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Tailored co-extruded cereals for seniors: fabrication, palatability, and *in vitro* digestibility

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ABSTRACT

The rise in life expectancies and gap between life span and health span necessitate innovative approaches, interventions, and food solutions to secure healthy aging. This study, conducted within the framework of the EAT4AGE project of the Joint Programming Initiative 'A Healthy Diet for a Healthy Life', focuses on the rational design of functional foods for older adults. A co-extruded cereal prototype, fortified with two bioactive moieties, maca root powder and olive leaf extract, aims to address nutritional gaps identified in older adults, with a particular emphasis on high-quality and highly digestible proteins. Analytical determinations reveal the cereals have rich macronutrient profile exceeding 12% (*m*/*m*) protein, 20% (*m*/*m*) fat, and low sugar (< 5%, *m*/*m*), surpassing commercially available products with texture analyses supporting improved hardness, reduced oral friction and oral comfort. An untrained consumer panel (n = 21, age 73 ± 5) confirmed high palatability in various metrics and overall acceptability that were also affirmed through a trained sensory panel. Lastly, the product digestion was explored through an age-tailored *in vitro* digestion model which consistently demonstrated high protein digestibility, surpassing 80%, across all product formulations. Further, calculation of the *in vitro* digestible indispensable amino acid score of the product affirms its high nutritional quality. Thus, this study underscores the potential of designing palatable foods that could help promote a balanced and sustainable diet towards healthy aging.

1. Introduction

The National Institutes of Health (NIH), U.S identified a pronounced rise in human lifespan that is yet to be matched by a concomitant rise in human health span[1-2]. This is accompanied by a rise in efforts to exhaust the potential of plant-based foodstuffs towards healthier and more sustainable diets[3-4]. Thus, development of tailored nutritional solutions can significantly affect the independence, quality of life and healthy aging of older adults by addressing many of the adversities of aging[5-6]. Such solutions need to address relevant age-related physiological decline, e.g. changes in salivation, reduced mandibular forces, problems swallowing, slower gastric emptying, decreased excretion of digestive fluids and altered microbiota along with decreased appetite and early satiety[5,7-10]. In

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addition, design of foods for the older adults' mandates tackling age-related mental decline and changes in eating patterns, wherein potential sensory deterioration may underlie malnourishment and insufficient protein intake and damper health[5,11-13].

Today, there is a growing consumer and commercial interest in food and dietary solutions that can effectively meet the unique preferences, acceptance and needs of seniors[5,12]. This led to an ERA-Net ERA-HDHL call for the "Development of targeted nutrition for prevention of undernutrition for older adults" (PREVNUT). This challenge was met by several consortia, one of which was the EAT4AGE consortium that targeted the development of palatable nutrient-dense functional foods. Within this project, a literature survey pin-pointed actual nutritional gaps in older adults, with high quality protein, dietary fibres and specific micronutrients, like calcium and iron set as viable targets[11,14-17]. In fact, protein was found to be of great nutritional importance, as a robust dietary interventional tool to affect sarcopenia of aging, healthy muscle ageing and even help regulate appetite[17-20]. To this end, senior consumption of dietary proteins has been suggested to be increased to 1.0–1.2 g/(kg BW·day) (compared to 0.8 g/(kg BW·day) recommendation for healthy adults) with indications that animal proteins, e.g. dairy proteins, exhibiting improved digestibility and nutritional value in older adults compared to plant-based protein sources, e.g. soy[16-17]. However, animal proteins are not devoid of limitations and plant-based proteins prove to be advantageous in various aspects, notably as part of recommended dietary choices leaning towards healthier and eco-friendlier planetary diets[3-4,21-22].

In addition, this work sought to offer seniors with value-added products by incorporating functional food bioactives[23-25]. Out of the various possibilities, this work identified and focused on macamides found in the maca root (maca) and oleuropein in olive leaf extract (OLE)[26-28]. Maca was selected for its various indications of beneficial antioxidant, hormone-balancing and therapeutic properties that are relevant to aged individuals, these include enhanced fertility[29] and alleviation of symptoms associated with menopause[30]. Supplementation with OLE was selected due to demonstrated safety[31] and indications that its supplementation reduces mitochondrial superoxide production and increases mitochondrial biogenesis[32], contributing to an overall positive impact on cellular health.

Applying consumer-oriented considerations for the development of functional foods for seniors gave rise to a pragmatic solution in the form of a shelf-stable extruded cereal product. This relied on indications that consumption of extruded cereal products and plant-based products has increased over the years due to their considerable shelf life, affordability, sustainability, and ease of use as ready-to-eat foods[33-34]. Moreover, manufacturing of such plant-based products, produced via extrusion, may help tackle challenges of allergens, antinutritional factors (ANF) and improve their overall limited digestibility[35-37]. For instance, extrusion process has been shown to promote denaturation of proteins and inactivation of thermo-labile ANF moieties, thereby enhancing *in vitro* protein digestibility and positively influencing protein quality[36-37].

This rational food engineering approach sought to open opportunities for tailoring food compositions to tackle the challenges of maximizing palatability, oral comfort, lubricity while optimizing nutrient bioaccessibility and bioavailability to seniors[38-41]. In fact, a growing number of recent studies demonstrate that the altered digestive functions of seniors affect their ability to render nutrients bioaccessible[5,42-45]. Furthermore, interactions between food components may interfere with nutrient digestibility and absorption[46-48]. Consequently, research into the digestive fate of tailored food products is an essential milestone towards their bio-efficacious development[5,40,44].

Thus, this research sought to design and study the potential digestive fate of a co-extruded cereal product in seniors (age > 65). Moreover, the product was formulated to be a functional food with two added bioactive moieties: maca and OLE from the olive oil industry. This study focused on the palatability of the functional product, its oral processing and the potential digestibility of its proteins in healthy seniors. The underlying hypothesis was that seniors have altered food preferences and digestive capabilities which affect their perception and breakdown of the tailored cereal product. For this purpose, we adapted a two-armed study combining *in vitro* and *in vivo* measures.

2. Materials and methods

To investigate the potential digestive fate of the functional cereals in healthy older adults, we employed a battery of methods delving into the oral processing of the product and its gastro-intestinal digestibility using and *in vitro* setup, guided by a recently published consensus protocol[9].

2.1 Materials

Chickpea, rice and teff flours, coconut oil and tapioca starch were acquired in a local supermarket (Shufersal Ltd., Israel). OLE with 20% oleuropein (Doctor Phyto, Israel) was acquired from Bio-Gaya (Bio-Gaya, Israel). Maca powder (Beit Shaked Kfar, Israel) was acquired in a local supermarket. Roasted sesame bran and sesame paste (tahini) were generously donated by Al-Arz (Al Arz, Israel). Magnesium carbonate, corn grits, cocoa, saccharin, vanillin, NUTRIOSE® and erythritol were kindly provided by the International Beer Breweries Ltd. Pilot Laboratory. Fruitlift™ is an all-natural sweetening solution containing 90% fruit components, fully compatible with extrusion processing (ensuring no "caking" and no stickiness). All the above ingredients were food grade, and used as received without any purification.

Standard 1 000 mg/L single element standards by Agilent technologies mineral solutions for mineral composition analysis was used. Pepsin from porcine gastric mucosa (P7000), trypsin from porcine pancreas (T0303), α-chymotrypsin from bovine pancreas (C4129), pancreatin from porcine pancreas (P7545); all enzymes batches were studied for their specific activity (including periodical tests to monitor changes during shelf life) and used according to the INFOGEST protocol and it overall rationale[49]. Porcine bile extract (SC-214601) was purchased from Santa Cruz Biotechnology (USA). Sodium glycodeoxycholate, taurocholic acid sodium salt hydrate, phenylmethylsulfonyl fluoride (PMSF), pepstatin-A (CAS# 26305-03-3, microbial, ≥ 90% high performance liquid chromatography (HPLC)) were purchased from Sigma Aldrich (Rehovot, Israel). Calcium chloride dehydrate was obtained from Spectrum Chemical Manufacturing Corp. (New Brunswick, NJ, USA). All chemicals and enzymes used in the present study for determination of total amino acid were purchased from Merck (Zug, Switzerland). Gels were stained by InstantBlue™ stain (Bio Consult, Israel). All other chemicals and reagents used in the study were of analytical grade.

2.2 Formulation and co-extrusion of cereal products

Acknowledging "anabolic resistance" in older adults[5], the product was designed to offer easily digestible, high-quality proteins in a shelf-stable and accessible food product. This relied on studies showing protein bioaccessibility and digestibility of plant proteins are improved by their controlled processing[36-38,50]. Thus, a calorie-dense product was crafted to comprise a shell made from 5 gluten-free flours that also offer a well-balanced amino acid profile along with sesame peels, a by-product of tahini production and a rich source of dietary fibers[51]. To enhance functionality, the product was also supplemented with two key bioactive moieties whose bioactivity is supported by clinical studies, maca was added to the shell and OLE added to the oily filling[27,30,32,52-53]. The first additive, maca powder, was selected for its various beneficial indications relevant to hormonal regulation and immunomodulation in seniors[54-55]. Similarly, OLE was selected due to indications that its supplementation has antidiabetic and antioxidant effects as well as beneficial effects on mitochondrial function[32,56].

The complex hard shell comprised teff, chickpea, maize, tapioca, and rice with Fruitlift™ for sweetening and maca root powder as functional ingredient. The filling was a mixture of sesame paste, coconut oil, flavorings (vanillin, cocoa powder, saccharin, erythritol) and OLE as a functional supplement. The production of three test products was pursued through co-extrusion at International Beer Breweries Ltd. that enabled the production and packaging of the food-grade ready-to-eat products (formulae in Table 1). Co-extrusion of the plant-based cereal product was optimized for production and three formulae were produced: a control or reference version without any functional ingredients in the shell or the filling (Ref + ref), a product with 0.03% (*m*/*m*) Maca-added to the shell (Maca + ref) and a product with 0.03% (*m*/*m*) Maca and 0.015% (*m*/*m*) OLE in the shell and filling (Maca + OLE), respectively.

Table 1
Composition of co-extruded plant-based cereal products.

	Ingredients (amount in 1 kg product)	Ref + ref	Maca + ref	Maca + OL
Shell	Corn grits (g)	360	340	340
	Chickpea (g)	200	200	200
	Rice (g)	110	100	100
	Tapioca (g)	100	100	100
	FruitLift solution (g)	100	100	100
	Teff (g)	80	80	80
	Maca root powder (g)	–	50	50
	Sesame peels (g)	20	20	20
	Cocoa (g)	10	10	10
	Salt (g)	3	3	3
	Magnesium carbonate (g)	3	3	3
Filling	Tahini (g)	600	600	600
	Erythritol (g)	150	150	150
	NUTRIOSE® (g)	150	150	150
	Coconut oil (g)	80	80	80
	Cacao (g)	20	20	20
	Vanillin (g)	0.3	0.3	0.3
	Saccharin (μg)	40	40	40
	OLE (mL)	–	–	15

Note: The pivotal process parameters for co-extrusion encompassed inlet and outlet temperatures of 13 and 100/105 °C, respectively, coupled with a pressure of 553 mPa, a die diameter of 6 mm (*L*/*D* = 28) in a 36 mm twin-screw extruder. Initial moisture content set at 13%–15% (*m*/*m*) and a final target of 7.2% (*m*/*m*).

The products were produced via co-extrusion using a 36 mm twin-screw extruder (*L*/*D* = 28, main die 6 mm diameter and core filling nozzle diameter 4 mm) in a pilot plant complying with food-grade national health and safety regulations. The screw elements, including kneading blocks and reverse screw elements were selected to offer high shear at 220 r/min. Feed was conveyed into the extruder with a twin-screw weight feeder (Shandong Light M&E Co.) and adjusted to a feed rate of 10 kg/h. The die temperature was 105 °C and samples were collected after 3 min of processing. The samples were air dried on trays at room temperature, then approximately 15 g of product were packed in aluminium bags sealed with heat welding of the polyethylene component. All samples were stored at room temperature until further analysis.

2.3 In-vitro characterization of the plant-based cereal prototypes

2.3.1 Proximate analyses

Protein content was determined by the Kjeldahl method and by automated CHNS analysis system enabling Dumas protein quantification. CHNS Elemental analysis was performed using Flash 2000 Organic elemental analyzer (Thermo Scientific, USA). Samples of 2–4 mg were weighed with 8–10 mg of vanadium in a tin crucible. Combustion temperature was 950 °C , carrier gas was He (99.999%) with a rate of 140 mL/min. Adding O_2 at 250 mL/min for 5 s. Standards were cystine, 2,5-bis-2(5-tert-butyl-benzoxalyl)thiophene (BBOT), sulphanilamide, methionine, nicotinamide. A conversion factor of 5.7 was used for the cereal products[57]. The fat content was determined according to AOAC method 996.01[58]. All analyses were performed in triplicate. Regulatory labeling values were calculated based on individual ingredient composition and customized software analysis pack (Tzameret software, Department of Nutrition, Israel Ministry of Health).

2.3.2 Instrumental single compression test

Instrumental single compression tests were carried out using a TA1 texture profile analyzer (Lloyd Instruments Ltd., AMETEK, Israel) equipped with a cylindrical plate (115 mm diameter, working force 50 N, pre-speed and test speed set to 3 mm/s and 1 mm/s, respectively)[59-61]. A single sample of each of the three products was placed on the plate and its deformation was observed for a compression up to 90% of the original product thickness[59]. The parameters hardness (N) and Young's modulus were obtained from the instrumental single compression tests[62].

2.3.3 Tribological measurements

The evaluation of the lubrication properties of the simulated bolus samples was conducted at a controlled temperature of 37 °C using a mini-traction machine (MTM2) (PCS Instruments, UK). This equipment offers the advantage of measuring the friction coefficient across a broad speed range, enabling a comprehensive mapping of the Stribeck curve. The tribopair configuration involved a ball (19.0 mm diameter) on disc contact, where both surfaces were composed of polydimethylsiloxane (PDMS) with a Young's modulus of 2.4 MPa and an average surface roughness (Ra) of approximately 50 nm[63].

Prior to the experiments, the PDMS surfaces were cleaned in an ultrasonic bath with a solution of 3% surface-active cleaning agent (Decon 90®, East Sussex, UK), a solution of 10% isopropanol, followed by rinsing with isopropanol. After such treatment, the surface of the PDMS retained its natural hydrophobic characteristic.

For measurement, pure sliding conditions of 50% slide-roll ratio (SRR) was used and three tests of ascending sliding speed (0.1–1 000 mm/s) were completed, and the average reported. A volume-reducing insert was used allowing for a sample size of 15 mL. Experiments were performed at 37 °C. A normal force of 2 N was used as in-mouth friction have been reported to be between 0.1 and 10 N[64]. Hence, 2 N would allow low contact pressure, which is of relevance in oral processing applications.

2.3.4 Microstructure characterisation by confocal scanning laser microscopy (CLSM)

The microstructures of the simulated sample boli were characterized before being subjected to tribological stress using a Zeiss LSM 880 inverted confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Approximately 5 μL of Nile red solution (0.1 g/100 mL in dimethyl sulfoxide) and Fast Green solution (1.0 g/100 mL in MilliQ water) were used to fluorescently stain the lipid (excited at 514 nm) and protein phases (excited at 633 nm), respectively. The fluorescently labeled sample boli were placed on a concave confocal microscope slide, fixed with a glass coverslip, and imaged using an oil-immersion 63× lens and pinhole diameter of 1 Airy Unit to filter out most of the light scattering.

2.3.5 Simulated oral processing

2.3.5.1 Preparation of model saliva

The model saliva was prepared following the composition previously described[65]. Briefly, to prepare 1 L of model saliva, 1.59 g/L NaCl, 0.328 g/L NH_4NO_3, 0.64 g/L KH_2PO_4, 0.20 g/L KCl, 0.31 g/L $K_3C_6H_5O_7 \cdot H_2O$, 0.02 g/L $C_5H_3N_4O_3Na$, 0.20 g/L H_2NCONH_2, 0.15 g/L $C_3H_5O_3Na$ and 3.00 g/L porcine gastric mucin type II were dissolved in distilled water. After adjusting the pH to 7.0 using 1 mol/L NaOH, the volume was made up to 1 L using a volumetric flask. Porcine gastric mucin was used due to its ability to simulate the rheological properties of human saliva. It is noteworthy, however, that bovine submaxillary mucin is the optimal source of commercially available mucin for lubricating properties[66], and therefore this is a limitation of the current study.

2.3.5.2 Simulated bolus formation

To simulate oral processing, food boli were prepared as described in Menard et al.[9]. Briefly, samples were mixed with model saliva at a final insalivation ratio, food:saliva = 1:1 (*m*:*m*), which was determined from spit boli obtained from INRAE. Subsequently, the samples underwent mincing using a Kitchen Craft No. 5 meat mincer equipped with a 5 cm mincing disc and a 0.5 cm mesh size for one pass. After recovering the bolus, additional model saliva was introduced to achieve a final insalivation ratio of 1:4 (*m*:*m*). The mixture was agitated using a magnetic stirrer at 100 r/min for 10 min to ensure particle dispersion while preserving the bolus structure, thereby achieving a consistency suitable for subsequent characterization.

2.4 Comprehensive in vivo sensory evaluation

2.4.1 Food comfortability

Food comfortability tests were evaluated by 21 untrained volunteers (mean age: 73 ± 5; 13 females, 8 males), with specific criteria established to ensure a representative sample of older adults. This study cohort was recruited to meet levels for laboratory acceptability testing[67] and using a previously developed questionnaire[68]. Participants were recruited from the community and each participant gave written consent to participate in the experiments as well as a declaration that he/she complied with the study's inclusion criteria (over 65, overall independent community-dwelling, healthy and in good present state, allergy-free, and not suffering from dry mouth or other oral or gastrointestinal problems). The subjects who met all the requirements were invited to an information session, where they received an explanation of the study setup and signed the informed consent form. During the sessions, the volunteers were invited to eat a portion of the three formulae cereal products prior to each of the 5 parts of the questionnaire (water was available ad libitum). The evaluations were performed under normal light at room temperature (approximately 25 °C).

2.4.2 Sensory evaluation of products

Ten experienced assessors, highly trained in the use of quantitative descriptive analysis (QDA) and temporal dominance of sensations (TDS) participated in this part of the research. The formal assessments were conducted in individual booths under white light with proper ventilation, while general discussions among panelists were conducted in a well-ventilated discussion room. The sensory laboratory was designed according to guidelines in ISO 8589:2007(E) featuring separate booths and electronic registration of data (Eye Question, v. 3.8.6, Logic 8, The Netherlands), standardized lighting and a separate ventilation system.

2.4.2.1 QDA

Nofima's trained panel conducted the sensory profiling on the cereal samples using QDA as described previously[69]. The vocabulary for the products was established during a preliminary session involving two of the samples. This pre-trial session, lasting 1 h, involved the assessors in agreeing upon descriptors, their definitions, and reference samples. By the end of the pre-trial, all assessors demonstrated the ability to distinguish among samples, showed consistency in their evaluations during subsequent trials, and reached a consensus within the group. The final list of attributes included odor, flavor, texture and mouthfeel attributes: sweetly odor, chocolate odor, nutty odor, roasted odor, grain odor, drawer odor, rancid odor, sweet taste, salty taste, bitter taste, chocolate flavor, nutty flavor, roasted flavor, grain flavor, drawer flavor, cloying flavor, rancid flavor, crispness, juiciness, fatness, stickiness and astringency.

Each panelist received each cereal sample in plastic cups labeled with randomly assigned 3-digit codes. The samples were presented at

room temperature in a sequential monadic fashion following a balanced presentation order. The evaluations were conducted in two replicates and lasted approximately 1.5 h.

2.4.2.2 TDS

The assessment process followed the TDS methodology outlined previously[70]. TDS allows for the dynamic sensory description of food products, during the whole consumption period. Initially, assessors were reminded of the concept of dominant sensations during food consumption. Subsequently, they tasted the samples and noted the attributes they perceived to differentiate the samples from a temporal perspective (during the whole consumption period). The most relevant attributes for the dynamic description of the products were selected through discussion and consensus among the panelists.

The sensory lexicon created for the cereal samples included eight flavor and texture attributes with definitions sourced from ISO 5492:2008 (bitter, chocolate, roasted, grain, drawer, rancid, crispy, and sticky). In the formal assessment, assessors evaluated the samples one by one, served in small plastic cups labeled with unique 3-digit codes. Water rinses were required between samples.

Assessors were instructed to place the sample in their mouth, press the "START" button, and choose the dominant sensations they experienced while consuming the sample by clicking on one of the eight attributes displayed on screen. When they were ready to swallow, they pressed "STOP" and spat out the sample. Assessors could choose from among the attributes as many times as necessary during the consumption of the samples, even re-selecting an attribute more than once during the test; only one attribute at a time could be selected as the dominant sensation.

2.5 In vitro semi-dynamic digestion adjusted to older adults

Since data on oral processing in older adults is scant[9], this part of the work initiated with estimating the salivary mineral composition of seniors (n = 10, age: 75 ± 5) by inductively coupled plasma optical emission spectroscopy (ICP-OES) with outcomes given in Table S1. This affirmed that the calcium composition of seniors doesn't differ from that of young adults[49], validating that defined recently for *in vitro* digestion (IVD) modeling[9]. The bolus features, however, can be impacted by other aging-related changes in oral processing[5,71]. For this reason, oral bolus from senior volunteers (n = 4) was selected to be used in the semi-dynamic IVD model.

Samples were orally processed *in vivo* (four healthy volunteers, mean age = 75 ± 2) right before being subjected to semi-dynamic IVD. Each volunteer chewed a sample of 15 g until a point where they felt ready to swallow but instead the oral bolus was spit it into a sterile cup. Subsequently, a gargle with 15 mL of water was performed to dislodge any remaining residue adhering to the teeth. As controls, the pre-extruded product blend (Mix, Mix + maca) or a protein-free cookie (comprised solely of fat and carbohydrates) were mixed in a 1:1 ratio (m:m) with SSF solution and 75 μL $CaCl_2$ (0.3 mol/L) instead of being chewed by the volunteers.

Semi-dynamic IVD. The simulation of human gastrointestinal digestion was carried out following an adjusted form that combines 2 standardized methods for simulated IVD[49,72], similar to that depicted in past studies[73-74].

Simulated adult digestion. Measured oral bolus aliquots (30 mL) were placed in a stirred double jacketed vessel (6.1418.250, Metrohm, Switzerland) ((37 ± 1) ℃ and 250 r/min) which was controlled by a dual auto titration unit (Titrando 902, Metrohm, Switzerland). Then, 28 mL preheated simulated gastric fluid (SGF) (pH 3, 37 ℃) were added, followed by the addition of 2 mL freshly prepared pepsin solution (final concentration of 2 000 U/mL) and 15 μL $CaCl_2$ (0.3 mol/L). Once mixed in the vessel, the TIAMO control software was activated to follow a semi-dynamic digestion protocol and generate a 2 h gastric pH gradient (using 0.3 mol/L HCl), as previously reported[75]. At the end of this gastric phase, half of the gastric digesta was collected and mixed with simulated intestinal fluid (SIF) (1:1, *V*/*V*). Trypsin (100 U/mL), α-chymotrypsin (25 U/mL), pancreatic α-amylase (200 U/mL), sodium glycodeoxycholate and taurocholic acid sodium salt hydrate (final concentration of 10 mmol/L in total) and 60 μL $CaCl_2$ (0.3 mol/L) were added, and the intestinal phase protocol was initiated. This phase maintained at pH 6.25 (using 0.3 mol/L NaOH) for 2 h at (37 ± 1) ℃. A total of ten aliquots of 1 mL were aspirated during the digestion experiments: six from the gastric phase at time lapses of 0, 15, 30, 60 and 120 min (abbreviated as G0, G15, G30, G60 and G120); four samples from the intestinal phase at time lapses of 15, 30, 60 and 120 min (abbreviated as D15, D30, D60 and D120). Upon collection, each aspirate was inactivated: gastric effluents by pepstatin-A (final concentration of 7 μmol/L) and intestinal effluents using the irreversible serine-protease inhibitor PMSF (final concentration of 0.5 mmol/L). All inactivated gastric and intestinal effluents were stored at −20 ℃ until further analyses. The pre-extruded product (Mix, Mix + maca) and a protein-free cookie (with only fat and carbohydrates) were digested in triplicates, as controls.

Simulated older adult digestion. Semi-dynamic IVD was performed similarly to adult digestion with modifications to mirror age-related physiological changes in gut functions, as detailed previously[5,9,42,76]. Each product sample was subjected to two different sets of experiments: one set included three replicate experiments done using oral contributions from a single individual, and another set of digestion experiments with three contributions collected from three different individuals. All oral boli were subjected to a semi-dynamic gastro-intestinal protocol concurring with a recent consensus protocol devised for older adults[9]. Briefly, this model included changes in the pH and duration of the gastric phase, as well as the activity of the digestive enzymes in the stomach (pepsin with final concentration of 1 200 U/mL) and small intestine (20% decrease for all enzymes) and the concentration of bile salts (6.7 mmol/L in total) and $CaCl_2$ in the intestinal (final concertation of 1 mmol/L). The composition of SSF, SGF, SIF concentrations were based on studies of Levi et al.[42,76] and detailed in Table 2. In the gastric phase, the pH gradient was adjusted to slightly different profile to stimulate the elevated gastric pH (major steps: pH 5 for 23 min, pH 4 for 52 min, pH 3 for 89 min, pH 2 for 16 min)[9]. The gastric phase was carried out for 3 h and the intestinal phase was kept at a constant pH 6.25 for 2 h. The schematic depiction (Fig. 1) elucidates and defines the specific digestive conditions for seniors, offering a valuable framework for future research endeavours in this field.

Table 2
Composition of stock solutions and simulated digestive fluids used in *in vitro* adult and senior digestion experiments.

Compound	Molecular weight (g/mol)	Stock (g/L)	Volume in stock (mL)	SSF (mL/L)		SGF (mL/L)		SIF (mL/L)	
				Adult	Senior	Adult	Senior	Adult	Senior
KCl	74.55	46.72	0.627	24.1	10	11	56	10.8	10.8
KH_2PO_4	136.09	68	0.5	7.4	40	1.8	1.8	1.6	1.6
$NaHCO_3$	84.01	84	1	13.6	8	25	13	85	85
NaCl	58.44	120	2.053		2	23.6	20	19.2	16
$MgCl_2(H_2O)_6$	203.3	30	0.148	1	2	0.8	4	2.2	2.2
$(NH_4)_2CO_3$	96.086	27.28	0.283	0.21		1.8	2		
Urea	60.06	22.5			10		0.6		4.8

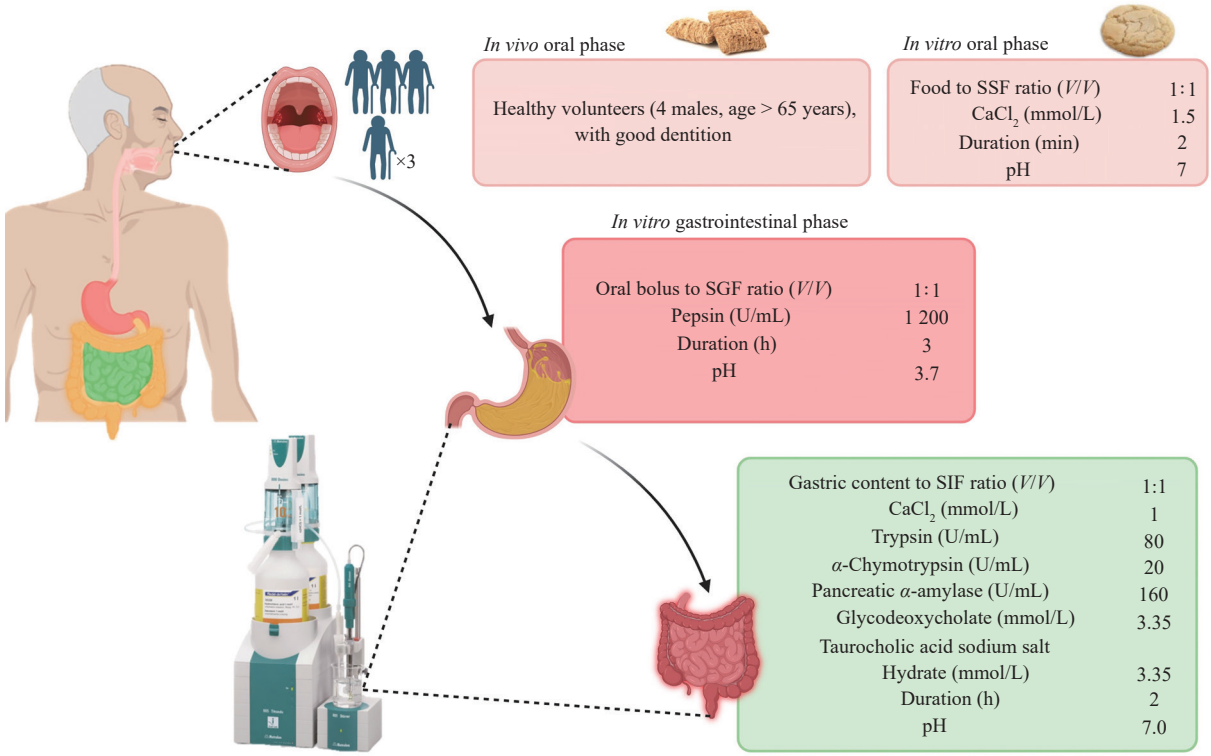


Fig. 1 Schematic illustration of simulated senior digestive tract experiments starting from *in vivo* or *in vitro* oral phase and ends in *in vitro* gastro-intestinal phase. Each digestion experiment was fed with one of four test substrates: controls, cookie (a protein-free blank), Mix and Mix + maca. The three formulae: Ref + ref, Maca + ref and Maca + OLE. Two sets of experiments were conducted for each product sample: the first set included three replicate experiments with oral contributions from a single individual, while the second set involved three replicate experiments with contributions from three different individuals. Created with BioRender.com.

2.5.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Differences in proteins digestion and peptides formation under the various digestion conditions were based on SDS-PAGE. This qualitative analysis was performed to enable determining protein dissipation during digestion alongside monitoring the breakdown patterns formed therein. The SDS-PAGE analysis followed the method described previously[74] with slight modifications. Samples were loaded onto 12% acrylamide gels and focused on the molecular weight range between 10–170 kDa using the protein size standard Spectra Multicolor Low Range Protein Ladder (Thermo Scientific).

2.5.2 Proteomic analysis of bioaccessible peptides

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were used to identify the bioaccessible peptides found in aspirated digesta samples. The protein samples were first subjected to 8.5 mol/L urea, 100 mmol/L ammonium bicarbonate, and 10 mmol/L dithiothreitol, followed by vortexing and centrifugation at 16 000 × *g* for 15 min at room temperature. Protein amount estimation was conducted using Bradford readings.

The samples underwent reduction (60 °C for 30 min) and modification with 35.2 mmol/L iodoacetamide in 100 mmol/L ammonium bicarbonate (room temperature for 30 min in the dark). Post-modification, samples were diluted to 6 mol/L urea and filtered using Omicon 10 kD (centrifugal filter Amicon® Ultra,

0.5 mL, 10 kDa). Peptides from the filtrate were desalted using a homemade C_{18} stage tip, dried, and resuspended in 0.1% formic acid, 2% acetonitrile. Peptides were analyzed by LC-MS/MS using a Q Exactive HF mass spectrometer (Thermo) coupled with a capillary HPLC (Evosep). The peptides were loaded onto an 8 cm, ID 150 μm, 1.9-micron Endurancse column EV1109 (Evosep) and eluted with the built-in Xcalibur 30 SPD method (44 min). MS was performed in positive mode, involving repetitively full MS scans (*m*/*z* 300−1 500) and high-energy collision dissociation (HCD) of the 18 most dominant ions (>1 charges) selected from the full MS scan. Dynamic exclusion with a 20 s duration was enabled. Protein Discoverer 2.4 (Thermo) with the Sequest search engine was utilized for MS data analysis. The search encompassed proteomes from various sources, including UniProt and NCBI NR databases, with specified mass tolerances. Oxidation on methionine and protein N-terminus acetylation were considered as variable modifications, while carbamidomethyl on cysteine was considered a static modification. Label-free analysis was employed for data quantification, and peptide-level false discovery rates (FDRs) were filtered to 1% using the target-decoy strategy amino acids.

All samples were found to contain over 1 200 different peptides sequences. The peptides sequences were classified according to their length and the percentage of each length group was evaluated in each sample.

2.5.3 *In vitro digestible indispensable amino acid score (DIAAS)*

In vitro digestibility and DIAAS determinations were carried out as described previously[77]. Similar experiments were also conducted on samples subjected to *in vitro* senior digestion to evaluate total *in vitro* digestibility and DIAAS values under these conditions[9]. A protein-free blank (cookie) was digested in parallel to serve as control and all samples were subjected to analyses in triplicate. The total amino acids of each substrate were determined as described in ISO 13903:2005[78]. Samples were hydrolyzed for 24 h with 6 mol/L HCl, then derivatized with AccQ-Tag Ultra reagent (Waters, 2007) before the amino acid profile was determined by ultra-high-performance liquid chromatography (UHPLC) (AccQ-Tag Ultra (2.1 mm × 100 mm, 1.7 μm) Waters) coupled with a UV detector (Vanquish, Thermo Scientific, Reinach, Switzerland). Digestibility percentages for both total and individual amino acids of the substrates subjected to IVD were determined through measuring the digestible fraction of the food (supernatant) and dividing it by the total food aliquot (supernatant + pellet (undigested fraction)), according to the formula (1):

$$\textit{In vitro}\ \text{digestibility}\,(\%) = \frac{\mathrm{Fs}-\mathrm{Cs}}{(\mathrm{Fs}-\mathrm{Cs})+\max(0;\mathrm{Fp}-\mathrm{Cp})}\times 100 \quad (1)$$

Where Fs represents food supernatant mass, Cs represents cookie supernatant mass, Fp represents food pellet mass, Cp represents cookie pellet mass.

Then, the calculation of *in vitro* digestible indispensable amino acid (*in vitro* DIAA) per gram of food protein was calculated according to the formula (2):

$$\textit{In vitro}\ \text{DIAA content}\,(\text{mg/g}) = \text{IAA}\,(\text{mg/g}) \times \textit{in vitro}\ \text{digestibility of IAA} \quad (2)$$

The *in vitro* DIAA ratio (DIAAR) per gram of food protein was calculated, considering the reference protein for older child, adolescent, adult, given by FAO[79], according to the formula (3):

$$\textit{In vitro}\ \text{DIAAR}\,(\%) = \frac{\textit{In vitro}\ \text{DIAA in dietary protein}\,(\text{mg/g})}{\text{The same dietary IAA in the reference protein}\,(\text{mg/g})} \quad (3)$$

DIAAR was computed for each individual IAA and the DIAAS for a specific food corresponds to the lowest DIAAR among them.

2.6 *Statistical analysis*

All experiments were conducted in duplicates or triplicates, and the results are presented as the calculated mean and standard deviation. Statistical analyses were performed using various methods tailored to specific measurements. For general statistical analyses, a one-way ANOVA ($P < 0.05$) with a Tukey's Multiple Comparison test and Kruskal Wallis H Test were carried out using MATLAB and Excel. For tribological measurements and microstructure characterization of products, a one-way ANOVA followed by the Tukey's pairwise comparison with a 95% confidence interval was performed using SPSS software (IBM, SPSS Statistics, version 24).

The TDS was illustrated as dominance curves *vs*. time, employing standardized times (from t_0 to t_{100}). Two crucial levels were outlined for interpretation: the "chance level" (P_0), representing the dominance rate an attribute could obtain by chance, and the "significance level" (P_s), denoting the minimum dominance rate for the attribute occurrence to be considered significantly higher than the chance level P_0[80]. For the analysis of QDA data, an ANOVA with assessor (random) and product (fixed) effects was applied. The Tukey post hoc test was utilized to compare attribute means among different products.

3. Results and discussion

3.1 *In vitro characterization of the plant-based cereal prototypes*

3.1.1 *Proximate analyses*

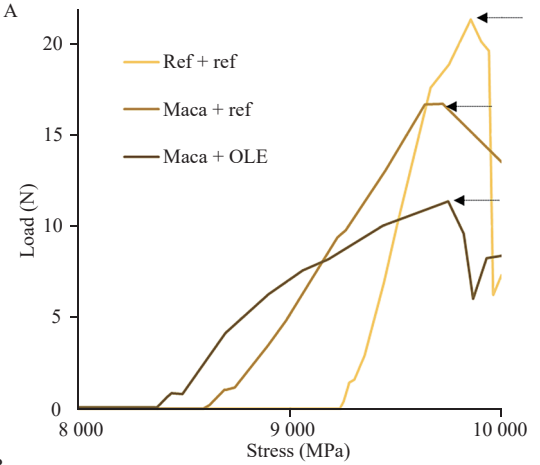
This work developed food prototypes that could be classified as ultraprocessed foods whose association with adverse effects to health are vividly discussed these days[81-84]. Yet, proximate analyses summarized in Table 3 confirmed that the prototypes boast a protein content exceeding 12% (*m*/*m*), a fat content of approximately 20%, and minimal sugar (less than 5%). Interestingly, this nutritional profile surpasses that of commercially available counterparts, which typically offer around 6% (*m*/*m*) protein, ~15% fat and a very high sugar content of ~40% (*m*/*m*). Accumulating evidence on the beneficial role of dietary fiber and whole grains[85-90] was also addressed in the product formulation which was calculated to contain 17% (*m*/*m*) dietary fiber. Further, studies highlight the important role of micronutrients like iron (Fe), zinc (Zn), copper (Cu), and selenium (Se) in the nutrition of older adults[5,15,91]. Nevertheless, cereals often fall short in providing adequate quantities of these essential nutrients. Thus, the inclusion of legumes, like chickpeas, offered an opportunity to compensate for the amino acid profile and this inherent drawback of micronutrient

content of cereals, namely as it pertains iron content as depicted in Table 3. Combining these complementary sources enhances the overall nutritional profile of the extruded cereal products, thus, highlighting the importance of product formulation over processing[81]. However, the success of such novel and healthier food choices also mandates matching their nutritional potential with consumer adoption that stems from the palatability.

3.1.2 *Instrumental single compression test*

One of the major sensorial traits of cereal products is their distinct texture and descriptors, such as crunchiness, chewiness and sogginess which can be elemental in the development of future foods for seniors[10]. As a first step, instrumental single compression measurements were initiated and sought to measure the impact of supplementation on the mechanical properties of the products (Fig. 2). These analyses revealed that addition of the functional supplements (maca and OLE) significantly lowered sample hardness ($P < 0.05$) to values lower than that reported for an extrudate of maize-millet fortified with soy flour[92]. This would imply a possible improvement in their chewiness and oral comfort for seniors. In addition, the supplementation of maca involved a reduction in corn grits content (Table 1) and product hardness (Fig. 2) which coincides with recent studies correlating diminished grits content with a subsequent reduction in product hardness[93-94]. These findings underscore the intricate influence of formulation adjustments on the characteristic features of snacks. Furthermore, the examination reveals minimal variance in Young's modulus among the tested products (Fig. 2B). In comparison to other items, our product demonstrates a lower stiffness than snacks[95] and crackerbread[96], again implying a propensity for improved oral comfort in older adults. Thus, further experiments were held to improve the insight into the oral processing and palatability of the products.

A



B

Index	Ref + ref	Maca + ref	Maca + OLE
Hardness (N)	21 ± 2[a]	16.0 ± 0.9[a,b]	11.2 ± 0.3[b]
Young's modulus (MPa)	0.72 ± 0.13[a]	0.61 ± 0.05[a]	0.47 ± 0.03[a]

Fig. 2 Texture profile analysis (TPA) for plant-based prototypes shows enhanced oral comfort through functional materials addition. (A) Strength to strain TPA graph. (B) Summary table of textural properties values. Values in the same line followed by a different letter are significantly different ($P < 0.05$).

3.1.3 *Characterization of samples through tribological measurements*

The lubrication properties of the products were evaluated using a ball-on-disk tribometer featuring hydrophobic elastomer surfaces resembling bio-tribological contacts (Fig. 3). The Stribeck curve

Table 3
Proximate analyses of plant-based cereal prototypes ($n = 3$) (30 g portion size).

Index	Pre-extrusion			Post-extrusion	
	Mix	Mix + maca	Ref + ref	Maca + ref	Maca + OLE
Calories* (kcal)			126	126	~126
Total fat (g)			5.112 ± 0.006[a]	5.7 ± 0.2[a]	6.0 ± 0.3[a]
Protein (g)	3.15 ± 0.06[a]	3.24 ± 0.03[a]	3.83 ± 0.02[b]	3.75 ± 0.06[b]	3.9 ± 0.1[b]
Sodium (g)	0.03 ± 0.02	0.08 ± 0.03	0.06 ± 0.03	0.06 ± 0.02	0.06 ± 0.02
Total Carbohydrate* (g)			8	8	8
Dietary fiber* (g)			5 (20%)**	5 (20%)	5 (20%)
Total sugar (g)			1.3 ± 0.2[a]	1.0 ± 0.2[a]	1.2 ± 0.2[a]
Iron (mg)	9 ± 2	8 ± 1	6.8 ± 1.8 (85%)	5.0 ± 0.2 (63%)	4.0 ± 0.3 (50%)
Calcium (mg)	32.99 ± 2.91	51 ± 7	32 ± 5 (3%)	37.90 ± 2.65 (3%)	32 ± 2 (2%)
Magnesium (mg)	30.2 ± 3.1	41 ± 7	40 ± 5 (11%)	49 ± 4 (13%)	41 ± 4 (11%)
Phosphorus (mg)	36 ± 3	49 ± 5	60 ± 4 (9%)	78 ± 3 (11%)	67 ± 4 (10%)
Potassium (mg)	93.20 ± 9.22	150 ± 18	108.76 ± 11.78 (4%)	142.50 ± 6.81 (5%)	108.3 ± 9.3 (4%)
Zinc (mg)	0.66 ± 0.07	0.59 ± 0.05	0.94 ± 0.31 (10%)	0.965 0 ± 0.295 1 (10%)	0.9 ± 0.3 (10%)
Copper (mg)	0.13 ± 0.05	0.092 ± 0.008	0.3 ± 0.1 (0%)	0.164 ± 0.011 (0%)	0.14 ± 0.02 (0%)
Selenium (mg)	19 ± 2	24.937 ± 4.281	23.71 ± 5.31 (43%)	32 ± 4 (58%)	28 ± 4 (51%)
Manganese (mg)	1.0 ± 0.6	0.53 ± 0.05	0.917 3 ± 0.462 1 (45%)	0.48 ± 0.02 (24%)	0.31 ± 0.05 (15%)

Note: Values in the same line followed by a different letter are significantly different ($P < 0.05$). * Theoretical values based on individual ingredients composition (Tzameret software program used by the Israeli Ministry of Health). ** Percentage of average RDA for men and women aged 51–70 years.

graphically illustrates the tribological behavior and lubrication properties, traditionally divided into three regimes: boundary, mixed, and hydrodynamic.

The friction behavior of water shows that lubrication occurred in the boundary and mixed regimes, whereas the lubrication behavior of model saliva showed boundary, mixed and the onset of hydrodynamic regime, indicating that both lubricants and the surface properties influenced the lubrication properties. For Maca + OLE boli, the friction curve displayed the three regimes, a boundary plateau at lower speeds (< 10 mm/s), a mixed regime (10–100 mm/s), and a hydrodynamic lubrication regime (> 100 mm/s) (Fig. 3A). In contrast, both Ref + ref and Maca + ref boli exhibited superimposed behavior with low coefficient of friction (μ) at entrainment speeds < 100 mm/s, corresponding to the mixed regime, and an increase in μ when entrainment speeds exceeded 100 mm/s, indicative of the hydrodynamic lubrication regime (Fig. 3A). These frictional curve behaviors mirror the lubrication characteristics observed in some edible oils and plant-derived oleosomes[97-98] suggesting that some oil might have been released from the cereal matrix. Considering that all products had similar composition (Table 3, $P > 0.05$), the differences in oil release and its effect on the lubrication profile might have been influenced by the fracture properties of the products, where Maca + OLE presented significantly reduced sample hardness (Fig. 2B, $P < 0.05$). The impact of mechanical properties on chewing and bolus formation has been previously discussed, noting that the formation of larger bolus particles tends to decrease as the hardness of food increases[99]. Consequently, it is plausible that a reduced breakdown of the food matrix could contribute to a reduction in the release of oil from the matrix.

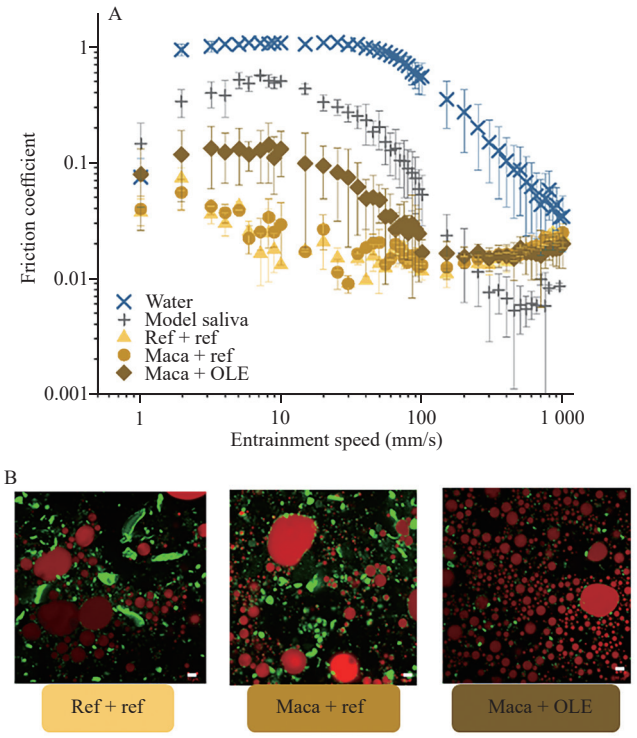


Fig. 3 (A) Mean friction coefficients as a function of entrainment speeds and (B) CLSM images of Ref + ref, Maca + ref, Maca + OLE. Protein aggregates are stained fluorescently green using Fast green whilst fat droplets are stained fluorescently red using Nile red. The friction curve of water and model saliva are added as reference. Data represent average of triplicate measurements on three samples ($n = 2 \times 3$).

As determining bolus particle size using the Mastersizer instrument was considered inappropriate due to potential shear degradation caused by the impeller, and because conventional laser diffraction instruments assume spherical particles leading to an overestimation, especially for the volume-weighted mean diameter, which emphasizes larger particles within the distribution, confocal microscopy images of the bolus samples were acquired to observe the relative distribution of protein and fat components (Fig. 3B). In both, Ref + ref and Maca + ref boluses, substantial protein structure fragments and prominent fat coalescence areas were evident. In contrast, Maca + OLE bolus displayed smaller protein fragments and more uniformly distributed smaller oil droplets. Based on these observations, it is likely that in both Ref + ref and Maca + ref boluses, the observed low μ at entrainment speeds < 100 mm/s is associated with the formation of a lubricating oil layer on the surfaces. At entrainment speeds exceeding 100 mm/s, some small particles might have entrained into the contact, becoming dominant and resulting in higher μ. For Maca + OLE the friction behavior was likely governed by the small, dispersed oil droplets and soluble substances forming the boundary lubricating regime. As speed increased (> 10 mm/s), bigger boli particles/droplets might entrained between contact surfaces, forming a lubricating film and decreasing μ with increasing speed. At high entrainment velocities (> 100 mm/s), complete separation of surfaces occurred, and large particles entering the gap increased the friction coefficient (Fig. 3A). Thus, these experiments helped quantitate some of the characteristics of oral boli of the products and establish the possible benefits that arise from the use of the functional supplements. Yet, these instrumental measures needed to be confirmed in the target population through corresponding sensory evaluations of oral comfortability and palatability.

3.2 *Comprehensive in vivo sensory evaluation*

3.2.1 *Food comfortability*

Mastication is a key step in the indulgence humans extract out of food and it has repercussions to the product's commercial success as well as its subsequent breakdown in the consumer's gut. Thus, food comfortability was evaluated in a human cohort using a questionnaire following a procedure described previously[68,100] and the results are plotted on different spider plots (Fig. 4). First, self-reports on bolus formation (Fig. 4A), mouth pain (Fig. 4B), texture sensation (Fig. 4C) and flavor (Fig. 4D) were assessed and found not to be adverse nor modulated significantly by the addition of the supplements. Particularly noteworthy is the observed self-reported ease in performing important masticatory operations (cutting, chewing, and swallowing) towards bolus formation, coupled with a relatively short total time (Fig. 4A) and very low reports on mouth pain (Fig. 4B). Together with the instrumental texture results (Fig. 2), the findings indicate enhanced oral comfort of the functional product which could be particularly beneficial for seniors with physical limitations hindering the consumption of complex or tough foods[5]. Analysis of the perceived properties of the products reveal that the products were characterized as being crunchy and melting (Fig. 4C). This suggests that the filling does not compromise the shell integrity and hardness due to possible moistening. The absence of extreme flavors (Fig. 4D) and low scores for bitterness and acidity, coupled with the low sugar content (Table 3), may contribute to this outcome. These positive perceived properties were also

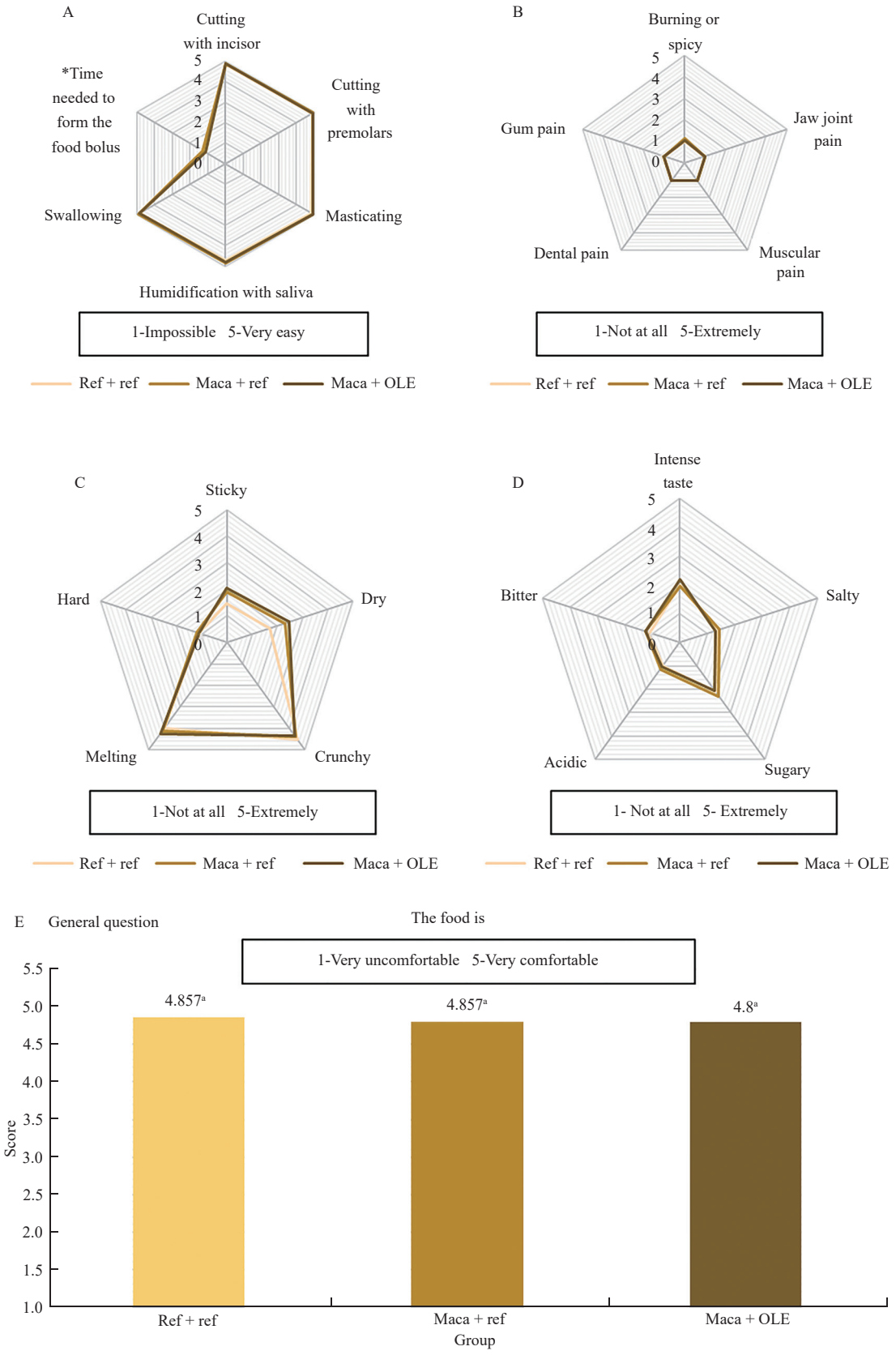


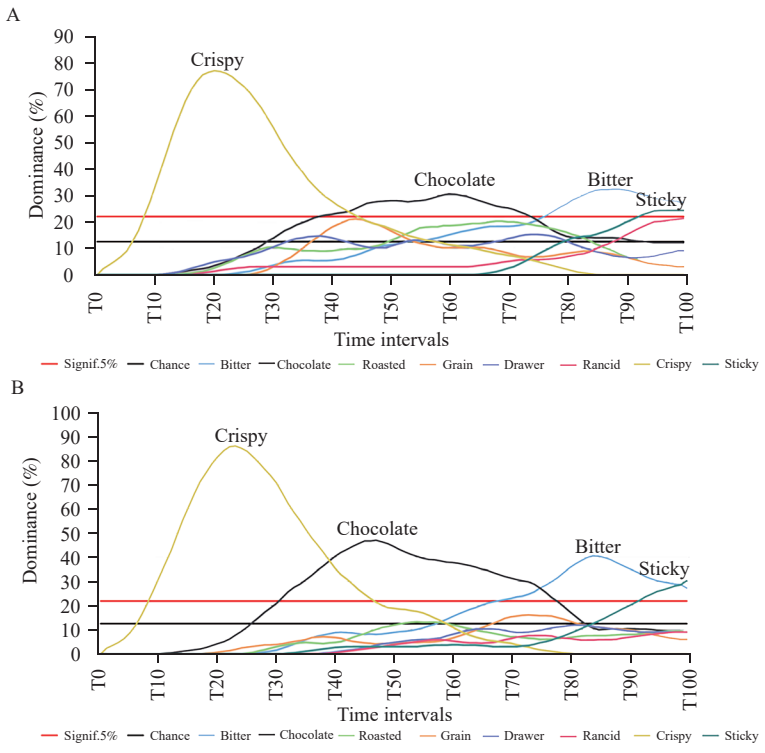
Fig. 4 Results from the questionnaire on the perception of food comfortability. (A) Bolus formation and time needed (scale 1–5: "Impossible" to "Very easy" and "Impossible" to "Very brief"). (B) Pain perception (scale 1–5: "Extremely" to "Not at all"). (C) Texture perception (scale 1–5: "Extremely" to "Not at all"). (D) Flavor perception (scale 1–5: "Extremely" to "Not at all"). (E) General food comfort (scale 1–5: "Very uncomfortable" to "Very comfortable"). Values followed by a different letter are significantly different ($P < 0.05$). All sections consistently use a 5-point scale.

expressed in the high scoring of the products' oral comfort (Fig. 4E), with scores exceeding 4.8 out of 5.0. This adds an extra layer of evidence to reinforce the overall positive reception of the products and their potential palatability to the target customers.

3.2.2 *Sensory descriptions by the trained panel via QDA and TDS*

QDA provided a comprehensive description of sensory properties from a static perspective, while TDS identified the dominant properties over the consumption period (dynamic perception). The outcomes demonstrated were very much aligned, with minor differences observed among the samples (Fig. 5).

According to TDS (Figs. 5A–B), crispiness–a desirable characteristic for this product – dominated at the beginning of oral processing for Maca + ref and Maca + OLE, persisting for approximately 50% of the eating duration. Chocolate flavour prevailed in the middle of the consumption process for all samples, especially Maca + OLE (Fig. 5C). Toward the end, bitterness and stickiness became more prominent, although these attributes received low to medium scores (0.2 – 0.4), suggesting either limited consensus among evaluators, or that there were no clear dominating attributes in the evaluation. The results indicated that the combination of flours and the oil-based filling resulted in a somewhat sticky bolus, occurring toward the end of consumption. However, this was not a major concern, as research suggests that boluses with more saliva tend to have a dry and sticky texture, which can be beneficial for oral issues in the older adult demographic[101]. Moreover, prolonged chewing associated with sticky foods is essential for both eating and overall well-being. Recent research highlights the cognitive benefits of chewing, particularly in the hippocampus, a region of the central nervous system responsible for spatial memory and learning[102]. The panel described the maca sample as significantly less roasted, chocolatey and less juicy, and with significantly more drawer odour (Fig. 5). The older adult panel, however, did not notice the differences in dryness (Fig. 4), which makes sense as the trained panel is more sensitive because of the training, and they found a significant but minor



C

	Maca + ref	Maca + OLE	*P*-value
Sweetly odour	4.62^{A}	4.85^{A}	0.095
Chocolate odour	3.89^{A}	4.03^{A}	0.266
Nutty odour	3.40^{A}	3.53^{A}	0.319
Roasted odour	3.92^{B}	4.31^{A}	0.039
Grain odour	4.54^{A}	4.60^{A}	0.693
Drawer odour	5.21^{A}	4.28^{B}	0.017
Rancid odour	3.86^{A}	3.00^{A}	0.112
Sweet taste	4.27^{A}	4.43^{A}	0.413
Salty taste	3.38^{A}	3.31^{A}	0.588
Bitter taste	5.48^{A}	5.29^{A}	0.075
Chocolate flavour	3.88^{B}	4.19^{A}	0.007
Nutty flavour	3.36^{A}	3.33^{A}	0.883
Roasted flavour	4.28^{A}	4.72^{A}	0.093
Grain flavour	4.63^{A}	4.79^{A}	0.308
Drawer flavour	4.83^{A}	4.26^{A}	0.124
Cloying flavour	4.08^{A}	3.20^{A}	0.077
Rancid flavour	3.78^{A}	2.88^{A}	0.108
Crispness	6.19^{A}	6.08^{A}	0.356
Juiciness	4.21^{B}	4.74^{A}	0.011
Fatness	3.78^{A}	3.87^{A}	0.526
Stickiness	5.55^{A}	5.50^{A}	0.619
Astringency	4.51^{A}	4.37^{A}	0.228

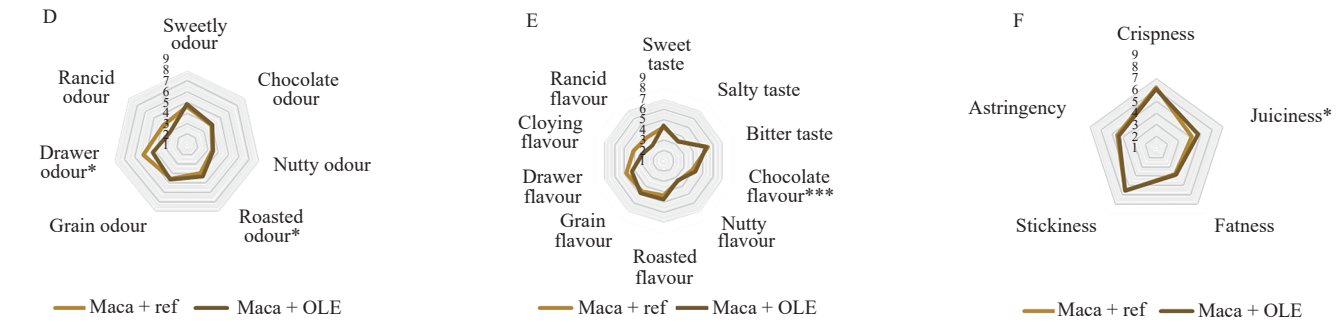


Fig. 5 TDS, QDA, and spider plots derived from the trained panel sensory description of two products (Maca + ref, Maca + OLE). TDS curve of (A) Maca + ref and (B) Maca + OLE. (C) QDA results of the two formulae. Values followed by a different letter are significantly different ($P < 0.05$). (D–F) Spider plots of QDA results (odour, taste/flavour, texture). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

difference. In terms of bitterness, maca and OLE are usually bitter and earthy flavours, the panel rated both samples similarly in terms of bitterness. However, their inclusion had no discernible effect on product acceptance or characteristics, as supported by the tasting tests conducted on older adult participants in Israel (Fig. 4). The trained panel evaluated the samples in accordance with the data of oral comfort as described by the older adult subjects, revealing minimal significant differences among various samples.

3.3 Studying protein breakdown using a semi-dynamic IVD model

3.3.1 SDS-PAGE results

Age-related changes have been shown to affect digestive parameters[5]. In turn, altered glycemic index functions may divert the digestive fate of foods compared to that assessed in healthy young adults. Therefore, this work evaluated the IVD proteolysis of the products using a recently agreed protocol for recreating digestion in older adults[9]. Utilizing SDS-PAGE analysis as a primal qualitative tool, we dissected protein digestion of the various products and compared between their digestion in young and older adults (Figs. 6 and 7, respectively). First, we investigated the impact of adding functional ingredients (Maca + OLE) on protein breakdown trajectories in seniors. Studies indicate that food antioxidants may interact with proteins thereby hindering their accessibility to digestive breakdown[47-48]. The findings here revealed no significant differences in the protein breakdown patterns, with a sustained gastric digestion that is practically completed during the intestinal phase (Fig. 6). Moreover, bands corresponding to most proteins and large peptides, observed during the gastric phase, had dissipated during the intestinal phase. Yet, extrusion processing may impact protein nutritional quality, with studies highlighting both beneficial and deleterious effects of processing on protein digestibility[37,50]. The structural changes induced by high-temperature, short-duration, and high-pressure conditions of extrusion may enhance protein digestibility by denaturing proteins and exposing enzyme-accessible sites or inactivation of anti-nutritional moieties[50]. Thus, further tests focused on the protein digestibility of pre- and post-extruded plant-based cereals, as determined from analysis of the bioaccessible soluble aliquots of digestive effluents (Fig. 7). It is important to note, the pre-extruded product (Mix + maca) exhibited faint protein bands that were attributed to the lack of release of the proteins from the food matrices, i.e. proteins were not released to the gastric lumen or collected for the SDS-PAGE analyses, both in older and young adults (Figs. 7A and C). Contrary, visible protein bands were noted in the corresponding gels of processed samples (Figs. 7B and D), which suggests a higher bioaccessibility of proteins in these processed samples, both for young and older adults. This would concur with previous studies supporting extrusion processing potential to unleash the nutritional potential of plant proteins[36-37,50]. When comparing the digestive trajectories observed during digestion of the processed samples (Figs. 7B and D), protein breakdown was found to be more delayed under the senior's gut conditions compared to that of a young adult. In fact, protein bands were found to endure no longer than 30 min of gastric digestion under the conditions of a young adult (Fig. 7D) while such bands were found to persist for up to 180 min of gastric digestion under conditions of older adults (Fig. 7B). This noticeable divergence between the two target populations emerged within just 15 min of gastric digestion, signifying a delayed digestion in seniors. This distinction underscores the influence of physiological changes on the digestive proteolysis patterns of food products in young and older adults which could have nutritional ramifications. Therefore, further in-depth qualitative view of protein breakdown profiles was sought.

3.3.2 Proteomic analysis of bioaccessible peptides

From the nutritional perspective, proteins are broken down into bioaccessible peptides and amino acids that are up taken into the body where they may exercise their biological function. First, LC-MS/MS analysis was used to identify the bioaccessible peptides present in aspirated digesta samples (Fig. 8). These analyses provide a comparative look into the bioaccessible peptide distributions recovered from the digestion of pre-extrusion and post-extrusion products as well as differentiating between the adult and older adults' patterns, according to peptide amino acid length. These revealed no significant differential patterns of bioaccessible peptides between adults and seniors (Fig. 8). This could be attributed to the dense matrix of cereal products that may limit

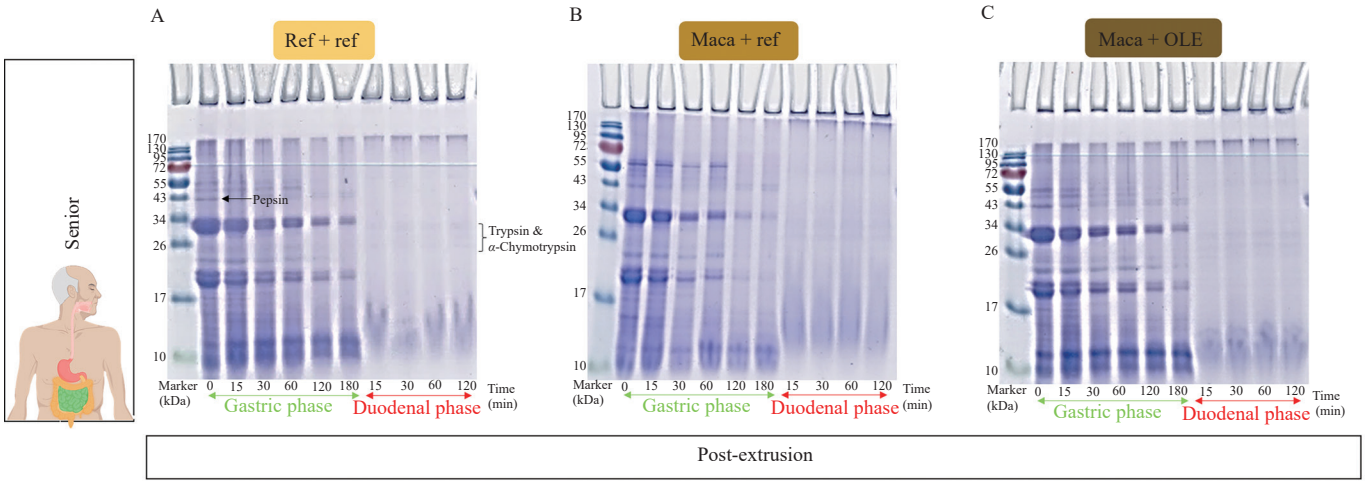


Fig. 6 Consistent protein breakdown pattern: SDS-PAGE analyses of senior digestion for post-extruded products. (A) Ref + ref, (B) Maca + ref, (C) Maca + OLE.

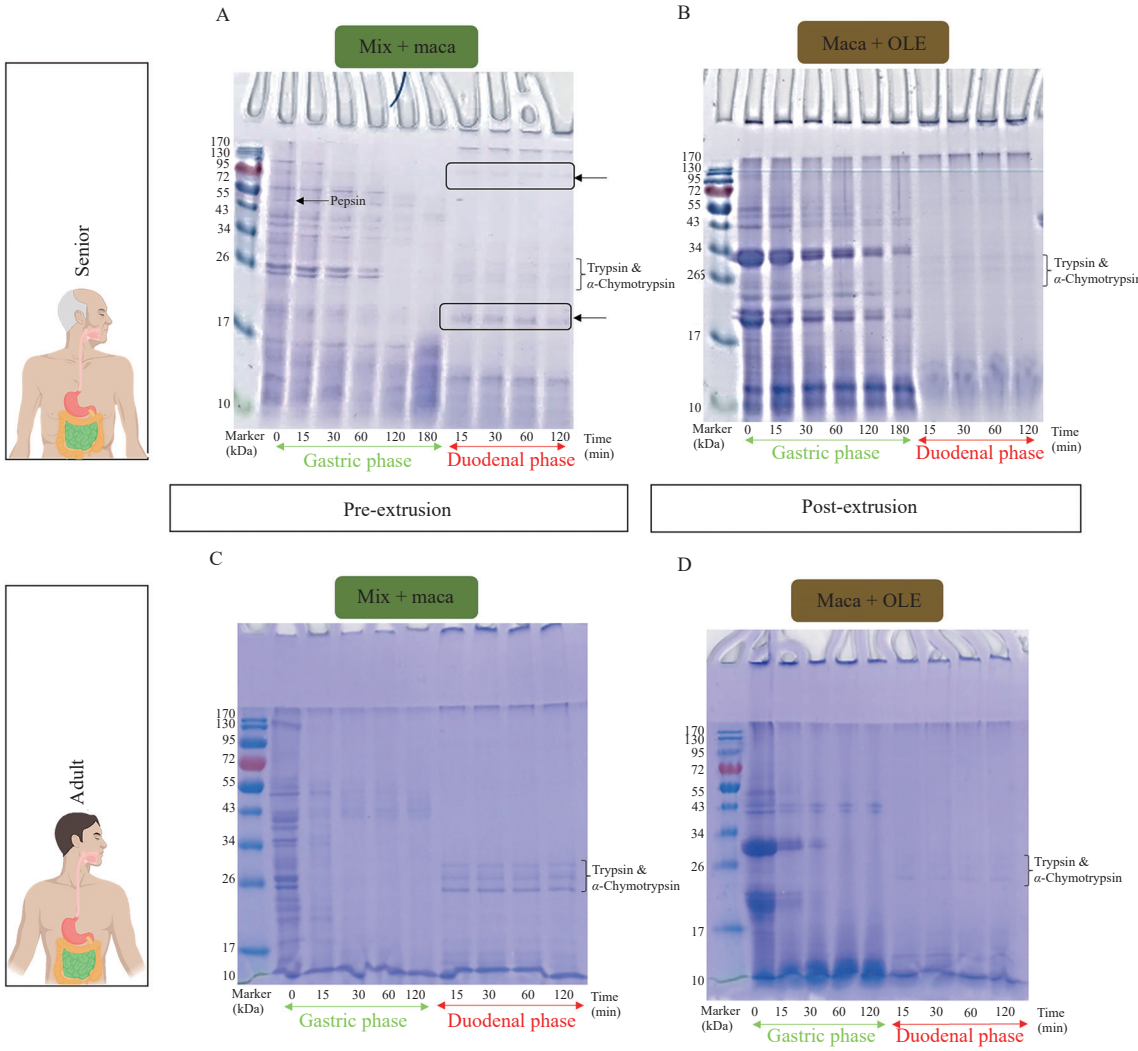


Fig. 7 Comparative SDS-PAGE analyses of digesta samples shows enhanced proteolysis in post-extruded products (Maca + OLE) and reduced proteolysis in senior IVD *vs*. adult. (A, C) Protein breakdown patterns of the pre-extruded (Mix + maca) and (B, D) post-extruded product (Maca + OLE) under (A–B) senior and (C–D) adult IVD conditions.

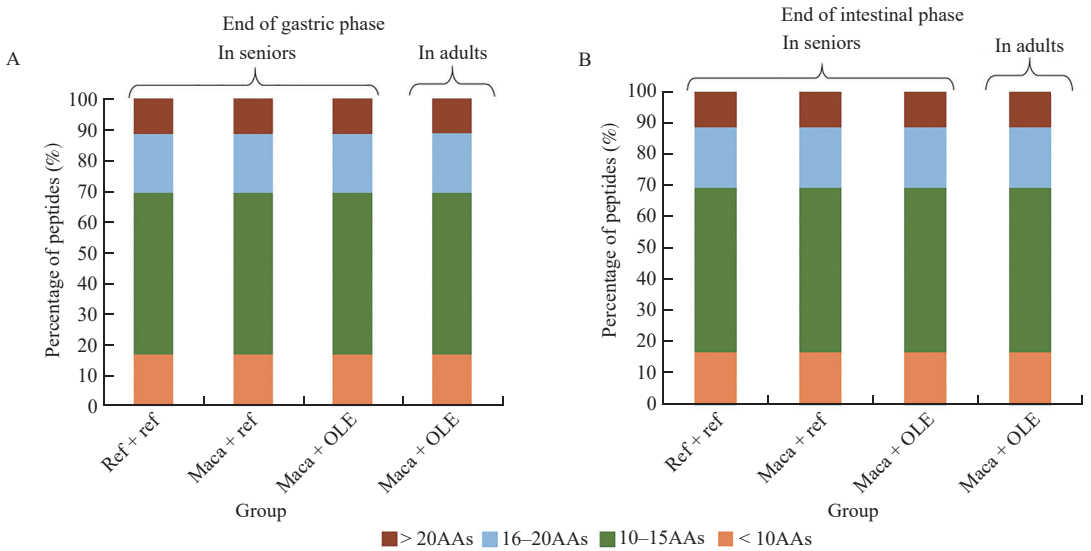


Fig. 8 Uniform peptide distribution across LC-MS/MS samples collected from (A) the end of the gastric phase and (B) the end of the intestinal phase in IVD model under senior and adult conditions of the different formulae. Columns for adult are in full color, and for senior are dashed. AAs, amino acids.

pepsin's access to protein substrates, as reported in the past for proteinaceous gels[103-104]. The intricate nature of the cereal product matrix may obscure physiological variations between adults and the older adults, potentially making any differences in digestibility less conspicuous in our findings. While SDS-PAGE analyses highlighted a delayed proteolysis in seniors for proteins between 10–170 kDa, these analyses indicate that at the low molecular weight peptide level below 10 kDa (up to 52 amino acids) there are no pronounced differences.

A similar investigation comparing meat digestion in seniors and adults yielded analogous results[43], supporting the notion that the properties of the food matrix may obscure effects arising from age-related physiological differences in gut functions. However, two points should be emphasized: 1) the absence of apparent differences noted herein do not necessarily imply equal digestion, as the IVD findings do not account for the full kinetics of digestive breakdown[105-106]; 2) similarities in the bioaccessible peptide lengths does not necessarily mean similarities in the amino acid sequences of these peptides. Moreover, these analyses focused on peptide lengths between 10 up to 52 amino acids, thus, overlooking insights into free amino acids which are absorbable and of nutritional importance[107-110].

3.3.3 *In vitro DIAAS*

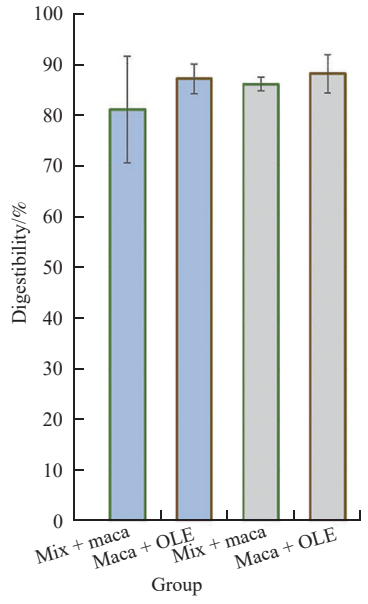
From the nutritional perspective, protein quality is assessed through scoring its ability to provide the body with amino acids. Practically, this is done through various methods with DIAAS being the most recently advocated method[77,79,111-112]. The newly introduced *in vitro* DIAAS methodology provides a robust framework for assessing the protein quality of human foods, leveraging ileal digestibility as a key metric and circumventing the need for ethically challenging and time-consuming human trials[77]. This *in vitro* scheme was used in this study and the resulting digestibility data of the functional product (before and after processing) are given in Fig. 9 and Table S2. Comparing the product's digestibility in adults or seniors (over 65 years) gave rise to two main observations. One was that there were no significant differences in the product's digestibility and nutritional quality in adults or seniors (Figs. 9A and B). The second observation is that processing increased the product's digestibility to obtain highly digestible proteins (total protein digestibility exceeding 80%, Fig. 9B). This showcases the potential for producing a nutritionally rich cereal product with sustainable plant-based protein sources. Moreover, these digestibility values surpass the typical digestibility range of plant-based sources, which are typically between 40% and 80%[36-37,77].

Furthermore, the DIAAS values were determined by considering the lowest *in vitro* DIAA ratios for older child, adolescents, and adults for each analyzed substrate. The resulting lowest limiting AA and their respective value were as follows: in the pre-extruded product, Mix + maca exhibited *in vitro* DIAAS values of lysine (84 ± 46) and tryptophan (64 ± 35) for adults and seniors, respectively. For the post-extrusion product, Maca + OLE recorded values of lysine (34 ± 3) and lysine (33.9 ± 0.3) for adults and seniors, respectively. These findings highlight that co-extrusion may decrease the bioaccessibility of specific amino acids, notably lysine and tryptophan which concurs with previous reports[50,113]. This affirmed that although processing may compromise the bioaccessibility levels of specific amino acids, this did not come at the expense of an overall low digestibility of the product. Thus, this work confirmed that the combined formulation of a cereal product with four different flours yielded a palatable product with high digestibility scores, even when assessed under the age- deteriorated conditions of the older adults' gut.

4. Conclusion

Nutrition is expected to play a pivotal role in promoting healthy aging, and age-tailored functional foods could help capitalize on this notion. To this end, food research and development efforts need to

A



B

Sample digestibility (%)	Adult		Senior	
	Mix + maca	Maca + OLE	Mix + maca	Maca + OLE
HIS	86 ± 16	89 ± 3	89 ± 1	90 ± 1
SER	72 ± 30	79 ± 6	77 ± 6	82 ± 3
ARG	80 ± 19	86 ± 3	81 ± 2	88 ± 1
GLY	82 ± 24	95 ± 3	95 ± 1	96 ± 1
ASP	82 ± 21	89 ± 3	91 ± 8	88 ± 1
GLU	86 ± 14	89 ± 2	88 ± 4	88 ± 1
THR	78 ± 23	84 ± 4	84 ± 4	87 ± 2
ALA	83 ± 18	87 ± 3	88 ± 4	88 ± 1
PRO	84 ± 14	86 ± 5	88 ± 2	87 ± 2
LYS	74 ± 28	87 ± 5	84 ± 10	86 ± 3
TYR	71 ± 19	76 ± 5	76 ± 6	82 ± 3
CYS	59 ± 33	57 ± 11	31 ± 31	55 ± 14
MET	95 ± 3	96 ± 3	97 ± 2	97 ± 2
VAL	76 ± 24	82 ± 4	83 ± 4	87 ± 2
ILE	77 ± 22	82 ± 4	83 ± 3	86 ± 2
LEU	83 ± 15	86 ± 3	87 ± 2	89 ± 1
PHE	84 ± 16	87 ± 3	87 ± 1	90 ± 1
TRP	68 ± 28	66 ± 35	46 ± 26	68 ± 13
Total digestibility (%)	81 ± 19[a]	87 ± 3[a]	86 ± 4[a]	88 ± 1[a]

Fig. 9 High *in vitro* digestibility highlighted in results. (A) Comparative protein digestibility percentages in different cereal products pre- and post-extrusion under adult and senior IVD models. (B) Table presenting digestibility percentages of individual amino acids. Different letters denote significant differences ($P < 0.05$).

support dietary shifts towards healthier and more sustainable food choices, such as those suggested in the EAT-Lancet report[4]. As an example of relevant EAT4AGE consortium outputs, this work developed plant-based prototypes of co-extruded cereals designed for superior macronutrient profiles that surpass existing commercial products and are pleasurable to the consumer. With *in vitro* digestibility exceeding 80%, this project makes a valuable addition to the table of choices for a balanced and sustainable diet for the aging demographic. Yet, bridging the gap between our various analyses and real-world *in vivo* efficacy requires further investigation which could be jointly pursued by industry and academia together. In fact, this work joins rising efforts to develop nutritional solutions that seek to tackle concerns over the health gap of the soaring global aging population. Overall, this research emphasizes a nutritional paradigm that tailoring food solutions for seniors, or any specific target population, need to intersect nutritional values with consumer preferences for enjoyment. Thus, this work hopes to stimulate an avenue of endeavors towards innovative, nutritionally optimized products that seek to boost human health and quality of life.

Ethical statement

The Technion Ethics Committee granted this research its institutional review board (IRB) permission (form No. 139-2022) and approved the untrained consumer panel as well as the ethical collection of human salivary and bolus samples as part of it (both conducted in Israel). Each participant signed informed permission papers after being made aware of the study's objectives and methods, being checked to see if they met the requirements for inclusion and passing a questionnaire.

Conflict of interest

The authors report no conflict of interest.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250421.

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