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Combining high-protein ingredients from pseudocereals and legumes for the development of fresh high-protein hybrid pasta: Enhanced nutritional profile

Nutritional profile of high-protein hybrid pasta

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DRAFT

Keywords:

plant-protein, faba bean, buckwheat, lupin, protein quality, antinutritional compounds

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Abstract

BACKGROUND: The fortification of wheat based staple foods, such as pasta, with pseudocereal and legume flours has received growing research interest in recent years. While it is associated with many challenges regarding technological and sensory quality of the products, it promises a substantial improvement of the nutritional value of pasta. However, investigations of the nutritional quality of fortified pasta often focus on the carbohydrate/starch fraction and information on changes in protein quality is relatively scarce. This study evaluates the nutritional profile of a high-protein hybrid pasta (HPHP) formulation where a combination of three high-protein ingredients (HPIs) from buckwheat, faba bean and lupin is used to partially replace wheat semolina. The formulation's macronutrient composition, protein quality and the contents of antinutritional compounds are assessed in comparison to regular wheat pasta.

RESULTS: The HPHP formulation represents a more favourable macronutrient profile compared to regular wheat pasta, particularly in relation to the isocaloric replacement of wheat starch by non-wheat protein. Furthermore, a more balanced amino acid profile, improved N utilisation and increased protein efficiency ratio (*in vivo*) were determined for HPHP which conclusively suggests a substantially enhanced protein quality. The cooking process was shown to significantly reduce levels of vicine/convicine and trypsin inhibitor activity originating from HPIs. The small remaining levels seem to not adversely affect HPHP's nutritional quality.

CONCLUSION: This significant upgrade of the pasta's nutritional value identifies HPHP, and similar hybrid formulations, as a healthy food choice and valuable alternative to regular wheat pasta, specifically for a protein supply of adequate quality in mostly plant-based diets.

DRAFT

1. INTRODUCTION

The focus of research concerning durum wheat (*Triticum durum*) pasta has shifted in recent years. While technological quality, texture properties and sensory still represent the main topics of interest, nutrition and health aspects of pasta have gained increasing attention. This is in close relation to the evolving market potential of functional foods that have the ability to improve wellness and health of consumers.[1] According to Jenkins et al.[2] there is a necessity to transition from diets with high nutrient density to diets with high nutritional density, particularly in the context of decreasing physical activity. This refers to an increased intake of essential macro- and micronutrients at lower total caloric intakes. Pasta has been described as a promising vehicle for the addition of both macro- and micronutrients through the incorporation of functional ingredients.[3] The fortification with pseudocereal and legume ingredients, which are particularly rich in protein and micronutrients, offers an upgrade of nutritional quality and nutritional density of pasta; for example due to increased protein content and enhanced protein quality.[4,5] While protein intakes in many parts of the world exceed the average daily requirement, this is often related to an overconsumption of animal protein or food in general.[6] Many dietary guidelines recommend a substantial reduction of protein from animal sources and increased intake of plant-based protein.[6] Furthermore, the urgently required transition to a food system with improved environmental sustainability largely relies on a shift to predominantly plant-based human diets.[6] Several studies have investigated the impact of pasta fortification with pseudocereal and legume ingredients on technological, sensory and nutritional product quality.[7–13]

However, many of these articles report major challenges with regard to textural properties, cooking quality and sensory characteristics. From a nutritional point of view, the research was often focused on the carbohydrate/starch fraction (e.g. starch digestibility) and information on changes in protein quality of fortified pasta is rather scarce.[14] One of the concerns regarding nutritional quality when pseudocereal and legume ingredients are incorporated in foods refers to antinutritional compounds (ANCs). These compounds are molecules which are naturally present in plants and can, for example, decrease a product's sensory quality (primarily tannins and saponins), protein digestibility (trypsin inhibitors) and the bioavailability of minerals (phytate).[5,15,16] The pyrimidine glycosides vicine and convicine, which can trigger favism, are also considered ANCs and are mainly found in faba beans.[17,18] The analysis of important ANCs should be considered when the nutritional quality of pseudocereal and legume containing foods is evaluated. In this study, a combination of three high-protein ingredients (HPIs; from the pseudocereal buckwheat and the legumes faba bean and lupin) was used to partially replace wheat semolina and to produce a high-protein hybrid pasta (HPHP). This HPHP formulation was subjected to a thorough assessment of its nutritional quality with a focus on macronutrient composition, starch digestibility (*in vitro*), protein quality (amino acid profile, *in vitro* and *in vivo* protein digestion) and ANCs. Technological and sensory quality of this formulation were previously validated by Hoehnel et al.[19] and found to be similar to regular wheat pasta. While favourable texture attributes and organoleptic properties remain the most important determinators for consumer acceptance of foods, also the products' nutritional

value and potential health benefits have received growing interest from consumers. The objective of this study is to compare the nutritional quality of the HPHP formulation to regular wheat pasta and to identify whether the combination of HPIs from buckwheat, faba bean and lupin leads to an enhanced nutritional value in addition to the previously confirmed adequate technological and sensory quality[19].

2. EXPERIMENTAL

2.1. Materials

Reference wheat pasta (RWP) and high-protein hybrid pasta (HPHP) were produced from the same ingredients as specified in Hoehnel et al.[19]. Buckwheat flour (protein content 22.52 %DM, lipids 2.78 %DM, ash 3.22 %DM, fibre 1.56 %DM, carbohydrates by difference 69.91 %DM, total starch 54.72 %DM, obtained by dry fractionation), faba bean flour (protein content 61.25 %DM, lipids 3.81 %DM, ash 5.43 %DM, fibre 0.35 %DM, carbohydrates by difference 29.17 %DM, total starch 7.77 %DM;[20] obtained by dry fractionation) and lupin protein isolate (protein content 94.51 %DM, lipids 2.94 %DM, ash 5.62 %DM)[20] were provided by Fraunhofer Institute IVV, Freising, Germany. Wheat semolina (protein content 17.4 %DM, lipids 2.13 %DM, ash 1.37 %DM, fibre 4.21 %DM, carbohydrates by difference 74.86 %DM) was purchased from W G Buchanan & Son Ltd, Ireland; and salt by Glacia British Salt Ltd, UK. The following ingredients were used for the preparation of diets for *in vivo* nitrogen balance trials: casein (C) from Lacpol Co., Poland; soya protein isolate (SPI) ISOPRO 900 HI

(non-GMO) from EDMIR-POL Co., Poland; soya flour (SF) SOPRO TB 200 from EDMIR-POL Co., Poland; α-cellulose (C8002) from Sigma Aldrich, Missouri, USA; soya oil from ZPT Co., Poland; choline chloride from SIGMA, Poland; cholesterol from PPH Standard Co., Poland; sucrose from POCH SA Co., Poland; and corn starch from Avebe, The Netherlands. For *in vitro* digestion trials, enzymes were purchased from Sigma-Aldrich, Missouri, USA: pepsin from porcine gastric mucosa; EC 3.4.23.1; P7000; 727 U/mg and pancreatin from porcine pancreas; 4 x USP; P1750. All other chemicals were also purchased from Sigma-Aldrich, Missouri, USA unless stated otherwise.

2.2. Recipe Adaptation and Pasta Production

Pasta (spaghetti) samples were produced according to Table 1 and the procedure described by Hoehnel et al.[19]. In brief, the HPHP formulation was established based on the recipe of the reference wheat pasta (RWP), by partial replacement of wheat semolina by HPIs. The impact of 15 plant-based HPIs from cereals, potato, pseudocereals and legumes on the quality of high-protein pasta was screened in a series of preliminary trials. The levels of wheat semolina replacement were calculated to reach a protein level of 20 % of calories provided by protein (20 %E). The results identified three HPIs (buckwheat flour, faba bean flour and lupin protein isolate) as most suitable with regard to technological pasta quality. Response surface methodology was used to determine a combination of these three HPIs to produce pasta with optimised technological quality. Due to its allergenicity and its limited capacity to compensate for the lack of lysine in wheat

semolina protein, the amount of lupin protein isolate in the formulation was kept to a minimum (see section 3.2). The fresh pasta product obtained after extrusion is referred to as 'raw pasta' throughout this work. In order to prepare the product which is further referred to as 'cooked pasta', the optimal cooking time (OCT) of RWP and HPHP determined by Hoehnel et al.[19] was applied as cooking time. After cooking (tap water, no salt added), pasta was drained (not rinsed) and left to cool down. The strands were cut into small pieces of approx. 1 cm length, frozen at -80 °C and freeze-dried. Freeze-dried pasta was milled prior to nutritional analysis. Results are expressed as contents per dry matter considering the moisture of the freeze-dried pasta powders unless stated otherwise.

2.3. Compositional Analysis

Compositional analysis was performed as previously described by Hoehnel et al.[21]. In brief, analysis of the following compositional data was performed by Concept Life Science Ltd., UK based on the indicated validated methods: energy (calculated considering protein, lipids, available carbohydrates and fibre), protein (Dumas method, modified after AOAC 1977.992.15; nitrogen-to-protein conversion factor 6.25), ash (removal of organic matter by oxidation at 550 °C, based on ISO 936:1998), lipids (low resolution proton nuclear magnetic resonance (NMR), based on MQC-23-35 Oxford Instruments application note), fatty acid profile (GC-FID of fatty acid methyl esters; triglyceride conversion factor 0.956), total dietary fibre (gravimetric method, based on AOAC 991.43), sodium (flame photometry after removal of organic matter). Moisture was measured based on

the air-oven method (AACC 44-15.02) using a moisture analyser (Mettler Toledo, Ohio, US). The contents of total, digestible and resistant starch were analysed with the enzyme kit K-RAPRS (Megazyme, Ireland).

2.4. In Vitro Starch Digestion

A starch digestion was performed *in vitro* to obtain the hydrolysis index (HI) value of HPHP by monitoring the release of reducing sugars throughout the digestion in comparison to RWP. The HI value is calculated by dividing the area under the sugar release curve of HPHP (30 to 240 min) by the area under the sugar release curve of RWP (30 to 240 min). The digestion (pepsin treatment at pH 1.5 followed by incubation with pancreatic α-amylase at pH 6.9 for 5 hours) was carried out following the procedure reported by Brennan and Tudorica[22] with some modifications. During α-amylase incubation, samples were collected every 30 minutes and their contents of reducing sugars were determined spectrophotometrically with 3,5-dinitrosalicylic acid (DNS; 10 g/L) as colouring reagent (100 µL of both DNS and sample solution were mixed and then further diluted with 1 mL buffer; absorbance read at 546 nm after 15 min incubation at 110 °C). The level of released reducing sugars was expressed relative to the level digestible starch in the sample.

2.5. Amino Acid Analysis

Determination of protein amino acid composition was performed by Mérieux NutriSciences CHELAB S.r.l., Italy based on ionic chromatography with postcolumn ninhydrin derivatisation (fluorescence detection; UV

detection for tryptophan) after adequate extraction and protein hydrolysis (separate procedures for tryptophan, sulphur-containing amino acids and remaining amino acids).

2.6. Antinutritional Compounds

Antinutritional compounds in pasta samples were analysed as described by Hoehnel et al.[21] for bread samples. In brief, trypsin inhibitors were extracted from the freeze-dried pasta samples (raw and cooked) with a sodium acetate buffer (0.1 M, pH4.9). Trypsin inhibitor activity (TIA) was determined following the method described by Joehnke et al.[23] with some modifications described by Hoehnel et al.[21]. For analysis of vicine and convicine, freeze-dried pasta (raw and cooked) were extracted with boiling methanol according to procedure reported by Petersen et al.[24]. Quantification was achieved by micellar electrokinetic capillary chromatography (MCEC) with vicine as external standard as described by Bjergegaard et al.[25].

2.7. In Vitro Protein Digestion

In vitro simulation of gastro-pancreatic protein digestion was performed as described by Hoehnel et al.[21] based on a previously reported static multi-step method to determine *in vitro* protein digestibility (IVPD)[23,26]. In short, sample amounts containing 50 ± 1 mg protein were subjected to enzymatic hydrolysis: pepsin digestion (37 °C, pH 1-2, 1 h) followed by a sequential pancreatin digestion (37 °C, pH 7-8, short-term: +1 h, medium-term: +3 h, long-term: +24 h). The enzyme/substrate ratio (w/w) was kept constant at 1:50 (pepsin stage) and 1:10 (pancreatin stages). IVPD in % was

168 determined using a trinitrobenzenesulfonic acid (TNBS) assay. Results are
169 expressed as the concentration of free α-amino groups in the digested sam-
170 ples in relation to an alanine standard solution representing 100 % protein
171 digestibility.

172 *2.8. In Vivo Nitrogen Balance*

173 The animal protocol used in this study was approved by the local insti-
174 tutional Animal Care and Use Committee (Olsztyn, Poland) and the study
175 was performed in accordance with EU Directive 2010/63/EU for animal
176 experiments. *In vivo* nitrogen balance was investigated according to the
177 procedure described by Hoehnel et al.[21]. The experiment was performed
178 with growing male Wistar rats (average bodyweight of 173.2 g), which were
179 randomly divided into groups of seven animals. The following diets were
180 used for experimental feeding: a standard control diet based on casein as
181 main protein source (supplemented with 0.2% DL-methionine), a second
182 control diet based on soya protein isolate (without any supplementation),
183 a third control diet based on soya flour (without any supplementation); and
184 the experimental diets containing RWP and HPHP (Table 2). All experi-
185 mental diets represent modifications of the AIN-93G diet for laboratory
186 rodents recommended by the American Institute of Nutrition[27] (dietary pro-
187 tein level was lowered to approx. 11 % to measure protein digestibility and
188 utilisation rate). In order to determine nitrogen (N) digestibility and utili-
189 sation, faeces and urine of all rats (7 per diet group) were thoroughly col-
190 lected for 5 days (after a 9-day preliminary period). The total N content of
191 each diet, faecal sample and urinal sample was analysed in duplicate

(AOAC 979.09). Additionally, bodyweight (BW) gain (BWs recorded at beginning and end of the study) and diet intake (daily record) were monitored for all rats to enable calculation of the protein efficiency ratio (PER).

2.9. Statistical Analysis

All measurements were performed in triplicate unless stated otherwise. Data analysis was performed using RStudio, version 1.2.1335 with R version 3.6.1 (RStudio Inc, USA; R Core Team, r-project). One-way analysis of variance (ANOVA) with post-hoc pairwise Tukey's test was used to identify significant differences ($p < 0.05$ unless stated otherwise). When available, values are given as the mean ± standard deviation or uncertainty (amino acid profile).

3. RESULTS AND DISCUSSION

The nutritional profile of the cooked pasta formulations RWP and HPHP was evaluated with a focus on macronutrient composition, ANCs and protein quality. The analysis of ANCs and *in vitro* digestibility was additionally performed with raw pasta samples in order to monitor the effect of heat treatment and aqueous extraction during cooking on nutritional pasta quality.

3.1. Macronutrient Composition and In Vitro Starch Digestibility

The macronutrient composition of RWP and HPHP was analysed to assess compositional changes caused by the partial replacement of wheat semolina by plant-based HPIs (Table 3). An increased protein level and

decreased carbohydrate content, amongst other minor differences to RWP, characterise the macronutrient profile of HPHP. The HPHP formulation contains with 23.2 %DM approximately 6 %DM more protein than RWP with 17.3 %DM. The content of total starch accounts for 64.70 %DM in HPHP and 72.10 %DM in RWP. Hence, the partial replacement of wheat semolina by HPIs primarily causes a substitution of wheat starch by non-wheat protein. The determined energy contents are with 393.0 kcal/100 g DM (RWP) and 396.8 kcal/100 g DM (HPHP) very similar in both formulations. Therefore, the compositional changes in HPHP can additionally be considered an isocaloric replacement of wheat starch by non-wheat protein. It has been reported that the isocaloric replacement of dietary carbohydrate by protein in diets reduces blood lipid concentrations and blood pressure.[28] This effect was attributed to the reduced intake of carbohydrate rather than the increased intake of protein.[28] However, the metabolic effects of dietary carbohydrate are influenced by its glycaemic index (GI).[29] Consequently, the isocaloric replacement of dietary carbohydrate by protein might be more or less effective (with regard to blood lipid concentrations, blood glucose or other metabolic conditions) depending on the GI of the replaced carbohydrate. Appel et al.[28] observed a positive influence on blood lipid concentrations and blood pressure when medium GI (between 68 and 75) carbohydrate was replaced by protein. Even though pasta contains refined wheat starch, which is a rapidly digestible carbohydrate with high GI, pasta is considered a low GI food.[14] This is related to its unique and dense structure, which seems to prevent or delay enzymatic degradation of starch.[13,14] While in other carbohydrate foods, like bread, the

incorporation of plant-based protein ingredients has been reported to significantly lower GI,[30] only small effects or no significant changes have been reported for pasta.[8,13,31] The HI values determined in this study (Table 3) suggest a decreased starch digestibility of HPHP (86.0 %) compared to RWP (100 %). This trend is also evident with regard to the sugar release curves obtained from *in vitro* starch digestion trials which are displayed in Figure 2. This indicates a lower GI of HPHP in comparison to RWP, but additionally a lower GL due to the significantly lower level of digestible starch in HPHP than in RWP (Table 3). This suggests HPHP as favourable pasta formulation compared to RWP, since diets with high GI and/or GL have been associated with elevated risk for heart disease, diabetes and certain types of cancer.[32–34] The isocaloric replacement of wheat starch by non-wheat proteins in HPHP also brings the ratio of calories provided by protein up to 23.4 % compared to the lower ratio of 17.6 % in RWP. This is particularly important with regard to European legislation (regulation (EC) No 1924/2006[35]), where a protein level of at least 20 % of calories provided by protein is specified as requirement for a 'high in protein' nutritional claim made on foods. Besides non-wheat proteins, also a considerable amount of non-wheat starch is brought into the HPHP formulation by buckwheat flour; accounting for approx. 9.4 %DM (calculated based on composition of raw materials and HPHP recipe). Buckwheat is known to contain substantial amounts of resistant starch (RS), which has been linked to several health benefits and could further improve HPHP's nutritional value.[36,37] However, the contents of RS determined for RWP and HPHP are both relatively low and HPHP contains with 0.56 %DM even slightly less

RS than RWP with 0.91 %DM (Table 3). This is in line with the literature where cooking has been reported to cause a substantial decrease of RS in buckwheat groats.[36] Other minor compositional changes include the contents of lipids, ash and fibre. The HPHP formulation contains with 1.97 % slightly more lipids than RWP with 1.33 %DM. However, this can only be considered a small difference and both formulations are low in fat (threshold for 'low fat' nutritional claim in European legislation[38]: 3 g of fat per 100 g of solids). Furthermore, studies have shown that the fatty acid balance of a diet is more critical than the total fat intake.[39] Although dietary recommendations refer to the whole diet, RWP's and HPHP's contribution to a balanced fatty acid profile can be compared. A desired ratio of SFA:MUFA:PUFA in a diet seems to approximate 1:1.3:1.[39] Both formulations contribute with 1:1.3:2.6 (RWP) and 1:1.4:2.3 (HPHP) similarly elevated amounts of PUFA to complement other low-PUFA diet components (e.g. milk fat). The increased ash content of 1.4 % in HPHP (1.0 %DM in RWP) is likely related to a higher concentration of minerals caused by the incorporation of HPIs. Vogelsang-O'Dwyer et al.[40] determined the mineral composition of the lupin protein isolate used in the present study and reported high levels of the nutritionally valuable minerals Ca, Fe and Zn. Tazrart et al.[8] and Petitot et al.[12] found substantially elevated levels of Ca, Fe and Zn in pasta fortified with faba bean flour. The determined fibre content for HPHP is with 3.8 %DM slightly lower than for RWP with 4.8 %DM. However, the analytical standard method applied in this study (AOAC 991.43) has been reported to not sufficiently quantitate food components like resistant starch, fructans and galactooligosaccharides (GOS),

which are considered as dietary fibre according to more recent definitions.[41] Ispiryan et al.[42] characterised the FODMAP profile of selected cereal-product ingredients and reported a high GOS content (raffinose, stachyose and verbascose) of 4.87 %DM for the faba bean flour used in the present study. Considering this, it could be expected that the amount of total dietary fibre in HPHP was underestimated and might be equal to or higher than in RWP.

3.2. Amino Acid Profile

Wheat based staple foods, like regular wheat pasta, represent an important source of plant-based dietary protein. However, wheat protein has low quality which is related to its unbalanced amino acid (AA) profile and a lack of indispensable AA; specifically lysine.[43] Besides changes in the macronutrient profile, the incorporation of pseudocereal and legume HPIs in HPHP also introduces a shift in the AA pattern. The AA contents of RWP and HPHP are presented in Table 4 and expressed in percent relative to the formulations' protein content in order to allow for a direct comparison of the AA profiles. Amongst the dispensable AAs, the contents of asparagine/aspartic acid, glutamine/glutamic acid, proline and arginine differ substantially between RWP and HPHP. Wheat is rich in glutamine/glutamic acid and proline but contains relatively small amounts of asparagine/aspartic acid and arginine.[43] Buckwheat, faba bean and lupin represent a complementary pattern with respect to these AAs.[37,40,44] Therefore, reduced levels of glutamine/glutamic acid and proline and raised levels of asparagine/aspartic acid and arginine are present in HPHP. The profiles of

indispensable AAs in RWP and HPHP also exhibit several differences, specifically with regard to threonine, tryptophan, lysine and sulphur-containing AAs (SAAs). The incorporation of HPIs leads to a small increase in the level of threonine in HPHP compared to RWP which is related to high contents of this AA in faba bean, lupin and particularly in buckwheat.[40,44,45] The tryptophan level was below the limit of quantification (LOQ; equals 0.29 %Protein for RWP) and above the limit of determination (LOD; equals 0.58 %Protein for RWP) for RWP. For HPHP, a tryptophan content of 0.84 %Protein was determined. While wheat semolina, faba bean and lupin all contain similar amounts of this AA, buckwheat is relatively rich in tryptophan.[37,40,43,44] Therefore, an increased tryptophan level is achieved in HPHP due to the use of buckwheat HPI in addition to legume HPIs. Lysine represents the indispensable AA with the biggest discrepancy between RWP and HPHP. The HPHP formulation contains 3.90 %Protein which represents a by 34 % increased lysine level in comparison to RWP with 2.58 %Protein. In contrast to this increase observed for lysine, the results show that the substitution of wheat semolina by HPIs leads to a decrease in SAAs, which account for 3.39 %Protein in RWP and 2.99 %Protein in HPHP. These differences in indispensable AAs might seem small when levels expressed in %Protein are compared. But some of these differences represent a significant improvement of the pasta's amino acid balance. This becomes apparent when AA levels are expressed relative to a reference pattern of AA intake for adults recommended by WHO[46] (Figure 3). The comparison of the AAs in RWP and HPHP with the reference pattern shows that lysine does not reach the recommended level (= 1). Thus, lysine

represents the limiting AA in the protein from both formulations. However, the increased lysine content in HPHP reaches 87 % of the recommended level as opposed to 57 % in RWP. Furthermore, the incorporation of buckwheat flour secures an adequate level of tryptophan in HPHP by raising its content to 140 % of the required amount. This makes the HPHP AA profile, which nearly covers the recommended intake of all indispensable AAs, much more balanced. Amino acid scores (AASs) represent the content of the limiting AA of a protein calculated relative to the reference pattern and are commonly used to interpret protein quality. Table 5 summarises the AASs of RWP, HPHP and the raw materials used for their production. The AASs indicate that the combination of buckwheat flour, faba bean flour and lupin protein isolate for partial wheat semolina substitution leads to an upgrade of the protein quality of all protein sources. Only buckwheat flour represents an exception and possesses a desirable AA profile on its own. The partial substitution of wheat semolina by legumes has previously been reported to improve the AA profile of pasta protein; specifically through increased lysine levels.[9] However, an optimal substitution ratio (legume:wheat) has been discussed with regard to SAAs.[9] While lysine levels rise with increased legumes:wheat ratios, SAA contents drop; due to low levels of SAAs in legumes. The fact that only a moderate decrease in SAA content was observed for HPHP in this study is related to the high level of SAAs in buckwheat which even exceeds that of wheat semolina.[43,45] The use of AASs to evaluate protein quality is based on the assumption that the considered daily intake of 0.66 g protein per kg bodyweight is entirely covered by the concerning protein source. In a real diet, AA deficiencies of one

protein source can potentially be covered by another. Nevertheless, the assessment of a protein's ability to cover AA requirements is considered an adequate approach to compare proteins from different sources and their nutritional quality.

3.3. Antinutritional Compounds

The activity/contents of trypsin inhibitors and the pyrimidine glycosides vicine and convicine have been determined for both raw and cooked RWP and HPHP (Table 6). Trypsin inhibitors are some of the most relevant ANCs with regard to a product's protein quality. These molecules can form complexes with the intestinal enzyme trypsin (or chymotrypsin) thereby inhibiting their proteolytic activity and potentially causing a decrease in protein digestibility.[15] They have also been reported to cause other adverse physical conditions like pancreatic hypertrophy and increased pancreatic secretory activity.[47] The TIAs determined for raw and cooked HPHP were higher than those for RWP (both raw and cooked). While the lupin protein isolate applied in HPHP reportedly only has a very low remaining TIA,[40] the HPIs from faba bean and buckwheat are likely to cause the higher TIA in HPHP. Vogelsang-O'Dwyer et al.[44] found a TIA of 2.34 TIU/mg (based on dry matter) in the faba bean flour applied in HPHP. Also the low digestibility of buckwheat protein has been associated with a high trypsin inhibitor activity.[5,48] The results in the present study additionally show that the cooking process leads to a substantial decrease in TIA in both RWP and HPHP formulation. A very low TIA of 0.38 TIU/mg was measured for raw RWP and no TIA was detected in cooked RWP. For HPHP, the

cooking process caused a more than threefold reduction of TIA from 3.36 TIU/mg in raw HPHP to 0.91 TIU/mg in cooked HPHP. Several articles have addressed the impact of various processing techniques on TIA in foods.[16,47,49] Thermal treatments such as boiling, cooking and roasting have been mentioned as the most efficient procedures to inactivate legume trypsin inhibitors. The mechanism of this inactivation primarily relies on conformational changes of their active site which prevents complex formation with and inhibition of trypsin. Soybean trypsin inhibitors, for example, seem to be sufficiently inactivated by boiling at 100 °C for approx. 9 min.[49] Similar conditions were applied in the cooking procedure of HPHP. The achieved reduction of TIA is in agreement with results reported in literature, where usually a decrease to 15-51 % residual TIA in cooked pasta is reached.[10,11] Zhoa et al.[11] even reported no residual TIA in cooked spaghetti fortified with 15-20 % pea and lentil flour. The small remaining TIA in this study could be related to the lack of a heat drying process (which is performed in most studies investigating pasta quality) after extrusion of RWP and HPHP which could contribute to trypsin inhibitor inactivation. Furthermore, trypsin inhibitors originating from buckwheat seeds were found to be relatively thermostable (specifically at acidic pH) when subjected to heat treatment at 100 °C for 30 min.[50] Faba beans are known to contain the thermostable ANCs vicine and convicine.[51] These pyrimidine glycosides can trigger an adverse physical condition called favism in human individuals that are deficient in glucose-6-phosphate dehydrogenase (G6PD).[18] Since triggered favism leads to acute haemolytic anaemia in G6PD deficient consumers,[18] it is important to monitor vicine and convicine

levels in faba bean containing foods. Recent efforts in plant breeding with the objective to reduce pyrimidine glycoside contents led to cultivars with levels as low as 0.01-0.02 %DM. However, in faba bean seeds from most varieties, a VC (sum of vicine and convicine) level of approx. 1 %DM is present.[17,51] While for RWP, expectedly, no vicine and convicine were detected, a VC level of 0.300 %DM (vicine 0.161 %DM; convicine 0.139 %DM) was determined in raw HPHP. This is in agreement with the findings of Vogelsang-O'Dwyer et al.[44] who reported a VC content of 1.25 %DM in the faba bean flour applied in HPHP (expected amount of VC in HPHP approximates 0.50 %DM based on calculation considering recipe and ingredient composition). Cooked HPHP contains only 0.050 %DM of VC (vicine 0.028 %DM; convicine 0.022 %DM) which represents an 83 % reduction of VC content in HPHP induced by the cooking procedure. Besides enzyme treatment, also aqueous extraction at elevated temperatures, has been mentioned as efficient technique to eliminate or reduce vicine and convicine contents in faba beans.[17,51,52] A recent study by Gallo et al.[53] investigated the consumption of large quantities of faba beans (500 g) with a low VC content of 0.016 % (in wet weight as ingested) by G6PD deficient men. Favism was not triggered and the results suggested that this level of VC intake is safe for G6PD deficient individuals. In the context of these findings, also the consumption of approx. 300 g cooked HPHP (exceeds the daily intake of cooked pasta recommended by dietary guidelines of European countries[54]) can be considered safe. While the results in the present study confirm a substantial reduction of TIA and VC content due to cooking of pasta, also the dilution of both ANCs due to incorporation

of the HPIs in a complex pasta formulation should be addressed. The separate consumption of protein from sources with complementary AA patterns (cereals, pseudocereals, legumes) can in theory provide a balanced AA intake. However, higher ANC levels in raw materials, as opposed to hybrid formulations like HPHP, can impair protein digestibility and, therefore, bioavailability of AA from some of these sources.

3.4. Protein Digestibility and Utilisation

In vitro digestion was performed with raw and cooked pasta in order to evaluate the effect of the cooking procedure on the samples' susceptibility to degradation by pepsin and pancreatin. The results are presented in Table 7. The pepsin treatment and subsequent short term treatment with pancreatin mimics the protein digestion of RWP and HPHP in the human gastrointestinal tract. Medium and long term treatment with pancreatin were carried out in order to obtain an indication of the maximum achievable protein degradation under the same conditions. Two major trends were observed. For pepsin treatment as well as short and medium term pancreatin treatment of both formulations, raw pasta reached significantly (except RWP after medium term pancreatin treatment) higher IVPDs than cooked pasta. This trend was clearly detected even for the HPHP formulation, where a substantial reduction of TIA (by 73 %) was observed due to the cooking process, which could have been expected to cause an increase in protein digestibility. Petitot et al.[14] who reviewed the impact of pasta processing on starch and protein digestibility concluded that thermally induced protein aggregation could increase resistance to pepsin/pancreatin

degradation of protein in cooked pasta. It is also possible that, similar to observations reported for *in vitro* starch digestion[13,14], the pasta structure created during the cooking process (coagulated protein network entrapping starch granules) slows down enzymatic degradation of proteins. The fact that IVPDs of cooked pasta after long term pancreatin treatment are not smaller than for raw pasta further supports this theory. According to these results, changes in structure and substrate accessibility induced by the cooking procedure seem to have a larger impact on protein digestibility in HPHP than TIA. Furthermore, the difference in IVPD between raw and cooked pasta seems to be more pronounced for HPHP than for RWP. This is in line with the previous findings of Hoehnel et al.[19]. Their results of technological quality analysis of RWP and HPHP suggested a thicker protein network in HPHP than RWP; and a strengthened protein network in HPHP through interactions between wheat and non-wheat proteins. The second clear trend with regard to IVPDs concerns the difference between RWP and HPHP. Both raw and cooked HPHP reach higher IVPDs for all digestive treatments (except long term pancreatin treatment) than their RWP equivalent. Again, higher IVPDs were observed despite higher detected TIAs for these samples. This indicates that TIA levels might have been too low to affect enzymatic protein degradation. Also Laleg et al.[55] and Tazrart et al.[8] found slightly increased protein digestibility of pasta formulations fortified with legume ingredients when compared to wheat semolina controls. A higher protein digestibility in formulations containing pseudocereal and legume ingredients could be related to a higher abundance of target AA for the cleavage by trypsin and chymotrypsin (lysine, arginine,

AAAs, tryptophan) as already suggested by Hoehnel et al.[21]. The results of *in vivo* nitrogen balance trials performed with rats are presented in Table 7. The most important variables are N intake (which is used to calculate relative N losses in urine and faeces) as well as N digestibility, N utilisation and PER, which are indicative of the pastas' nutritional value. No significant difference was observed for the N intake of rats which were fed diets containing either RWP or HPHP as protein source. Since the content of RWP and HPHP in the experimental diets was adjusted so they contained the same amount of protein, this indicates that similar amounts of total diet were consumed by RWP and HPHP rats. The values determined for N digestibility were not significantly different between RWP (88.0 %) and HPHP (88.4 %). *In vitro* digestion, however, indicated higher digestibility of HPHP. According to literature, *in vitro* and *in vivo* digestibility data usually show good correlations; but it has also been reported that legumes tend to reach higher digestibility *in vitro* than *in vivo*.[56] The protein digestibility corrected amino acid score (PDCAAS) represents another commonly used indicator of protein quality and is calculated considering faecal N digestibility and AASs (discussed in amino acid section above). Due to the similar N digestibility of RWP and HPHP, PDCAAS values follow the same trend as AAS values. While RWP has a PDCAAS of only 0.50, HPHP reaches a value of 0.79 owing to its higher lysine level. The significantly lower urinary N loss measured for HPHP rats results in a by 55.6 % increased N utilisation rate for HPHP (43.1 %) compared to RWP (27.7 %). This is likely related to the improved AA balance of HPHP. The lack of one or more indispensable AAs, amongst absorbed AAs, has been reported

to cause a plateau in AA retention while AA which are present in excess of the limiting AA (relative to the required AA pattern) are excreted with the urine after oxidisation.[46,57] Furthermore, a positive correlation between balance of dietary AAs and AA utilisation was found in both animal and human studies and a higher degree of AA imbalance led to limited protein synthesis.[58] In agreement with the higher N utilisation rate, also the PER determined for rats with HPHP diet (2.13 g/g) was significantly higher than that determined for RWP rats (1.37 g/g). The PER reflects a protein's value with respect to AA requirements for growth. Both *in vitro* and *in vivo* models to determine protein quality for human nutrition have their limitations and true protein quality depends on the AA profile which is absorbed and utilised to achieve specific metabolic actions in the human body.[57,59] However, AA profile and values like IVPD, N digestibility, N utilisation rate and PER provide a valid comparison of protein from RWP and HPHP and conclusively indicate improved protein quality in HPHP.

4. CONCLUSION

The present study evaluates the nutritional profile of a high-protein hybrid pasta (HPHP) formulation. In this formulation, a combination of three HPIs from buckwheat, faba bean and lupin was used to partially substitute wheat semolina. The nutritional value of the pasta was assessed in comparison to regular wheat pasta as a reference with a focus on protein quality. The results confirm that HPHP represents a pasta formulation with an overall enhanced nutritional profile. The improved macronutrient composition is primarily characterised by an isocaloric replacement of dietary

carbohydrate by plant-based protein. With regard to ANCs brought into the pasta formulation by pseudocereal and legume HPIs, the results show that the cooking procedure realises a substantial reduction of trypsin inhibitor activity and contents of vicine and convicine. The small remaining activity/contents seem to not adversely affect nutritional quality of HPHP. All measures indicative of protein quality determined in this study (AA composition, IVPD, N digestibility, N utilisation, PER) conclusively suggest improved protein quality of HPHP compared to RWP. The results also indicate that specifically the combination of pseudocereal and legume HPIs to replace wheat semolina is beneficial to achieve a balanced AA profile. In addition to its enhanced nutritional profile, HPHP has been shown to possess technological and sensory quality similar to RWP by Hoehnel et al.[19] which identifies HPHP as an attractive alternative to regular wheat pasta in currently consumed diets. Furthermore, HPHP and formulations of its kind represent an increased potential of wheat based staple foods to contribute to a sufficient intake of high-quality protein in future predominantly plant-based diets.

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ABBREVIATIONS

ANC	Antinutritional compound
HPI	High-protein ingredient
HPHP	High-protein hybrid pasta
RWP	Reference wheat pasta
%DM	Percentage based on dry matter
C	Casein
SPI	Soya protein isolate
SF	Soya flour
%E	Percentage based on energy
OCT	Optimal cooking time
HI	Hydrolysis index
DNS	3,5-Dinitrosalicylic acid
TIA	Trypsin inhibitor activity
MCEC	Micellar electrokinetic capillary chromatography
IVPD	In vitro protein digestibility
TNBS	Trinitrobenzenesulfonic acid
BW	Body weight
PER	Protein efficiency ratio
ANOVA	Analysis of variance
proteinE	Percentage of calories provided by protein
GI	Glycaemic index
GL	Glycaemic load
SFA	Saturated fatty acids
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
GOS	Galactooligosaccharides
FODMAP	Fermentable oligo-, di-, mono-saccharides and polyols
%Protein	Percentage based on protein
AA	Amino acid
SAA	Sulphur containing amino acids
AAA	Aromatic amino acids
LOQ	Limit of quantification
LOD	Limit of determination
AAS	Amino acid score
TIU	Trypsin inhibitor unit
G6PD	Glucose-6-phosphate dehydrogenase
VC	Sum of vicine and convicine
PDCAAS	Protein digestibility corrected amino acid score

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Figure Legends

Figure 1: Photographs of RWP and HPHP (as reported by Hoehnel et al.[19]): **A** – raw pasta; **B** – cooked pasta.

Figure 2: Sugar release curves of RWP and HPHP obtained from in vitro starch digestion; values expressed as amount of reducing sugars relative to digestible starch.

Figure 3: Profile of indispensable amino acids of reference wheat pasta and high-protein hybrid pasta expressed relative to the requirement pattern (WHO 2007[46]) and based on an average intake of 0.66 g protein/kg; the level of tryptophan in RWP was below the limit of quantification (LOQ) and above the limit of detection (LOD) which equals a range between 0.48 and 0.97 calculated relative to tryptophan in the reference pattern.

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Tables

Table 1: Recipes for RWP and HPHP (as reported by Hoehnel et al.[19]), values given in % based on recipe unless stated otherwise

Ingredient	**RWP**	**HPHP**
Semolina	76.54	57.55
Buckwheat flour	-	13.02
Faba bean flour	-	3.97
Lupin protein isolate	-	2.01
NaCl	0.38	0.38
Water	23.08	23.08
Total	**100.00**	**100.00**

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Table 2: Composition of diets for *in vivo* nitrogen balance trials, values given in % of diet

Component of diet	**C**	**SPI**	**SF**	**RWP**	**HPHP**
Casein	11.15				
DL-Methionine	0.20				
Soya protein isolate		10.80			
Soya flour			19.69		
Reference wheat pasta				66.66	
High-protein hybrid pasta					48.80
Cellulose	8.00	8.00	8.00	8.00	8.00
Soya oil	8.00	8.00	8.00	8.00	8.00
Mineral mix[1]	3.50	3.50	3.50	3.50	3.50
Vitamin mix[2]	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20	0.20	0.20
Cholesterol	0.30	0.30	0.30	0.30	0.30
Sucrose	5.00	5.00	5.00	5.00	5.00
Corn starch	62.65	62.29	54.31	7.34	25.20

[1] AIN-93G-MX: mineral mixture as specified by Reeves[27]

[2] AIN-93G-VX: vitamin mixture as specified by Reeves[27]

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Table 3: Composition of reference wheat pasta (cooked) and high-protein hybrid pasta (cooked); contents expressed in %DM unless stated otherwise

Component	**RWP**	**HPHP**
Moisture [%], raw pasta	18.39 ± 0.71[a]	18.13 ± 0.83[a]
Moisture [%], cooked pasta	43.21 ± 1.70[a]	46.57 ± 1.37[a]
Energy [kcal/100 g DM]	393.0	396.8
Protein	17.3	23.2
proteinE[†] [%E]	17.6	23.4
Ash	1.0	1.4
Lipids	1.33	1.97
SFA	0.26	0.40
MUFA	0.34	0.56
PUFA	0.67	0.92
Total carbohydrates[‡]	80.4	73.5
Total dietary fibre	4.8	3.8
Available carbohydrates[‡]	75.6	69.7
Total starch	72.10 ± 0.27[a]	64.70 ± 0.79[b]
Digestible starch	71.18 ± 0.26[a]	64.14 ± 0.79[b]
Resistant starch	0.91 ± 0.01[a]	0.56 ± 0.00[b]
Sodium	0.091	0.098
Sodium expressed as salt (NaCl)	0.23	0.24
Hydrolysis index (HI) [%]	100[§]	86.0 ± 0.7

Moisture and total, digestible and resistant starch: means ± standard deviation (different letters in the same row indicate significant differences at $p < 0.05$)

[†] Calculated based on energy content, protein content and 4 kcal/g protein

[‡] Calculated by difference

[§] HI of HPHP calculated as areas under its sugar release curve (30 to 240 min) relative to the area under RWP's sugar release curve (30 to 240 min)

Table 4: Amino acid composition of protein of reference wheat pasta and high-protein hybrid pasta

Content [%Protein]	**RWP**	**HPHP**
Indispensable and conditionally indispensable AAs		
Histidine	2.39 ± 0.29	2.56 ± 0.31
Isoleucine	3.94 ± 0.48	4.22 ± 0.51
Leucine	7.56 ± 0.92	7.36 ± 0.89
Lysine	2.58 ± 0.32	3.90 ± 0.47
Cystine	1.91 ± 0.23	1.74 ± 0.13
Methionine	1.49 ± 0.18	1.26 ± 0.10
Cystine + Methionine (SAAs)	3.39 ± 0.42	2.99 ± 0.23
Phenylalanine	4.84 ± 0.59	4.96 ± 0.60
Tyrosine	2.65 ± 0.33	2.56 ± 0.31
Phenylalanine + Tyrosine (AAAs)	7.49 ± 0.92	7.52 ± 0.92
Threonine	3.10 ± 0.38	3.68 ± 0.45
Tryptophan	<LOQ†	0.84 ± 0.31
Valine	4.78 ± 0.58	4.43 ± 0.54
Total indispensable AA	35.82 ± 4.30	37.51 ± 4.63
Dispensable AAs		
Asparagine/aspartic acid	4.39 ± 0.53	6.88 ± 0.84
Glutamine/glutamic acid	30.35 ± 3.68	25.35 ± 3.08
Glycine	3.74 ± 0.46	4.48 ± 0.55
Alanine	3.42 ± 0.42	3.31 ± 0.40
Serine	4.78 ± 0.58	5.13 ± 0.62
Proline	10.01 ± 1.22	7.90 ± 0.96
Arginine	4.07 ± 0.50	7.32 ± 0.87
Total dispensable AA	60.77 ± 7.37	60.26 ± 7.32

Amino acid contents ± uncertainty values

† Content was below limit of quantification (LOQ) of tryptophan (equals 0.58 %Protein for RWP) and above limit of determination (LOD; equals 0.29 %Protein for RWP)

Table 5: Amino aicd scores (AASs) for pasta formulations and their raw materials

Protein source	AAS	Limiting AAs
RWP	0.57	Lysine; Tryptophan (<0.97)
HPHP	0.87	Lysine
Semolina[†]	0.57	Lysine
Buckwheat flour[†]	- (1.13)[§]	- (Leucine) [§]
Faba bean flour[‡]	0.66	SAAs
Lupin protein isolate[‡]	0.70	SAAs; Valine (0.93); Lysine (0.98)

[†] Calculated from amino acid composition; determined as for RWP and HPHP (data not shown)

[‡] Calculated from amino acid composition; determined as for RWP and HPHP and reported by Vogelsang-O'Dwyer et al.[40,44]

[§] Not strictly limiting (≥ 1), but represents AA with lowest level relative to reference pattern

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Table 6: Contents of antinutritional compounds of reference wheat pasta and high-protein hybrid bread, contents refer to dry matter as indicated

	RWP		**HPHP**	
Antinutritional compound	Raw	Cooked	Raw	Cooked
Trypsin inhibitor activity (TIA) [TIU/mg]	0.38 ± 0.01^{c}	n.d.	3.36 ± 0.34^{a}	0.91 ± 0.03^{b}
Vicine [%DM]	n.d.	n.d.	0.161 ± 0.005^{a}	0.028 ± 0.004^{b}
Convicine [%DM]	n.d.	n.d.	0.139 ± 0.006^{a}	0.022 ± 0.002^{b}

Means ± standard deviation with different letters in the same row were significantly different at $p < 0.05$
n.d. – not detected

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Table 7: *In vitro* digestibility and *in vivo* nitrogen balance

	RWP		**HPHP**	
Variable	Raw	Cooked	Raw	Cooked
***In vitro* protein digestibility (IVPD)** [%]				
Pepsin 1 h	1.9 ± 0.1^{b}	1.3 ± 0.2^{c}	2.3 ± 0.1^{a}	1.6 ± 0.1^{bc}
Pancreatin 1 h (short term)	16.4 ± 0.2^{b}	14.5 ± 0.3^{c}	20.0 ± 0.4^{a}	16.5 ± 0.6^{b}
Pancreatin 3 h (medium term)	22.0 ± 1.4^{c}	21.7 ± 0.8^{c}	25.2 ± 2.6^{a}	23.6 ± 1.2^{b}
Pancreatin 24 h (long term)	28.5 ± 2.9^{a}	32.8 ± 0.4^{a}	29.2 ± 1.4^{a}	31.0 ± 1.6^{a}
***In vivo* nitrogen balance**				
N intake [g/5 d]		1379 ± 157^{a}		1505 ± 157^{a}
N in faeces [g/5 d]		165 ± 16^{a}		174 ± 18^{a}
N faecal [% N intake]		12.0 ± 1.1^{a}		11.6 ± 0.7^{a}
N in urine [g/5 d]		829 ± 79^{a}		681 ± 76^{b}
N urinary [% N intake]		60.3 ± 1.6^{a}		45.3 ± 2.4^{b}
N digestibility [%]		88.0 ± 1.1^{a}		88.4 ± 0.7^{a}
N utilisation [%]		27.7 ± 2.6^{b}		43.1 ± 2.2^{a}
PER [g/g]		1.37 ± 0.21^{b}		2.12 ± 0.09^{a}

Means ± standard deviation with different letters in the same row were significantly different at $p < 0.05$

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Figures

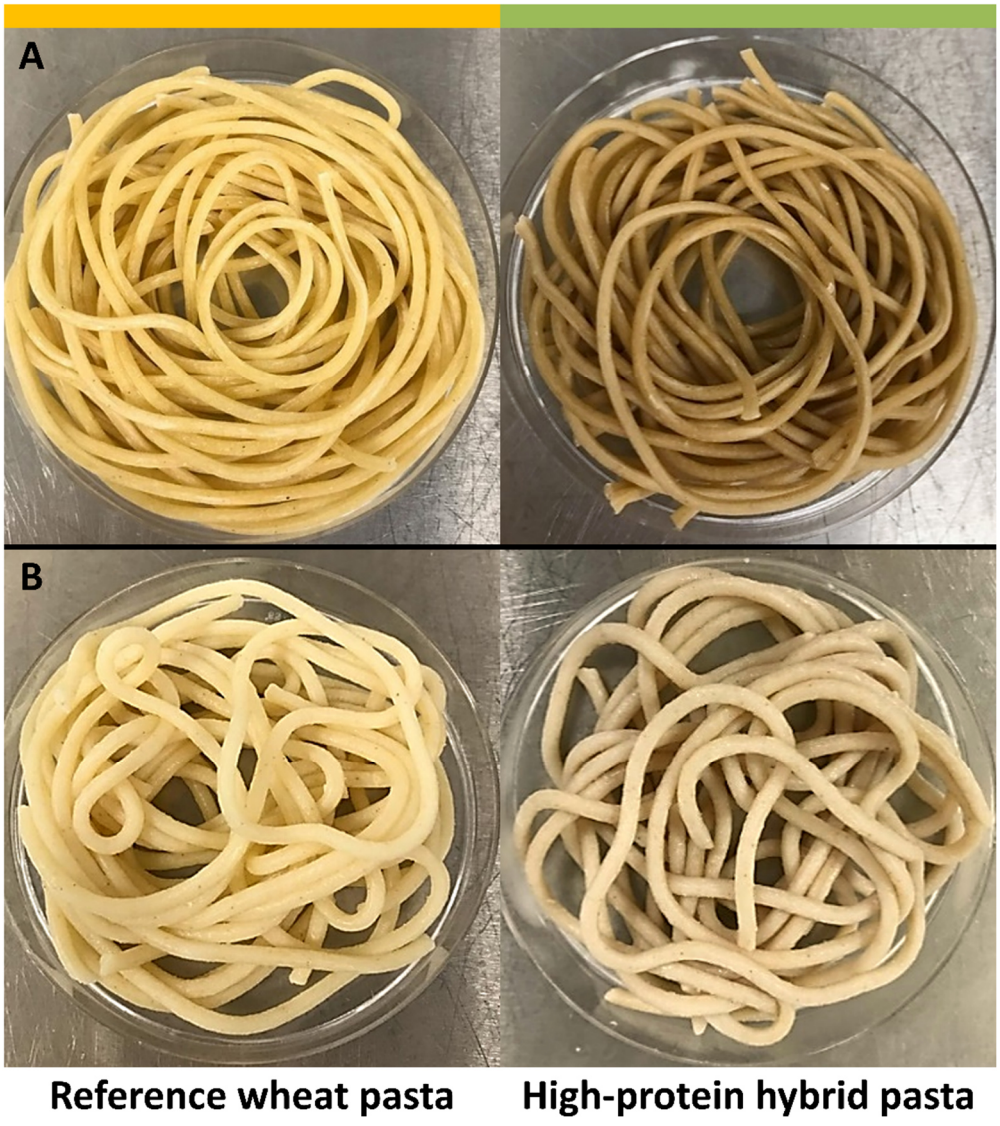


Figure 3: Photographs of RWP and HPHP (as reported by Hoehnel et al.[19]): **A** – raw pasta; **B** – cooked pasta.

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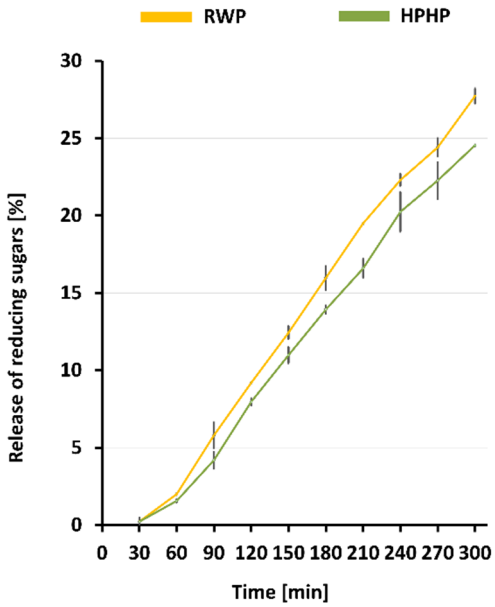


Figure 4: Sugar release curves of RWP and HPHP obtained from in vitro starch digestion; values expressed as amount of reducing sugars relative to digestible starch.

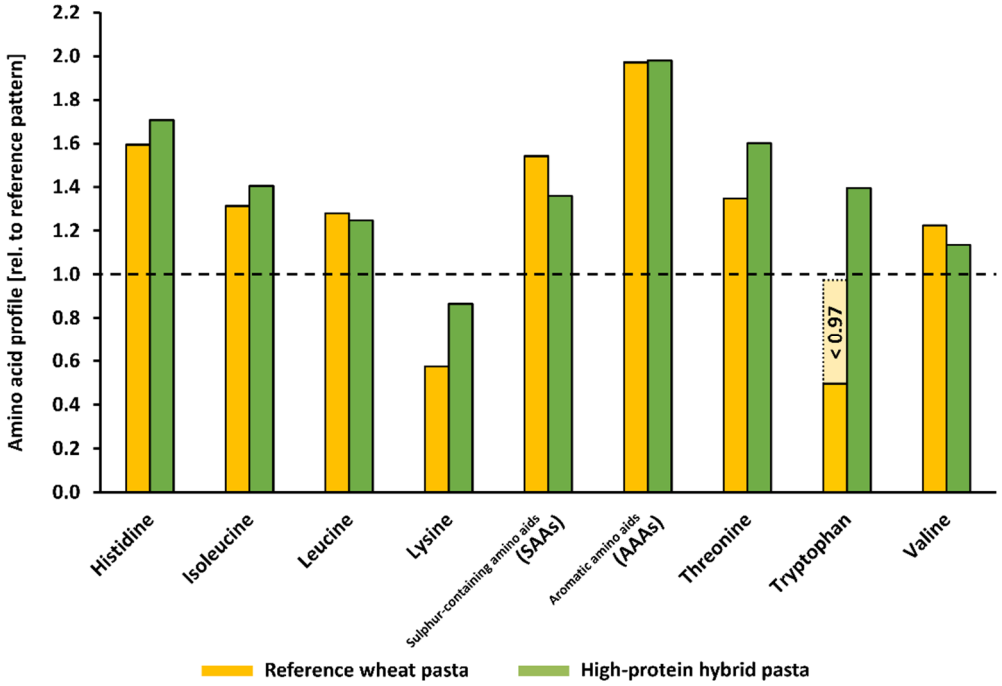


Figure 3: Profile of indispensable amino acids of reference wheat pasta and high-protein hybrid pasta expressed relative to the requirement pattern (WHO 2007[46]) and based on an average intake of 0.66 g protein/kg; the level of tryptophan in RWP was below the limit of quantification (LOQ) and above the limit of detection (LOD) which equals a range between 0.48 and 0.97 calculated relative to tryptophan in the reference pattern.