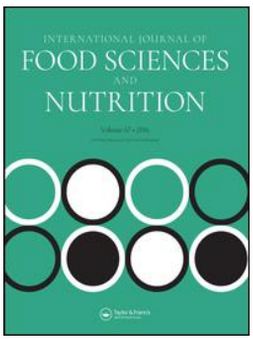

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A controlled human intervention trial to study protein quality by amino acid uptake kinetics with the novel Lemna protein concentrate as case study

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ABSTRACT

A human intervention trial was conducted to study amino acid uptake of the novel Lemna protein concentrate (LPC) in comparison to whey (WPC). The study was a cross-over, double-blind, controlled trial in which 12 healthy participants received 20 grams of LPC and WPC in randomised order. The LPC consumption resulted in a significant lower postprandial increase in almost all individual amino acids, total amino acid (TAA) and total essential amino acids (TEAA) compared to WPC based on area under the curve (AUC) calculations. When the AUC after WPC consumption was set at 100%, LPC showed a relative AUC of 60.4% for TAA and 66.3% for the TEAA. Interindividual variation for LPC was high with an uptake of TEAA of LPC compared to WPC ranging from 18.2 to 94.2%. Human intervention trials can partly replace animal trials as they fully reflect the human situation and provide estimates on individual variations.

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Lemna; duckweed; protein concentrate; *in vivo* protein digestibility; amino acid uptake; 3Rs

Introduction

The rapid population growth and increasing standards of living are expected to lead to an increasing demand for proteins. In order to provide sufficient dietary protein for human consumption, a transition towards a more plant-based protein diet is required. This resulted in the development of novel sustainable proteins that need authorisation before entering the food market. Food authorities request data on protein quality and bioavailability for which animal studies measuring Protein Digestibility Corrected Amino Acid Score (PDCAAS) or Digestible Indispensable Amino Acid Score (DIAAS) are currently the standard. However, an important trend in scientific research is to reduce the use of animals for safety and health analysis studies, especially when there are good alternatives. On top of that, animal models do not fully reflect the human situation and do not provide information about individual differences between consumers.

The most reliable methods to analyse protein digestion in humans make use of labelling strategies (Edwards et al. 2002; Gaudichon et al. 2002; Trommelen et al. 2020), but these methods are costly and cannot easily be implemented as standard protocol worldwide. Measuring the appearance of postprandial amino acids levels is relatively easy to perform and is already used by many science groups all over the world. Some groups used whey as a reference to study the relative uptake of their protein of interest (Tang et al. 2009; Pennings et al. 2011; He et al. 2013; Detzel et al. 2016; Gorissen et al. 2016; Vangsoe et al. 2018). Including such a reference protein allows the comparison of protein sources across literature, although also whey can show some variation in digestibility. Results of human postprandial amino acid uptake kinetic trials are often only expressed as averages of total amino acids, total essential amino acids, or individual amino acids. The data of these studies are hardly explored to come up with an average digestibility score and often do not report the first rate limiting amino acid which is required when

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determining a DIAAS value. Also the inter-individual variation found within these studies are not fully examined. As shown by the study of Zeevi on glucose responses (Zeevi et al. 2015), more attention should be given to inter-individual variation of nutrient uptake and how people respond to food interventions as this can lead to precision nutritional advises in the future.

Species belonging to the duckweed family (Lemnoideae) are seen as an interesting alternative protein source due to their enormous growth capacity, adaptation to a broad spectrum of growth conditions, balanced amino acid composition, content of vitamins and phytochemicals, and easiness to cultivate on a shallow layer of water allowing cultivation in automated (vertical) farming systems (Appenroth et al. 2017; Zeinstra et al. 2019). Although plants from the duckweed family are currently consumed in Southeast Asia, Lemna plant material is not yet allowed on the European food market. Not only the plants themselves, but also isolated proteins from Lemnoideae plant material might be an interesting alternative protein source for the future. However, digestibility data and information on tolerance of human subjects to this protein source are still missing.

Therefore, a human intervention study was conducted to analyse the amino acid uptake kinetics of Lemna protein concentrate (LPC) in blood in comparison to the benchmark whey protein concentrate (WPC). Special attention was given to curve fitting of the individual amino acid postprandial profiles and the interindividual variation between the subjects. Next to that, we explored a strategy to come to a "digestibility score" and a calculated human *in vivo* DIAAS value, which could partly replace animal-based PDCAAS or DIAAS values in the future. We also generated an overview of the current literature that later can be expanded to compare the protein quality for human consumption of different type of proteins.

Materials and methods

Ethical consideration

The study was conducted according to the principles of the Declaration of Helsinki (64th WMA Assembly, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO 1998). The study was approved by the local ethical committee (METC 18/29, NL66859.081.18) and registered at ClinicalTrials.gov: NCT03823222.

Study design

The study was a cross-over, double-blind, controlled trial in which study participants visited the research facility on two occasions under fasting conditions. All study participants were asked not to drink alcohol or perform heavy exercise the day before each study day. The evening before the test day the participants consumed a standardised ready-to-prepare meal. After 8 p.m., they were only allowed to drink water, no other food or other drinks were allowed. The next morning they visited the research facility under fasting conditions. During the test visits, subjects received the two protein sources in randomised order with a washout period of one week between visits. Blood was collected via a catheter before and 15, 30, 45, 60, 75, 90, 120, 150 and 180 minutes after protein consumption. Treatment orders [AB or BA] were randomised over the study subjects by block randomisation. Sex and age was stratified among groups. The main study parameter was amino acid uptake kinetics of blood free amino acids calculated as maximum peak value, time to peak and Area Under Curve (AUC).

Study population

We included 12 healthy men and women with the following inclusion criteria: age between 18 and 50 years, body mass index (BMI) between 18 and 25 kg/m^2. The average age of the subjects was 28 ± 10 years old and eight females and four male participants were included. All participants signed the informed consent and were fully compliant to the study protocol. There were no drop outs of subjects.

Intervention

Study participants received Lemna protein concentrate (LPC) produced by ABC Kroos BV (production on 29 August 2018, batch MK10343, packed on 12 October 2018), and whey protein concentrate (kindly donated by Nutricia Research, Utrecht, the Netherlands). Protein content of the products was calculated based on Dumas. The LPC contained 64% protein and 31.25 g product was therefore administered to provide a dose of 20 g of protein. Whey protein contained 80% protein and therefore 25 g was administered. See for more information on the composition of WPC and LPC Supplementary Table S1. The proteins were suspended in 400 ml water and offered in a shaker. The shaker was covered with foil so the participants could not see the colour and texture of the product.

The study coordinator checked whether intake of the provided shake was completed according to protocol.

Amino acid analysis of the intervention product

Analysis of amino acid content of the intervention products was performed with HPLC-UV/FLU Biochrom amino acid analysers using classical ion-exchange liquid chromatography with post-column Ninhydrin derivatization and photometric detection. Li-Citrate as well as Na-Citrate buffer system elution was used to cover the total amino acid analysis field. Quantifications were performed with suitable internal standard using methods based on EP2.2.56, USP <1052> and EC directive L152/2009, Annex III.

Protein quantifications of the intervention product

The Dumas method in analytical chemistry was used to quantitatively determine nitrogen content in chemical substances. The used method was based on the protocol of J. Adler-Nissen (Enzymatic hydrolysis of food proteins, London Elsevier) and performed in triplicate. A conversion factor of 6.25 was used for LPC and 6.38 for WPC.

Amino acid analysis in blood

In total, 19 individual plasma amino acids were analysed in the blood samples. Sample preparation was adapted from Reverter (Reverter et al. 1997). Aliquots of 40 µl of (EDTA) plasma were kept on ice and were diluted with 50 µl of 250 µM Norvalin in 0,1 N HCl as internal standard. The samples were deproteinized by addition of 10 µl cold 5-sulphosalicillic acid, followed by centrifugation 10 min 13000xg at 4 °C. Standard solutions of five levels (6.25–300 µM) for all amino acids was prepared with 125 µM Norvalin as internal standard. Derivatization of both samples and standards was achieved by mixing 60 µl borate buffer, 20 µl of sample, and 20 µl of AccQ tag reagent. The AccQ-tag Ultra method originally designed for UPLC, was adapted for use on an Acquity ARC UHPLC. A Xbridge BEH C18 2.5 µm 3.0 × 150 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. 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 amino acids except Gln and Arg. The derivatives of these amino acids elute overlap in a single peak.

Blood glucose and insulin

Plasma glucose and insulin concentrations were measured (Hospital laboratory GV) in a large subset of the same series of blood samples.

Questionnaire

Before the evening meal on the day, they received the protein, study participants were instructed to complete a questionnaire related to satiety, thirstiness and gastro-intestinal complaints. This was done on a 7-point scale ranging from "not at all present" to "strongly present". The following gastro-intestinal symptoms were included in the participant diary: bloated feeling, belching, flatulence, nausea, abdominal pain, diarrhoea and constipation. The questionnaire was repeated on the subsequent two days at the same time point. Adverse events (AEs) were registered by a medical doctor. AEs were classified under the responsibility of the MD according to ICD-10 coding.

Data analysis

The data analysis consisted of several steps: in the first step, the time profiles for the amino-acid levels in blood were described by parametric curves (modified Wood curve). Separate curves were fitted to the time profile from each amino acid and each study participant. Based on the fitted curves, two categories with respect to amino acid uptake could be distinguished, namely examples where a clear peak above the baseline d is visible, and examples where such a peak is not visible. These two categories were named responders and non-responders, respectively. More specifically, non-responders were defined as cases with very bad or unrealistic fits, that is, $m \leq 0$, $AUC \leq 0$, or $max(y) \leq 0$. Next, variables summarising the time profile such as area under the curve (AUC), the peak height, and the time to the maximum were obtained from each fit. As a final step, the summarising variables of interest were used in mixed models to investigate whether there were any differences between the responses to the two protein interventions.

Time curves for amino acid levels in blood of individual participants were described by the following equation: $y(t) = d + at^{mc}e^{-ct}$. In this equation, $y(t)$ is the amino acid level at time t, d is the level of the

baseline, a is a scaling factor, m describes the time to the maximum of the curve, and c describes the shape of the decreasing part of the curve (note that mc describes the shape of the left part of the curve, the increase). This function, without parameter d, was originally coined by Wood (Wood 1967) to describe lactation in cattle. The modified Wood curve used here was proposed for analysis of cortisol time profiles in calves (Engel et al. 2003).

The four parameters describing the curve, a, c, d and m, were estimated by nonlinear least squares using the best solution from 500 random initializations. The AUC, time to maximum and peak height were obtained from the estimates of these parameters (Rook et al. 1993).

A linear mixed model was used to study the variables summarising the time curves (AUC, time to maximum, peak height) in more detail. The model comprised fixed effects for protein intervention and test week and a random effect for participants. The Kenward–Roger F test was used to assess the significance of protein intervention effect. A p-value <0.05 was considered significant. Results for the differences between the two protein interventions are presented in the form of 95% confidence intervals (CIs). Note that the analysis of AUC-values focussed on ratios, by fitting the mixed model to the logarithm of the AUC-values. Parameter estimates and confidence intervals were back transformed to the original scale. All statistical analyses have been implemented in an R package, response, which will be made available as open-source software (Wehrens and Engel in preparation).

Glucose and insulin data were statistical analysed using SPSS using a repeated measures analysis of variance. Data from the questionnaire was analysed using a paired t-test. For these tests, a p-value of $p < 0.05$ was considered as statistically significant.

Results

LPC amino acid profile

The individual amino acids of the LPC product used for this human trial were analysed and compared with values from WPC (Figure 1). Results indicate that WPC and LPC differ in the amount of some of the amino acids; isoleucine, threonine, lysine, cysteine are considerably lower in LPC compared to WPC while phenylalanine, glycine, and arginine showed higher amounts in LPC compared to WPC. The sum of all nine essential amino acid of LPC are 94.8% compared to WPC.

Next, the amino acid composition of LPC and WPC was compared with the FAO recommendations based on the requirements of 6–36 months children (Figure 2). This resulted in an amino acid score of 0.99 for the used WPC product with histidine as first rate limiting amino acid and an amino acid score of 1.14 for LPC with lysine as first rate limiting amino acid. LPC can therefore be considered to have an intrinsic good balanced amino acid profile for human nutrition.

Postprandial amino acid response

The area under the curve (AUC) was calculated for the postprandial response of each amino acid per

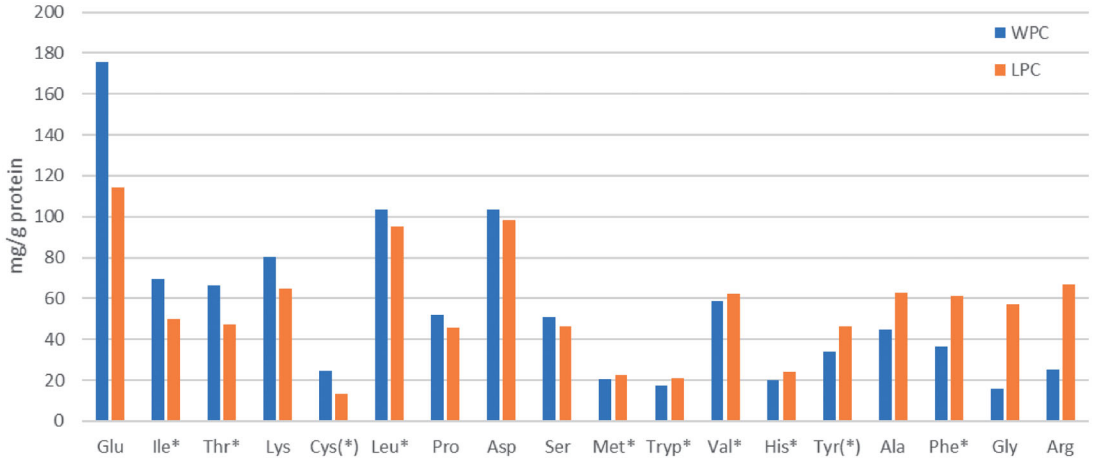

Figure 1. Bar plot of the individual amino acids in LPC compare to WPC. Amino acids are ordered according to the difference in amounts in the two proteins: amino acids much more present in WPC than in LPC are on the left. Essential amino acids are labelled with * and amino acids that are conditional essential amino acid and part of DIAAS calculations (*).

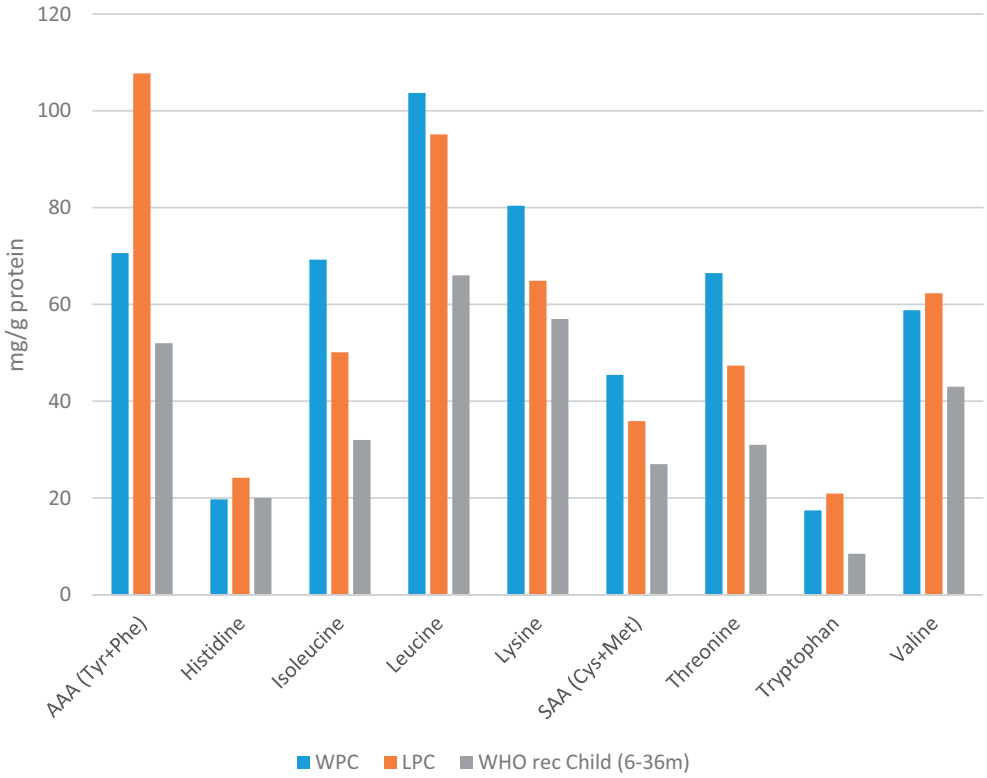

Figure 2. Bar plot of the individual amino acids compared to the recommendation for child (6–36 months) as suggested by FAO guideline document (FAO 2103).

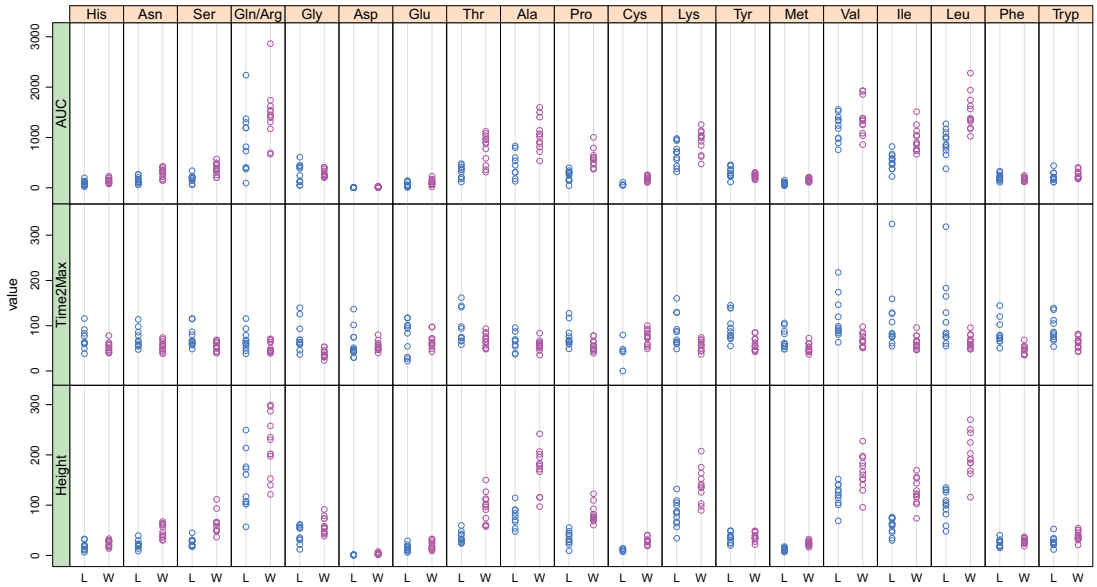

Figure 3. Area under the curve (AUC) time to max and peak height values for each individual amino acids and for both protein interventions expressed per subject. L = LPC and W = WPC each individual subjects is represented by a ring.

participant for the two intervention WPC and LPC as shown in Figure 3 (upper panel). The baseline was taken as the zero level and nonresponders were not taken into account. Each dot corresponds to a participant. In general, the AUC values of LPC are lower than those of WPC.

The output of the absolute differences between LPC and WPC AUC studied by Kenward–Roger *F*-test is shown as Supplementary Figure S1. Significant differences between LPC and WPC in AUC-values were found for all amino acids, except glycine, phenylalanine and tyrosine.

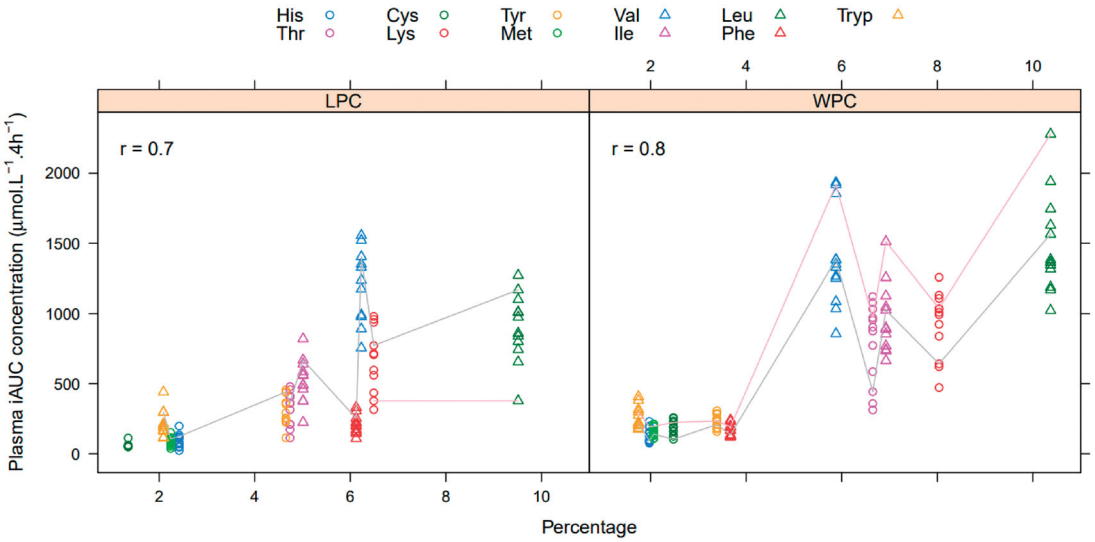

Figure 4. Relationship between amino-acid prevalence in the two protein concentrates and the fitted AUC values for the nine essential amino acids plus cysteine and tyrosine. Data points for participant 11 are connected with pink line segments; data points for participant 3 with grey segments.

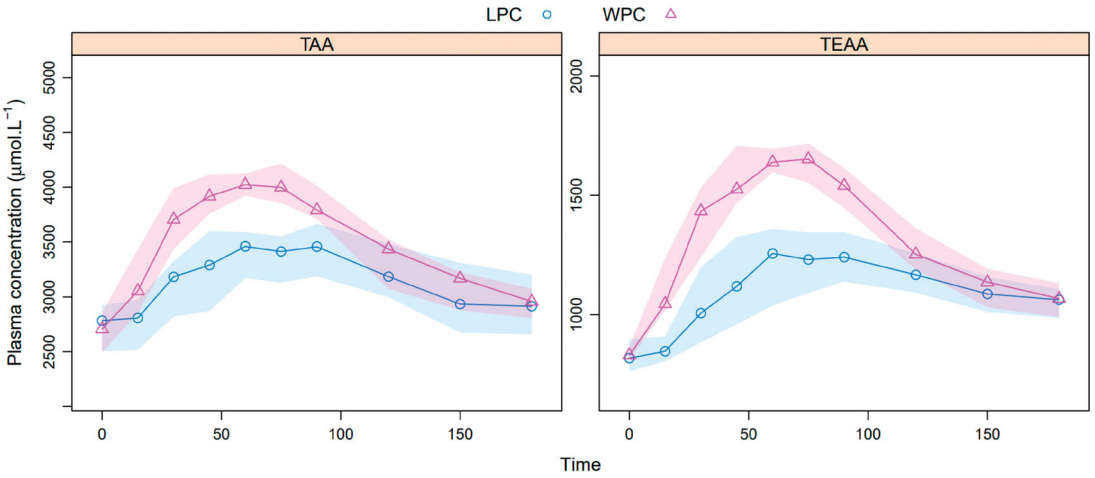

Figure 5. Postprandial change in amino acids after intake of either WPC (open triangles) or LPC (open circles). Panel A indicate the total amino acids (TAA) and panel B the total of nine essential amino acids (TEAA). Data are presented as median with interquartile ranges. The mixed model analysis indicated $p < 0.001$ for intervention time and intervention × time for both TAA and TEAA. $n = 12$.

Next, we compared the time to peak of the postprandial amino acid values between the two protein sources. For most amino acids, the time to peak was earlier after intake of WPC compared to LPC. This was further analysed using the same mixed model approach outlined for comparison of AUC values (Figure 3 middle panel, and Supplementary Figure S1). In general, the time to peak from uptake of amino acids from WPC was around 1 hour, while time to peak values for LPC were 10 minutes up to 1 hour later. These differences were significant, except for alanine, aspartic acid, glutamine/arginine and glutamic acid. Comparison of peak maxima of the individual amino acids between both interventions leads to largely the same conclusion as found for the AUC-values. Figure 3, lower panel, shows the maxima for each combination of protein source and amino acid for each of the 12 subjects. Higher peak values are observed for WPC. Mixed model analysis confirmed that these differences were significant for all amino acids except for phenylalanine and tyrosine (Supplementary Fig S1).

Next we studied the relationship between the level of essential amino acid in blood and those present in the protein concentrates (Figure 4). In general, the graphs show a good correlation between the delta

Table 1. Total postprandial amino acid AUC and total of 9 essential amino acids (His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val) AUC after oral intake of both protein concentrate for each of the 12 subjects.

	TAA			TEAA (9)		
Subjects	WPC	LPC	LPC relative to WPC (%)	WPC	LPC	LPC relative to WPC (%)
1	11620.9	7684.6	66.1	6305.8	4896.1	77.6
2	7131.7	5521.7	77.4	4247.3	3409.8	80.3
3	8301.9	9468.9	114.1	5446.1	5132.9	94.2
4	10510.0	9469.5	90.1	6204.3	5543.1	89.3
5	12298.5	5631.1	45.8	8051.1	4529.8	56.3
6	9458.0	4656.5	49.2	5544.9	3552.0	64.1
7	15439.7	7709.1	49.9	8040.4	5800.8	72.1
8	10182.2	6161.0	60.5	5799.5	3915.2	67.5
9	8850.1	6686.6	75.6	5004.2	3327.2	66.5
10	10142.4	4988.8	49.2	6321.8	3962.6	62.7
11	12107.2	2518.1	20.8	8465.2	1543.7	18.2
12	6128.8	3312.4	54.0	3607.0	2792.4	77.4
Total	122171.4	73808.2	60.4	73037.6	48405.6	66.3
mean	10180.9	6150.7		6086.5	4033.8	
SD	2515.0	2186.6		1505.8	1224.0	
CV	24.7	35.6		24.7	30.3	

postprandial AUC of the amino acids and those present in the protein concentrate with *r* values of 0.7 for LPC and 0.8 for WPC. The large variability in spreading for individual amino acids is remarkable – some amino acid shows a huge interindividual variation, others are fairly constant.

Total amino acids and interindividual variation

We calculated the total amino acids AUC and total of nine essential amino acids (His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val) AUC per protein concentrate, as these are often reported in literature and will enable a comparison with published data. In Figure 5, the median interquartile ranges for all participants together are plotted for both protein concentrates in time. Both total amino acids (Figure 5(a)) and total essential amino acid (Figure 5(b)) resulted in significant lower values for LPC compared to WPC.

Data were also analysed on an individual subject basis for which individual amino acid at each time point were added together, Wood curves fitted and AUC calculated (Table 1). Subjects showed a large variation in amino acid appearance in the blood after intake of these two protein concentrates. In some cases, like subject 3, the AA uptake kinetics were highly similar for both sources, while for another subject (e.g. subject 11) LPC intake resulted in much lower amino acid uptake (in line with results as shown in Figure 5). Next to that, this analysis indicated that subject 9 and 12 are for both protein sources at the lower quartile of this population and might be persons with a general lower capacity to digest proteins and absorb amino acids. Comparing the mean AUC of all subjects revealed that when LPC and WPC are given to persons in equal amounts, LPC reaches to 60.4% of the total amino acids and 66.3% of the total essential amino acids compared to WPC. Besides, the interindividual variation (CV) for LPC is higher than for WPC indicating more interindividual variation in efficiency to digest and absorb this plant-based protein compared to the WPC.

In vivo DIAAS

To calculate a human *in vivo* DIAAS value, we used the overall digestibility of LPC essential amino acids compared to WPC (60.4%) and multiply that with the amino acids as present in the original LPC protein powder as shown in Figure 2. This explorative approach assumes that WPC is digested and absorbed for 100% and results in a human *in vivo* DIAAS value of 69% for LPC based on the recommended amino acid scoring patterns for child (6–36 months) and 82% in comparison with older children/adolescents/adults with for both target groups lysine as first rate-limiting amino acid (Figure 6).

Similar human bioavailability studies were performed in which whey was used as a reference (Table 2). We tried to summarise these studies in order to compare different protein sources. This overall comparison can only be by estimation as whey composition and digestibility can vary in these studies. Based on these studies, we ranked the AUC of TEAA of the products compared to the used whey preparation which was set at 100% for each study. The results of LPC were added to this literature overview indicating that LPC ranks above casein and just below casein hydrolysate.

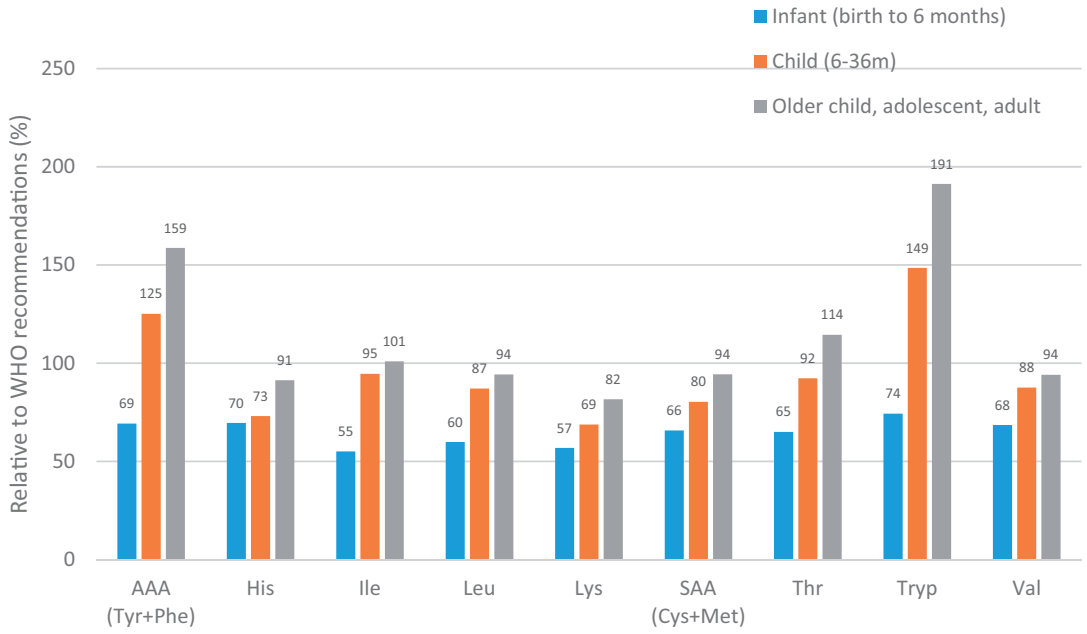

Figure 6. Explorative calculated human *in vivo* DIAAS values for LPC based on a general mean amino acid digestibility of 60.4% multiplied with the amino acids as present in the LPC protein sample and then compared with the FAO recommendations for the three target groups as indicated (FAO 2013).

Table 2. Overview of the percentage of amino acid uptake of different sources of protein compared to whey as found in the literature based on postprandial analysis in humans.

Product	TEAA relative to whey (%)	CV	Reference
Whey	100	35.3/21.9/23.6	Detzel et al. 2009; Vangsoe et al. 2018; this study
Soy protein	90	–	Tang et al. 2009
Beef protein isolate	79	92	Detzel et al. 2016
Soy protein	72	18.1	He et al. 2013; Vangsoe et al. 2018
Lesser meal worm protein	69	24.5	Vangsoe et al. 2018
Casein Hydrolysate	68	–	Penning et al. 2011
Lemna Protein Concentrate	66.3	30.3	This study
Casein	61	–	Penning et al. 2011
Casein	60	–	Gorissen et al. 2016
Casein	49	–	Tang et al. 2009
HMW potato protein	46	–	He et al. 2013
Wheat protein hydrolysate	45	–	Gorissen et al. 2016
LMW potato protein	21	–	He et al. 2013

–: not reported or difficult to extract from the publication.

Glucose and insulin responses

We analysed effects towards the glucose metabolism of the LPC in comparison to WPC. Figure 7 shows the change in glucose (Figure 7(A)) and insulin (Figure 7(B)) levels after intake of both protein concentrates. The healthy subjects showed a temporary increase in insulin levels without a glucose peak. WPC intake resulted in a higher insulin increase, accompanied by a stronger reduction in blood glucose levels, compared with LPC. The effect between LPC and WPC differed significantly with a $p = 0.01$ for the glucose response and a $p < 0.01$ for insulin.

Questionnaire

Subjects also had to complete a questionnaire on the day of the trial and on two consecutive days. Results of the questionnaire are presented in Supplementary Table S2 showing no significant effect between both protein products on satiety, thirstiness and health parameters like gastric complains.

Discussion

Here we studied in a human cross-over trial whether LPC can be seen as an nutritious addition to the range of plant-based protein sources that can support a shift to towards a more sustainable diet. Protein quality and digestibility studies are in almost all cases performed by the PDCAAS and the newly developed DIAAS method in animal trials (FAO 2013). The PDCAAS value is seen as the gold standard but with several concerns and drawbacks as pointed out by

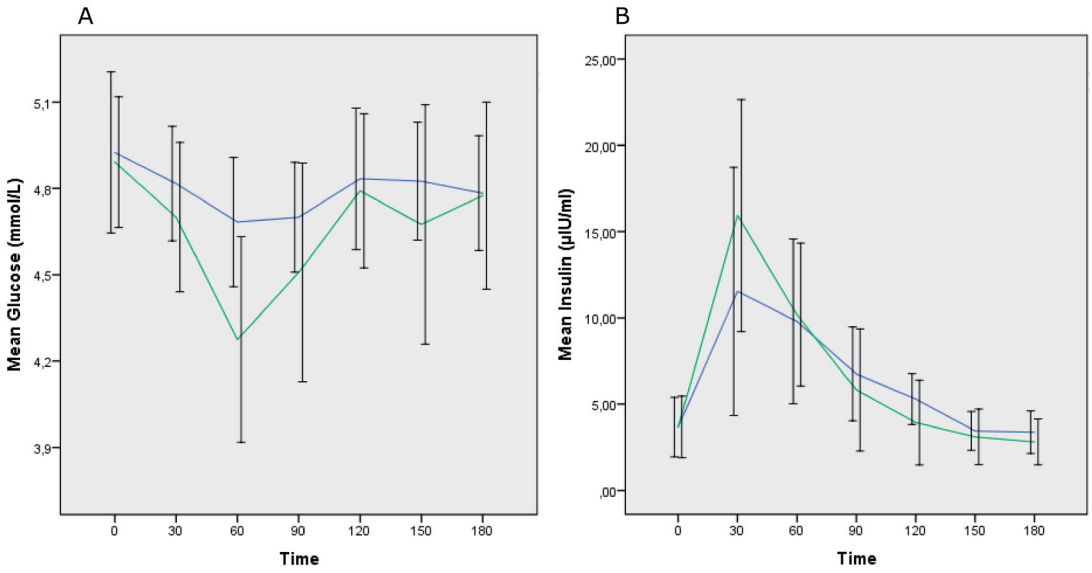

Figure 7. Effect of the WPC (green line) and LPC (blue line) interventions on postprandial blood glucose (panel A) and insulin levels (panel B). $n = 12$.

Schaafsma (2005). The DIAAS is calculated based on the proteins remaining at the end of the ileum and compared to FAO recommendations which is then reported as single value together with the first rate-limiting amino acid. This method also has its limitations as it is fully based on the remaining proteins and peptides in the intestine. However, proteins can also have been utilised by intestinal bacteria, and proteins in the intestine can in part be derived from other, non-food sources such as degraded intestinal cells. Furthermore, this method is depending on internal markers and protein-free control interventions and based on cannulated pigs or sacrificed animals. Specially this last issue raised concerns among producers and consumers and led to alternative methods such as *in vitro* models for which the Infogest protocol is a good example (Brodkorb et al. 2019). Controlled human intervention trials are an even better method to study digestion and protein bioavailability, although we must be aware that circulating amino acids in blood are a result of absorption and clearance rates in tissues (such as muscles). Using standardised fasting protocols and delta values compared to baseline, we have been able to show clear postprandial changes as a result of protein intake, resulting in a strong correlation between the consumed protein amino acids and postprandial amino acids (Figure 4). We also plead to perform these analyses in a cross-over design including as reference protein whey to be able to compare results over different experiments using this reference protein. It has to be said that postprandial plasma amino acids levels are a result of processes in the body and therefore amino acids might not only be low because of resistance towards intestinal enzymes and low bioavailability, but also due to fast metabolisation. A (dual) tracer approach to measuring *in vivo* protein quality will be more accurate (Edwards et al. 2002; Gaudichon et al. 2002; Trommelen et al. 2020) but also more costly and measuring ileal digestibility in patients with an ileostomy (Moughan et al. 2005) presents its challenges and will be difficult to implement as standardised protocol worldwide.

The 20 g protein used in this intervention was determined by Dumas method which might not be a fully correct quantification method to equal amino acids between both study products. As the conversion factor for LPC was not yet established and the protein powder might contain other sources of N, these data might give an under-prediction of the amino acid uptake of LPC as we potentially started with less true protein compared to WPC. Therefore, a total amino acid quantification to determine protein concentration is preferred; however, the Dumas method is used in practice, and therefore, we decide to standardise both products via Dumas and hope that other groups will follow this approach.

DIAAS calculations take into account the first rate-limiting amino acid compared to the FAO recommendations. However, in human plasma samples, the first rate-limiting amino acid and the exact value is more difficult to determine due to individual variation in body composition, blood volume, muscle mass (that will influence disappearance rate), lower stability of some of the amino acids, fast metabolisation and the continuous in- and efflux rate of amino acids in

tissues. Human protein digestibility trials therefore have a low reporting rate of the potential first rate-limiting amino acid and to what potential *in vivo* DIAAS score this would lead. Here, we used the overall digestibility of the essential amino acid compared to WPC (60.4%) and multiplied that with the amino acids levels as present in the original protein concentrate to calculate a human *in vivo* DIAAS value. We recognise that this is not a very exact approach but it also leads to the question how exact does a digestibility score for proteins for human consumption has to be. PDCAAS or DIAAS values are important for optimising animal feed (focussed on very efficient feed conversions) or pet food that are fed via a very restricted and not variable diet. Same counts for infant formulas. In contrast, older infants and adult humans consume a variable diet with a large variety of different protein sources. We argue that for the average population of consumers our approach for protein quality may be sufficient and that for specific applications for infants or the vulnerable population additional experiments should be carried out in which animal studies might be appropriate.

Whey protein is generally classified as a fast protein that reaches its maximum level of postprandial amino acids at one hour after consumption, while at three hours the level almost returns to baseline (Boirie et al. 1997). Casein is more classified as a slow protein which requires often more time to return to baseline (Tang et al. 2009; Pennings et al. 2011; He et al. 2013; Gorissen et al. 2016). LPC also showed a delayed peak of the postprandial amino acids and also a reduced peak height. Based on the postprandial amino acid curves found in our study, we might conclude that after three hours the LPC can still provide a bit more amino acids. For future trials, we propose to extend the postprandial analysis of other plant-based proteins to at least four hours to be able to study the full postprandial responses.

Another advantage of performing protein quality studies in humans is the insight you gain on personal variation. Until now, results of human intervention trials are reported as means and SD over the subject population in the study. This way of reporting also leaves us with a lot of unexplored knowledge in the field of personal differences in protein bioavailability, and insights that could enable us improving personalised nutritional advices. In the case of protein digestibility, it is known that people vary in digestive enzyme amounts and activities, stomach pH, transepithelial transporters, intestinal surface area etc. which all will influence the digestion and uptake of nutrients (Dallas et al. 2017; Walther et al. 2019). Here, we observed a larger inter-individual variation among the subjects receiving LPC compared to WPC and that some persons were very low in uptake of amino acids from LPC. This could indicate that plant-based proteins might result in larger bioavailability variation between persons. Some subjects are for both protein source at the lowest quartile of amino acid uptake which could indicate that for those consumers a higher protein intake might be advisable. To confirm this hypothesis an in-depth study is required in which the same protein products (both difficult and easy to digest) are given repeatedly to the same persons and tested whether this results in robust classification of persons with different uptake efficiency and not due to day-to-day variation or lingering effect of intensive sports activities on previous day(s).

A large part of the nutritional value of a novel alternative protein is already fixed in the amino acid composition of the protein source. Several plant-based proteins have a slight imbalance in amino acid composition for optimal human nutrition. Cereals, seeds and nuts are often low in lysine while legumes have an under-representation of the sulphur amino acids, cysteine and methionine (Edelman and Colt 2016). In agreement with the FAO recommendations for children, LPC has an intrinsic good balanced amino acid composition which is in agreement with other publications based on *Lemna minor* plants (Zeinstra et al. 2019) and other species from the Lemnoideae (Edelman and Colt 2016; Appenroth et al. 2017; Kaplan et al. 2019). As proteins in green leafy plant material can consist up to 50% of RuBisCO (Feller et al. 2008), it can be assumed that LPC will contain for a large part RuBisCO proteins. Barbeau and Kinsella (1988) analysed the composition of RuBisCO from several plant origins (alfalfa, spinach and tobacco) and concluded that it is a nutritious protein with a calculated amino acid score of 87% and a good digestibility, although this will also be influenced by antinutritional factors that can be present in plant materials and are crop specific (Gilani et al. 2005). The used protein extraction method was not specialised for isolation of intact RuBisCO. Based on gel electrophoresis (data not shown), it can be assumed that it was composed of a heterogeneous mixture of intact and completely or partially degraded proteins. This partial hydrolysis may of course have had a role in the relatively good bioavailability, as found for other protein hydrolysates (Tang et al. 2009; Pennings et al. 2011; Detzel et al. 2016).

And finally, performing human intervention studies also provide us with information on safety and tolerance of a novel product in combination with the protein bioavailability. Previously, we showed that Lemna plant material consumed as a single intake of 20 g protein (~ 1111 g plant Fresh Weight) did not induce any adverse effects among 12 healthy study participants. Also the group of Shai performed several controlled trials with duckweed material: a single intake study of Wolffia at a level of 30 g protein (410 g fresh weight) in a meal (Kaplan et al. 2019) and an intervention with 75 g fresh weight Wolffia for three consecutive days (Zelicha et al. 2019). In both studies, no adverse effects were observed, nor negative effects on health parameters were reported. More recently, the same group performed a long-term human intervention trial in which up to 100 subjects consumed *Wolffia globosa* not giving any adverse effects, but instead they showed that it amplified the beneficial cardiometabolic effect of a Mediterranean diet (Tsaban et al. 2021). Zeinstra et al. 2019 found that oral intake of a single large bolus of Lemna plant material hardly altered glucose and insulin levels of the subjects. Also intake of *Wolffia globosa* showed smaller changes in glucose responses compared to yoghurt (Zelicha et al. 2019). The results from the current study are in line with these observations as they indicate that LPC induces less pronounced changes in glucose and insulin levels compared to WPC and therefore can fit within a diet to reverse or prevent type-2 diabetes. Based on these outcomes, and together with the knowledge that duckweed (very often not specified which species of the Lemnoideae family) is eaten for decades in Asian countries, we consider that the LPC in general can be safe for human consumption. However, it is known that plants from the Lemnoideae family can efficiently accumulate compounds from the cultivation medium, like heavy metals (Bokhari et al. 2016). When developing large-scale production facilities and bringing the product on the market, it is of importance to carefully monitor these risk factors and control the cultivation process.

Conclusions

In conclusion, a human cross-over trial can study protein quality by analysing individual amino acid uptake kinetics in blood and can result in an estimate for a human *in vivo* DIAAS value. This method can be easily performed in many trial facilities. The observed high interindividual variation for the plant-based protein LPC warrants further research towards the reproducibility of the individual response, with a potential implication for optimising protein intake for those that can suffer from malabsorption of specific type of proteins. We advocate that this human trial-based strategy for quality and bioavailability analysis of novel proteins should be accepted by food authorities reducing the necessity of performing animal experiments.

The following are available on line, Figure S1: Kenward-Roger *F*-test for difference between LPC and WPC AUC-values, maxima and time to peak. Table S1: Composition of the whey and lemna protein concentrates. Table S2: Results of the questionnaire on satiety, thirstiness and gut health parameters on the afternoon after the trial (day 0) and the two consecutive days.

Author contributions

I.M.v.d.M. and J.J.M initiated the project. I.M.v.d.M., J.J.M and D.E. arranged the financing of the research. J.J.M., I.M.v.d.M. and D.E. designed and organised the trial. E.O. supported the human trial, handled subjects, questionnaires and sample processing. R.T.M.v.d.D. performed the amino acid analysis. D.E., J.M., J.E., R.W. and G.A.H.d.J. performed data analysis and statistics. J.M. and I.v.d.M. wrote the manuscript. D.E., E.O., R.T.M.v.d.D., J.E., R.W., G.A.H.d.J contributed and approved the manuscript.

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Disclosure statement

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