Protein digestibility and techno-functional performance of milk-alternative prototypes based on combinations of lentil and cereal protein

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Abstract

Lentil protein isolate was combined with proteins from oat, rice, brewer's spent grain (BSGP) and wheat to achieve plant-based milk alternatives (PBMA) with improved protein quality and functionality. Due to the complementary amino acid (AA) profile of pulse protein which is high in lysine, and cereal protein which is high in sulphur amino acids, their combination at an optimised ratio resulted in a protein blend with a significantly improved indispensable amino acid score (IAAS) compared to the single ingredients. All protein combinations with lentil except for wheat resulted in a full IAAS for adults. The *in-vitro* protein digestibility was assessed using the static INFOGEST digestion model to calculate the proxy *in-vitro* DIAAS (PIVDIAAS) of the emulsions. Techno-functional properties such as particle size, rheological behaviour and physical stability were investigated. The PIVDIAAS of the combined protein emulsions was found to be 0.72, 0.78, 0.83, 0.98 for Lentil+Wheat, Lentil+Oat, Lentil+BSGP and Lentil+Rice emulsions, respectively, compared to 0.48, 0.25, 0.5, 0.67 and 0.81 determined for the emulsions based on lentil, wheat, oat, BSGP and rice alone, respectively. The emulsions based on the combination of lentil and cereal protein also showed improved physical stability regarding sedimentation and creaming, and a higher whiteness index of the emulsions. It could be shown that the combination of lentil and cereal protein is a promising strategy to achieve PBMAs with improved protein quality and techno-functionality.

Introduction

The large environmental footprint of dairy farming and a heightened awareness for the ethical implications of industrial animal husbandry have led to an increased demand for plant-based milk alternatives (PBMAs). From a nutritional perspective, commercially available PBMAS often have lower mineral content and higher glycaemic index compared to dairy products. Additionally, they often fall short when compared to dairy due to lower protein content, digestibility and quality (1). Protein quality is determined by its ability to meet the metabolic needs of the human body for indispensable amino acids (IAAs) and nitrogen. The FAO amino acid score (AAS) pattern indicates the minimum concentration of amino acids in mg/g protein to meet these metabolic needs and varies depending on the age of the consumers (2) (see **Table 1**). A comprehensive study of the nutritional profile of PBMAs compared to dairy found that none of the tested PBMAs fulfilled the AAS pattern requirements for older children and adults, while dairy contained all IAAs at sufficient levels. The soy-based products contained the highest amount of IAAs but were lacking in sulphur amino acids (SAAs) and valine, almond-based PBMAs were low in lysine, SAAs and valine, oat-based products were found to be lacking in lysine, and rice-based PBMAs were low in isoleucine, leucine, lysine and valine. Protein content in the PBMAs was also found to be extremely low, with percentages <0.5% in most of the analysed oat, rice and coconut-based products (3). Soy is the primary plant-based source of lysine in dairy alternatives, however, consumer concerns regarding genetically modified crops, soy allergies, and a quest to improve biodiversity amongst food crops have led to intensified research into the application of other grain legumes, such as lentils (4,5). Lentils are high in protein and lysine, with similar protein functionality to soy (6), and show potential as nitrogen crops in nemoral and boreal climates that could lessen the dependence of soy imports in the northern hemisphere (7,8). In combination with cereal proteins, which are low in lysine but high in SAAs, lentils pose a promising alternative source of high-quality protein to both soy and dairy protein. Furthermore, by applying lentil protein isolate and cereal protein isolates and concentrates in this study, it was also possible to produce PBMA prototypes with a higher protein content, similar to that found in cow's milk.

To fully assess the nutritional quality of a protein, the AAS is not sufficient due to the varying digestibility of food proteins, i.e. how much of the protein is digested, absorbed and available to

the human metabolism. Protein digestibility of foods is highly dependent on various factors such as protein structure, matrix effects such as fibre, accessibility of the protein to digestive enzymes, and antinutritional factors such as trypsin inhibitors, lectins, phytates and tannins (9). The Digestible Indispensable Amino Acid Score (DIAAS) quantifies protein quality by the amount of each individual IAA absorbed in the ileum, the terminal part of the small intestine. True ileal digestibility is ideally determined in humans by naso-ileal sampling, and an *in-vivo* alternative is the use of ileum-fistulated growing pigs (2). These methods are invasive, costly and raise ethical concerns, and *in-vitro* models such as the INFOGEST method that allows for a low-cost, reproducible assessment of the ileal digestibility of food proteins have been developed as alternatives (10–12). DIAAS values obtained with the static INFOGEST *in-vitro* digestion method have been shown to have a high correlation with *in-vivo* data from humans and pigs (13–15). As the analysis of individual AAs is costly, the proxy *in-vitro* DIAAS (PIVDIAAS) has been evaluated in this study, i.e. the results of the overall nitrogen digestibility of the PBMAs have been applied for all IAAs instead of the digestibility values of each AA, as proposed by Sousa et al. (13). With the results of the AA quantification of the lentil and cereal protein ingredients and the protein digestibility of the emulsions as measured with the static INFOGEST method, the PIVDIAAS of emulsions based on lentil and cereal proteins, and emulsions based on a combination of these ingredients for optimised AAS, could be assessed. In addition, functional properties crucial to the development of PBMAs that meet consumer demands, such as rheological behaviour, particle size distribution, physical stability and colour, were evaluated and synergistic effects of the combined protein ingredients assessed (16).

Table 1 Amino acid scoring pattern in mg of each indispensable amino acid or category of amino acids per g of dietary protein, depending on the age of the consumer (2).

Age *(yr)*	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
	(mg/g protein requirement)								
0 - 0.5	21	55	96	69	33	94	44	17	55
0.5 - 3	20	32	66	57	27	52	31	8.5	43
>3	16	30	61	48	23	41	25	6.6	40

His – histidine, Ile – iseoleucine, Leu – leucine, Lys – lysine, SAA – sulphur AA (cysteine + methionine), AA – aromatic AA (phenylalanine + tyrosine), Thr – threonine, Trp – tryptophan, Val – valine.

Materials and Methods

Raw Materials

Lentil protein isolate was produced by isoelectric precipitation as described by Alonso-Miravalles et al. (2019) and was provided by Fraunhofer IVV, Munich, Germany. Oat bran protein concentrate, commercial name 'PrOatein', was purchased from Lantmännen Oats (Kimstad, Sweden); wheat protein hydrolysate (Nutralys® W) was obtained from Roquette Frères (Lestrem, France); rice protein hydrolysate concentrate was purchased from A. Costantino & C. S.p.A. (Favria, Italy). Brewer's spent grain protein hydrolysate from barley and rice (EverPro™ Brewers' Grain Protein Isolate) was kindly provided by EverGrain Ingredients LLC, AB InBev (St. Louis, MO, USA). Sunflower oil was purchased from Tesco, Ireland, and sucrose was purchased from Sigma-Aldrich (St Louis, MO, USA).

Base Characterisation of the Protein Ingredients

Starch content was measured using the enzyme kit K-RARPS (Megazyme, Ireland), and FODMAP content was analysed by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), performed on a Dionex™ ICS-5000+ system (Sunnyvale, USA) as described by Ispiryan et al. (2019). Base compositional analysis (dry matter, fat, ash, sugars, dietary fibre, amino acids) was performed by an accredited external laboratory (Chelab S.r.l.,Resana, Italy) using validated methods based on ISO and AOAC standards (19). Total, soluble, and high molecular weight dietary fibre were analysed according to AOAC method 2017.16, and amino acid quantification was performed by ion chromatography with ninhydrin post-column derivatisation, or HPLC-UV for tryptophan. The protein content of the protein ingredients (PIs) was calculated as the sum of amino acids.

Protein Profile of the PIs

Protein was extracted from the PIs at a protein concentration of 2% in a reducing extraction buffer containing 5 M Urea, 2 M thiourea, 2 % SDS, 1.7% 2-mercaptoethanol and 0.1 M Tris/HCl (pH 8.8) by shaking at 500 rpm for 16 h at room temperature. After centrifugation at 14,000×g for 20 min, an aliquot of the supernatant was diluted with NuPAGE® LDS Sample buffer (4×) and Reducing Agent (10×) (Invitrogen, CA, USA), heated for 10 min at 70 °C and centrifuged at 14,000g for 2 min. Proteins were separated on NuPAGE® Bis-Tris gels (4-12%) (Invitrogen) with MES running buffer at 200 V, with a Precision Plus Protein™ Dual Xtra Prestained Protein Standard (Bio-Rad, CA, USA) as a molecular weight standard. Gels were stained using

InstantBlue® Coomassie (Abcam, Cambridge, United Kingdom) for 20 min and analysed using the software GelAnalyzer 23.1.1 (www.gelanalyzer.com, Istvan Lazar Jr.&Istvan Lazar Sr.)

Calculation of protein combinations

Based on the amino acid composition of the PI, the ratio of lentil to cereal protein on a g AA per 100 g of protein basis was determined using the Simplex method of linear programming (Excel ToolPak add-in, Microsoft, Redmond, USA) (20). The objective was to maximise the lysine content in the blend, with the constraints set to the fulfilment of the minimum requirements for all IAA (2). The ratios were then calculated on an ingredient as is basis by taking into consideration the varying protein and DM contents of the different PIs.

Preparation of Milk-alternative Prototypes

The protein emulsions were prepared by hydration, homogenization and pasteurization using a method described by Boeck et al. (2021), with minor modifications. 1.5% (w/w) of sunflower oil and the necessary amount of a single PI or a combination of lentil and cereal PI to reach a final net protein content of 3.5% (w/w, calculated as sum of amino acids) were used. Protein and fat contents were chosen to reach similar values to those found in semi-skimmed cow's milk. 1.9% (w/w) of sucrose was used to achieve sweetness levels comparable to those of bovine milk, based on the relative sweetness of lactose (22)Reference 23 Schaafsma et al. deleted.

***In-vitro* protein digestibility and determination of proxy *in-vitro* DIAASs**

In-vitro digestion simulation of the protein emulsions was performed using the static INFOGEST method with minor modifications (12). The activity of the pepsin from porcine gastric mucosa, and bovine bile salt concentration was assayed as described by Brodkorb et al. (2019), while the trypsin activity of the porcine pancreatin suspension was assayed after ultrasound treatment and centrifugation as outlined by Sousa et al. (Sousa et al., 2023) . Due to the low starch content of the PBMAs, the typically short oral phase of liquid foods and a previously shown small effect of salivary enzyme on the starch digestion kinetics in *in-vitro* digestion simulations, salivary amylase enzyme was not included in the simulated salivary fluid (SSF) (Brodkorb et al., 2019; Egger et al., 2019; Gwala et al., 2020). No gastric lipase was used in the simulated gastric fluid (SGF) as only the digestibility of dietary protein was the focus of the analysis (11,23). The volume of the omitted enzymes was replaced by water. For the digestion simulation, 5 mL of protein emulsion, or water for the enzyme blank, was used, and every digestion was performed in triplicate on separate days for each sample.

For the addition of enzymes and simulated digestion fluids, and incubation conditions of the *in-vitro* digestion simulation, the protocol of Brodkorb et al. (2019) was followed. Immediately after the intestinal phase, methanol (-20 °C) was added to reach a final concentration of 80% and the indigestible proteins were precipitated for 1 h at -20 °C. Separation of digestible and indigestible fractions, monitoring of the tube and digest weights, and drying of the food pellets were performed as described by Sousa et al. (2023). Total nitrogen in the digestible and indigestible fractions was determined by Kjeldahl (24). The *in-vitro* protein digestibility (IVPD) was calculated by the nitrogen ratio in the digestible and indigestible fractions, corrected by the nitrogen in the enzyme blank, by applying formula 1 (13):

$$\text{IVPD } [\%] = \frac{(\text{Fs}-\text{EBs})}{(\text{Fs}-\text{Ebs})+\max(0;\text{Fp}-\text{Ebp})} \times 100\% \qquad [1]$$

Fs = nitrogen in supernatant of digested protein emulsion; Ebs = nitrogen in supernatant of digested enzyme blank; Fp = nitrogen in pellet of digested protein emulsion; Ebp = nitrogen in pellet of digested enzyme blank

Proxy *in-vitro* DIAAS (PIVDIAAS) values were calculated based on the amount of the limiting IAA in the single or blended protein ingredients, the IVPD, and the reference protein for either children from the age of 6 months to three years, or adults. The following formula was used (13,25):

$$\text{PIVDIAAS } [\%] = \frac{\text{mg of limiting IAA per g of food protein x IVPD } [\%]}{\text{mg of that AA in 1 g of reference protein}} \times 100\% \quad [2]$$

Rheology

The flow behaviour of the protein emulsions at 20 °C and 80 °C was analysed using a controlled stress rheometer (MCR301, Anton Paar, Graz, Austria) with a concentric cylinder measuring system (C-CC27-T200/SS, Anton Paar). A shear ramp was performed by equilibration to a defined temperature for 2 min, linearly increasing shear rate $\dot{\gamma}$ from 0 to 100 s^{-1} over 60 s and holding at 100 s^{-1} for 3 min, measuring shear stress τ every 4 s.

The flow behaviour index n and consistency coefficient K were calculated on the upward ramp from 10-100 s-1 by linear regression, fitting the data to the power law model using formula 3 (26):

$$\tau = K \times \dot{\gamma}^{n} \qquad [3]$$

Goodness of fit of the power law model was evaluated by the coefficient of determination (R2), and the difference of predicted and experimental data was calculated as the relative percentage error PE (27):

$$PE\ [\%] = \frac{100}{n}\sum_{i=1}^{n}\left[\frac{\tau_{observed}-\tau_{predicted}}{\tau_{observed}}\right] \qquad [4]$$

Apparent viscosity (η) was calculated at a shear rate of 100 s^{-1}, which correlates to viscosities found in-vivo in the mouth (28). Reference 29 Sonne et al. removed

Physical Stability

The rate of separation of the protein emulsions was measured using a LUMISizer (LUM GmbH, Berlin, Germany), by centrifugation and analysing the change in light transmission through the sample caused by creaming and sedimentation. The samples were centrifuged for 90 min at 1000×g at 15 °C, simulating storage at cool conditions for two months (29). The relative height of the cream and sediment layer after centrifugation, and the separation rate (calculated as the gradient of the cumulative light transmission over time plots) were determined.

Droplet and Particle Size Analysis

Both the dry PIs and the emulsions were measured on a Mastersizer 3000 (Malvern Panalytical Ltd, Malvern, UK). The analysis of the dry PIs was carried out using a dry feed cell by dispersion in air at a pressure of 1.5 bar, sample refractive index of 1.45 and a laser obscuration between 2-4%. The particle and droplet size distribution of the emulsions were measured in a wet cell with ultrapure water as the continuous phase (refractive index 1.33), with the particle refractive index set to 1.45 and measurements performed at a laser obscuration of 7 ± 0.5%. The emulsions were shaken by hand to ensure representative sampling, and diluted 1:10 with ultrapure water prior to analysis (29). Median particle diameter ($D_{[50]}$), particle diameter at the 10th and 90th percentile ($D_{[10]}$, $D_{[90]}$), the volume-weighted De Brouckere mean diameter $D_{[4,3]}$, and the surface area-weighted Sauter mean diameter $D_{[3,2]}$ were measured. The span was calculated to characterize the particle distribution width using formula 5 (30):

$$Span = \frac{D_{[90]}-D_{[10]}}{D_{[50]}} \qquad [5]$$

Flocculation

The flocculation index (FI) was determined using a method described by Nawaz et al. (2021) using the Mastersizer 3000 at the analysis parameters described above. Flocs in the emulsions were

disrupted by diluting 1:10 with a sodium dodecyl solution (SDS) solution (1% in ultrapure water), and using SDS solution of the same concentration as the continuous phase. The flocculation index was calculated by the ratio of $D_{[3,2]}$ of the emulsion diluted with water to the $D_{[3,2]}$ of the emulsion diluted with SDS solution, using formula 6 (32). The closer the FI is to 1, the more pronounced the flocculation of particles.

$$\mathrm{FI} = \frac{\mathrm{d}_{[3,2]\mathrm{water}} - \mathrm{d}_{[3,2]\mathrm{SDS}}}{\mathrm{d}_{[3,2]\mathrm{water}}} \quad [6]$$

Foaming behaviour

Foaming behaviour of the emulsions was evaluated by frothing in a graduated cylinder with a high-shear mixer (Ultraturrax T10, IKA Werke GmbH & Co. KG, Staufen, Germany) for 30 s at maximum speed. Foam capacity was expressed as the volume of foam immediately after foaming as a percentage of the original sample volume. Foam stability was calculated as the volume of foam after 1 h storage at room temperature as a percentage of the initial foam volume (33).

Colour

The colour of the emulsions in the CIE L*a*b* colour space was measured with a Chroma Meter (CR-400 with a D65 illuminant; Minolta, Osaka, Japan). The whiteness index was calculated using equation 7 (34):

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad [7]$$

Statistical Analysis

Three independent batches of each emulsion were produced, and analyses were performed at least in triplicate, with results presented as mean ± standard deviation. Standard deviations for results calculated per dry matter were calculated by Gaussian propagation of uncertainty. To determine differences between the mean results of analyses at a significance level $p < 0.05$, all data was first tested for normality using Shapiro Wilk test, and for homogeneity of variances by Bartlett's test. One-way ANOVA followed by Tukey's post-hoc test was performed for normally distributed data with homogenous variances. If the variances of the data were not homogenous, Welch's Test followed by Dunnett's T3 post-hoc test were used. In the case of non normally distributed data, the non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test was used. To assess the effect of blending of the PIs on protein digestibility, expected digestibility values for each lentil-cereal blend were estimated by bootstrap resampling of the digestibility

measurements of the emulsions with the individual PIs, and calculating the weighted mean digestibility values at the protein ratios used for the blends. The observed mean digestibility of the blends was then compared to the bootstrap-generated expected values using independent t-test. Statistical analysis was performed using GraphPad Prism (version 9.4.0; GraphPad Software Inc., San Diego, USA).

Results and Discussion

Base Characterisation of PIs

The results of the compositional analysis of the PIs are summarised in **Table 2**. Protein contents ranged from 52 (Rice) to 91 g/100 g DM (BSGP). The Oat PI showed the highest amount of dietary fibre, most of which was high molecular weight fibre, likely from oat bran beta-glucan. The Lentil PI also contains relatively high amounts of soluble fibre but no insoluble fibre, due to the isolation method via isoelectric precipitation, in which insoluble fibre gets removed during processing (17). The soluble fibres in lentils are mostly oligosaccharides such as raffinose, stachyose and verbascose, which is also reflected in the higher FODMAP content of the Lentil PI compared to the other PIs (35). While these oligosaccharides can have prebiotic effects, they can also cause flatulence and intestinal discomfort (36). A low FODMAP diet can be beneficial for patients with irritable bowel syndrome (IBS) (37). At the PI concentration used in the emulsions, a typical 200 mL serving of lentil-based PBMA prototype exceeds the cut-off level for FODMAPs in a low-FODMAP diet, due to the total content of the oligosaccharides raffinose, stachyose and verbascose of 0.24 g/serving. However, all PBMA prototypes based on a combination of lentil and cereal protein are below the recommended FODMAP cut-off levels (38). Detailed results of the FODMAP analysis of all PIs can be found in **Supplementary Table 1**.

Starch was present in high amounts in the Oat PI (21.1 g/100 g DM), and moderate amounts in the Wheat and Rice PIs (8.4 and 3.5 g/100 DM, respectively). Starch can cause a sandy mouthfeel and excessive sedimentation in PBMA and is usually enzymatically degraded to glucose for improved sweetness, mouthfeel and physical stability (1). To keep processing conditions consistent for all PIs in this study, no enzymatic processing has been performed on any PIs.

As expected, the size range of proteins in the PIs varied greatly between the protein isolates and the hydrolysates (**Figure 1)** The Lentil PI, produced by isoelectric precipitation and spray-drying, shows a typical full protein profile ranging from 5 to 106 kDa (39). In the Oat PI, the main bands

were observed at 23 and 30 kDa, corresponding to the acidic and basic subunits of oat globulin, as previously reported for protein isolated from oat bran (40). Reference Shahbal et al removed. The Wheat, BSGP and Rice PIs have undergone enzymatic hydrolysis and therefore consist of smaller proteins compared to the Lentil and Oat PIs. The Wheat PI is partially hydrolysed, with only a low amount of larger proteins and the majority of proteins ranging from 4 to 54 kDa, with strong bands at 38, 14 and 9 kDa. This protein profile is typical for wheat gluten proteins hydrolysed by commercially available proteases (41). The BSGP PI proteins are between 2 and 10 kDa, with a weak larger band at 33 kDa, which might correspond to Protein Z, a barley albumin highly resistant to enzymatic degradation (42). The proteins in the Rice PI are the most hydrolysed, ranging from 2 to 6 kDa.

Table 2 Composition of protein ingredients in g/100 g DM, or g/100 g for dry matter.

	Lentil	Oat	BSGP	Rice	Wheat
Macronutrients					
Protein*	79.64 ± 0.76	56.64 ± 0.37	91.18 ± 4.15	51.72 ± 1.96	84.04 ± 3.11
Sugars§	1.17 ± 0.01	0.8 ± 0.01	0.37 ± 0.01	2.87 ± 0.02	0.47 ± 0.03
Fat	5.24 ± 0.16	15.74 ± 0.10	0.08 ± 0.03	0.14 ± 0.04	6.27 ± 0.37
Starch	<LoQ	21.12 ± 0.03	<LoQ	3.47 ± 0.06	8.38 ± 0.15
Dietary Fibre					
TDF**	5.63 ± 1.38	6.12 ± 1.30	1.99 ± 0.48	2.73 ± 0.18	2.72 ± 0.47
SDFS†	5.63 ± 1.38	0.75 ± 0.18	<LoQ	1.46 ± 0.35	1.58 ± 0.38
HMWDF‡	<LoQ	5.37 ± 1.29	1.99 ± 0.48	1.27 ± 0.30	1.14 ± 0.28
FODMAPs¶	3.09 ± 0.03	0.18 ±0.00	0.14±0.05	0.13 ± 0.00	0.92 ± 0.00
Ash	6.56 ± 0.04	4.49 ± 0.30	6.24 ± 0.36	17.34 ± 0.33	0.88 ± 0.06
Dry Matter	94.10 ± 0.10	93.08 ± 0.02	95.63 ± 2.71	92.61 ± 0.38	93.88 ± 0.38

*Measured as sum of AAs; **Total dietary fibre acc. to AOAC 2017.16, sum of soluble and insoluble dietary fibre; †Soluble dietary fibre that remains soluble in the presence of 78% ethanol; ‡High molecular weight dietary fibre, sum of insoluble dietary fibre (IDF) and fibre that precipitates in the presence of 78% ethanol (SDFP); §Sum of glucose, sucrose, and fructose (maltose not detected); ¶Sum of fructose in excess of glucose, polyols (xylitol, sorbitol, and mannitol), and oligosaccharides (raffinose/stachyose, verbascose, and fructans).

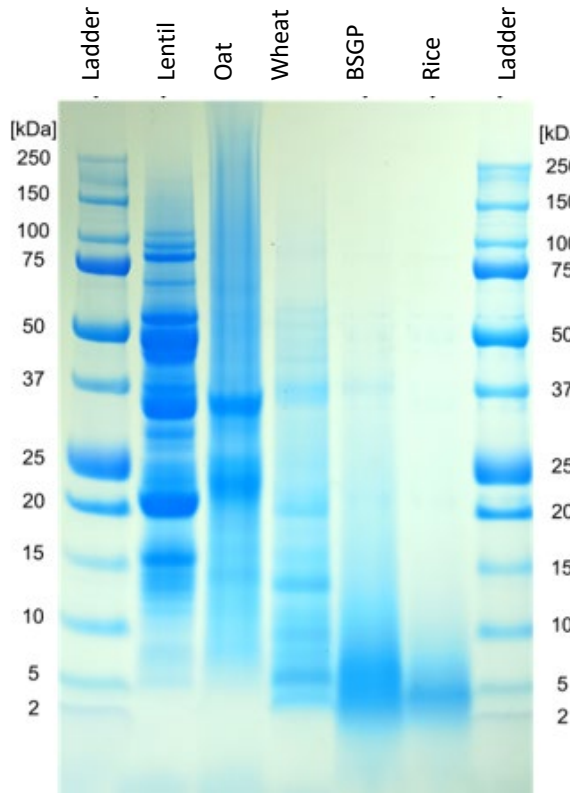


Figure 1 Profile of the proteins extracted from the protein ingredients on SDS PAGE under reducing condition.

Amino Acid Profiles of the Single and Blended PIs

The results of the amino acid quantification of the protein ingredients can be found in **Table 3a**. Comparing the content of IAA per 100 g of protein in the PIs to the adult IAA scoring pattern, it was found that Lentil PI was low in valine, tryptophan and SAA. In the Oat and BSGP PIs, the content of lysine was too low, the Rice PI was lacking in lysine and isoleucine, and the Wheat PI contained too low amounts of valine, lysine and isoleucine. A visualisation of the IAA compared to the FAO scoring pattern can be found in **Figure 2a-e.** The results of the AA analysis of the PIs were used to calculate the ratio of protein from lentil and cereal PI in which the requirements of the scoring pattern are fulfilled for all IAA while maximising lysine content. These optimized percentages of cereal protein in the lentil-cereal blends were determined to be as follows: Lentil+Oat: 40% oat protein; Lentil+BSGP: 47% BSGP protein; Lentil+Rice: 76% rice protein; Lentil+Wheat: 38% wheat protein. The calculated values of the amino acid contents of the PI blends are shown in **Table 3b,** and a comparison to the FAO scoring pattern is shown in **Figure**

2f-i. In the Oat, BSGP and Rice blends with Lentil, the IAA scoring pattern could be fulfilled to at least 100% in all IAA profile according to the scoring pattern. As both Lentil and Wheat PIs contained valine at lower amounts than recommended in the scoring pattern, a full IAA profile could not be achieved by a combination of these PIs. However, the combination of Wheat and Lentil in the determined ratio accomplished an overall highly improved IAA profile compared to that of the single PIs. In the Lentil PI, the SAA, tryptophan and valine requirements were fulfilled to 63.2%, 80.3% and 90.2%, and in the Wheat PI, the isoleucine, lysine and valine requirements were fulfilled to 90.8%, 29.6% and 81.8% respectively. In combination, isoleucine, SAA, tryptophan and valine requirements are fulfilled to 98.7%, 92.3%, 98.9% and 87.0% respectively, and lysine requirements are fulfilled completely, showing an overall improvement of the IAA scoring pattern fulfilment. A comprehensive study on the composition of 60 commercially available plant-based milk-alternatives was published by Moore et al. (2023). Comparing these reported amino acid contents on a g IAA per 100 g of protein basis to the scoring pattern revealed that none of the studied milk-alternative proteins fulfil the scoring pattern requirements, and only seven of the plant-based drinks contained 3.5% protein or more, all of which were soy-based (Moore, 2023). The combined lentil and cereal beverages therefore showed highly improved IAA scoring pattern results compared to plant-based milk-alternatives currently on the market. However, it needs to be noted that the scoring pattern results of the PBMAs is based on calculations based on the AA analysis of the ingredients and their ratio used in the PBMAs, and the AA content of the PBMAs was not analysed directly.

The amount of each PI added to the emulsions to reach 3.5% net protein, while considering the differing protein and dry matter contents of the PIs, can be found in the **Supplementary Figure 1**.

Table 3 Amino acid content (g/100 g DM) of the protein ingredients as measured by ion chromatography with ninhydrin post-column derivatisation, or HPLC-UV for tryptophan **(a)**, and calculated amino acid contents (g/100 g DM) of the blends of lentil protein isolate and the respective cereal protein ingredients **(b)**.

a)

	Measured AA content of ingredients				
	Lentil	Oat	BSGP	Rice	Wheat
Valine	2.57 ± 0.35	2.83 ± 0.28	5.49 ± 0.58	2.13 ± 0.22	2.67 ± 0.27
Tryptophan	0.38 ± 0.04	0.70 ± 0.08	1.28 ± 0.15	0.33 ± 0.04	0.70 ± 0.08
Threonine	2.55 ± 0.35	2.14 ± 0.21	3.53 ± 0.37	1.66 ± 0.17	2.23 ± 0.22
Phenyl-alanine	3.94 ± 0.54	3.44 ± 0.34	5.11 ± 0.53	2.14 ± 0.22	4.37 ± 0.44
Cysteine	0.53 ± 0.06	1.12 ± 0.17	1.20 ± 0.18	0.38 ± 0.06	1.51 ± 0.20
Methionine	0.50 ± 0.06	0.92 ± 0.15	1.89 ± 0.25	0.77 ± 0.13	1.11 ± 0.18
Lysine	4.90 ± 0.68	1.91 ± 0.19	4.02 ± 0.42	1.88 ± 0.19	1.16 ± 0.12
Leucine	5.88 ± 0.81	4.57 ± 0.46	6.66 ± 0.70	3.17 ± 0.32	5.08 ± 0.51
Isoleucine	2.21 ± 0.30	2.01 ± 0.20	3.88 ± 0.40	1.33 ± 0.14	2.23 ± 0.22
Histidine	1.77 ± 0.24	1.25 ± 0.13	1.94 ± 0.21	0.91 ± 0.09	1.47 ± 0.15
Aspartic acid	8.89 ± 1.23	4.52 ± 0.45	8.40 ± 0.87	4.52 ± 0.45	2.58 ± 0.27
Glutamic acid	14.03 ± 1.95	13.86 ± 1.40	21.23 ± 2.18	9.66 ± 0.96	31.96 ± 3.20
Alanine	3.17 ± 0.44	2.54 ± 0.26	4.59 ± 0.48	2.68 ± 0.27	1.96 ± 0.19
Arginine	6.73 ± 0.93	4.53 ± 0.45	5.30 ± 0.55	4.87 ± 0.49	2.85 ± 0.29
Glycine	3.05 ± 0.42	2.48 ± 0.26	3.86 ± 0.40	2.08 ± 0.22	2.72 ± 0.28
Proline	2.99 ± 0.41	3.09 ± 0.31	8.89 ± 0.92	1.99 ± 0.21	10.19 ± 1.02
Serine	4.47 ± 0.62	2.80 ± 0.29	4.10 ± 0.42	2.79 ± 0.28	4.27 ± 0.43
Tyrosine	2.59 ± 0.36	2.11 ± 0.21	3.50 ± 0.37	2.19 ± 0.22	2.72 ± 0.28

b)

	Calculated AA content of blends			
	Lentil+Oat	Lentil+BSGP	Lentil+Rice	Lentil+Wheat
Valine	2.68 ± 0.23	3.73 ± 0.33	2.2 ± 0.19	2.61 ± 0.25
Tryptophan	0.52 ± 0.04	0.74 ± 0.07	0.34 ± 0.03	0.49 ± 0.04
Threonine	2.36 ± 0.22	2.94 ± 0.26	1.81 ± 0.16	2.44 ± 0.24
Phenyl -alanine	3.72 ± 0.34	4.41 ± 0.39	2.45 ± 0.2	4.09 ± 0.39
Cysteine	0.8 ± 0.09	0.8 ± 0.09	0.4 ± 0.05	0.88 ± 0.08
Methionine	0.69 ± 0.08	1.05 ± 0.12	0.72 ± 0.11	0.71 ± 0.07
Lysine	3.56 ± 0.38	4.55 ± 0.42	2.39 ± 0.2	3.59 ± 0.45
Leucine	5.29 ± 0.5	6.19 ± 0.55	3.64 ± 0.3	5.6 ± 0.56
Isoleucine	2.12 ± 0.19	2.88 ± 0.25	1.48 ± 0.13	2.22 ± 0.22
Histidine	1.54 ± 0.15	1.84 ± 0.16	1.06 ± 0.09	1.67 ± 0.17
Aspartic acid	6.92 ± 0.71	8.69 ± 0.78	5.27 ± 0.43	6.68 ± 0.81
Glutamic acid	13.95 ± 1.24	16.89 ± 1.45	10.41 ± 0.86	20.3 ± 1.69
Alanine	2.88 ± 0.27	3.74 ± 0.32	2.76 ± 0.24	2.75 ± 0.3
Arginine	5.74 ± 0.55	6.16 ± 0.57	5.19 ± 0.43	5.37 ± 0.62
Glycine	2.79 ± 0.26	3.37 ± 0.3	2.25 ± 0.19	2.93 ± 0.29

Proline	3.04 ± 0.27	5.34 ± 0.47	2.16 ± 0.18	5.51 ± 0.45
Serine	3.72 ± 0.37	4.32 ± 0.39	3.07 ± 0.26	4.4 ± 0.43
Tyrosine	2.37 ± 0.22	2.95 ± 0.26	2.26 ± 0.19	2.63 ± 0.25

Results ± standard deviation of duplicate measurements (a), or calculated results ± standard deviation as determined by Gaussian propagation of uncertainty (b).

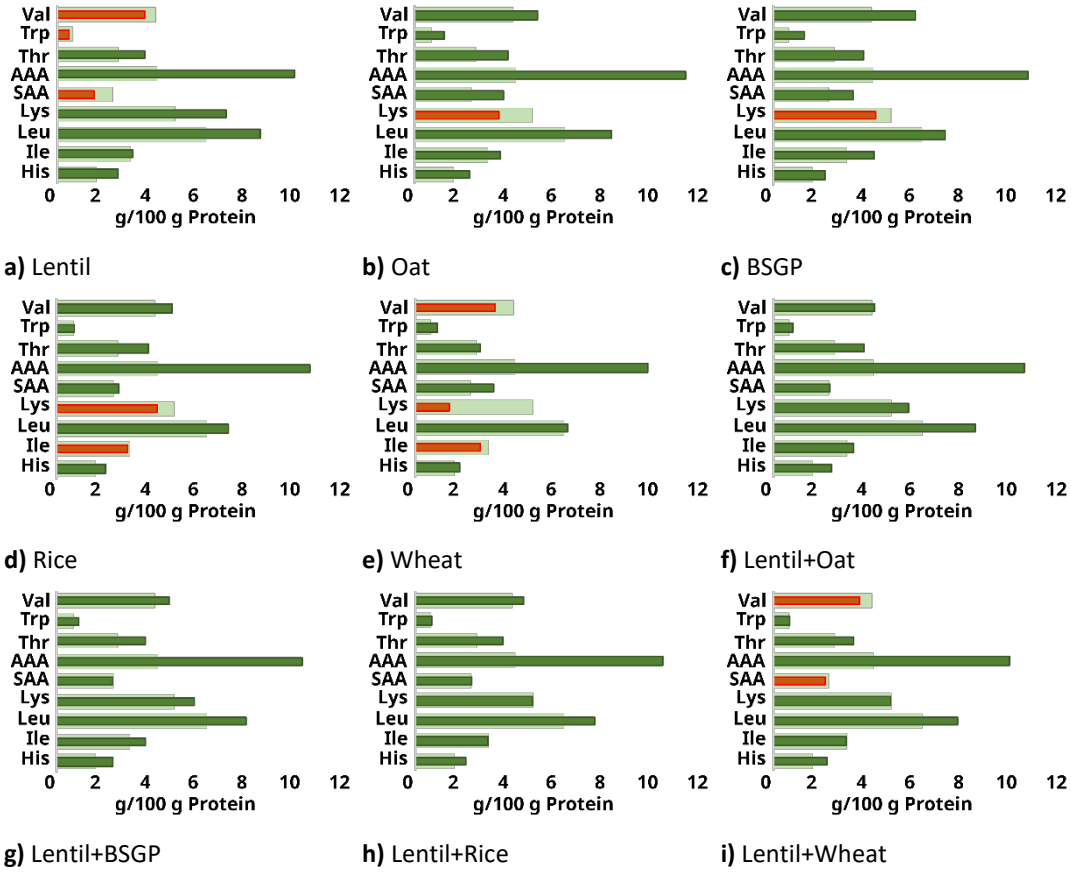


Figure 2 Contents of the IAA as measured by HPLC in g per 100 g protein (protein content calculated as sum of amino acids) of PIs (**a-e**), the calculated contents of IAA of the blends of lentil and the respective cereal PI in the determined optimal protein ratios (**f-i**) The recommended IAA scoring pattern for children over 3 years, adolescents and adults (FAO, 2013) is shown as light green bars. IAA contents not fulfilling the scoring pattern are shown in red. Error bars omitted for clarity, see **Table 3** for mean values ± standard deviation of AA content in g/100 g PI (DM).

In-Vitro *Protein Digestibility (IVPD)*

The results of the IVPD by Kjeldahl quantification of the nitrogen ratio in the digestible and indigestible fractions of the emulsions after INFOGEST digestion simulation are shown in **Figure 3.** The IVPDs of the Lentil, BSGP, and Oat emulsions of 75.1, 76.0 and 71.8 %, respectively, were lower than those reported for these ingredients in preceding studies. However, a direct comparison with previous in-vitro digestibility studies is difficult due to the possible influence of differing digestion simulation protocols, unless the harmonised INFOGEST method was used (12). An IVPD of 96.2 % and 90% was reported for wheat and oat bran protein isolates, respectively (44,45), while a study on hydrolysed brewers' spent grain protein reported an IVPD

of 90.1% (46). The higher IVPD values reported in these studies could be attributed to the use of pure protein isolates for digestion simulations which may overestimate the IVPD, as opposed to protein embedded in a food matrix such as the PBMAs.(13). A study on commercial plant-based milk alternatives reported an IVPD of 25 and 21% for an oat and soy drink, respectively (47),while the IVPD of the Wheat emulsion was found to be 84.53 %, which is similar to previously reported findings for wheat protein isolates (48,49). The PD of plant protein isolates can be influenced by the processing conditions during manufacture, such as pH and alkali concentration during extraction (50), drying methods (51), heat treatment (52), and protein pre-hydrolysis. Interestingly, although the protein in both the Rice and BSGP PIs is highly hydrolysed, the IVPD of the Rice emulsions is significantly higher than that of the BSGP emulsions. Increased IVPD by protein hydrolysis has been demonstrated for soy, rapeseed, and rice bran protein (53–55), while studies on chickpea and lentil protein hydrolysate showed no negative effects of hydrolysis on IVPD(56–58).

The IVPDs of the PI blend emulsions were found to be between 77.5% (Lentil+Oat) and 100 % (Lentil+Rice), which is higher than the theoretical values calculated using the experimental IVPD results of the single ingredient emulsions (illustrated as red bars in Figure 3). The difference was found to be statistically significant by independent t-test. It has been reported previously that the IVPD of single proteins cannot predict the IVPD of combined protein sources, and both synergistic and antagonistic effects on IVPD by the combination of proteins have been observed (59–62). The synergistic effect in the Lentil+Oat, Lentil+BSGP and Lentil+Rice emulsions can be classified as a type III response, where the IVPD of the blend is higher than that of the single ingredients. In contrast, the Lentil+Wheat emulsion showed only a slight synergistic effect compared to the theoretical IVPD value ($P = 0.0268$), representing a type IV response, with the IVPD of the blend being intermediate to that of the individual proteins (59).

The occurrence of synergistic effects on IVPD by the combination of protein sources is dependent on the protein source as well as the ratio of the combined proteins (60,62), but further research

is necessary to understand the underlying mechanisms.

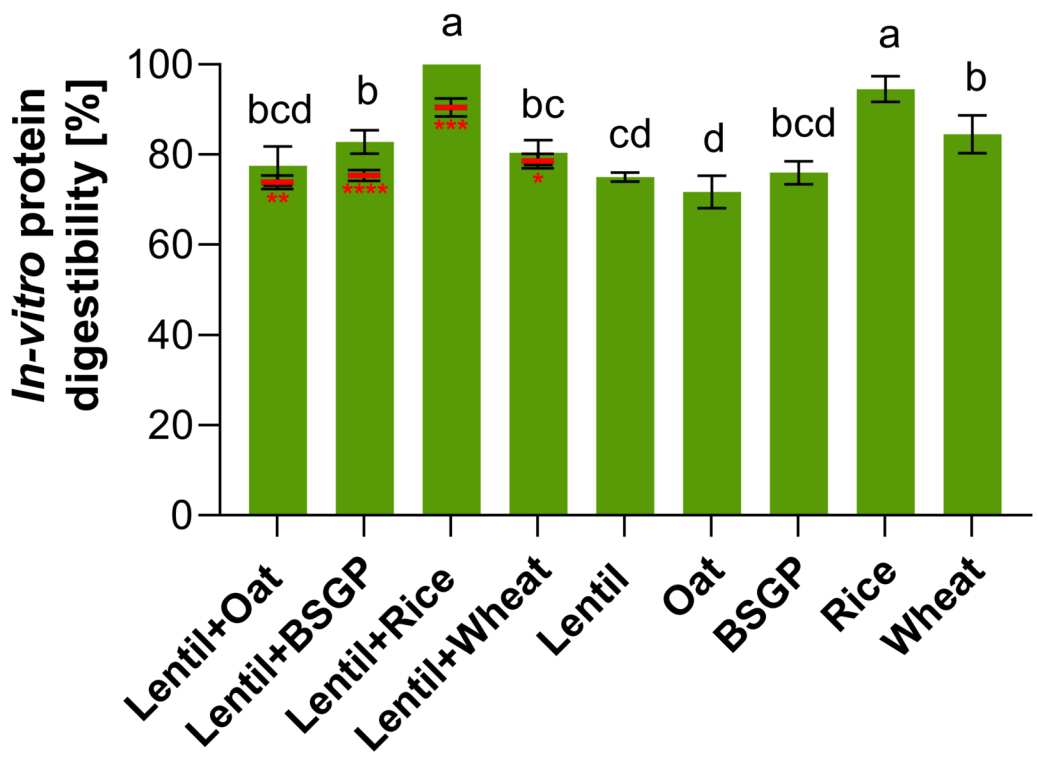


Figure 3 Results of the digestibility of the protein in the emulsions from the INFOGEST in-vitro digestion simulation. Expected mean digestibility values calculated by weighted average digestibility of the emulsions with individual PIs at the protein ratio used for the blended emulsions are shown as red bars. Independent t-test was performed to signify difference of measured and expected IVPD of blended emulsions: *P = 0.0268, **P = 0.0022, ***P = 0.0002, ****P < 0.0001.

Proxy* In-Vitro *DIAAS (PIVDIAAS)

With the results of the amino acid quantification of the PIs and the measured IVPD from the INFOGEST, the PIVDIAAS was calculated using the reference values for older children, adolescents and adults (**Figure 4)**. The PIVDIAAS is determined by both protein digestibility and the fulfilment of the FAO AA scoring pattern. The combination of lentil and cereal ingredients in their optimized ratios to meet the scoring pattern significantly increased the PIVDIAAS of the combined PBMAs compared to those made from single ingredients. Due to the very low amount of lysine in the Wheat PI, PIVDIAAS of the Wheat emulsion was found to be the lowest at only 25 %. *In-vivo* studies based on the caecal amino acid digestibility in rats found the DIAAS of gluten to be 25% as well (63). The PIVDIAAS of the Lentil emulsions was found to be 48%, which correlates well with a study reporting the DIAAS of cooked red lentils determined in-vivo in rats based on true faecal protein digestibility of 53%. In the combined Lentil+Wheat emulsion, due to the complementary amino acid profiles of the individual PIs, the PIVDIAAS was increased to

72%. The PIVDAAS of the Oat, BSGP and Rice emulsions was found to be 50, 67 and 81%, respectively, with lysine as the first limiting amino acid. In combination with Lentil, their PIVDIAAS increased to 78, 83 and 98% for Lentil+Oat, Lentil+BSGP and Lentil+Rice, respectively. According to FAO recommendations, a food product can be considered a "good" quality protein if the DIAAS is above 75 (2). Of the single ingredient emulsions, only the Rice emulsion could attain this threshold, while all of the blended emulsions, except for Lentil+Wheat, could be regarded as a "good" quality proteins. Together with the protein content of 3.5% of the emulsions, which fulfils the criteria of minimum 5% of the nutrient reference value (NRV) per 100 mL for labelling as "source of protein", these emulsions could therefore be labelled with the claim of "source of good quality protein". However, the results in this study are based on *in-vitro* data, and while high correlation for the nitrogen digestibility as measured with the INFOGEST model and *in-vivo* digestibility has been shown (13), the FAO advises that DIAAS evaluation should be performed through *in-vivo* studies in either humans or pigs (2). Another limitation of this study was that the digestibility of each amino acid was not individually assessed. As shown in previous *in-vivo* studies, true ileal digestibility of the IAA (25,64) can vary depending on the protein source, but for highly digestible foods such as soy milk, pea protein emulsion or tofu, the DIAAS is mostly determined by amino acid composition (9).

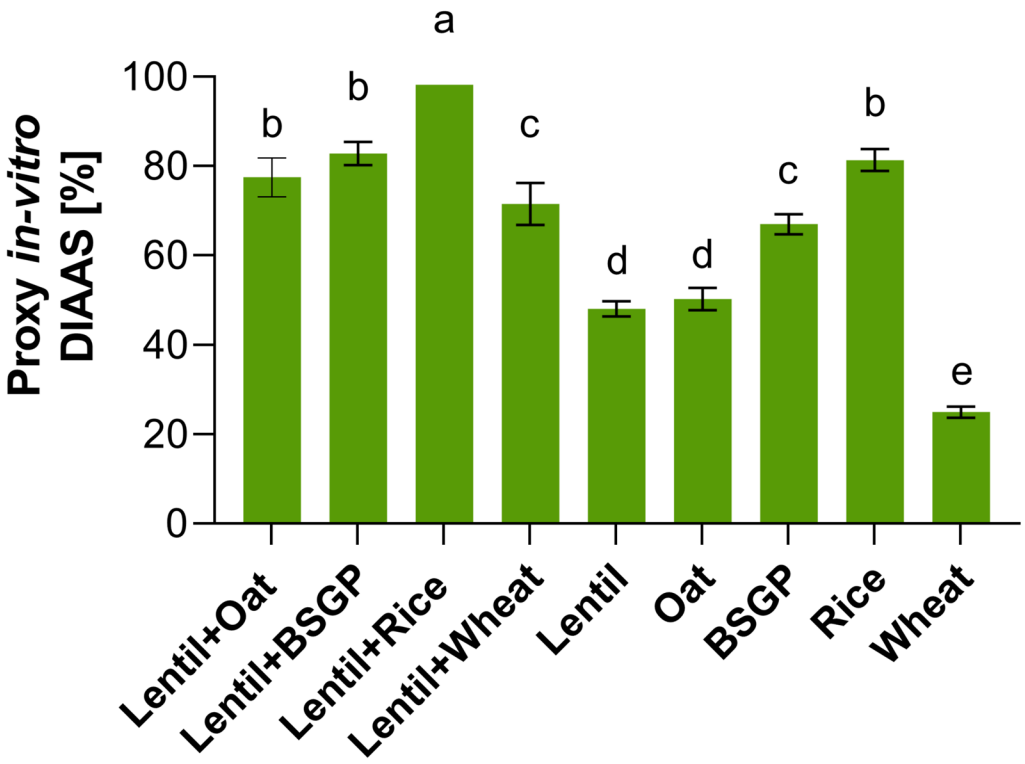


Figure 4 Proxy *in-vitro* DIAAS calculated using the AA contents of the PIs and *in-vitro* protein digestibility results from the nitrogen digestibility as determined by Kjeldahl method after INFOGEST digestion simulation.

Particle and Droplet Size Distribution, and Physical Stability of emulsions

The particle size distribution of the dry PI was measured by dispersion in air, while the particle and droplet size distribution of the emulsions was measured by dispersion in water, with both measurements conducted using static-light scattering analysis. The results of the size distribution analyses are shown in Table 4**.** The Oat and Wheat PIs showed the highest mean particle sizes with a $D_{[4,3]}$ of 139 and 423µm, respectively. However, their $D_{[3,2]}$ values were similar at 43.0 and 39.6 µm. The De Brouckere mean diameter $D_{[4,3]}$ gives the volume-weighted average diameter and is more sensitive to larger particles in the distribution, while the Sauter Mean diameter $D_{[3,2]}$ is the surface area-weighted diameter and influenced more by smaller particles compared to the $D_{[4,3]}$ (65). The significantly larger $D_{[4,3]}$ of the Oat PI while the $D_{[3,2]}$ is similar to that of the Wheat PI, suggests that the mean particle size of the Oat PI is skewed towards larger particles. This is confirmed by the much larger $D_{[90]}$ of the Oat PI (1663 µm) compared to the Wheat PI (326 µm), while their $D_{[10]}$ values are similar at 20.9 µm and 15.9 µm, respectively, and could be due to agglomeration of the Oat PI during the drying process. The BSGP and Rice PI showed non significantly different $D_{[4,3]}$ values of 63.3 µm and 63.5 µm, respectively, but the Rice PI showed a higher span due to the presence of both higher $D_{[90]}$ and

lower $D_{[10]}$ values. Lentil PI exhibited the smallest particle size of all PIs with a $D_{[4,3]}$ of 14.2 µm. In emulsion systems, particle size distribution is determined by both undissolved PI particles and the size of oil droplets and oil-protein aggregates. Emulsion stability is highly dependent on the particle size of the oil droplets, with creaming in aqueous phases increasing rapidly at oil droplet sizes above 0.5 µm (16). As all emulsions have undergone identical homogenization conditions, the oil droplet size is dependent on the emulsifying properties of the PIs, which can stabilize the oil droplets and prevent oil droplet coalescence. The emulsifying properties of proteins are dependent on their hydrophobicity, the surface charge of the protein depending on the pH of the solution and the isoelectric point of the protein, molecular weight, and protein solubility (66). Compared to the dry PIs, particle sizes in the emulsions were found to be much smaller due to disassociation of agglomerated particles and dissolution of soluble particles. As the Rice and BSGP PIs are highly hydrolysed and soluble at neutral pH, the particle size of the Rice and BSGP emulsions is defined by the oil droplets with no undissolved particles. Droplet size in the Rice emulsion was significantly larger than the BSGP emulsion, with median droplet sizes $D_{[50]}$ of 7.68 µm and 0.26 µm, respectively. The Rice emulsion also showed a very high flocculation index compared to the BSGP emulsion (0.40 and 0.07, respectively), indicating that the higher oil droplet size in the Rice emulsion is due to the aggregation of individual droplets, and not full coalescence. The larger particle size of the Rice emulsion compared to the BSGP emulsion is consistent with the results of the physical stability measurements by light transmission analysis under centrifugation (**Figure 5a-i)**. In the Rice emulsion, a rapid increase in light transmission over the whole length of the cuvette and a pronounced decrease at the top of the cuvette indicate a highly unstable emulsion separating into a cream layer and a clear solution (**Figure 5h**). In contrast, the BSGP emulsion transmission profile (**Figure 5g**) shows a less pronounced cream layer, and only a gradual increase of light transmission over the length of the cuvette, indicating a higher emulsion capacity compared to the Rice PI. It has been shown that partial enzymatic hydrolysis of plant proteins can increase their emulsifying capacity by increased protein solubility, and the exposure of hydrophobic peptide side groups which increases the amphiphilic properties. However, excessive hydrolysis into small peptides inhibits the formation of an emulsifying protein layer around the fat droplets, which lowers the emulsifying capacity of the hydrolysate (67,68). Other important factors influencing the emulsifying properties of hydrolysates are the choice of enzyme (67,69), deactivation temperature of the enzymes (70), pH of the food matrix (71) and protein source (72,73). The tendency of a protein-stabilised

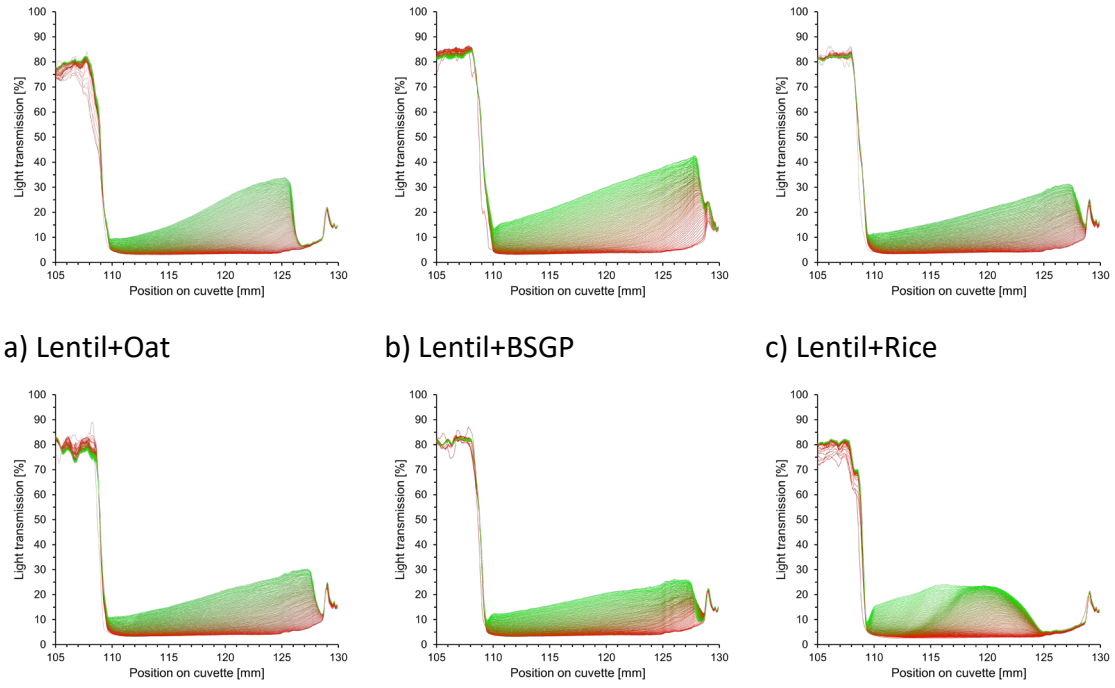
460 emulsion to flocculate depends on numerous factors such as protein surface hydrophobicity,
461 ionic strength and pH of the emulsion, and adjusting these variables could therefore improve
462 the stability of the Rice emulsion (74). The Wheat emulsion showed the largest particle sizes
463 among all tested emulsions in terms of size distribution parameters, with particularly high values
464 for the lower percentile diameter $D_{[10]}$ (6.80 µm), and flocculation index (0.94). The isoelectric
465 point and thus the lowest solubility of native wheat gluten has been found around neutral pH,
466 lowering the emulsifying potential of the protein (75,76). Hydrolysis of gluten protein lowers the
467 isoelectric point, making gluten emulsions at neutral pH more stable (77). However, the partial
468 hydrolysis of the Wheat PI does not appear to sufficiently enhance protein solubility at neutral
469 pH to stabilize the emulsion. **Figure 5i** shows complete separation of the Wheat emulsion into a
470 clear solution and a significant sediment layer. The absence of creaming in the Wheat emulsion
471 suggests that the protein adsorbed strongly to the oil droplets. However, the strong interaction
472 between the proteins near their isoelectric point caused the formation of large, flocculated
473 aggregates with a density higher than the continuous phase, leading to sedimentation. The Oat
474 emulsion showed relatively high $D_{[4,3]}$ and low $D_{[3,2]}$ values (5.67 µm and 0.44 µm, respectively),
475 indicating a skewness towards small particles in the emulsion. The Oat emulsion was also found
476 to have the biggest span of all emulsions, while flocculation index was low. Combined with the
477 results from the Lumisizer, that show strong sedimentation but no pronounced creaming layer,
478 this indicated that the sediment consists of large undissolved particles and not aggregated
479 micelles. Oat protein's isoelectric point is around pH 5 (78), and while the oat protein solubility
480 at pH 7 was still the lowest of all PIs (see **Table 6**), the emulsification potential of the soluble oat
481 proteins was high enough to inhibit complete phase separation. The Lentil emulsion showed
482 small particle size, no flocculation and good physical stability under centrifugation, with neither
483 a pronounced sedimentation nor creaming (**Figure 5e**). The isoelectric point of the Lentil PI is
484 around a pH of 4.5, and solubility at pH 7 is almost at 100%, providing good emulsifying power
485 in the conditions found in milk alternatives. In combination with Lentil, the particle size of the
486 emulsions was decreased compared to the emulsions from cereal PIs only. The flocculation index
487 of the Lentil+Wheat and Lentil+Rice emulsions was decreased significantly compared to the
488 respective single cereal ingredient emulsions, indicating that the lentil protein stabilized the oil
489 droplets sufficiently to overcome aggregation caused by the cereal proteins. The physical
490 stability of all emulsions was improved by the combination with Lentil PI (**Figure 5a-d**), with
491 significantly decreased creaming and/or sedimentation. Compared to mean particle sizes found

in commercial PBMAs, the particle sizes in the studied emulsions was similar, with Jeske et al. (2017) reporting a $D_{[4,3]}$ between 0.7 µm and 81.5 µm in PBMAs based on cereals, legumes and nuts. While the Wheat and Rice emulsions completely separated under simulated storage in the Lumisizer, combination with Lentil stabilized the emulsion sufficiently to remain in a turbid emulsified state.

Table 4 Particle size distribution of emulsions and dry PIs, and flocculation index (FI) of emulsions.

		$D_{[4,3]}$* [µm]	$D_{[3,2]}$** [µm]	$D_{[90]}$† [µm]	$D_{[50]}$‡ [µm]	$D_{[10]}$§ [µm]	Span [µm]	FI¶ [-]
Emulsions	Lentil+Oat	3.2 ± 0.3^{cd}	0.2 ± 0.0^{c}	10.4 ± 0.6^{c}	0.4 ± 0.1^{d}	0.1 ± 0.0^{b}	25.7 ± 2.4^{a}	-0.1 ± 0.2^{c}
	Lentil+BSGP	0.4 ± 0.0^{d}	0.2 ± 0.0^{c}	0.7 ± 0.1^{d}	0.2 ± 0.0^{d}	0.1 ± 0.0^{b}	2.6 ± 0.1^{b}	-0.1 ± 0.1^{c}
	Lentil+Rice	0.6 ± 0.0^{d}	0.2 ± 0.0^{c}	0.9 ± 0.0^{d}	0.3 ± 0.0^{d}	0.1 ± 0.0^{b}	2.9 ± 0.1^{b}	0.1 ± 0.1^{c}
	Lentil+Wheat	0.6 ± 0.0^{d}	0.2 ± 0.0^{c}	0.8 ± 0.1^{d}	0.3 ± 0.0^{d}	0.1 ± 0.0^{b}	3.0 ± 0.1^{b}	0.1 ± 0.0^{c}
	Lentil	0.5 ± 0.0^{d}	0.2 ± 0.0^{c}	0.7 ± 0.0^{d}	0.2 ± 0.0^{d}	0.1 ± 0.0^{b}	2.7 ± 0.1^{b}	0.0 ± 0.1^{c}
	Oat	5.7 ± 0.3^{c}	0.4 ± 0.0^{c}	14.7 ± 1.2^{bc}	3.8 ± 0.3^{c}	0.2 ± 0.0^{b}	3.9 ± 0.1^{b}	0.2 ± 0.1^{bc}
	BSGP	0.3 ± 0.0^{d}	0.2 ± 0.0^{c}	0.7 ± 0.0^{d}	0.3 ± 0.0^{d}	0.1 ± 0.0^{b}	2.2 ± 0.0^{b}	0.1 ± 0.0^{c}
	Rice	8.9 ± 1.4^{b}	2.2 ± 0.4^{b}	17.9 ± 2.1^{b}	7.7 ± 1.4^{b}	0.6 ± 0.1^{b}	2.3 ± 0.1^{b}	0.4 ± 0.1^{b}
	Wheat	19.7 ± 3.0^{a}	5.1 ± 0.4^{a}	35.1 ± 5.5^{a}	18.3 ± 2.9^{a}	6.8 ± 0.8^{a}	1.6 ± 0.1^{b}	0.9 ± 0.0^{a}
Dry PI	Lentil	14.2 ± 0.0^{d}	8.5 ± 0.1^{e}	28.6 ± 0.21^{e}	10.9 ± 0.1^{e}	4.3 ± 0.0^{d}	2.2 ± 0.0^{c}	
	Oat	423 ± 54^{a}	43.0 ± 2.1^{a}	1663 ± 163^{a}	68.7 ± 2.8^{b}	20.9 ± 0.7^{a}	23.9 ± 1.5^{a}	
	BSGP	63.3 ± 3.3^{c}	23.5 ± 1.6^{c}	141 ± 5^{d}	48.1 ± 3.9^{c}	10.6 ± 0.8^{c}	2.7 ± 0.2^{c}	
	Rice	63.5 ± 5.9^{c}	11.4 ± 1.5^{d}	174 ± 12^{c}	30.7 ± 6.5^{d}	4.2 ± 0.5^{d}	5.7 ± 1.0^{b}	
	Wheat	139 ± 2^{b}	39.6 ± 0.9^{b}	326 ± 4^{b}	97.1 ± 2.4^{a}	15.9 ± 0.3^{b}	3.2 ± 0.1^{c}	

*$D_{[4,3]}$= volume-weighted mean diameter (De Brouckere), **$D_{[3,2]}$= surface area-weighted mean diameter (Sauter), †$D_{[90]}$=particle diameter at 90th percentile, ‡$D_{[50]}$=median particle diameter, §$D_{[10]}$=particle diameter at 10th percentile, ¶FI=Flocculation index. Different letters in column sections indicate statistically significant difference between samples ($p < 0.05$).



a) Lentil+Oat b) Lentil+BSGP c) Lentil+Rice

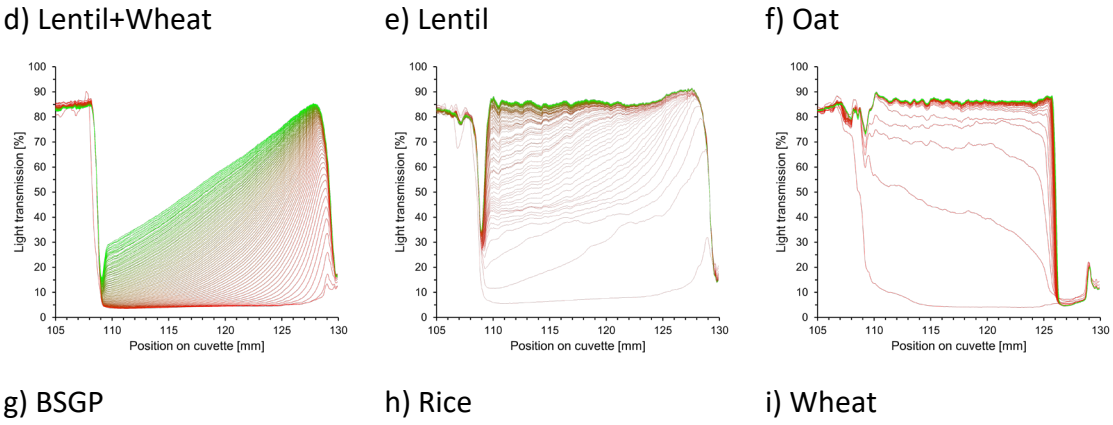


Figure 5 Light transmission through the emulsions over the length of the measurement cuvettes during centrifugation. Left-hand size of the plot represents the top of the measurement cuvette. Each line represents one measurement in 30 sec intervals with a colour gradient from red to green, with bottom red being the first measurement and and top green line being the last measurement (average values of triplicates for each line, error bars omitted for clarity).

Rheological Behaviour

The viscosity of plant-based emulsions is crucial for emulsion stability, as the speed of phase separation is inversely correlated with the viscosity of the surrounding phase as per Stoke's law (16). The apparent viscosity of the emulsions measured at 20 °C and a shear rate of 100 s^{-1}, and the flow behaviour, consistency index and goodness of fit indicators can be found in **Table 5**. All single ingredient and combined emulsions showed similar apparent viscosities of between 1.76 and 3.6 mPa.s, except for the Oat emulsion, which was found to have a higher apparent viscosity of 7.24 mPa.s compared to the other emulsions. The apparent viscosities of the experimental emulsions are comparable to those previously reported for PBMA prototypes, such as from oat (80), almond and hazelnut (81), lentil (82), and soy (83). In commercial plant drinks, similar apparent viscosities have been found for a majority of tested products, but with higher viscosities of 20 mPa.s to 48 mPa.s for almond, hazelnut and hemp-based products. However, as the tested commercial products contained hydrocolloid thickeners and the apparent viscosity was measured at a lower shear rate, a direct comparison of values is difficult, however. Stabilizers are frequently added to PBMAs to increase the viscosity of the continuous phase for improved emulsion stability and decreased sedimentation (84). Flow behaviour of the emulsions as assessed by linear regression of the shear ramp data to the power law model. Goodness of fit of the model to the data is shown by high R^2 values (0.9992-0.9999) and low PE values show only minor differences between the experimental and theoretical data (-0.0017%-0.0376%),

indicating good suitability of the model. The flow behaviour index n indicates whether a fluid exhibits ideal/Newtonian (n=1), shear-thinning (n<1) or shear-thickening behaviour (n>1). Flow behaviour of the emulsions were found to be close to Newtonian, ranging between 0.91 and 1.07, and are therefore close to the Newtonian behaviour exhibited by cow's milk, whereas PBMAs containing hydrocolloid thickeners often show sheer thinning behaviour which can pose technological challenges (79,85). The consistency index (K) specifies the resistance of a fluid to flow, and increases with the amount of insoluble particles in the system (80). It was found to be highest in the Oat emulsion (11.69) and lowest in the BSGP and Rice emulsions (1.3), corresponding to the nitrogen solubility of the PIs.

Table 5 Apparent viscosity and Power law constants of the protein emulsions at 20 °C, and indicators of goodness of fit of Power law model (R2 and PE).

Emulsion	$\eta_{100\ s}$-1* [mPa.s]	n** [-]	K† [Pa.s^n]	$R^{2‡}$ [-]	PE§ [%]
Lentil+Oat	3.60 ± 0.13^{b}	1.01 ± 0.00^{b}	3.39 ± 0.15^{c}	0.9999	-0.0038
Lentil+BSGP	2.25 ± 0.09^{e}	0.97 ± 0.02^{b}	2.63 ± 0.30^{d}	0.9996	-0.0237
Lentil+Rice	2.52 ± 0.31^{cde}	0.96 ± 0.03^{b}	3.22 ± 0.48^{c}	0.9995	-0.0161
Lentil+Wheat	2.38 ± 0.11^{de}	1.02 ± 0.01^{ab}	2.16 ± 0.16^{d}	0.9999	-0.0086
Lentil	2.60 ± 0.03^{d}	1.01 ± 0.03^{ab}	2.55 ± 0.37^{d}	0.9997	-0.0129
Oat	7.24 ± 0.87^{a}	0.91 ± 0.01^{b}	11.69 ± 0.50^{a}	0.9999	-0.0017
BSGP	1.76 ± 0.03^{g}	1.07 ± 0.02^{ab}	1.27 ± 0.12^{e}	0.9997	-0.0188
Rice	1.84 ± 0.02^{f}	1.09 ± 0.01^{ab}	1.25 ± 0.03^{e}	0.9998	-0.0376
Wheat	2.92 ± 0.06^{c}	0.91 ± 0.01^{b}	4.27 ± 0.16^{b}	0.9992	-0.0166

*$\eta_{100\ s}$-1 = Apparent viscosity at shear rate 100 s^{-1}; **n= Flow behaviour index; †K= Consistency index; ‡R^2= Coefficient of determination; §PE= Relative Percentage error. Different letters in columns indicate statistically significant difference between samples ($p< 0.05$).

Foaming Properties and Colour

High foam capacity and stability are crucial quality parameters of PBMAs for their application in coffee beverages. These foams are stabilized proteins forming a layer on the air bubbles dispersed in the continuous phase. The foaming properties of proteins are therefore dependent on their surface hydrophobicity, surface charge, solubility, protein structure and molecular size (86,87). The results of the foam behaviour analysis can be found in **Table 6**, with foaming capacity indicating the initial sample expansion after foaming, and foam stability was assessed by calculating the percentage of sample expansion retained after 1 hour, relative to the initial expansion. The emulsions containing hydrolysates and partial hydrolysates (BSGP, Rice and Wheat) showed the highest foam capacities, but low foam stability. The smaller molecular sizes

of the hydrolysates appear to enable fast adsorption to the air bubbles during foaming but prevent the formation of a stable film that prevents air bubble coalescence. The Lentil and Oat emulsions were found to have lower foam capacity than the hydrolysate-based emulsions, but produced more stable foams. The combination of Lentil+Rice emulsion showed both improved foam capacity and stability, while for the other hydrolysates, neither foaming capacity nor stability changed at a statistically significant level compared to the emulsions based on the hydrolysates alone.

Whiteness indices of PBMAs comparable to that of cow's milk are another key factor to meet consumers' expectations. Both the Rice and BSGP emulsions had very low whiteness indices (36.9 and 46.7 respectively), while Lentil and Oat showed the highest whiteness values (71.3 and 73.9). A study on 17 commercially available PBMAs found an average whiteness index of 66.9 (79), so both the Lentil+Oat and Lentil+Wheat emulsions showed satisfactory whiteness values of 71.8 and 72.7, respectively.

Table 6 Foam capacity (FC), foam stability (FS), whiteness index (WI), and nitrogen solubility (NS) of emulsions.

	FC * [%]	FS ** [%]	WI † [-]	NS ‡ [%]
Lentil+Oat	57 ± 2^{b}	54 ± 15^{ab}	71.8 ± 0.4^{b}	79.0 ± 4.3^{b}
Lentil+BSGP	176 ± 13^{a}	42 ± 2^{b}	58.6 ± 0.5^{d}	97.9 ± 1.4^{a}
Lentil+Rice	166 ± 11^{a}	43 ± 2^{b}	57.0 ± 0.4^{e}	99.3 ± 1.2^{a}
Lentil+Wheat	194 ± 7^{a}	22 ± 4^{c}	72.7 ± 0.6^{ab}	94.8 ± 4.1^{a}
Lentil	82 ± 6^{b}	68 ± 8^{a}	71.3 ± 0.3^{b}	98.4 ± 2.0^{a}
Oat	65 ± 10^{b}	78 ± 7^{a}	73.9 ± 0.3^{a}	30.2 ± 1.3^{c}
BSGP	173 ± 18^{a}	45 ± 3^{b}	46.7 ± 0.4^{f}	100 ± 0.1^{a}
Rice	146 ± 4^{a}	10 ± 3^{c}	36.9 ± 0.9^{g}	98.6 ± 1.9^{a}
Wheat	169 ± 4^{a}	33 ± 2^{bc}	67.2 ± 0.0^{c}	71.6 ± 1.2^{b}

Average values ± standard deviation. Different letters in columns indicate statistically significant difference between samples ($p < 0.05$).

Conclusions

This study has demonstrated that with the combination of lentil protein isolate and cereal proteins in PBMA prototypes, their protein quality and techno-functional properties could be improved significantly. By blending the PIs at an optimized ratio based on their complementary AA profile, PBMA prototypes that meet or exceed the AAS pattern for adults were achieved, with current PBMAs that are commercially available today not usually fulfilling these requirements (3). The *in-vitro* digestibility assessments suggested good protein

digestibility of the emulsions, and the PIVDIAAS of all PBMAs based combinations of lentil and cereal was significantly increased compared to those based on the single ingredients. The combination of lentil protein isolate and rice hydrolysate resulted in the highest PIVDIAAS. While protein hydrolysis can enhance digestibility, careful control of processing parameters is crucial to prevent the formation of bitter-tasting peptides, which could negatively affect consumer perception (88). Other parameters crucial for consumer acceptance, like physical stability, including sedimentation and creaming, and whiteness were improved by the synergistic effects of the lentil and cereal PIs, but could be further enhanced by the application of emulsifiers, stabilizers or proteolytic, amylolytic or cross-linking enzymes (89). Future studies that further investigate the protein quality of these PBMAs by *in-vitro* DIAAS assessment based on individual AA digestibility, and validation with *in-vivo* studies could lead to a more comprehensive understanding of the nutritional value of these products. Taking advantage of the complementary AA profile of legume and cereal protein is a currently underutilised strategy that could substantially contribute to the development of more sustainable and nutritionally balanced dairy alternatives, and continued research is essential to fully exploit this potential.

Data Availability Statement

The data supporting this article have been included as part of the Supplementary Information.

Conflicts of Interest

There are no conflicts to declare.

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CRediT authorship contribution statement

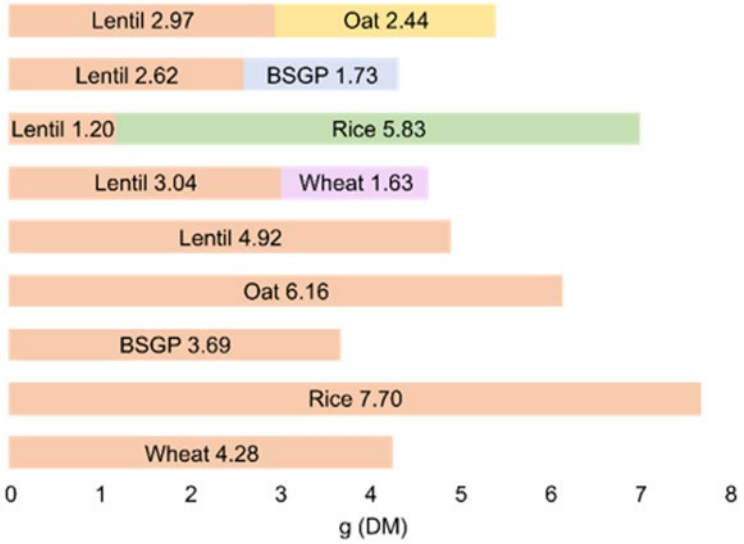
Theresa Boeck: Writing - original draft, Formal analysis, Methodology, Investigation, Data Curation, Validation, Visualisation. Laura Nyhan: Investigation, Writing - review & editing. Emanuele Zannini: Project Administration, Funding Acquisition, Writing - review & editing. Elke K. Arendt: Conceptualization, Resources, Validation, Writing - review & editing, Supervision.

Supplementary Material

Supplementary Table 1 Results of the sugar and FODMAP analysis of the PIs by HPAEC-PAD, in g/100 g DM.

			Lentil	Oat	BSGP	Rice	Wheat
Glucose			0.11 ± 0.01	0.03 ± 0.01	0.29 ± 0.01	2.33 ± 0.02	0.28 ± 0.04
Fructose			0.05 ± 0.01	n.d.*	0.07 ± 0.00	0.34 ± 0.00	0.13 ± 0.00
Sucrose			1.01 ± 0.02	0.77 ± 0.00	0.01 ± 0.00	0.2 ± 0.01	0.06 ± 0.00
FODMAPs	EF**		-	-	-	-	-
	Polyols	Xylitol	0.02 ± 0.00	n.d.	n.d.	0.01 ± 0.00	n.d.
		Sorbitol	0.01 ± 0.00	n.d.	n.d.	0.09 ± 0.00	0.21 ± 0.00
		Mannitol	0.62 ± 0.01	n.d.	n.d.	0.01 ± 0.00	0.03 ± 0.00
		∑ Polyols	0.65 ± 0.01	n.d.	n.d.	0.11 ± 0.01	0.24 ± 0.00
	Oligo-saccharides (OS)	Raffinose/ Stachyose	2.01 ± 0.03	0.17 ± 0.00	n.d.	0.02 ± 0.00	0.01 ± 0.00
		Verbascose	0.43 ± 0.01	0.01 ± 0.00	n.d.	n.d.	n.d.
		Fructans	-†	n.d.	0.14 ± 0.05	n.d.	0.67 ± 0.03
		∑ OS	2.44 ± 0.03	0.18 ± 0.00	0.14 ± 0.05	0.02 ± 0.00	0.68 ± 0.03
	∑ FODMAPs		3.09 ± 0.03	0.18 ±0.00	0.14 ± 0.05	0.13 ± 0.00	0.92 ± 0.00

*n.d., not detected or levels below 0.0005 g / 100 g DM. **Fructose in excess of glucose. †Fructans not determined as they are not present in legumes.



Supplementary Figure 1 Amount of PI (g DM) per 100 g emulsion in single-ingredient and blended emulsions.

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