphenols alone.[61] Studies on fermented baked products, including sourdough breads, generally report α-amylase inhibition values below 20% following *in vitro* digestion.[62] In this context, the α-amylase inhibition observed for the innovative crackers in the present study (approximately 30–50%), at both low and high inclusion levels, exceeds that commonly reported for fermented cereal-based baked products and may therefore be functionally relevant in modulating starch digestion. In contrast, all samples exhibited relatively weak α-glucosidase inhibitory activity, with values ranging from 3% to 8%. The highest inhibition was observed for FF (approximately 8%), followed by C25FF and CF, while no significant differences were detected among the other enriched crackers ($p > 0.05$). The slightly higher α-glucosidase inhibitory activity of FF and C25FF compared with their unfermented counterparts may be attributed to increased levels of glycosylated phenolic compounds, including glucosides of protocatechuic and sinapic acids, as well as kaempferol glucoside. This interpretation is consistent with the findings of Zhu *et al.*,[59] who reported greater α-glucosidase inhibition for glycosylated polyphenol fractions than for free polyphenols in fermented chickpea-based beverages.

Table 4 Activity of bioaccessible fractions of crackers and flours against the digestive enzymes α-amylase, α-glucosidase, and dipeptidyl peptidase-IV (DPP-IV)

Sample	α-Amylase	α-Glucosidase	DPP-IV
CF	30.2 ± 1.6^{d}	6.1 ± 0.6^{b}	20.8 ± 0.08^{a}
FF	46.6 ± 0.2^{b}	8.4 ± 0.3^{a}	19.4 ± 0.2^{b}
CW	—	—	3.8 ± 0.3^{e}
C10CF	49.2 ± 1.1^{a}	3.4 ± 0.2^{c}	11.8 ± 0.1^{d}
C25CF	32.8 ± 0.3^{c}	4.1 ± 0.5^{c}	11.7 ± 0.1c
C10FF	50.2 ± 0.5^{a}	4.0 ± 0.3^{c}	12.0 ± 0.1^{d}
C25FF	45.4 ± 0.1^{b}	8.3 ± 0.8^{a,b}	12.2 ± 0.1^{d}

CF: cooked chickpea flour; FF: fermented cooked chickpea flour; CW: 100% wheat-based cracker; C10CF: 10% cooked chickpea flour-enriched cracker; C25CF: 25% cooked chickpea flour-enriched cracker; C10FF: 10% fermented cooked chickpea flour-enriched cracker; C25FF: 25% fermented cooked chickpea flour-enriched cracker. Data are shown as % of inhibition (mean ± SD). Different letters indicate differences among the bioaccessible fractions within the same column for each digestive enzyme assessed by One-way ANOVA and Tukey's *post hoc* ($p < 0.05$).

Among the tested flours, CF exhibited the highest DPP-IV inhibitory activity (21%), followed by FF (19%), with the difference being small but statistically significant. This result is consistent with our previous characterization of chickpea flours fermented with different LAB strains,[20] in which unfermented flour contained a higher abundance of peptides with predicted DPP-IV inhibitory potential than most fermented counterparts. Although LAB fermentation is often reported to enhance the release of DPP-IV inhibitory peptides from legume substrates, the higher activity observed for CF may reflect more efficient peptide release during simulated gastrointestinal digestion or the effects of processing steps, such as mixing and thermal treatment, which can promote protein denaturation and the generation of short bioactive peptides.[63–65] In enriched crackers, DPP-IV inhibitory activity was, on average, 3.2-fold higher than in the control wheat-based cracker (CW), with C25CF showing the strongest effect. These findings align with previous studies by Lammi *et al.*[66] and Bollati *et al.*,[67] which reported DPP-IV inhibition values ranging from 8% to 82% for legume protein hydrolysates, particularly from soybeans and peas. Chickpea proteins are especially rich in hydrophobic amino acids (approximately 35% of total residues), a feature that enhances their potential as DPP-IV inhibitors, given the enzyme's preference for peptide bonds adjacent to hydrophobic residues such as proline and alanine.[68] Overall, DPP-IV inhibition is nutritionally relevant because it contributes to the preservation of incretin hormones, including glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which play a key role in postprandial glucose regulation. Collectively, these results support the potential of chickpea-based crackers to modestly modulate enzymes involved in postprandial glucose metabolism. Nevertheless, further *in vivo* and clinical studies are required to determine whether these *in vitro* effects translate into physiologically meaningful benefits relevant to glycaemic control and diabetes-related metabolic outcomes.

3.4 Cytotoxicity effect of bioaccessible fractions on Caco-2 cells

Caco-2 cells were exposed to both undiluted and serially diluted bioaccessible fractions to evaluate their potential cytotoxicity based on the effect on cellular viability. As shown in Fig. S2, neither diluted nor undiluted digests had cytotoxic effect on Caco-2 cells, with cell viability ranging from 68% to 197%. After 24 h and 48 h of exposure, cell viability was generally homogenous, except for FF, C10CF, and C25CF, which on average showed increased viability after 48 h compared to 24 h, by 1.1-, 1.4-, and 1.7-fold, respectively. Based on these results, undiluted bioaccessible fractions were selected to evaluate polyphenol bioavailability and their impact on Caco-2 cell layer permeability.

3.5 Transport of polyphenols in Caco-2 cell line

To study the transport of polyphenols and their potential uptake, the bioaccessible fractions were incubated on Caco-2 cells for 4 h. Their concentrations (ng mL^{-1}) at different transport time points, along with their overall bioavailability, are shown in Fig. 2A, B and C, respectively. As depicted in the heatmap, distinct transport patterns over time (rate) were observed among the various samples, likely determined both by the different matrices (ingredient *vs.* cracker) and by the specific phenolic compound profiles. CF exhibited peaks of total permeated polyphenols after 1 h and 3 h, while the fermented counterpart showed a gradual release over time with a maximum peak at 3 h. At the end of the transport (4 h), the FF had the highest amount of polyphenols permeated compared to CF. Among the monitored compounds, only *p*-hydroxybenzoic acid and pyrogallol were consistently detected across all time points aligning with previous findings.[69]

Noteworthy, the majority of polyphenols were still present in the apical compartment after 4 h.

The presence in the apical compartment of different polyphenols, along with other compounds yet after 4 h, suggest a saturation of specific transporters in the cell monolayer, thereby hindering or preventing permeation to the basolateral side.[70] The CF showed the highest bioavailability of *p*-hydroxybenzoic acid and sinapoyl D-glucoside, with values of 79% and 44%, respectively (Fig. 2A). Conversely, the FF exhibited higher bioavailability of 4-hydroxyphenylacetic acid (62%) compared to CF and contained pyrogallol, which was not detected in the unfermented counterpart. Interestingly, pyrogallol produced through the microbial fermentation, exhibited a bioavailability of approximately 65%, with the permeated concentration reaching a plateau between 2 h and 3 h. This finding is particularly relevant, as pyrogallol is known to reinforce the mucus barrier and attenuate gliadin-induced cytotoxicity and inflammation, in previous *in vitro* studies.[71] Among the two ingredients, no significant differences were observed for protocatechuic 4-*O*-glucoside, showing the highest overall bioavailability among the phenolic compounds. Concerning the crackers, only 3 polyphenols out of 9 were detected following the transport in the basolateral compartment, with differences in abundance depending on both the inclusion level and type of flour (Fig. 2B and C).

Interestingly, for both fermented and unfermented chickpea flour enriched crackers, the predominant permeated polyphenols were the glucosides of protocatechuic and sinapic acids.

This observation is consistent with Guo *et al.*,[72] who showed that lentil-derived polyphenols with a higher degree of glycosylation can permeate the Caco-2 monolayer *via* active transport mediated by the sodium-dependent glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2), both of which are expressed in Caco-2 cells.

These transporters may actively aid in the absorption of mono-glucoside phenolics because of the preferential transportation of glucose/galactose moieties. Consequently, mono-glucoside phenolics have a faster and higher absorption rate than other phenolics lacking active transport and are regarded as competitive substrates for glucose transport by SGLT1 at the same time.[73] Furthermore, aglycone polyphenols may interact with glycosidases expressed by Caco-2 cells, thereby potentially

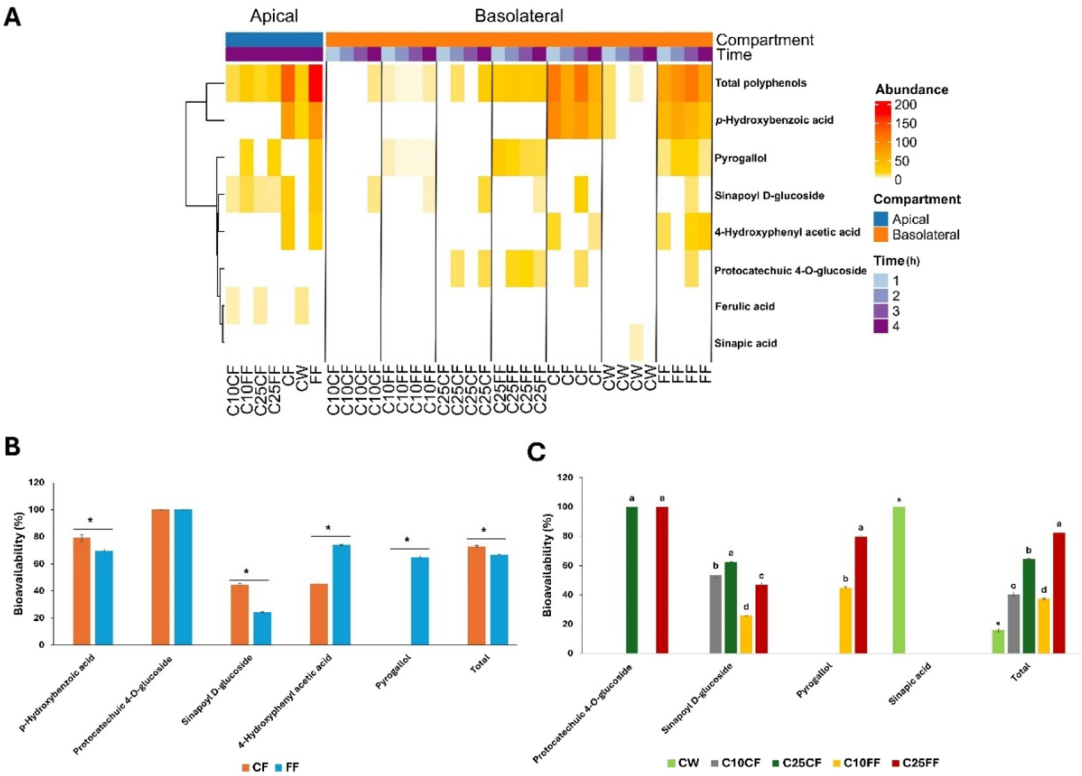

Fig. 2 (A) Heatmap showing the abundance of polyphenols (ng mL^{-1}) detected in the apical (4 h) and basolateral (1 h, 2 h, 3 h, 4 h) compartments over time. The coloured bar across the top of the graph depicts the apical (blue) and basolateral (orange) compartment. The color scale represents the polyphenol abundance with red and white indicating the highest and the lowest abundance, respectively. (B) *In vitro* bioavailability of polyphenols from flours. The results are expressed as % of bioavailability (mean ± SD). * indicates differences among the samples for each phenolic compounds assessed by *T*-test ($p < 0.05$). (C) *In vitro* bioavailability of polyphenols from crackers. Different letters indicate significant differences among the samples for each phenolic compounds assessed by One-way ANOVA and Tukey *post hoc*. CF: cooked chickpea flour; FF: fermented cooked chickpea flour; CW: 100% wheat-based cracker; C10CF: 10% cooked chickpea flour-enriched cracker; C25CF: 25% cooked chickpea flour-enriched cracker; C10FF: 10% fermented cooked chickpea flour-enriched cracker; C25FF: 25% fermented cooked chickpea flour-enriched cracker.

reducing their deglycosylation activity.[74] However, further studies should investigate the specific transport mechanisms of these polyphenols. Consistent with what was observed in the bioaccessible fractions following digestion, the crackers formulated with FF, contained a higher amount of bioavailable polyphenols ranging from 40–80% compared to the wheat-based control (17%). Thus, incorporating the innovative ingredients to crackers led to a significant enhancement of the nutritional value of the final products, such effect was more pronounced in the prototypes made with the innovative FF. Indeed, at both inclusion levels of FF, the bioavailable fractions of the crackers showed appreciable amount of pyrogallol, whose concentration in the basolateral compartment ranged from 2–5 ng mL^{-1} and 8–10 ng mL^{-1} for C10FF and C25FF, respectively, showing a decreasing trend from 1 h to 4 h. Unlike the CF-enriched prototypes, the polyphenols from FF-based crackers showed a gradual permeation pattern over time, with a peak concentration after 4 h (7–32 ng mL^{-1}). Specifically, the C25FF showed a gradual but sustained release of polyphenols over time probably due to the more complex matrix that slowed diffusion while prolonging availability. In general, the release of polyphenols was more abundant in prototypes with higher amount of innovative FF, suggesting that the transport kinetics and efficiency are matrix dependent.[75] On the other hand, the crackers C10CF and C25CF, showed the highest bioavailability of sinapoyl D-glucoside (52% and 65%, respectively), mirroring the CF ingredient. Protocatechuic 4-*O*-glucoside was only found in crackers formulated with the highest inclusion levels (C25CF and C25FF), suggesting that, since it was not detected in the apical compartment after 4 h, enzymatic degradation or metabolic conversion may have occurred *via* enzymes expressed by Caco-2 cells, such as Phase II conjugation enzymes.[76] The same metabolic fate may be hypothesized for 4-hydroxyphenylacetic acid, which was not identified in neither the apical nor basolateral compartments. Moreover, it may be hypothesized that following fermentation and/or digestion the breakdown of chemical bonds occurred along with the formation of new aggregates (dietary fiber–polyphenol, melanoidin–polyphenol, starch-polyphenols, or peptides–polyphenol), which may have limited the availability of polyphenols for cellular absorption.[77–79] Such complexes may act as carriers, allowing polyphenols to reach the large intestine intact, where they may modulate gut microbial composition and promote the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*.[80] This hypothesis is also sup-

ported by the residual TAC observed in the crackers following digestion. Overall, this dual protective and prebiotic role extends the health potential of phenolic-rich matrices beyond their immediate bioavailability in the small intestine. Thus, these results demonstrate that the more complex food matrix can modulate polyphenol bioavailability beyond the structural characteristics of individual compounds.[69]

3.5 *trans*-Epithelial electrical resistance and Caco-2 barrier integrity

To assess whether the transport of polyphenols through the monolayer affected the permeability and therefore the integrity of the monolayer, TEER was measured at 0 h, 2 h, and 4 h. A higher TEER value corresponds to lower permeability, and *vice versa*; therefore, its measurements can provide insights into the modulation of tight junctions' integrity.[81]

The permeability-time curves in Fig. 3 show that the TEER values changed over time from baseline (0 h), ranging between 57% and 124%. After 2 h of exposure, cells treated with FF exhibited a greater reduction in TERR than those treated with CF, suggesting an increase in epithelial permeability. This effect became more pronounced after 4 h and may reflect the progressive permeation of polyphenols across the monolayer, with a maximum detected at 3 h. Consistently, a statistically significant inverse Pearson correlation was observed between the amount of permeated polyphenols and the TEER values over time ($p < 0.05$, $r = -0.47$).

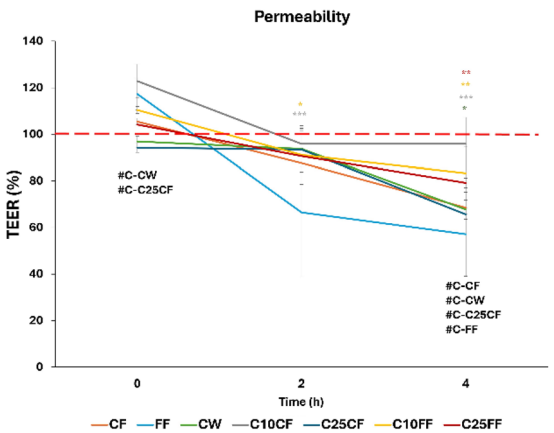

Fig. 3 Permeability-time curves obtained measuring the trans-epithelial electrical resistance (TEER) of Caco-2 cells at time 0 h, 2 h, and 4 h of exposure to dimethyl sulfoxide as control (C) (red dashed line), cooked chickpea flour (CF) (orange line), fermented cooked chickpea flour (FF) (blue line), 100% wheat-based cracker (CW) (green line), 10% cooked chickpea flour-enriched cracker (C10CF) (grey line), 25% cooked chickpea flour-enriched cracker (C25CF) (dark green line), 10% fermented cooked chickpea flour-enriched cracker (C10FF) (yellow line), and 25% fermented cooked chickpea flour-enriched cracker (C25FF) (dark red line). The results are expressed as % of control TEER (mean ± SD). (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ indicated differences between the samples compared to their baseline point (0 h) whereas # indicated differences among the samples and the C at the corresponding time assessed by One-way ANOVA and Tukey *post hoc*.

These findings agree with previous studies reporting that specific flavonoids and flavan-3-ols readily diffuse across Caco-2 monolayers and increase paracellular permeability.[82,83] Although sustained increases in epithelial permeability may theoretically facilitate the transport of both beneficial and potentially harmful compounds, the TEER variations observed in this study are indicative of a transient and functional modulation of tight junction dynamics rather than epithelial damage. This effect is likely associated with the action of phenolic compounds present in FF as previously described.[84,85] Indeed, the modulation of tight junction proteins by polyphenols is considered to be temporary and functional rather than cytotoxic, which is consistent with the absence of cytotoxic effects observed in our experiments. The physiological activity of flavonoids has been linked to their ability to modulate intracellular signalling pathways and enzyme activities, thereby influencing tight junction dynamics and epithelial barrier function.[86] In contrast, crackers exerted a milder effect on epithelial permeability. TEER values for C10CF and C10FF remained relatively stable over time, maintaining approximately 95% and 80% of their baseline values, respectively. Accordingly, these treatments induced only limited modulation of epithelial permeability, with no significant differences compared with the control (red dashed line; $p > 0.05$).

4. Conclusions

This study demonstrated that partially replacing wheat flour with cooked chickpea flour, particularly at a 25% inclusion level, either in its native or *Lent. diolivorans* 13-4A-fermented form, results in innovative crackers with enhanced nutritional properties. Specifically, the use of fermented cooked chickpea flour significantly increased total polyphenol content, improved polyphenol bioaccessibility, and conferred moderate inhibitory effects on digestive enzymes involved in glucose metabolism.

Notably, pyrogallol emerged as a distinctive polyphenol in the fermented flour and its corresponding cracker formulations, highlighting the potential of fermentation to modify and enrich the polyphenolic profile of legume-based products. Alongside synapoyl D-glucoside and protocatechuic 4-*O*-glucoside, pyrogallol was also among the most bioaccessible and bioavailable compounds following digestion, reinforcing the functional benefits of fermentation.

Overall, these findings support the application of *Lent. diolivorans* 13-4A-fermented chickpea flour as a promising functional ingredient for the development of health-promoting baked goods. Further *in vivo* studies are warranted to confirm the physiological relevance and stability of the observed effects.

Author contributions

Manuela Flavia Chiacchio: investigation, writing – original draft, formal analysis, visualization. Silvia Tagliamonte: investigation, writing – review & editing, visualization. Angela

Pazzanese: investigation. Giuseppe Blaiotta: conceptualization, resources, review & editing. Cristina Juan: conceptualization, resources, funding acquisition, review & editing. Ana Juan-Garcìa: conceptualization, resources, funding acquisition, review & editing. Paola Vitaglione: conceptualization, supervision, project administration, funding acquisition, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d5fo05351j.

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