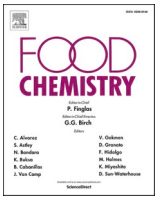
Food Chemistry 404 (2023) 134538

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



In vitro protein bioaccessibility and human serum amino acid responses to white bread enriched with intact plant cells

Natalia Perez-Moral [a], Shikha Saha [a], Ana M. Pinto [b], Balazs H. Bajka [b], Cathrina H. Edwards [a,*]

[a] Food Innovation and Health, Quadram Institute Bioscience, Norwich Research Park, NR4 7UQ Norwich, UK
[b] Biopolymers Group, Departments of Biochemistry and Nutritional Sciences, Faculty of Life Sciences & Medicine, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK

ARTICLE INFO

Keywords:
Legume plant cells
Protein
Amino acids
Bioaccessibility
Bioavailability
INFOGEST *in vitro* digestion
Randomised controlled trial

ABSTRACT

The cell structure and low glycaemic benefits of pulses are compromised by conventional flour-milling. *Cellular* chickpea powders ('CCPs') are a new alternative to pulse flours. Here we investigated the *in vitro* bioaccessibility of essential amino acids ('EAAs') from CCP-enriched bread products and determined the effect of their consumption on serum amino acid responses in healthy humans ($n = 20$, randomised cross-over design). Breads were prepared with 0, 30 and 60 % of the wheat flour replaced by CCP (intact cells containing encapsulated protein). We found that significant proportion of EAAs from encapsulated protein became bioaccessible during *in vitro* duodenal digestion, and that *in vivo* serum EAA responses from healthy human participants were significantly higher following consumption of CCP-enriched breads. Furthermore, the EAA profile of *in vitro* digestion products were well-correlated with *in vivo* peak serum EAAs responses. We conclude that CCP-enrichment of wheat bread improved the amount and diversity of bioavailable EAAs.

1. Introduction

Pulses (dry, edible non-oilseed legumes including beans, chickpeas, peas and lentils) have been identified by the EAT Lancet Commission (Willett et al., 2019) and FAO (Calles, Xipsiti, & del Castello, 2019) as sustainable sources of plant-protein and are critically import to future food security. Currently however, dietary intake of pulses is well below the level recommended by reference diets. Thus, there is a need to develop new pulse-enriched food products that support population and planetary health while also appealing to the mass market (McDermott & Wyatt, 2017).

Pulses contain 17–30 % protein, mainly in the form of salt soluble globulins (legumin and vicilin) and water soluble albumins, with some glutelin and prolamine (alcohol-soluble, glutamin and proline) (Boye, Zare, & Pletch, 2010). Pulse proteins are rich in essential amino acids (EAAs, also known as 'indispensable amino acids'), particularly lysine, which is lacking in cereal proteins (Herreman, Nommensen, Pennings, & Laus, 2020). However, the nutritional value of pulses is more precisely dependant on the extent to which these amino acids ('AAs') are absorbed ('bioavailability'), which in turn depends on their release from protein and its surrounding food matrix into the intestinal lumen ('bioaccessibility') during digestion. Pulse protein digestibility is typically reported to be around 65–70 % (Nosworthy et al., 2017), however there are large variations which seem to depend on their botanical origin as well as processing treatments and digestion methodologies (Adhikari, Schop, de Boer, & Huppertz, 2022; Gu et al., 2022). Previous studies have tended to use protein isolates, pulse flours or homogenised preparations for digestibility testing, however such materials lack the physical structure of whole cooked pulses, including the intact plant cell walls, which may influence protein bioaccessibility (Bhattarai, Dhital, Wu, Chen, & Gidley, 2017; Duijsens et al., 2022; Gwala, Pallares Pallares, Pälchen, Hendrickx, & Grauwet, 2020; Han, Moughan, Li, & Pang, 2020; Rovalino-Córdova, Fogliano, & Capuano, 2019) during digestion.

Notably, the intact primary plant cell walls of legume cotyledons have been shown to protect intracellular starch and protein (within legume cotyledon tissue) from processing conditions (Edwards et al., 2015; Melito & Tovar, 1995) and digestive enzymes (Edwards, Ryden, Mandalari, Butterworth, & Ellis, 2021; Pälchen et al., 2021; Pallares Pallares et al., 2021; Rovalino-Córdova et al., 2019; Verkempinck, Pallares, Hendrickx, & Grauwet, 2020; Xiong, Devkota, Zhang, Muir, &

Abbreviations: CCP, Cellular chickpea powder; EAA, Essential amino acid; AA, Amino acid; FAA, Free amino acid; SP, Small peptides; LPP, Large polypeptides and proteins; TCA, Trichloroacetic acid.

* Corresponding author.
E-mail address: cathrina.edwards@quadram.ac.uk (C.H. Edwards).

https://doi.org/10.1016/j.foodchem.2022.134538
Received 14 July 2022; Received in revised form 16 September 2022; Accepted 4 October 2022
Available online 7 October 2022

Dhital, 2022). Furthermore, recent human studies have shown that food containing intact pulse cells significantly attenuate postprandial glycaemia (Bajka et al., 2021; Golay et al., 1986; Petropoulou et al., 2020) and enhance satiety (Pälchen, Bredie, et al., 2022), compared with food prepared from conventional pulse flours or 'acellular' ingredients. Together, these previous studies indicate that intact plant cells (usually lacking in pulse flour and other refined pulse ingredients) may be important to deliver some of the cardiometabolic health beneficial effects of whole pulse consumption (Kim et al., 2016).

Together, these findings have stimulated interest in the development of novel cellular ingredients, which show promise as a new means of developing food products with low glycaemic properties (Berry et al., 2020; Edwards et al., 2020; Pallares Pallares et al., 2021; Verkempinck et al., 2020; Xiong et al., 2022). However, such ingredients rely on the low bioaccessibility of starch from within legume cells, and there is a need to understand the implications for bioaccessibility and bioavailability of the co-located protein. Our hypothesis was that replacing wheat flour with encapsulated legume protein would alter the amount and types of bioaccessible and bioavailable amino acids from bread, and that the effects on EAA release during *in vitro* (INFOGEST) digestion would be reflected in postprandial amino acid responses measured in human serum. We have recently reported on the beneficial low glycaemic effects of using novel intact legume plant cell powders as more slowly digested pulse-based alternatives to refined ingredients in bread products (Bajka et al., 2021), however, the implications for protein digestion and nutrition have not yet been studied. Our aims were therefore to i) investigate the release of amino acids during simulated *in vitro* digestion of breads enriched with chickpea cells, and ii) compare these to postprandial serum amino acid responses following human consumption.

2. Materials and methods

2.1. Bread rolls

Three different bread types, denoted B0, B30 and B60, were prepared by replacing 0, 30 or 60 % of white wheat bread flour with a proprietary cellular chickpea powder 'CCP' (tradename PulseON®, patent pending WO 2019/155190/A1, Pulseon Foods Ltd, Chesterfield, UK) (Edwards et al., 2020). Full details of bread preparation, macronutrient composition and palatability have been described elsewhere (Bajka et al., 2021), but the present study is the first to report details of their protein digestibility and EAA bioaccessibility and bioavailability.

2.2. In vitro digestion

Each bread type was subjected to simulated *in vitro* digestion following the INFOGEST 2.0 method (Brodkorb et al., 2019). In each digestion, 0.228 g of bread dry matter (crumbs, 1–2 mm size, obtained after blending and sieving) and 0.572 mL water were mixed with simulated fluids that mimic the conditions of electrolytes, pH, bile salts and enzymes in the oral, gastric and duodenal phase. Individual enzymes i.e. salivary amylase, pepsin, trypsin, chymotrypsin, pancreatic amylase and bile salts at the specified activities or concentration were added to an individual tube for each time point. Digestions were performed in triplicate for B30 and B60 and in duplicate for B0. A blank digestion containing all the enzymes and fluids with water instead of breadcrumbs was included in each run to account for the background values of the fluids, enzymes and bile salts employed. Digestions were performed to obtain a sample of the digesta at the end of the oral phase, after 30 min and 60 min of gastric phase and at 0, 5, 10, 20, 30, 60 and 120 min of small intestinal digestion. At each time point, the digestion was stopped by addition of 0.3 M sodium carbonate (to inhibit amylases and pepsin) and 0.1 M pefabloc (to inhibit proteases trypsin and chymotrypsin in intestinal samples). The samples were frozen at −70 °C immediately afterwards.

2.2.1. Fractionation of in vitro digesta

Samples of digesta collected at each time point were treated as per Fig. 1 to fractionate the solubilised components present in digesta supernatant into free amino acids (FAA), small peptides (SP), large polypeptides and solubilised proteins (LPP), using a modified method based on previously published methods (Pälchen et al., 2021; Roux et al., 2020). Basically, the *in vitro* digesta samples obtained at each time point were centrifuged at 3000×*g* for 10 min at 4 °C to exclude undigested food and big particulates from the supernatant. The resulting supernatant was then split into 3 aliquots which were differently processed (Fig. 1) and analysed for amino acids (AAs) content. The AAs present as 'Free Amino Acids' (FAA), 'Small Peptides' (SP), or 'Large polypeptides and proteins' (LPP) were then calculated from each fraction by applying the following equations (**Equation 1ABC**):

$$FAA = FAA_{fraction1} \quad (1A)$$

$$SP = (FAA + SP)_{fraction2} - FAA_{fraction1} \quad (1B)$$

$$LPP = (FAA + SP + LPP)_{fraction3} - (FAA + SP)_{fraction2} \quad (1C)$$

Equation 1 - Calculation of amino acids present as Free Amino Acids (FAA), Small Peptides (SP) and 'Large proteins and polypeptides' (LPP) using the amino acid content of protein fractions referred to in Fig. 1.

For determination of AAs present as FAA (*'fraction 1' in* Fig. 1), the aliquoted supernatants were analysed directly by LC-MS/MS *(see Section 2.2.2)*. For determination of AAs present as FAA or contained within SP (*'fraction 2' in* Fig. 1), the aliquoted supernatant was diluted 1:1 with Trichloroacetic acid ('TCA') 6.4 % before mixing and centrifuging at 5000g for 30 min to exclude precipitated peptides and proteins and splitting the supernatant into three × 1 mL aliquots, which were each subjected to different hydrolysis conditions (*a, b* or *c,* described below), and, as recommended by the AOAC method 982.30 E(a,b,c) for amino acid analysis, the highest value of each AA obtained by any of these hydrolyses was taken: *a*) acid hydrolysis by incubation with 1 mL of 6 M HCl at 110 °C for 24 h to analyse most amino acids; *b*) basic hydrolysis by incubation with 1 mL saturated 0.4 M $Ba(OH)_2$ at 110 °C for 24 h (to analyse tryptophan) and *c*) pre-oxidation by incubation with 1 mL of freshly prepared performic acid (9:1 formic acid:30 % hydrogen peroxide) left to oxidize for 20 h at 4 °C before adding 0.17 g $Na_2S_2O_5$ to decompose the performic acid, and then followed by an acid hydrolysis in 2 mL of 6 M HCl at 110 °C for 24 h (to analyse sulphur-containing amino acids methionine and cysteine). For determination of AAs contained in FAA, SP and LPP (*'fraction 3'* in Fig. 1) the supernatant was hydrolysed following the acid and basic conditions described above. After the hydrolyses described in *aliquots 2 and 3*, the solvent in each sample was evaporated from the samples using a centrifugal rotaevaporator (Genevac EZ-2 Elite Personal Evaporator) and the solid resuspended in 1 mL of ultrapure water before being filtered and analysed by LC-MS. Total Nitrogen was also measured by combustion using an Exeter CE440 CHN Elemental Analyser and multiplied by the standard protein conversion factor 6.25 (although other conversion factors could also be applied as suggested by Mariotti et al., 2008) to estimate the amount of protein in undigested samples ('before'), and pellets recovered at the end of the gastro-intestinal digestion (after 120 min small intestinal or 'duodenal' digestion).

2.2.2. Amino acid extraction and quantification

Extraction for amino acids analysis in plasma and digesta samples was adopted from Kok, Nix, Nys, & Fillet, 2019. Briefly, isotope labelled internal standards (canonical amino acid mix, Cambridge Isotope Laboratories, Inc. Massachusetts, USA) dissolved in 90 μl of 60 % acetonitrile were added to 10 μl sample/calibration standards, vortexed and kept at 4 °C for 5 min. Samples were then centrifuged at 13000 × g at 4 °C for 10 mins. Targeted amino acids in the supernatant were analysed using a HILIC column in an Agilent® 1260 Infinity LC system coupled to a 6490 triple quadrupole mass spectrometer with an Agilent® Jet

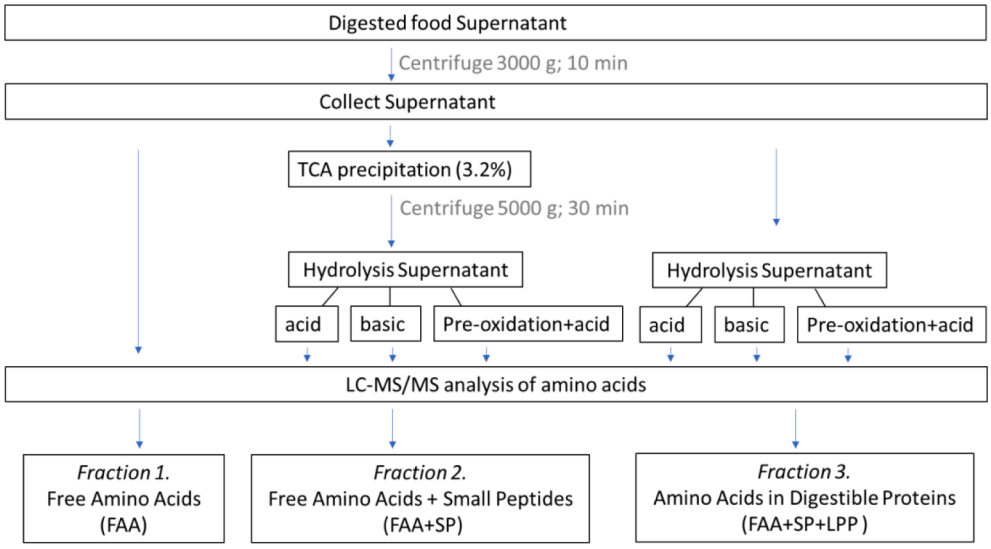


Fig. 1. Fractionation of *in vitro* digesta. This approach separates proteolytic products of digestion into fractions containing 1-free amino acids (FAA), 2-FAA and small peptides (SP), and 3-FAA, SP and large polypeptides and solubilised protein (LPP), which are relevant for estimations of AA bioaccessibility. Modified/Adapted method based on (Pälchen et al., 2021).

Stream source (Santa Clara, USA).

The amino acid separation was carried out according to the chromatographic method described by Prinsen and colleagues (Prinsen et al., 2016) using a programmed gradient mobile phase after injecting 1 μl of extracted sample. The amino acids were detected by multiple reaction monitoring (MRM) mode using positive electrospray ionization. The source conditions were as follows: dry gas temperature 200 °C; dry gas flow 16 L/min; nebulizer pressure 50 psi; capillary voltage 3500 V; sheath gas temperature 300 °C; sheath gas flow 11 L/min; nozzle voltage 1000 V; high pressure RF 150 V; and low pressure RF 60 V. Collision energies (CE) were optimized for the amino acids transitions of interest. All transitions were used as qualifiers by automatic detection on specific retention time windows. One transition was used as quantifier.

Quantification was performed using the concentration vs peak area ratio (the integrated peak area of the analyte relative to that of the internal standard); calibration curve and data were processed with MassHunter Workstation Quantitative Analysis software (version 10.0, Agilent Technologies). These analyses were performed for measurement of the 9 EAAs; Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Threonine (Thr), Tryptophan (Trp) and Valine (Val), and for the following non-EAAs; Alanine (Ala), Arginine (Arg), Asparagine (Asn), Aspartic acid (Asp), Betain ('Bet'), Cystine (Cys), Glutamic acid (Glu), Glutamine (Gln), Glycine (Gly), Hydroxy-Proline (Hyp), Ornithine ('Orn'), Proline (Pro), Serine (Ser), Tyrosine (Tyr). Throughout this manuscript, the term 'total amino acids' ('total AAs') refers to the sum of the EEAs and the aforementioned non-EEAs, which were selected based on their nutritional relevance.

2.2.3. Estimations of amino acid bioaccessibility

Bioaccessible EAAs are defined here as the proportion of EAAs that had been released from the food matrix and are in a form that is potentially available for absorption. Non-food sources of EAAs were subtracted from the total by using values obtained during 'blank' digestion runs. We consider that food-derived EAAs within the FAA fraction would likely be bioaccessible, but those within the SP fraction could also be considered bioaccessible, particularly if digested by brush border peptidases that are present *in vivo*, but were not included in the *in vitro* digestion model. To explore this further, we calculated EAA bioaccessibility in two ways using the measured EAAs within the FAA fractions without (**Equation (2A)**) or with inclusion of small peptides (**Equation (2B)**) fractions of digesta. The same equation and principles were also applied to calculate bioaccessibility of individual amino acids (i.e. using only the values for the AA of interest rather than the sum of all AAs). The basis for bioaccessibility calculations is defined in the Figure legends.

$$\boldsymbol{BioActotalEAAasFAA}(\%) = \frac{\boldsymbol{EAA}_{\boldsymbol{FAAt}}}{\sum \boldsymbol{EAA}_i} \boldsymbol{x} 100 \quad (2A)$$

$$\boldsymbol{BioActotalEAAasFAA + SP}(\%) = \frac{\boldsymbol{EAA}_{\boldsymbol{FAAt}} + \boldsymbol{EAA}_{\boldsymbol{SPt}}}{\sum \boldsymbol{EAA}_i} \boldsymbol{x} 100 \quad (2B)$$

Equation 2 – Total EAA bioaccessibility is calculated as the sum of measured amounts of food-derived EAAs (μg released per mg bread dry matter) in either the FAA fractions (EAA $_{FAAt}$) as in eqn. (1A), or within the SP + FAA fractions, eqn. (1B), after 120 min of duodenal digestion, divided by the total amount of EAA present in the original bread product (μg/mg bread dry matter).

2.2.4. Imaging

Images of breads were produced using a Zeiss LSM880 confocal laser scanning microscope and processed using ZEN Blue software. Samples of digested breads resuspended in water were mixed with 10 μl of 1 mg/mL solution of calcofluor-white and fast green FCF (Sigma-Aldrich Co, Poole, UK) to stain cell walls and protein respectively for 10 min before centrifuging for 5 min at 5000g. The pellet was resuspended and rinsed 3 times with water before placing an aliquot on a glass slide. The samples were imaged using laser excitations of 405 nm and 561 nm with detection wavelengths of 568–712 nm and 410–493 nm for calcofluor and fast green, respectively.

2.3. Human postprandial blood sample collection

This investigation used blood samples collected from healthy human participants (n = 20) following consumption of B0, B30 and B60 bread types as part of a previous study. This previous human study was conducted in accordance with the Declaration of Helsinki and approved by the relevant research ethics committee (HR-18/19–8431, BDM Research Ethics Subcommittee at King's College London) in the UK. All participants gave their written informed consent. The study was registered at clinicaltrials.gov as NCT03994276, and full details of the study protocol have been published elsewhere (Bajka et al., 2021).

In brief, the study followed a double-blind, randomised, controlled,

cross-over design, in which each participant consumed each bread type on separate study visits in random order. Each bread was served as a bread roll together with 20 g of no-added sugar strawberry jam (energy reduced strawberry jam with sweetener, Marillo Foods ltd., West Yorkshire, UK) and a drinking glass with water. For each visit, participants arrived having fasted for 12 h to the Metabolic Research Unit, Department of Nutritional Sciences, King's College London, where the study was taking place between August 2019 and January 2020. Upon arrival, a trained phlebotomist inserted a venous cannula in a vein in the antecubital fossa or a forearm vein for subsequent venous blood sampling. Blood samples used for amino acid analysis were collected into BD Vacutainer® SST serum tubes before test meal consumption (−15 min) and at 15, 30, 45, 60, 90, 120, 180 and 240 min postprandially. All samples were centrifuged at 1300 × g, 4 °C for 15 min and aliquots of the supernatant stored at −80 °C prior to amino acid analysis (see Section 2.2.2 for details of amino acid quantification).

2.4. Data and statistical analysis

Graphical and statistical analysis were performed using GraphPad Prism 9.0 for Windows (Version 9.3.1, GraphPad Software, LLC), except for radar plots, which were produced in Microsoft® Excel® for Microsoft 365 MSO (Version 2202, Microsoft Corporation). *In vitro* data is reported as mean of duplicate or triplicate runs, as specified in the methods. Food-derived AA concentrations measured in the digesta were expressed per 'mg bread dry matter' or 'per roll served' to facilitate comparison to the human study data. Human study data is reported as mean with SD, unless otherwise specified. Outliers were identified and excluded from group mean serum total AA data by performing ROUT (Q = 1 %). Postprandial responses (i.e. change from fasted values following meal consumption) were calculated by subtracting fasted values (-10 min) from measured values at subsequent time points within each individual. ΔPeak, defined here as the maximum postprandial rise in AA concentration, were then calculated for each individual participant for each AA following after each bread type. For statistical analysis, mixed-effects ANOVA was performed on repeated measures data, with time and treatment (bread type) as fixed-effects and individual differences as random-effects. Geisser-Greenhouse correction was applied for violation of sphericity. *Post-hoc* tests were performed when significant main effects were detected, and Tukey's correction for multiple-comparisons applied (multiplicity-adjusted *P*-values are reported). Pearson's two-tailed correlation test was performed to compare *in vitro* and *in vivo* EAA profiles. For graphical representation, the group means were plotted with error bars as SEM. The number of participants (*n*) who's data were included in each analysis is reported in the text or figure legends.

3. Results

3.1. Protein content and amino acid composition of ingredients and bread products

Protein content and amino acid composition of ingredients and bread products is shown in Fig. 2. The macronutrient composition of bread products is shown in Fig. 2**A**. The control wheat bread (B0) contained 17.0 g protein/100 g dry matter(DM), and replacing 30 or 60 % (w/w) of the wheat flour in the formulation with CCP increased the protein content to 20.2 and 25.5 g protein/100 g DM for B30 and B60 respectively (protein by Dumas method, N × 6.25, data supplied by ALS). These values were consistent with our in-house calculation of protein content from analysis of total Nitrogen of the bread products (17.1, 20.1, and 23.8 g protein/100 g DM for B0, B30 and B60, respectively) and within 5 % theoretical values calculated from the ingredient composition. Protein (17KJ/g) accounted for 16.8, 20.0 and 25.3 % of the total energy value in B0 (1715.2 KJ/100 g DM), B30 (1712.1 KJ/100 g DM) and B60 (1710.2 KJ/100 g DM), respectively. White wheat flour (17.1 g protein/100 g ingredient DM), CCP (20.3 g protein/100 g ingredient DM) and added wheat gluten (83.6 g protein/100 g ingredient DM) were the main ingredients of the bread recipes and the main protein source in the bread products, with a remaining < 7 % of protein coming from yeast and other sources **(**Fig. 2**B).**

Based on the EAA composition of these ingredients, we calculated that the proteins in the CCP (cellular chickpea powder) contained a higher proportion of EAAs (~33.5 % EAAs/total AAs) compared with wheat protein in white wheat flour (22.0 % EAAs) and gluten (25.2 % EAAs). The EAA composition (% of total protein basis) of ingredients is shown in the **radar plot,** Fig. 2**C.** Compared to the wheat proteins, CCP protein contained higher proportions of all other EAA, with exception of methionine and tryptophan which were a minor component of all ingredients. In the CCP ingredient, Leucine, Lysine, and Phenylalanine were the EAAs present in the highest amounts. Wheat flour was also high in Leucine, but low in Lysine. In the breads, EAAs accounted for ~ 29, 28 and 27 % of total AAs in B0, B30 and B60, and the AA composition reflected that of the ingredients (Fig. 2**D, radar plot).** Replacing bread

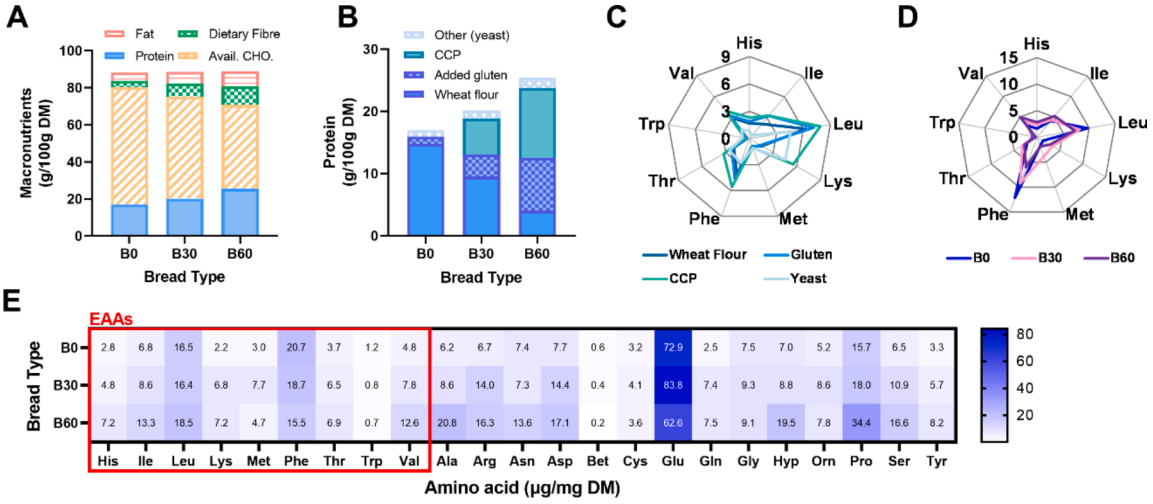


Fig. 2. Bread and ingredient composition. Macronutrient composition of bread (A) and contribution of ingredients to bread protein content (B) for breads B0, B30 and B60, in which CCP replaced 0, 30 or 60 % (w/w) of wheat flour, respectively. Radar plots show the essential amino acid composition (EAA) of the protein component (mg EAA/100 mg protein) of ingredients (C) and breads (D). Heatmap (E) shows the amino acid content per bread dry matter (DM) for all amino acids measured in each bread type, with darker colour intensity for higher values.

wheat flour with CCP and gluten increased the total EAA and non-EAA content (Fig. 2 **E**).

3.2. *In vitro digestion of breads*

The appearance of proteins and proteolytic products in the digesta following *in vitro* digestion of each bread type is shown in Fig. 3. In all bread products, a rapid release of protein occurred during the early gastric phase; this process occurred more rapidly and to a greater extent in in the control bread (B0, Fig. 3**A**) than in the chickpea-enriched breads (B30 and B60, Fig. 3**B and C**). At the end of the gastric phase (60 min), 61 % of the initial protein in B0 had been released from the food matrix, but was still primarily in the form of large proteins or polypeptides. For B30 and B60, the hydrolysis of the proteins in the gastric phase was lower accounting for 38 and 46 % of the total protein respectively, but here the released protein was mainly in the form of small peptides, with very low amounts of free amino acids released. In the duodenal phase, the amount of small peptides and free amino acids in the digesta increases for all bread products. The most rapid rate of change occurred within the first 20 min of meal exposure to duodenal conditions. The release of free AA in the small intestine was quicker in breads containing chickpea powder compared to breads made only with wheat flour, reaching stable values in B60 and B30 after 60 min of intestinal digestion whereas B0 followed a slower and lower production of AA, with the curve beginning to approach a plateau from around 90 min. At the end of the duodenal digestion, ~99 % of the protein from B0 had been released and digested into small peptides (65 % of initial protein), and free AAs (34 % of initial protein). For B30 and B60, ~90, and 88 % of the protein had been released and digested into small peptides (~62 %, 58 %) and free AAs (~28 %, 30 %). This implies a lower release, solubilisation and/or digestibility of protein in the chickpea-enriched breads.

The release of free essential AA was followed during the digestion at different time points (Fig. 3**DEF**). The levels of the other free essential AA released at the end of the oral and gastric phase were negligible with the exception of tryptophan (0.10; 0.20 and 0.34 ug tryptophan/mg bread dry matter were released at the end of oral phase in B0, B30 and B60). However, as soon as the digesta entered the *in vitro* duodenal phase, the presence of the nine EAAs released into the aqueous digesta started to increase. At D120 the amounts of free Phe, Leu and Lys increased with the increasing content of chickpea powder in composition of the breads. The release of Ile, Met and His was lower in B30 and B60 than in B0, whereas Val, Thr and Tyr were released at slightly lower amounts in B30. Overall, bread B60 released more free EAAs than the other two breads, only matching values of threonine in B0.

Based on the sum of amino acids present within small peptides and free amino acids fraction, ~ 99, 90 and 88 % of the total AAs in B0, B30 and B60 could be considered bioaccessible. A similar effect was observed with regard to the total N analyses, which showed that 96, 97 and 93 % of total N in B0, B30 and B60 had been released at the end of the duodenal digestion. Our data are also consistent with confocal imaging of bread samples collected from the oral, gastric and duodenal digesta (Fig. 3**G**) revealed a high proportion of cell wall encapsulated protein within breads containing CCP during oral and gastric digestion, but after 120 min of duodenal digestion, this encapsulated protein was no longer apparent within the cells.

Thus, a high proportion of the EAAs and N that constitutes the encapsulated CCP protein appears to be digested and released from the

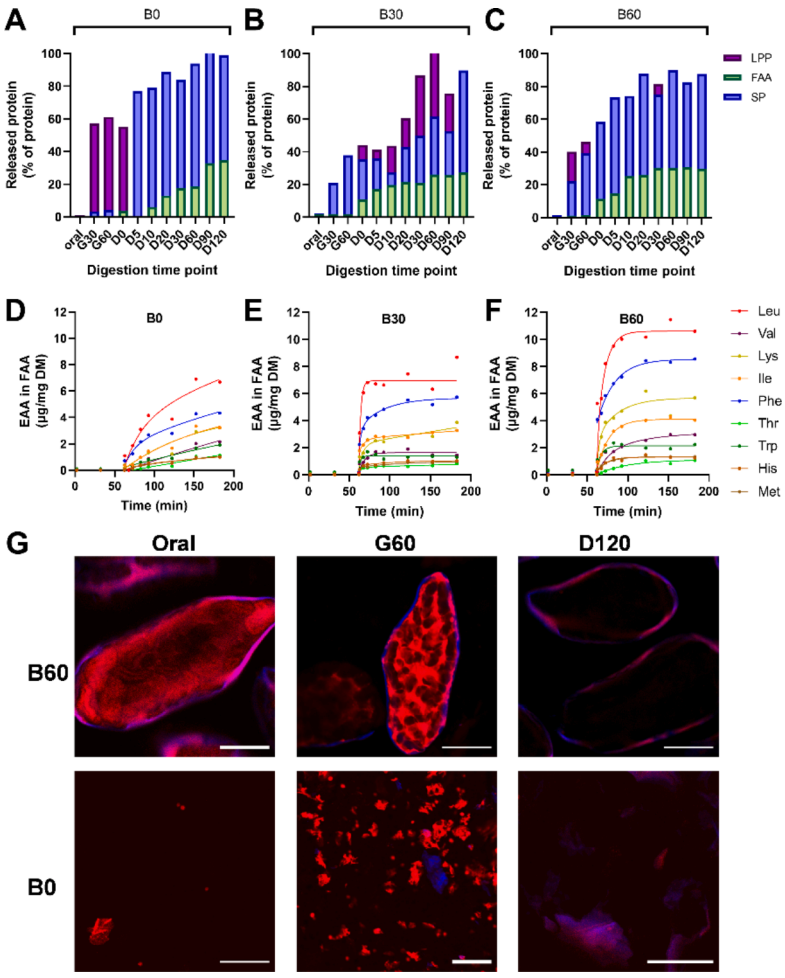


Fig. 3. Release of proteolytic products from *in vitro* bread digestion. Stacked bar charts (ABC) show free amino acids ('FAA'), small peptides ('SP') and large polypeptides and proteins ('LPP') measured at different time points in the digesta supernatant after oral ('O'), gastric ('G') and duodenal ('D') digestion for each bread type (B0, B30 and B60). XY scatter plots (DEF) show increasing concentration of individual EAAs within the FAA fraction with time, and the legend in F applies to DEF. Data points in DEF are overlayed with a trendline fitted for each individual EAA by robust non-linear regression to an exponential two-phase association equation. Confocal images (G) show protein (red) encapsulated within plant cell walls (blue) of CCP (as seen here in B60) or in the surrounding wheat matrix (B0) at different stages of digestion; Oral, -after 2 min oral; G60- after 60 min gastric; D120- after 120 min duodenal digestion. Scalebar 50 µm.

food matrix as SP and FAA within 2 h of duodenal digestion. Consequently, replacing wheat flour with encapsulated chickpea protein (CCP) resulted in higher amounts of bioaccessible EAAs.

3.3. Human serum amino acid responses to breads

Amino acid concentrations were measured in human serum for up to 4 h after consumption of B0, B30 and B60. Fig. 4 shows the mean total AA (Fig. 4**A**) and EAA (Fig. 4**B**) serum concentrations measured during the postprandial period after each bread type. EAAs accounted for ~ 27, 29 and 31 % of the postprandial rise in total serum AA for B0, B30, B60 at ΔPeak (Fig. 4**C**).

The highest total amino acid concentration was most commonly observed at 60 min, regardless of bread type. However, there were some different temporal patterns for individual amino acids (Fig. 4**. DEF**), for example, phenylalanine tended to peak later than 60 min while leucine peaked earlier, while lysine tended to peak later with higher CCP-content.

The maximum postprandial increase in serum EAA concentrations (ΔPeak) differed significantly between bread types. Total AA were 49 and 83 % higher following the B30 and B60 compared to the control. At an individual amino acid level (Fig. 4**G-O**), ΔPeak for Leucine increased significantly with increasing dose of CCP; isoleucine, phenylalanine and histidine were significantly higher for CCP enriched breads compared with the control bread, but the difference between 30 and 60 was not significant. For lysine, valine, tryptophan and methionine ΔPeaks for B60 was significantly higher than the control. ΔPeak for threonine was not significant between bread types**.** Postprandial responses for each individual amino acids (non EAA and EAA) are shown in **Supplementary** Fig. 1**.**

3.4. In vitro – In vivo comparisons

The serving-size adjusted bioaccessible EAAs are shown for each EAA in Fig. 5 alongside Δpeak EAA concentrations measured in human serum following consumption of the same bread rolls. For the *in vitro* data, EAAs within the FAA fractions would be expected to be bioavailable, however it is also possible that the EAAs within the small peptides would rapidly become bioavailable (e.g., through the action of brush-border peptidases that are not represented in the simulated digestion procedure). The FAA (Fig. 5**A**) and FAA + SP (Fig. 5**B**) fractions were therefore plotted separately for comparison of these to human serum levels of absorbed EAAs (Fig. 5**C**). The EAA profile of the bread products (Fig. 5**DEF**) was weakly correlated with the *in vivo* serum EAA profile

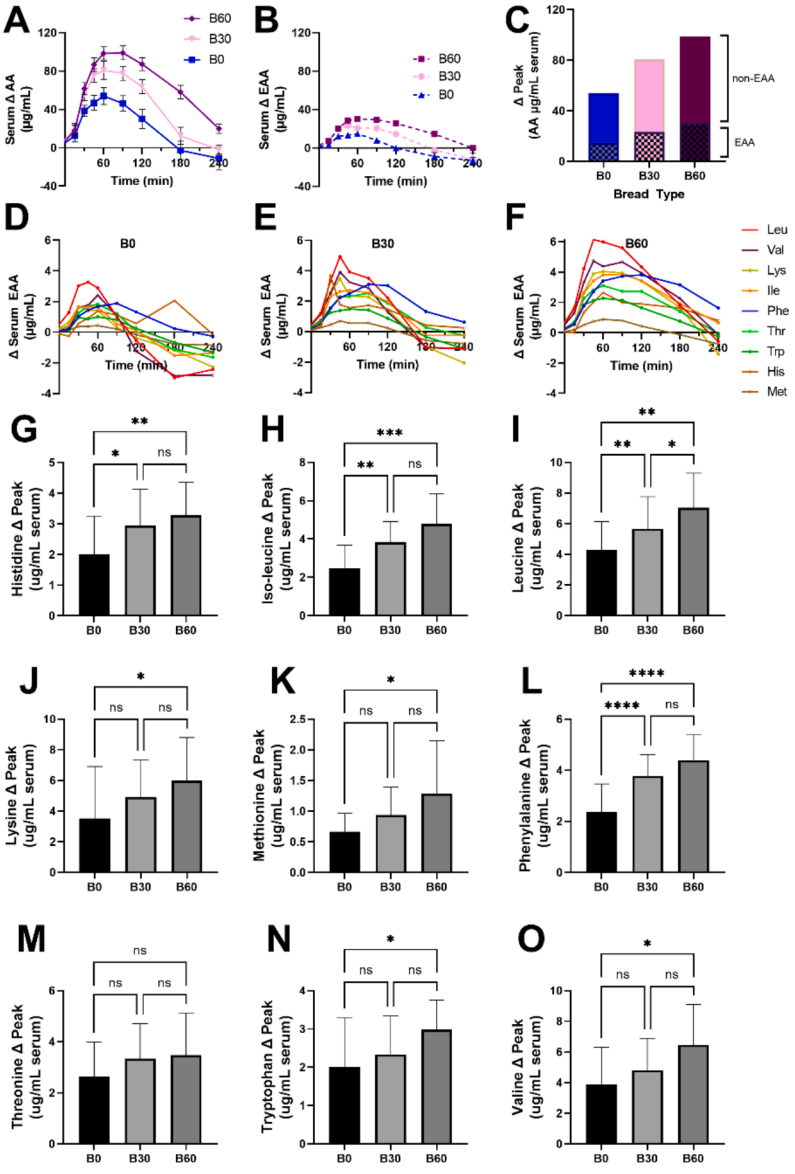


Fig. 4. Human serum amino acid response to breads. Postprandial responses (change from fasted baseline) following consumption by healthy humans of breads in which CCP replaced 0, 30 or 60 % (w/w) wheat flour (B0, B30 or B60), shown as group mean (n = 20 for B30 and B60, n = 19 for B60) with SEM for total measured amino acids (A) and essential amino acids (B). Stacked bar chart shows maximum postprandial rise from fasted concentrations (ΔPeak) for EAAs and measured non-EAAs (C). Mean postprandial responses are shown for each EAA and following each bread type in DEF. Bar charts (G-O) show effect of bread type on ΔPeak for each EAA. Significant differences determined by repeated measures ANOVA with Tukey's post-hoc test are annotated as follows; p < 0.01*, p < 0.05** and p < 0.001***, p < 0.0001****, ns- not significant.

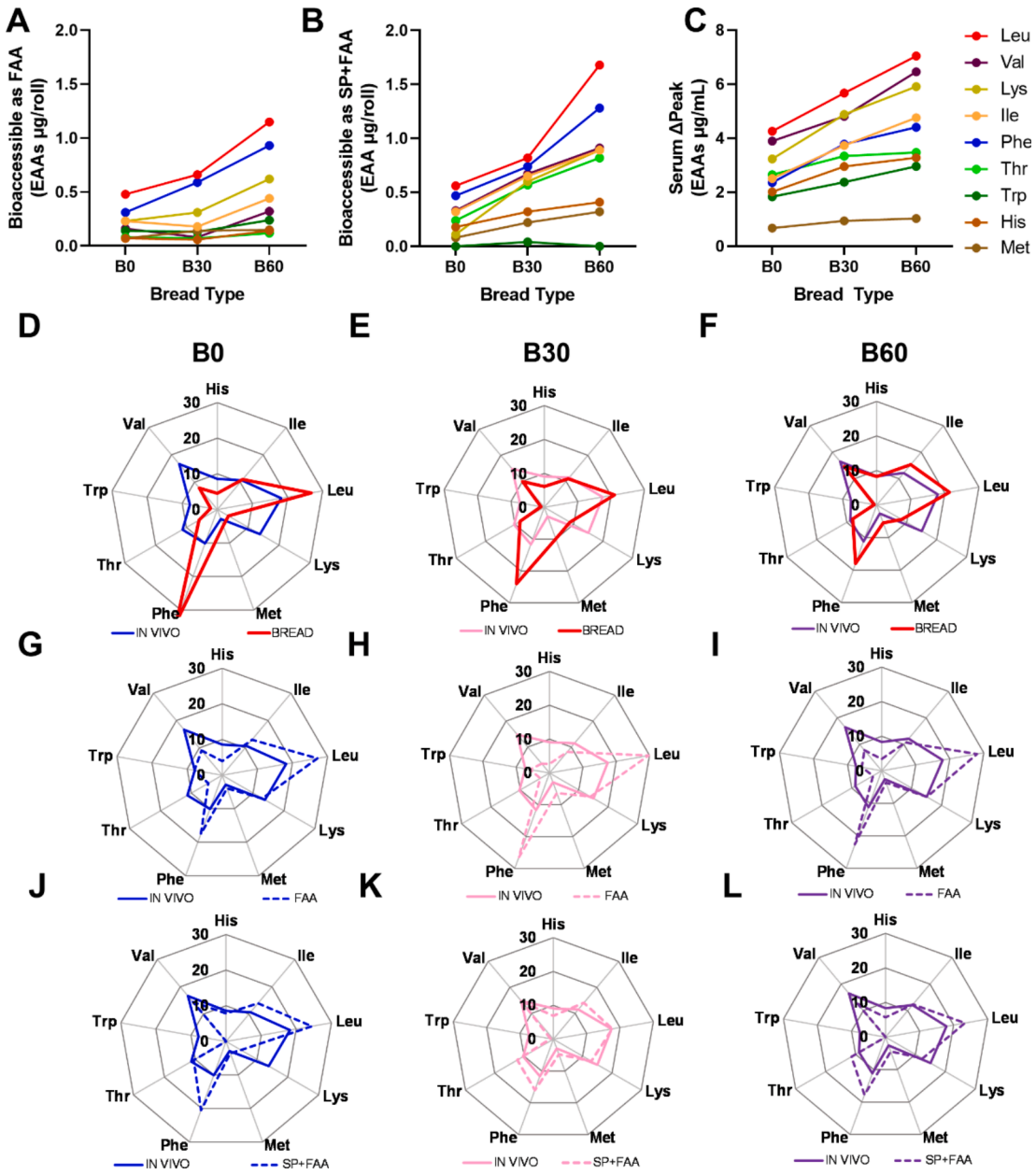


Fig. 5. Bioaccessible EAAs *In vitro* versus Serum EAAs *in vivo*. The amount of bioaccessible EAA within the free amino acid ('FAA', A) and/or small peptides fraction ('SPP', B) of digesta after 120 min of duodenal digestion per bread roll B0, B30 and B60 (as served basis) is shown alongside *in vivo* EAA Δpeak concentrations (C) measured in human serum (mean of n = 20 for B0 and B30, n = 19 for B60) after consumption of each bread type (1 roll per serving). Legend in C applies to ABC. Radar plots (D-L) show each EAA as a proportion of total EAA (y-axis = EAA %/total EAAs) measured *in vivo* (Δpeak, dashed lines), in the bread product (DEF), and released *in vitro* (solid lines) as either SP + FAA (GHI, calculated as per eqn. (2A)) or FAA (JKL, calculated as per eqn. (2B)) and resolved by bread type; B0 (DG), B30(EH) and B60(FI).

(Pearson's r = 0.467, p = 0.014). There was a significant positive correlation between EAA profiles of *in vitro* digesta and *in vivo* serum, with tendency for serum levels to be slightly better correlated to the *in vitro* data when EAAs within the small peptide fraction were included in the comparison (i.e. considered bioavailable) (Fig. 5. **JKL**, Pearson's r = 0.725, p < 0.001), than when only EAAs within the FAAs were considered (Fig. 5 **GHI**, Pearson's r = 0.607, p < 0.001). It is also noteworthy that *in vitro* measures of Phe and Leu bioaccessibility seemed to generally and systematically overestimate their bioavailability. Overall, there was good agreement between bioaccessible EAAs measured within the *in vitro* digesta and the serum EAA responses seen *in vivo*, regardless of bread type.

4. Discussion

Our studies demonstrate, for the first time, the effect of encapsulated legume protein enrichment of white wheat bread on bioaccessibility and bioavailability of EAAs. Encapsulated legume proteins in CCP-enriched breads were found to be digested into bioaccessible small peptides and free amino acids during simulated duodenal digestion. The EAA within these fractions of *in vitro* digesta was strongly correlated to the postprandial rise in serum EAAs following human consumption of CCP-enriched breads. Together, these findings indicate that the digestion of encapsulated legume protein liberates EAAs that are both bioaccessible and bioavailable. Thus, CCP-enrichment of white wheat bread improved their protein quality.

White wheat bread is a staple-food in many households worldwide and is therefore an appealing food vehicle for influencing nutritional status of the general public. Enrichment of wheat bread with pulses provides a route to enhancing the protein and dietary fibre intakes. Using novel cellular pulse flours, makes it possible to also lower the bread's glycaemic potency significantly, however, such ingredients require the protein and starch to be encapsulated by the cell wall, and the implications for bioavailability of EAAs during digestion had not yet been studied.

Here we show that CCP-enrichment of white wheat bread products improved the amount and types of bioaccessible and bioavailable EAA, even though the legume protein was encapsulated in the plant cells. The improved EAA profile of the CCP-enriched bread product were both due to the higher protein (and therefore total amino acid-) content of the CCP-enriched bread compared to wheat bread, and the higher ratio of EAA:non-EAA within the legume compared to cereal protein. Although the additional protein contributed by the CCP was encapsulated by cell walls at the time of ingestion, a nutritionally significant proportion of EEAs become bioaccessible in the form of small peptides and free amino acids released by action of proteolytic enzymes (e.g. trypsin and chymotrypsin) in the duodenum; this was reflected in the significantly higher serum EAA concentrations following human consumption of the CCP-enriched breads. Thus, the higher release of EAAs from CCP-enriched bread suggests that some intracellular protein hydrolysis is occurring, with smaller digestion products becoming bioaccessible and subsequently absorbed.

Not all the EAAs with the CCP-enriched bread products were bioaccessible, however; after 2 h of simulated duodenal digestion, we estimated that around 10–12 % of the EEAs in B30 and B60 were still trapped within inaccessible/undigested proteins. These breads contained a combination of wheat and CCP-derived protein, but considering that ~99 % of EAAs within wheat protein were found to be bioaccessible (as seen in B0), it seems likely that the inaccessible protein was associated with CCP component. This would suggest around ~27–34 % of the CCP protein to be inaccessible, and falls within the range of digestibility estimated for cooked cellular legume material in previous studies (Melito et al., 1995). The inaccessible fraction could be entrapped or intrinsically resistant to digestion by mammalian enzymes (Duijsens et al., 2022).

Another important aspect of our studies was the comparison of *in vitro* estimates of bioaccessible EAAs to serum EAA concentrations for the same bread products. The INFOGEST digestion model, used here, has been designed to simulate the biochemical and enzymic conditions of the human small intestinal lumen (Brodkorb et al., 2019), however it lacks brush-border peptidases which further digest small peptides. By comparing the EAA profiles of the free amino acid and small peptide fractions with serum responses, we found that inclusion of the EAAs within the small peptide fraction provided a better correlation to post-prandial serum EAA concentrations.

Performing these investigations on bread products allowed for important *in vitro- in vivo* comparisons and were highly relevant to real-life product applications of a novel food ingredient, however the complexity of the bread matrices limited depth of interpretation. Further mechanistic structure–function studies of proteolytic products released from isolated legume cells would therefore be a logical next step to complement the work presented here. There is also scope for exploring methodological aspects of the *in vitro* protocol, and future users could consider expanding the sample collection and analysis to enable calculation of Digestible Indispensable Amino Acid Score (DIAAS).

With regard to product applications, the commercial development of novel cellular pulse flours as functional food ingredients is gaining traction, and there is increasing understanding and evidence of their nutritional advantages over conventional pulse flours (i.e. in which the plant cells have been destroyed) (Edwards et al., 2020; Verkempinck et al., 2020; Xiong et al., 2022). In B30 and B60 bread products studied here, the protein accounted for 20 and 25 % of energy value of the food, and both would quality for a 'high protein' claim as defined by European Commission. In our previous studies, these bread products were also found to produce significantly lower glycaemic responses and prolonged satiety responses compared with wheat flour (Bajka et al., 2021), and provide more than double the amount of dietary fibre per serving of wheat bread, while having product quality characteristics (texture, sensory etc.) that were acceptable to study participants. Thus, enrichment of bread and other bakery products with novel cellular pulse flours represents a promising and tangible opportunity to improve nutritional status and cardiometabolic and gut health of the population.

5. Conclusion

A nutritionally significant proportion of encapsulated legume protein within CPP was bioaccessible during duodenal digestion of breads. Consequently, the replacement of refined wheat flour with intact chickpea cells improved both the amount and diversity of bioavailable EAAs. EAAs released as small peptides and free amino acids during INFOGEST simulated *in vitro* digestion were strongly correlated to the EAA profile of human serum following consumption of the bread products studied. Overall, enrichment of white wheat bread with novel cellular legume flour provides opportunities to improve both protein and carbohydrate quality for potential benefits to cardiometabolic and gut health.

CRediT authorship contribution statement

Natalia Perez-Moral: Conceptualization, Investigation, Data curation, Writing – original draft. **Shikha Saha:** Formal analysis, Data curation. **Ana M. Pinto:** Investigation, Data curation. **Balazs H. Bajka:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Project administration. **Cathrina H. Edwards:** Conceptualization, Writing – original draft, Writing – review & editing, Formal analysis, Visualization, Project administration, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cathrina Edwards, has patent #WO 2019155190 pending to King's College London.

Data availability

Data will be made available on request.

Acknowledgements

This research was funded by: Biotechnology and Biological Sciences Research Council, UK (BBSRC) Super Follow-On Fund grant BB/PO23770/1; the BBSRC Institute Strategic Programme BB/R012512/1 and its constituent project BBS/E/F/000PR10343; and BBS/E/F/00044427; and the BBSRC grant BB/W510701/1. We acknowledge the support of New Food Innovation Ltd. and Biopolymer Solutions Ltd. who produced the chickpea powder and provided ongoing support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134538.

References

Adhikari, S., Schop, M., de Boer, I. J. M., & Huppertz, T. (2022). Protein quality in perspective: A review of protein quality metrics and their applications. *Nutrients, 14*(5). https://doi.org/10.3390/nu14050947

Bajka, B. H., Pinto, A. M., Ahn-Jarvis, J., Ryden, P., Perez-Moral, N., van der Schoot, A., … Edwards, C. H. (2021). The impact of replacing wheat flour with cellular legume powder on starch bioaccessibility, glycaemic response and bread roll quality: A double-blind randomised controlled trial in healthy participants. *Food Hydrocoll, 114*, Article 106565. https://doi.org/10.1016/j.foodhyd.2020.106565

Berry, S. E., Valdes, A. M., Drew, D. A., Asnicar, F., Mazidi, M., Wolf, J., … Spector, T. D. (2020). Human postprandial responses to food and potential for precision nutrition. *Nature Medicine, 26*(6), 964–973. https://doi.org/10.1038/s41591-020-0934-0

Bhattarai, R. R., Dhital, S., Wu, P., Chen, X. D., & Gidley, M. J. (2017). Digestion of isolated legume cells in a stomach-duodenum model: Three mechanisms limit starch and protein hydrolysis. *Food & Function, 8*(7), 2573–2582. https://doi.org/10.1039/C7FO00086C

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International, 43*(2), 414–431. https://doi.org/10.1016/j.foodres.2009.09.003

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Calles, T., Xipsiti, M., & del Castello, R. (2019). Legacy of the international year of pulses. *Environmental Earth Sciences, 78*(5), 124. https://doi.org/10.1007/s12665-019-8106-6

Duijsens, D., Pälchen, K., De Coster, A., Verkempinck, S. H. E., Hendrickx, M. E., & Grauwet, T. (2022). Effect of manufacturing conditions on in vitro starch and protein digestibility of (cellular) lentil-based ingredients. *Food Research International, 158*, Article 111546. https://doi.org/10.1016/j.foodres.2022.111546

Edwards, C. H., Ryden, P., Mandalari, G., Butterworth, P. J., & Ellis, P. R. (2021). Structure–function studies of chickpea and durum wheat uncover mechanisms by which cell wall properties influence starch bioaccessibility. *Nature Food, 2*(2), 118–126. https://doi.org/10.1038/s43016-021-00230-y

Edwards, C. H., Ryden, P., Pinto, A. M., van der Schoot, A., Stocchi, C., Perez-Moral, N., … Ellis, P. R. (2020). Chemical, physical and glycaemic characterisation of PulseON®: A novel legume cell-powder ingredient for use in the design of functional foods. *Journal of Functional Foods, 68*, Article 103918. https://doi.org/10.1016/j.jff.2020.103918

Edwards, C. H., Warren, F. J., Campbell, G. M., Gaisford, S., Royall, P. G., Butterworth, P. J., & Ellis, P. R. (2015). A study of starch gelatinisation behaviour in hydrothermally-processed plant food tissues and implications for in vitro digestibility. *Food & Function, 6*(12), 3634–3641. https://doi.org/10.1039/C5FO00754B

Golay, A., Coulston, A. M., Hollenbeck, C. B., Kaiser, L. L., Wursch, P., & Reaven, G. M. (1986). Comparison of metabolic effects of white beans processed into 2 different physical forms. *Diabetes Care, 9*(3), 260–266. https://doi.org/10.2337/diacare.9.3.260

Gu, J., Bk, A., Wu, H., Lu, P., Nawaz, M. A., Barrow, C. J., … Suleria, H. A. R. (2022). Impact of processing and storage on protein digestibility and bioavailability of legumes. *Food Reviews International, 1–28*. https://doi.org/10.1080/87559129.2022.2039690

Gwala, S., Pallares Pallares, A., Pälchen, K., Hendrickx, M., & Grauwet, T. (2020). In vitro starch and protein digestion kinetics of cooked Bambara groundnuts depend on processing intensity and hardness sorting. *Food Research International, 137*, Article 109512. https://doi.org/10.1016/j.foodres.2020.109512

Han, F., Moughan, P. J., Li, J., & Pang, S. (2020). Digestible indispensable amino acid scores (DIAAS) of six cooked chinese pulses. *Nutrients, 12*(12), 3831. https://doi.org/10.3390/nu12123831

Herreman, L., Nommensen, P., Pennings, B., & Laus, M. C. (2020). Comprehensive overview of the quality of plant – And animal-sourced proteins based on the digestible indispensable amino acid score. *Food Science & Nutrition, 8*(10), 5379–5391. https://doi.org/10.1002/fsn3.1809

Kim, S. J., de Souza, R. J., Choo, V. L., Ha, V., Cozma, A. I., Chiavaroli, L., … Sievenpiper, J. L. (2016). Effects of dietary pulse consumption on body weight: A systematic review and meta-analysis of randomized controlled trials. *American Journal of Clinical Nutrition, 103*(5), 1213–1223. https://doi.org/10.3945/ajcn.115.124677

Kok, M. G. M., Nix, C., Nys, G., & Fillet, M. (2019). Targeted metabolomics of whole blood using volumetric absorptive microsampling. *Talanta, 197*, 49–58. https://doi.org/10.1016/j.talanta.2019.01.014

Mariotti, F., Tomé, D., & Mirand, P. P. (2008). Converting nitrogen into protein-beyond 6.25 and Jones' factors. *Critical Reviews in Food Science and Nutrition, 48*(2), 177–184. https://doi.org/10.1080/10408390701279749

McDermott, J., & Wyatt, A. J. (2017). The role of pulses in sustainable and healthy food systems. *Annals of the New York Academy of Sciences, 1392*(1), 30–42. https://doi.org/10.1111/nyas.13319

Melito, C., & Tovar, J. (1995). Cell walls limit in vitro protein digestibility in processed legume seeds. *Food Chemistry, 53*(3), 305–307. https://doi.org/10.1016/0308-8146(95)93937-M

Nosworthy, M. G., Neufeld, J., Frohlich, P., Young, G., Malcolmson, L., & House, J. D. (2017). Determination of the protein quality of cooked Canadian pulses. *Food Science & Nutrition, 5*(4), 896–903. https://doi.org/10.1002/fsn3.473

Pälchen, K., Bredie, W. L. P., Duijsens, D., Castillo, I. A., Hendrickx, M., Van Loey, A., & Grauwet, T. (2022). Effect of processing and microstructural properties of chickpea-flours on in vitro digestion and appetite sensations. *Food Research International, 157*, Article 111245. https://doi.org/10.1016/j.foodres.2022.111245

Pälchen, K., Michels, D., Duijsens, D., Gwala, S., Pallares Pallares, A., Hendrickx, M., … Grauwet, T. (2021). In vitro protein and starch digestion kinetics of individual chickpea cells: From static to more complex in vitro digestion approaches. *Food & Function*. https://doi.org/10.1039/D1FO01123E

Pallares Pallares, A., Gwala, S., Pälchen, K., Duijsens, D., Hendrickx, M., & Grauwet, T. (2021). Pulse seeds as promising and sustainable source of ingredients with naturally bioencapsulated nutrients: Literature review and outlook. *Comprehensive Reviews in Food Science and Food Safety, 20*(2), 1524–1553. https://doi.org/10.1111/1541-4337.12692

Petropoulou, K., Salt, L. J., Edwards, C. H., Warren, F. J., Garcia-Perez, I., Chambers, E. S., … Frost, G. S. (2020). A natural mutation in Pisum sativum L. (Pea) alters starch assembly and improves glucose homeostasis in humans. *Nature Food*. https://doi.org/10.1038/s43016-020-00159-8

Prinsen, H. C. M. T., Schiebergen-Bronkhorst, B. G. M., Roeleveld, M. W., Jans, J. J. M., de Sain-van der Velden, M. G. M., Visser, G., van Hasselt, P. M., & Verhoeven-Duif, N. M. (2016). Rapid quantification of underivatized amino acids in plasma by hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass-spectrometry. *Journal of Inherited Metabolic Disease, 39*(5), 651–660. https://doi.org/10.1007/s10545-016-9935-z

Roux, L. L., Chacon, R., Dupont, D., Jeantet, R., Deglaire, A., & Nau, F. (2020). In vitro static digestion reveals how plant proteins modulate model infant formula digestibility. *Food Research International, 130*, Article 108917. https://doi.org/10.1016/j.foodres.2019.108917

Rovalino-Córdova, A. M., Fogliano, V., & Capuano, E. (2019). The effect of cell wall encapsulation on macronutrients digestion: A case study in kidney beans. *Food Chemistry, 286*, 557–566. https://doi.org/10.1016/j.foodchem.2019.02.057

Verkempinck, S., Pallares, A. P., Hendrickx, M., & Grauwet, T. (2020). Processing as a tool to manage digestive barriers in plant-based foods: Recent advances. *Current Opinion in Food Science, 35*, 1–9. https://doi.org/10.1016/j.cofs.2019.11.007

Willett, W., Rockström, J., Loken, B., Springmann, M., Lang, T., Vermeulen, S., … Murray, C. J. L. (2019). Food in the Anthropocene: The EAT Lancet Commission on healthy diets from sustainable food systems. *The Lancet, 393*(10170), 447–492. https://doi.org/10.1016/S0140-6736(18)31788-4

Xiong, W., Devkota, L., Zhang, B., Muir, J., & Dhital, S. (2022). Intact cells: "Nutritional capsules" in plant foods. *Comprehensive Reviews in Food Science and Food Safety*. https://doi.org/10.1111/1541-4337.12904