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Comparing nutritional and digestibility aspects of sustainable proteins using the INFOGEST digestion protocol

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ABSTRACT

We studied several sustainable alternative protein concentrates and a whey protein concentrate as reference, to determine their protein composition, digestibility and quality using the harmonized INFOGEST static protocol. The proteins concentrates were analyzed to determine their conversion factor, degree of hydrolysis, true ileal digestibility, *in vitro* digestible indispensable amino acid score (IVDIAAS) and total absorbable amino acids and total essential amino acids. The results revealed that whey, blood plasma concentrate and yeast protein concentrate have a high mean true ileal indispensable amino acid *in vitro* digestibility (91.1–85.8%), closely followed by corn, pea, potato and proteins derived from lesser meal worms (ranging between 82.5 and 77.9%). Mycoprotein gave a much lower score in the mass balance, so that its other results could not be interpreted as being reliable. Whey, potato, blood plasma and yeast protein concentrates ranked highest in IVDIAAS (between 119 and 97.2), followed by lesser meal worm and pea (between 73.8 and 57.8) with corn protein concentrate having the lowest IVDIAAS due to underrepresentation of lysine. The method and data presented in this paper can form a start for further applying the INFOGEST *in vitro* digestion protocol to evaluate protein quality.

1. Introduction

With a growing population and increasing prosperity, the demand for food and especially animal-based proteins is increasing. As appropriate protein intake is essential for optimal functioning of the human metabolism and physiology (i.e. growth, development, muscle mass and function), global protein availability and supply must be secured. It is therefore important to make use of alternative sources to develop new protein concentrates and isolates, preferable with a low ecological foot print and of low costs. Many of these protein alternatives are being developed, such as those based on insects, aquatic plants and algae, cereals and legumes. Co-streams that previously were not valorised are also being explored, like animal offal, residue from oil pressings, by-products from the yeast and brewing industry, or unconsumed plant leaves and stems like those from sugar beet (Fasolin et al., 2019, Martin et al., 2019). Such alternative proteins will find their way to the market, especially when they have excellent functional properties in food product applications, like solubility, gelling, foaming and texturing properties and a neutral taste with no strong off-flavours (Zhao et al., 2020, Raikos et al., 2014), in addition to their demonstrated safety for

Abbreviations: PDCAAS, protein digestibility-corrected amino acid scores; 3R, reduce, refine and replace; DIAAS, digestible indispensable amino acid score; COST, European Cooperation in Science and Technology; BW, body weight; TID, true ileal digestibility; BPC, blood plasma protein concentrate; CPC, corn protein concentrate; LMWC2, lesser meal worm protein concentrate 2; MP, mycoprotein; NPP, pea protein concentrate; TPP1, potato protein concentrate 1; WPC, whey protein concentrate; YPC, yeast protein concentrate; SSF, simulated saliva fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; MW, molecular weight; PDA, photo diode array; SDS, sodium dodecyl sulfate; TNBS, 2,4,6-Trinitrobenzene sulfonic acid; DH, degree of hydrolysis; AA, amino acids; FAO, food and agriculture organization of the united nations; IVDIAAS, *in vitro* digestible indispensable amino acid score; ANOVA, Analysis of variance; LSD, least significant difference; His, histidine; Thr, threonine; Val, valine; Lys, lysine; Ile, isoleucine; Leu, leucine; Met, methionine; Cys, cysteine; Phe, phenylalanine; Tyr, tyrosine; Gln, glutamine; Arg, arginine; SAA, sulfur containing amino acids; AAA, aromatic amino acids; LAA, first rate limiting amino acid; AUC, Area under curve; TAA, total amino acids; EAA, essential amino acids; TEAA, total essential amino acids.

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human consumption.

Besides these functional aspects of alternative protein isolates and concentrates, it is highly important to know their digestibility and nutritional value, especially when the protein will be applied in food products for the vulnerable groups who have difficulties consuming enough protein. Although protein intake recommendations may vary per country, elderly people can have a protein intake far below the current recommended intake (Baum et al., 2016, Berryman et al. 2018). On the other hand, consuming less nutritious proteins can be beneficial, as overconsumption of proteins is very common in Western countries (DNFCS 2012–2016, https://www.rivm.nl/bibliotheek/rapporten/2020–0083.pdf, Berryman et al., 2018).

The gold standard to characterize protein quality is the rat-based PDCAAS which is currently challenged by the more accurate swine-based DIAAS method, considered to be more representative of the actual protein utilized by the host (Huang et al., 2018, Abelilla et al., 2018). However, in accordance with the 3R principle, consumers, governments, and industry aim to reduce and replace animal experiments whenever possible and therefore, dedicated *in vitro* models would be a pragmatic approach to study protein quality. In the past, many different *in vitro* simulation models of gastrointestinal food digestion have been published without any consensus in the methods used, making data comparisons between different protein sources impossible. Since 2014, the European COST INFOGEST consortium generated several consensus protocols for static and semi-dynamic *in vitro* digestion models, to guarantee comparability between research labs (Minekus et al., 2014, Brodkorb et al., 2019). The biological relevance and application in research of the static protocol has been demonstrated in several studies (Egger et al., 2016, Sanchon et al., 2018, Santos-Hernandez et al., 2020, Sousaa et al., 2020), but no study has yet used the protocol for the quantification of digestibility and DIAAS values for protein samples.

Here, we have applied the static digestion protocol to study the quality and digestibility of a range of alternative protein sources. Whey protein concentrate was taken as a reference, because of its quick and good digestibility (Rutherfurd et al., 2015).

2. Material and methods

2.1. Samples & chemicals

Chemicals and enzymes used were standard analytical grade, and were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless stated otherwise. Pepsin (P7012), bile (B8631) and pancreatin (P7545; 8 × USP specifications activity) were all from porcine origin. Enzyme activities were determined according to the Minekus et al., (2014) supplementary protocol. Bile activity was measured using the bile acid assay kit (MAK309). The AccQ-Tag Ultra derivatization reagents kit was obtained from Waters Corporation (Milford, MA, USA). An overview of the protein samples made available for this study is provided in Table 1.

2.2. Protein content

Quantitative determination of nitrogen content was estimated by the Dumas method in triplicate based on the protocol of Adler-Nissen et al. (Adler-Nissen et al., 1979). Liquid samples from *in vitro* digestion simulations (200 μL) where first dried at 60 °C. Methionine was used as a standard and cellulose as a negative control. Measurements were performed on a Flash EA 1112 GC (Interscience BV, Breda, The Netherlands).

2.3. Dry matter content

Determination of dry matter content was carried out by drying the protein concentrate powders in air at 105 °C for 24 h using an Heraus hot air oven (Tamson Instruments BV; Bleiswijk, The Netherlands). Samples where measured in duplicate.

2.4. In vitro digestion

Protein *in vitro* digestions were performed according to the static INFOGEST consensus protocol (Minekus et al., 2014) with minor adjustments. Simulated oral and gastric fluids were made according to protocol. The digestions were performed in a thermal jacket glass vessel at 37C in which the pH was constantly monitored and adjusted by pH stat titration (Titrando 888; Metrohm AG, Herisau, Switzerland). All digestions were magnetic bar-stirred. Based on Dumas protein quantification as provided by the suppliers or measured on the product (using 6.25 as conversion factor for all samples), 9.375 g of protein was weighted (based on Dumas) and completed with MQ untill 93.75 g, mixed and the suspension subsequently mixed with 93.75 ml of simulated saliva fluid (SSF), protein concentration 0.05 g/ml. Then, 150 ml concentrated (1.25*) of simulated gastric juice (SGF) was added and the pH was set to pH 3.0 by titration of 1 M HCl after which pepsin (2000 U/ml, Sigma P7012), end volume of gastric phase is 375 ml, concentration protein 0.025 g/ml. The protein digestion was incubated for two hours while keeping the pH constant. Next, 290 ml concentrated (1.25*) simulated intestinal juice (SIF) and bile (Sigma, B8631) were added and the pH was adjusted to pH 7.0 with 1 M NaOH, before adding pancreatin (Sigma, P7545) containing 100 or 10 U/ml trypsin (Units measured with TAME) end volume intestinal phase 750 ml, final protein concentration

Table 1
Overview of the protein samples used in this study.

Full name	Protein Source	Provided by	Abbreviation	Protein % *	PDCAAS literature	DIAAS # literature
Bovine plasma concentrate	Blood proteins of Bovine (*Bos taurus*)	Darling Ingredients (Irving, Texas, USA),	BPC	72	78.5[6]	NA
Corn protein concentrate	Corn (*Zea mays*) kernels	Cargill Inc. (Wayzata, MO, USA)	CPC	NA (83)	28.7–67[7, 8]	NA
Lesser mealworm concentrate 2	Lesser mealworm (*Alphitobius diaperinus*)	Protifarm NV. (Ermelo, The Netherlands)	LMC2	NA (68)	82[9]	NA
MycoProtein	Mycelium of *Fusarium venenatum*	Marlow Foods Ltd (Stokesley, North Yorkshire, UK)	MP	54	91–99.6[1, 2]	NA
Experimental Pea protein concentrate	Yellow peas (*Pisum sativum*)	Roquette Frères (Lestrem, France)	NPP	80	71–89[3,4]	62–82[3,4]
Potato protein concentrate 1	Potato tubers (*Solanum tuberosum*)	Royal Avebe U.A. (Veendam, The Netherlands),	TPP1	95	71–99[10,11]	NA
Whey protein concentrate	Whey of Bovine (*Bos taurus*)	Nutricia (Zoetermeer, The Netherlands)	WPC	74	100[3,4]	107–109[3,4]
Yeast protein concentrate	Yeast (*Saccharomyces cerevisiea*)	Lesaffre (Marcq-en-Baroeul, France),	YPC	NA (84)	82–90[5]	NA

NA: data not available; * based on supplier and between brackets concentration based on Dumas (CF 6.25); # calculated using the amino acid requirement pattern for the 0.5–3-y-old child FAO (2013). [1] Miller & Dwyer (2001); [2] Edwards & Cummings (2010); [3]Rutherfurd et al., (2015); [4]Mathai et al., (2017); [5]Pacheco et al., (1997); [6]Montero Castillo et al., (2015); [7]Mendes et al., (2017); [8]Zarkadas et al., (1995); [9]Jensen et al., (2019); [10]https://orgprints.org/5192/1/5192.pdf; [11]Schmidt (2016).

0.0125 g/ml. The digest was incubated at pH 7.0 for 2 h (see, for more detail, the result section). The sodium bicarbonate in the simulated intestinal juice was exchanged for sodium chloride to generate a more stable pH maintaining the same electrolyte concentration (Mat et al., 2016). All digestions were performed in duplicate.

Samples for measurements were taken before digestion, at start of gastric digestion and after 2 h gastric incubation, at start of intestinal phase and after incubation for 2 h, which is the end of the digestion. All samples were snap-frozen in liquid nitrogen and stored at −80 °C.

2.5. Ultrafiltration of digests

The protein digest was separated in a filtrate and retentate by ultrafiltration using a 5,000 MW Hydrosart Vivaflow filter (VF05H1; Sartorius AG, Göttingen, Germany). First, the digest was centrifuged (10,000xg for 15 min at 4 °C) and the supernatant used for ultrafiltration while the pellet was stored on ice. The supernatant, approximately 750 ml, was concentrated to 100 ml at 2 bar pressure, using the pump speed to control the pressure of the circulating supernatant. The concentrated supernatant volume was diafiltrated 6 times with 100 ml buffer (which had the same composition as the final buffer composition of the digests but without bile and enzymes) using the Hydrosart Vivaflow filter. All samples were kept on ice during ultra-filtration. After filtration, the > 5 kDa fraction was combined with the previously obtained pellet and together were classified as retentate. Both filtrate (fraction < 5 kDa) and retentate were stored at −80 °C until further use. Both digests were filtered separately.

2.6. Amino acid composition

Amino acid analysis of the source material, digests, retentate and filtrate was performed according to the Waters Co. AccQ-Tag Ultra Derivatization manufacturers protocol with slight modifications. Protein samples were dissolved in 20 mM phosphate buffer pH 7.3 (~2 mg/ml) and 100 μL of each sample was mixed with 100 μL 8 M methanesulfonic acid containing 0.1% tryptamine. The vial was capped and flushed with N_2 gas for 1 min. Each sample was hydrolyzed at 121 °C for 4 h in the dark. After hydrolysis, the sample was neutralized by adding 200 μL 4 M NaOH. As a standard, Norvalin was added and samples were diluted before measurement. Similarly, standard solutions for all amino acids were prepared with Norvalin as internal standard. For derivatization of both the standards and the samples, 70 μL of borate buffer was mixed with 10 μL of standard or sample. To each of the vials, 20 μL of AccQ-Tag Ultra derivatization reagent dissolved in acetonitrile was added after which the vials were immediately capped and mixed for 10 s. The vials were then heated at 55 °C for 10 min and stored at −20 °C prior to HPLC analysis.

The AccQ-tag Ultra method originally designed for UPLC, was adapted for use on an Acquity ARC UHPLC. An Xbridge BEH C18 2.5 μm 3.0x150 mm Column XP (Waters Corporation; Milford, MA, USA) at 55 °C was used in combination with the eluents A and B from the AccQ-Tag Ultra derivatization kit. Injections of 1 μL were eluted at a flowrate of 0.971 ml/min using a 200 μL gradient composition (99.9% A at t = 1.2 min to 10% A/90% B at t = 21 min) for 21 min followed by 8 min 99.9% A eluents. For detection, a Waters Co. 2998 PDA detector equipped with a micro bore flow cell was used and results were analysed using the Waters Co. Empower software. Baseline separation was obtained for all free amino acids standards except for the amino acids Gln and Arg which elute as a single peak. Analysis were performed in duplicate.

2.7. Calculation of protein conversion factor

Conversion factor was calculated as described by Sriperm et al. (Sriperm et al., 2011). Conversion factor k_a was calculated based on the AA content ($\sum E_i$) and divided by the calculated nitrogen content $\sum D_i$, which is the sum of the nitrogen present in each AA plus any produced ammonia nitrogen content. The nitrogen content of the ammonia was added to the total calculated amino acid nitrogen content since this is a side product of the acid hydrolysis performed to measure the AA. The amide linkage of Asn and Gln can be broken at low pH. During acid hydrolysis these residues of Asn and Gln are converted to aspartic acid and glutamic acid releasing amide nitrogen which forms free ammonia.

2.8. Degree of hydrolysis

The amount of free NH_2 groups was measured spectrophotometrically according to the method of Spellman et al. (Spellman et al., 2003). In vitro digested samples were centrifuged (3 min 14000 rpm, 4 °C) to remove insoluble fragments. Next, supernatants were diluted 40 times in MQ and mixed 1:1 (200 μL) with 2% SDS. The supernatants were heated at 75 °C for 15 min, cooled down and mixed (125 μL) with 1 ml 0.2125 M sodium phosphate buffer (pH = 8.2) and 1 ml 0.05% TNBS. Mixtures were incubated at 50 °C for 1 h after which the reaction was stopped by adding 2 ml 0.1 N HCl. The samples were cooled to room temperature, and the absorbance at 340 nm was measured in a UV-3100 spectrophotometer (VWR, Breda, The Netherlands). Iso-leucine was used as a standard. The free NH_2-groups were calculated per gram product after subtraction of a blanc, and expressed as *iso*-leucine amino equivalents (megv/g product). Non-digested protein samples were analysed the same way, but with a supernatant dilution factor of 2 in MQ. Analysis were performed in triplicate

The degree of hydrolysis (DH) was calculated by the following equation:

$$DH = h/h_{tot}*100\%$$

Hydrolysis equivalents (h) can be obtained by converting the *iso*-leucine amino equivalents by means of the following equation:

$$h = (\text{iso} - \text{leucine} - \text{NH2} - \beta)/\alpha$$

where α and β ares resp. 1.00 and 0.40 (Adler-Nissen, 1986)

h_{tot} can be calculated from the amino-acid composition of the protein concentrates used in the *in vitro* digestion simulations (Adler-Nissen, 1986).

2.9. True ileal digestibility (TID) calculation

The true ileal digestibility was calculated in several ways, either basing calculation on a start protein or digestive mixture including digestive enzymes (for which a correction is needed using an empty digest) and based on retentate (like done in an *in vivo* DIAAS) or filtrate. Analytic methods and sample handling of retentate (especially due to fast precipitation of insoluble protein aggregates or non-homogenous samples) can cause problems in amino acid analysis. These problems were less prominent when analysing well soluble filtrate. Besides, proteins become nutritious when digested and absorbed which is represented by the filtrate. We hypothesised that using filtrate is physiologically relevant and more direct than calculating the absorbed amino acid content from the difference between start and retentate. This resulted in tree types of calculation strategies depicted as formula A, B and C as explained below in more detail.

Calculation A was based on the filtrate divided by the start protein: Each individual amino acid (AA) in mg in the filtrate is subtracted with the same AA (mg) of the control 'empty' digest (containing and treated the same without the addition of a protein sample) and divided by that AA in the start protein sample.

$$\text{A; } TID_{AA}\ (\%) = ((\text{AA filtrate}_{prot} - \text{AA filtrate}_{control})/\text{AA Start}_{prot})*100$$

Calculation B was based on the filtrate divided by the protein in the digest (before filtration): the amount of an individual AA in the filtrate of a protein sample minus that AA of a control 'empty' digest was divided by the same AA in the digest (before filtration) minus the AA in digest

control.

B; TID_{AA} (%) = ((AA $filtrate_{prot}$ – AA $filtrate_{control}$)/(AA $digestion_{prot}$ – AA $digestion_{control}$))*100

Calculation C was based on the filtrate divided by the digest but without the subtraction of the control, in both filtrate and digest the same starting amount of digestive enzymes are present. This method is less sensitive, due to a wrong correction from an empty digest (as digestive enzymes could maybe hydrolyse each other when no other substrate is available).

C; TID_{AA} (%)= (AA $filtrate_{protein}$/ AA $digest_{protein}$)*100

2.10. Digestible indispensable amino acid score (DIAAS)

Based on the TID, the *in vitro* DIAAS (IVDIAAS) was calculated as suggested in the FAO report (FAO, 2013), using the recommended amino acids scoring patterns for young children (6 months to 3 years), and older children, adolescents and adults for which the scoring is based on the recommended pattern for 3 to 10 year old children as noted in FAO report. The IVDIAAS were calculated based of the three TID methods as follows:

IVDIAAS (%) = ((TID $filtrate_{A/B/C}$ * (mg of digestible dietary indispensable amino acid per 1 g of dietary protein)) / (mg of the same dietary indispensable amino acid per 1 g of the reference protein)) * 100.

2.11. Statistics

The TNBS data of the 10U and 100U trypsin digests was compared using a Kruskal-Wallis test in the software program SPSS (v25.0.02), applying the Shapiro-Wilk test. All other statistical analyses were performed in Graphpad Prism (v 8.3.1) using an ANOVA Fisher LSD test.

3. Results

3.1. Modification on the INFOGEST protocol

The static INFOGEST consensus protocol advises 2000 U/ml of pepsin in the gastric phase and 100 U/ml of trypsin in the small intestinal digestion phase. In preliminary (unpublished) experiments, in which pancreatin was added comprising the advised 100 U/ml trypsin, we encountered problems in quantifying the release of free AA from samples containing low concentrations of digestible proteins above the background level resulting from the 'empty' digest control. As the IVDIAAS calculations require the correction for these control values it is recommended to have a considerable amount of AA derived from the sample protein compared to the amount of protein added in the 'empty' digest in the form of enzymes (calibrated in activity units). It is therefore preferred to have either enzymes with a high activity or use lower amount of units in the digestion protocol.

The effect of a 10 times reduction of trypsin on the degree of hydrolyses (DH) of WPC was analysed by TNBS. The digestion with 100 U trypsin/ml resulted in 363 ± 29.7 μmol /g NH_2 while 10 U trypsin/ml resulted in 334 ± 41.3 μmol /g NH_2. These differences in DH between both enzyme concentrations were not statistically different ($p = 0.435$). Based on these results, it was decided to perform the *in vitro* digestions and further analyses using 10 U/ml trypsin together with the more standard digestive enzyme concentrations as described in the Material and Methods (M&M) section.

3.2. TNBS analyses show variation in start, digest and filtrate

Start sample, the digest and the < 5 kDa filtrate sample were analysed by TNBS to study presence, generation and fate of the primary amino groups (Fig. 1). Start samples did not contain many free amino groups, thus the increase in amino groups after digestion was generated by digestive hydrolysis. Data from LMC2 are not shown due to the release of a yellow colour which interfered with the TNBS measurement. From the data it can be concluded that the degree of hydrolysis is comparable between the proteins from different sources with no significant differences (two way ANOVA, Fisher LSD test). All proteins were hydrolysed by the digestive enzymes and led to small peptides and amino acids which end up mainly in the < 5 kDa filtrate fraction. MP (p-value < 0.0001), CPC (P-value = 0.0001), NPP (p-value = 0.008) and YPC (p-value = 0.037) have less small peptides and free AA in their digest leading to a lower NH_2 recovery in the filtrate compared to WPC (Two way ANOVA, Fisher LSD test p value < 0.05). Hence, during filtration of the digests, part of these proteins may have remained in the retentate (e.g. MP) while for other sources almost all of these peptides end up in the filtrate, an indication that these peptide fragments are small and likely available for uptake in the small intestine. The total degree of hydrolysis (DH) in the digest was calculated and can be found

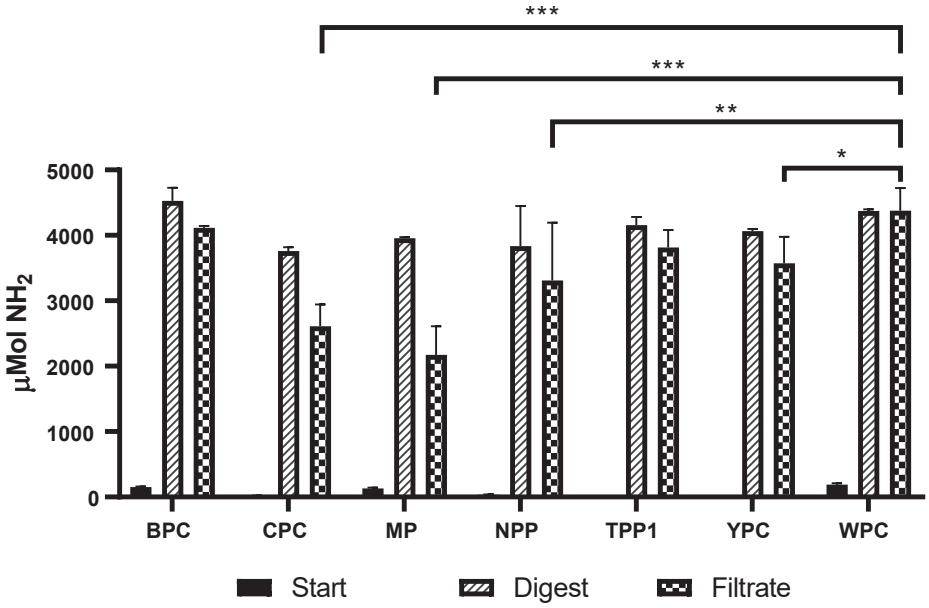

Fig. 1. TNBS analysis of the start protein samples, digested protein samples and the protein/peptides that end up in the < 5 kDa filtrate. * p value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

in Fig. 2. TPP1 was found to contain the highest DH, even significantly higher than WPC. Most of the other proteins have a DH similar to WPC ranging between 31.2% and 34.4%, except for MP which has a significantly lower DH (DH of 17.6 %) than WPC.

3.3. Protein conversion factor

Protein quantifications based on Dumas with a standard conversion factor of 6.25 can lead to an underestimation, but more often to an overestimation for the protein content due to the inclusion of other nitrogen sources like nucleic acids, urea, ammonia, glucosamine, phospholipids, nitrates and purine derivates. As the total AA contents were determined in the samples, a more correct conversion factor for Dumas analysis could be calculated resulting in the k_a factor as shown in Table 2. Results indicate that conversion factors are within the same range as in literature for most samples. Differences can be due to different strategies applied in extraction and processing of the protein concentrates.

3.4. Mass balance differs per protein source

Protein samples were digested and separated into a filtrate (<5kDa) and retentate, containing the soluble protein fraction > 5 kDa and insoluble proteins. All three fractions (digest, filtrate and retentate) were analysed for the total amount of separately quantified amino acids (AA) in order to obtain a mass balance and insight on potential loss of detectable proteins, see Table 3. Results on the mass balance indicate that WPC and BPC both have a high total recovery, while for other proteins the recovery ranged from 95 to 86% and a lower recovery for MP (74%). The mass balance allows to calculate also the total AA content ending up in the filtrate expressed as % relative to the unfiltered digested sample as an indication of digestibility. This results in a range from 85% (BPC) to 50% (MP) when based on total AA values. As WPC is often seen as one of the best digestible protein sources and used as reference, the values were also expressed relative to WPC. The results indicate that digestibility of proteins based on total AA are very high for WPC, BPC and YPC, high for NPP, CPC, LMC2 and TPP1 and lowest for MP.

3.5. True ileal digestibility

Next, the True Ileal Digestibility (TID) was calculated in different ways (see M&M section). It is our opinion that calculation based on method B, AA in filtrate in comparison to AA in the digest, will resemble best what part of the protein could be absorbed in the small intestine (Table 4). Other strategies of calculation like those based on using the start protein sample (method A) or those based on ratio including digestive enzymes (method C) are shown as supplementary Tables S1 and S2 respectively. The results indicate that, when comparing the individual amino acids with the average TID digestibility values, in particular the SAA show the largest deviation. Potentially this is due to the redox sensitivity of these sulphur containing amino acids which might result in an under representation. The AAA of CPC shows an unlikely high % of digestibility but this will not have a very high impact on the overall conclusion. Results indicate that WPC, BPC and YPC are highest (ranging from 91.1 to 85.8%) in digestibility of EAA, MP relatively lowest (57.0%) and all other proteins samples in between (ranging between 82.5 and 77.7 %). Other calculation methods used resulted in slightly different absolute percentages of digestibility, but did not result in major shifts in the ranking or classification of the protein samples studied.

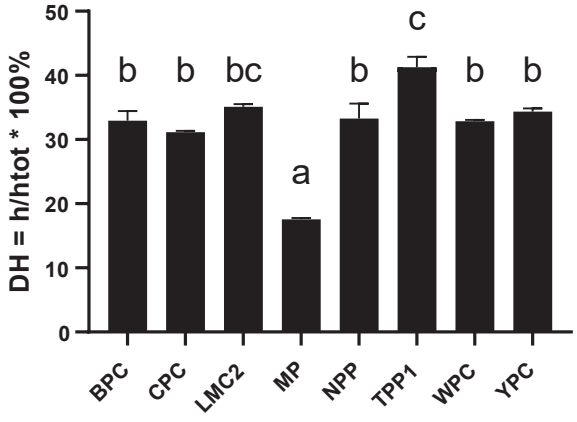

Fig. 2. Degree of hydrolysis of the digested protein samples. Based on ANOVA Tukey's multiple comparison test.

Table 2
Calculated conversion factors for the protein samples studied.

Protein source	Conversion factor literature	K_a mean (SD)
BPC	6.25[1]	5.99 (0.02)
CPC	5.6[2]–5.70[3]	5.94 (0.30)
LMC2	5.60[4]	5.49 (0.03)
MP	6.25[5]	5.63 (0.04)
NPP	5.36[2]	5.63 (0.04)
TPP1	6.24[6]	6.13 (0.06)
WPC	6.38[7]	6.34 (0.02)
YPC	5.78[8]	5.94 (0.09)

[1]Sosulski et al., 1990; [2]Mariotti et al., 2008; [3]Sriperm et al., 2011; [4]Janssen et al., 2017; [5]Lonchamp et al., 2020; [6]Van Gelder, 1981; [7]Grappin & Ribadeau-Dumas, 1992; [8]De la Hoz et al., 2014.

3.6. In vitro DIAAS

Based on the TID an *in vitro* DIAAS (IVDIAAS) was calculated making use of the most recent FOA/WHO recommendations (FAO, 2013). In these recommendations, DIAAS should be calculated based on requirements for children aged 6–36 months and supplying the first rate limiting AA which was subsequently calculated based on the three TID methods applied. As we expect an application of alternative protein in food for adults sooner than for young children, we also included the calculations based on the requirements of 3 years of age and older (Table 5). Based on the calculation method, small differences in absolute IVDIAAS values and in the ranking order for the sources was found, with method B and C showing highest similarities in outcome. Based on requirements for a child the sources TPP1, WPC and BPC all have an IVDIAAS score above 91.1. Lowest score is for CPC which is known to have a low lysine concentration which indeed was identified as the first rate limiting amino acid for CPC. For adults, the sources TPP1 and WPC have an IVDIAAS value above 100, independent of which calculation method was used, followed by BPC and YPC having both an IVDIAAS above 95. The protein concentrates NPP, MP and again CPC show the lowest IVDIAAS values, irrespective of recommendations for age groups.

3.7. TAA and TEAA in comparison to whey

Results of *in vivo* human protein digestibility studies are often expressed as area under curve (AUC) of the postprandial AA of which in most cases only the total AA (TAA) and total essential AA (TEAA) are provided. As study subjects can differ in their responses to interventions, a good design for such studies is a cross-over design in which a reference protein like WPC is compared to the protein(s) of interest and results are shown relative to WPC. To be able to compare our results with future human intervention studies, we also expressed the data as TAA and TEAA in the filtrate in absolute values and those relative to WPC (Table 6). Results indicate that the reference WPC and BPC shows the highest TEAA in the filtrate. YPC also provide high levels of bioavailable TEAA, followed by CPC and TPP1, while NPP, LMC2 and MP showed the

Table 3
Total AA values for the protein sample digests, filtrate and retentate and the calculated total recovery, recovery of filtrate.

Protein source	Digest (g)	Retentate (g)	Filtrate (g)	Total recovery (%)	Recovery filtrate (%)	Recovery filtrate relative to WPC (%)
BPC	11.39	1.89	9.64	101	85	101
	(0.05)	(0.01)	(0.25)	(2)	(2)	(3)
CPC	11.21	1.73	8.27	89	74	88
	(0.31)	(0.41)	(0.70)	(7)	(4)	(6)
LMC2	7.54	1.10	5.39	86	71	85
	(0.09)	(0.09)	(0.20)	(0)	(2)	(3)
MP	11.76	2.83	5.90	74	50	60
	(0.16)	(0.02)	(0.16)	(2)	(2)	(3)
NPP	10.42	1.88	7.69	92	74	88
	(0.10)	(0.00)	(1.55)	(14)	(14)	(16)
TPP1	9.40	1.57	6.56	86	70	83
	(0.09)	(0.68)	(0.34)	(3)	(4)	(6)
WPC	11.52	1.86	9.68	100	84	100
	(0.40)	(0.05)	(0.23)	(1)	(1)	(0)
YPC	11.03	1.73	8.71	95	79	94
	(0.07)	(0.00)	(0.00)	(1)	(0)	(0)

Table 4
Single AA TID score based on calculation method B. Data are expressed as mean and standard deviation %.

Protein Source	His	Thr	Val	Lys	Ile	Trp	Leu	SAA Met + Cys	AAA Phe + Tyr	Mean EAA	Mean TAA
BPC	93.7	89.4	92.2	89.6	92.9	95.5	90.5	70.8	100.9	90.6	89.1
	(1.2)	(2.1)	(1.0)	(0.9)	(1.9)	(0.5)	(2.9)	(2.1)	(3.1)	(8.1)	(12.2)
CPC	72.6	74.2	77.2	79.3	78.4	87.5	77.8	64.3	130.8	82.5	75.7
	(8.5)	(2.8)	(5.2)	(8.1)	(3.3)	(8.8)	(5.5)	(22.4)	(11.7)	(20.0)	(12.3)
LMC2	75.5	70.7	74.7	75.2	76.0	85.9	73.4	93.5	74.2	77.7	76.8
	(1.6)	(2.0)	(1.2)	(5.0)	(1.8)	(9.6)	(4.8)	(10.6)	(2.8)	(8.1)	(9.9)
MP	51.2	44.8	49.5	48.5	50.1	91.5	48.6	60.3	68.5	57.0	52.2
	(2.2)	(1.1)	(1.2)	(2.2)	(0.8)	(6.8)	(3.4)	(3.1)	(4.1)	(14.6)	(12.1)
NPP	75.5	77.1	77.5	79.3	79.4	82.0	77.5	73.2	98.3	80.0	76.5
	(17.4)	(17.6)	(16.9)	(15.6)	(14.9)	(14.2)	(14.4)	(7.5)	(18.7)	(13.3)	(12.0)
TPP1	75.4	70.2	70.7	75.2	72.2	80.3	70.9	87.6	98.7	77.9	73.0
	(0.3)	(5.9)	(7.4)	(7.2)	(5.3)	(0.7)	(5.8)	(1.4)	(6.3)	(10.1)	(11.2)
WPC	96.4	83.3	88.2	87.3	90.2	95.9	91.8	87.4	99.3	91.1	89.9
	(5.7)	(2.0)	(0.2)	(0.7)	(0.5)	(5.5)	(1.9)	(5.8)	(4.0)	(5.7)	(8.4)
YPC	84.5	84.5	83.5	88.3	83.1	84.7	83.2	79.6	100.7	85.8	82.9
	(3.6)	(3.2)	(2.1)	(5.9)	(2.8)	(1.1)	(2.8)	(4.5)	(5.0)	(6.4)	(4.9)

Table 5
In vitro DIAAS calculations for each of the three TID calculation methods, expressed as mean (standard deviation), based on FAO reference values for adults and child. Next to that the first limiting amino acid (LAA) when compared to an ideal protein composition is provided.

Protein	Calculation method A			Calculation method B			Calculation method C		
Source	Child[1]	Adult[2]	IAA	Child[1]	Adult[2]	IAA	Child[1]	Adult[2]	IAA
BPC	99.7	106.3	Ile	91.1	97.2	Ile	87.6	93.4	Ile
	(4.4)	(4.7)		(1.9)	(2.0)		(1.5)	(1.6)	
CPC	36.5	43.3	Lys	25.3	30.0	Lys	25.1	29.8	Lys
	(5.7)	(6.8)		(2.6)	(3.1)		(1.9)	(2.3)	
LMC2	56.8	66.6	SAA	68.2	73.8	Leu	66.9	72.4	Leu
	(6.9)	(8.1)		(4.5)	(4.8)		(3.7)	(4.0)	
MP	42.3	49.6	SAA	33.6	39.5	SAA	35.9	42.2	SAA
	(6.6)	(7.8)		(1.7)	(2.0)		(1.3)	(1.5)	
NPP	41.4	48.6	SAA	49.3	57.8	SAA	49.8	58.5	SAA
	(6.2)	(7.3)		(5.1)	(6.0)		(3.6)	(4.3)	
TPP1	102.5	120.3	SAA	92.6	110.7	His* /Leu#	92.9	116.2	His
	(19.1)	(22.4)		(0.4)	(9.1)		(0.2)	(0.2)	
WPC	89.9	112.4	His	95.2	119.0	His	92.8	116.0	His
	(3.6)	(4.4)		(5.6)	(7.0)		(4.8)	(6.0)	
YPC	82.8	97.2	SAA	83.9	98.5	SAA	84.4	99.0	SAA
	(12.1)	(14.2)		(4.8)	(5.6)		(3.3)	(3.8)	

[1] Child is based on requirements for young children 6–36 months (FAO, 2013).
[2] Adult is based on requirements of 3–10 year old child (FAO, 2013).
* His is IAA for child DIAAS and # leu is IAA for adult DIAAS for TPP1.

Table 6
Total AA and total essential AA found in the filtrate in absolute values and relative to WPC.

Protein	TAA		TEAA	
Source	g filtrate/ g digest[#]	Relative to WPC (%)	g filtrate/ g digest[#]	Relative to WPC (%)
BPC	0.87 (0.02)	100 (0)	0.41 (0.00)	100 (0)
CPC	0.75 (0.05)	86 (5)	0.35 (0.02)	85 (6)
LMC2	0.74 (0.02)	84 (3)	0.26 (0.00)	63 (2)
MP	0.49 (0.02)	56 (3)	0.21 (0.01)	53 (3)
NPP	0.75 (0.16)	86 (18)	0.28 (0.06)	70 (13)
TPP1	0.71 (0.05)	81 (6)	0.35 (0.03)	86 (7)
WPC	0.86 (0.01)	100 (0)	0.41 (0.00)	100 (0)
YPC	0.83 (0.03)	96(3)	0.37 (0.01)	92 (4)

[#] AA in digest and filtrate are corrected for AA in control digest and filtrate.

lowest relative bioavailability levels.

4. Discussion

4.1. The value and standardisation of in vitro digestion method

Here we studied the digestibility of several alternative protein sources for ileal digestibility and *in vitro* DIAAS calculations, based on a slightly adapted INFOGEST protocol. Although no *in vitro* method can fully simulate the *in vivo* gastrointestinal digestion and absorption kinetics, *in vitro* models are useful alternatives to animal and human models as part of a screening or evaluation strategy. Especially in case of comparative analysis of food ingredients and food products in order to answer questions related to digestibility, bioavailability, intestinal stability, release and formation of bioactive compounds and those involving food matrix (Egger et al., 2017, Fardet et al., 2019). One of the benefits of *in vitro* digestion methods is the absence of any ethical concerns such as valid for the current animal-based protein quality analyses. Moreover, the method used here is relatively cost efficient and can be performed with medium-throughput in almost any lab without the need of advanced equipment. As it is known that PDCAAS values can be overestimated because of limited bioavailability of specific forms of amino acids as well as bacterial assimilation of amino acids, that falsely enhance values of true protein digestibility (Marinangeli & House, 2017) it might be that a standardized *in vitro* method together with *in vivo* validation will result in good biological relevant predictions for human utilizations of proteins.

Despite the problems with correct AA quantification of resistant proteins agglomerates that can form in the lumen/retentate, we consider the mass balance between AA present in the digest and the subsequent retentate and filtrate an important quality parameter. In our mass balance (Table 3) almost all protein sources showed a high (>85%) recovery, except for MP which had a recovery of 74%. This could indicate that either the AA quantification was less reliable for this protein source due to interfering factors. We did not use the official AOAC method for amino acid analysis which requires three separate steps in which most amino acids are analysed after hydrolysis with hydrochloric acid, tryptophan analysis is performed after alkaline hydrolysis, cysteine and methionine after derivatisation as these are more sensitive for acid hydrolysis. The method as performed here makes use of methane sulfonic acid hydrolysis which provides the best recovery of all amino acids including labile amino acids cysteine, methionine and tryptophan. The stability of tryptophan is improved by addition of tryptamine during the acid hydrolysis making it possible to accurately determine tryptophan. This method is also an accepted method and can reduce costs for amino acids analysis. A more likely explanation for the low mass balance could be a quick sedimentation of aggregates resulting in a non-homogenous sample, or perhaps AAs stuck to the filter or surface of the used ultrafiltration material, which would have affected the TID calculation, ultimately resulting in a lower IVDIAAS. We therefore propose that for reliable interpretations, the mass balance must give a recovery of 80% or higher, so that the results obtained for MP in our study cannot be considered reliable. Colosimo et al. (Colosimo et al., 2020) reported some of the factors that influence the digestibility of MP in the INFOGEST system, including slower digestibility due to its complex nature and filamentous structure, as well as other variables such as sample weight used and particle size.

In general it could be concluded that WPC, BPC and YPC have a high TID digestibility and a balanced AA composition, which together results in a high IVDIAAS value. TPP1 showed a slightly lower digestibility but due to its well-balanced amino acid composition (summarised in Herreman et al., 2020), analysis resulted in a high IVDIAAS value. The opposite applies for CPC, which has a fairly good digestibility but, due to its low lysine concentration, the sample ends up with a low IVDIAAS value, confirming earlier data (Herreman et al., 2020). The digestibility results obtained with MP were not considered suitable to derive a reliable IVDIAAS value. We like to refer to several *in vitro* and *in vivo* studies that concluded a high digestibility of mycoprotein (Udall et al., 1984, Edwards & Cummings, 2010, Dunlop et al., 2017, Monteyne et al., 2020a, Monteyne et al., 2020b) although from none of these studies a DIAAS value could be concluded.

In communication about protein quality for human applications, we would advise using both the average digestibility, as based on the EAA in the TID, and the average IVDIAAS value in order to cover most aspects of protein quality for human consumption. In a diverse diet, the rate limiting amino acid(s) from one source can be compensated by another source, either together in one blend or as balanced products in a meal or diet. To provide or communicate only a DIAAS value is a great simplification and more developed for unilateral feeding strategies in animal production systems.

PDCAAS and DIAAS analysis of similar protein sources also have led to different absolute values in the past (Rutherfurd et al., 2015, Mathai et al., 2017) indicating that either the method or the variation in processing of material and batch-to-batch variation can have influence on the outcome of such evaluation for protein quality (Oberli et al., 2015). We included only two protein sources for which *in vivo* DIAAS values were available for comparison. Whey and NPP were determined to have *in vivo* DIAAS of 107 and 62 (Mathai et al., 2017), respectively, while our *in vitro* method resulted in IVDIAAS of 95.2 and 49.3 respectively. The first rate limiting amino acid as found *in vivo* was the same as *in vitro* (his for WPC and SAA for NPP). In general we could say that the *in vitro* method results in 10–25% lower DIAAS score but in line as was found *in vivo*. Regardless of which PDCAAS or DIAAS values the animal models provide, the most interesting evaluation is to compare the IVDIAAS values found with the nutritional value that the proteins provide in humans after intake. Therefore, more research is required to test the same products in this *in vitro* model and *in vivo* using controlled human bioavailability trials.

4.2. Limitation of the in vitro digestion model and analysis

A limitation of the used *in vitro* model is the lack of gastric emptying techniques or modelling approach and lack of the use of brush border enzymes. It is known that gastric emptying is an important factor in protein digestion and uptake (Ménard et al., 2018, Giezenaar et al., 2018). Although literature about some *in vitro* models for stomach movements has been published (Deng et al., 2020), these models are not widely available and no consensus on their validation has been performed to understand whether these models mimic gastric emptying in a biological valid way. Brush border enzymes, crude membrane scapings or S9 intestinal homogenates could be applied in *in vitro* digestions as published (Ringling & Rychlik, 2017, Boonpawa et al., 2017, Garcia-Campayo et al., 2018). However, these brush border enzymes are not yet part of the standardised INFOGEST protocol as first it would require consensus on the enzyme units to add, and quantification of enzyme activity in order to make such an additional step comparable between

labs. The INFOGEST consortium is currently working on further standardisation including the use of other digestive enzymes like lipase. Instead of using brush border enzymes located at the intestinal border we used the dialysis cut-off filter of 5 kDa, as also used by Verwei et al. (Havenaar et al., 2016) in the assumption that small peptide fragments, which are hydrolysed by the brush border enzymes to amino acids and di-tri peptides that are subsequently transported over the intestinal epithelium, are now passing the filter and end up in the filtrate. Other *in vitro* studies have considered < 10 kDa as potentially absorbable by the intestinal epithelium (Le Roux et al., 2020). Next to filtration, precipitation of proteins could be applied to separate amino acids and small peptides from larger peptides and protein using e.g. TCA or 80% MeOH. It would be interesting to study whether precipitation instead of filtration would result in a better mass balance for a product like MP and would circumvent the problems observed for this more complex product.

The standard INFOGEST protocol makes use of high concentrations of enzyme. The lower the activity of the enzymes, which differ batch to batch, the more enzymatic protein is added, which might be substantial compared to the protein content in a sample of interest. We reduced the pancreatin from 100 to 10 U/ml trypsin activity to ensure a better protein product: protein enzyme ratio and thus a more reliable quantify of the digestibility. Pilot experiments indicated that this lower amount of enzyme resulted in equal digestibility for a refence protein concentrate like whey. Only the dynamic changed as the protein was digested slower but after 2 h the digestions based on 100 and 10 units did not differed significantly. But still, food products with low protein digestibility or a complex food matrix that hinder enzyme accessibility using lower enzyme concentrations might face some challenges. When the *in vitro* model is used for complex products like the mycoprotein, but also composed products that are high in starch, fibres, fat or other compounds, it is wise not to limit the digestive enzymes to those used here in this study. It will be better to use the most complete set of digestive enzymes and include amylase, lipase etc in order to macerate the product network and simulate chewing in small particles to have a better release of proteins as will happen *in vivo*. Next to that, we expect that making use of a control digest based on a protein-free product (Moughan et al., 2005) instead of an 'empty digest' can reduce autolysis activity of the proteases and by that reduce background levels of amino acids that end up in the filtrate of the control digest.

4.3. Future of in vitro models and generation systematic overview

As stated by Marinangeli & House (Marinangeli & House, 2017) we also do not support strict DIAAS cut-off values for products eligible for protein content claims, as justification and rationale are lacking. Products providing bioavailable protein for human consumption should be weighted for their contribution to the total diet like its specific contribution to enrich protein intake at e.g. breakfast, lunch or in-between snacks, commonly found as events to improve protein intake (van Dongen et al., 2017). However, we do see the need to study the impact of moving towards *in vitro* assessments of digestibility, on nutrition messaging and policies from consumer, industry and food authorities perspectives. We plead for a wide spread of a consensus protocol by many labs and to start working on a systematic evaluation of (alternative) sources, effects of food processing and food matrix and to store data in open sources in order to support the development and assessment of protein rich products with high bioavailability dedicated for the vulnerable target groups in our society. It should be stressed that this method still requires further validation based on human trials in order to evaluate true nutritional value of products, meals and diets.

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Ethical statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript did not involved experimental animals or human patients.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

CRediT authorship contribution statement

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Declaration of Competing Interest

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

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104748.

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