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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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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 highprotein 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.

1 1. INTRODUCTION

2 The focus of research concerning durum wheat (*Triticum durum*) pasta 3 has shifted in recent years. While technological quality, texture properties 4 and sensory still represent the main topics of interest, nutrition and health 5 aspects of pasta have gained increasing attention. This is in close relation to the evolving market potential of functional foods that have the ability 6 to improve wellness and health of consumers.¹ According to Jenkins et al.² 7 there is a necessity to transition from diets with high nutrient density to 8 9 diets with high nutritional density, particularly in the context of decreasing 10 physical activity. This refers to an increased intake of essential macro- and 11 micronutrients at lower total caloric intakes. Pasta has been described as a 12 promising vehicle for the addition of both macro- and micronutrients through the incorporation of functional ingredients.³ The fortification with 13 14 pseudocereal and legume ingredients, which are particularly rich in protein 15 and micronutrients, offers an upgrade of nutritional quality and nutritional density of pasta; for example due to increased protein content and enhanced 16 17 protein quality.^{4,5} While protein intakes in many parts of the world exceed 18 the average daily requirement, this is often related to an overconsumption 19 of animal protein or food in general.⁶ Many dietary guidelines recommend 20 a substantial reduction of protein from animal sources and increased intake of plant-based protein.⁶ Furthermore, the urgently required transition to a 21 22 food system with improved environmental sustainability largely relies on a 23 shift to predominantly plant-based human diets.⁶ Several studies have in-24 vestigated the impact of pasta fortification with pseudocereal and legume 25 ingredients on technological, sensory and nutritional product quality.⁷⁻¹³

26 However, many of these articles report major challenges with regard to 27 textural properties, cooking quality and sensory characteristics. From a nutritional point of view, the research was often focused on the carbohy-28 29 drate/starch fraction (e.g. starch digestibility) and information on changes in protein quality of fortified pasta is rather scarce.¹⁴ One of the concerns 30 31 regarding nutritional quality when pseudocereal and legume ingredients are 32 incorporated in foods refers to antinutritional compounds (ANCs). These 33 compounds are molecules which are naturally present in plants and can, for 34 example, decrease a product's sensory quality (primarily tannins and saponins), protein digestibility (trypsin inhibitors) and the bioavailability of 35 minerals (phytate).^{5,15,16} The pyrimidine glycosides vicine and convicine, 36 which can trigger favism, are also considered ANCs and are mainly found 37 in faba beans.^{17,18} The analysis of important ANCs should be considered 38 39 when the nutritional quality of pseudocereal and legume containing foods is evaluated. In this study, a combination of three high-protein ingredients 40 41 (HPIs; from the pseudocereal buckwheat and the legumes faba bean and 42 lupin) was used to partially replace wheat semolina and to produce a highprotein hybrid pasta (HPHP). This HPHP formulation was subjected to a 43 44 thorough assessment of its nutritional quality with a focus on macronutrient 45 composition, starch digestibility (in vitro), protein quality (amino acid profile, in vitro and in vivo protein digestion) and ANCs. Technological and 46 47 sensory quality of this formulation were previously validated by Hoehnel et al.¹⁹ and found to be similar to regular wheat pasta. While favourable tex-48 49 ture attributes and organoleptic properties remain the most important de-50 terminators 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 quality¹⁹.

57

2. EXPERIMENTAL

58 2.1. Materials

59 Reference wheat pasta (RWP) and high-protein hybrid pasta (HPHP) were produced from the same ingredients as specified in Hoehnel et al.¹⁹. 60 61 Buckwheat flour (protein content 22.52 %DM, lipids 2.78 %DM, ash 62 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 63 content 61.25 %DM, lipids 3.81 %DM, ash 5.43 %DM, fibre 0.35 %DM, 64 carbohydrates by difference 29.17 %DM, total starch 7.77 %DM;²⁰ obtained 65 by dry fractionation) and lupin protein isolate (protein content 94.51 %DM, 66 lipids 2.94 %DM, ash 5.62 %DM)²⁰ were provided by Fraunhofer Institute 67 68 IVV, Freising, Germany. Wheat semolina (protein content 17.4 %DM, lipids 2.13 %DM, ash 1.37 %DM, fibre 4.21 %DM, carbohydrates by differ-69 70 ence 74.86 %DM) was purchased from W G Buchanan & Son Ltd, Ireland; 71 and salt by Glacia British Salt Ltd, UK. The following ingredients were 72 used for the preparation of diets for *in vivo* nitrogen balance trials: casein 73 (C) from Lacpol Co., Poland; soya protein isolate (SPI) ISOPRO 900 HI

74 (non-GMO) from EDMIR-POL Co., Poland; soya flour (SF) SOPRO TB 75 200 from EDMIR-POL Co., Poland; α -cellulose (C8002) from Sigma Al-76 drich, Missouri, USA; soya oil from ZPT Co., Poland; choline chloride from 77 SIGMA, Poland; cholesterol from PPH Standard Co., Poland; sucrose from 78 POCH SA Co., Poland; and corn starch from Avebe, The Netherlands. For 79 in vitro digestion trials, enzymes were purchased from Sigma-Aldrich, Mis-80 souri, USA: pepsin from porcine gastric mucosa; EC 3.4.23.1; P7000; 727 81 U/mg and pancreatin from porcine pancreas; 4 x USP; P1750. All other 82 chemicals were also purchased from Sigma-Aldrich, Missouri, USA unless 83 stated otherwise.

84 2.2. Recipe Adaptation and Pasta Production

85 Pasta (spaghetti) samples were produced according to Table 1 and the procedure described by Hoehnel et al.¹⁹. In brief, the HPHP formulation 86 was established based on the recipe of the reference wheat pasta (RWP), 87 88 by partial replacement of wheat semolina by HPIs. The impact of 15 plant-89 based HPIs from cereals, potato, pseudocereals and legumes on the quality 90 of high-protein pasta was screened in a series of preliminary trials. The 91 levels of wheat semolina replacement were calculated to reach a protein 92 level of 20 % of calories provided by protein (20 %E). The results identified 93 three HPIs (buckwheat flour, faba bean flour and lupin protein isolate) as 94 most suitable with regard to technological pasta quality. Response surface 95 methodology was used to determine a combination of these three HPIs to 96 produce pasta with optimised technological quality. Due to its allergenicity 97 and its limited capacity to compensate for the lack of lysine in wheat

98 semolina protein, the amount of lupin protein isolate in the formulation was 99 kept to a minimum (see section 3.2). The fresh pasta product obtained after 100 extrusion is referred to as 'raw pasta' throughout this work. In order to 101 prepare the product which is further referred to as 'cooked pasta', the optimal cooking time (OCT) of RWP and HPHP determined by Hoehnel et 102 al.¹⁹ was applied as cooking time. After cooking (tap water, no salt added), 103 104 pasta was drained (not rinsed) and left to cool down. The strands were cut 105 into small pieces of approx. 1 cm length, frozen at -80 °C and freeze-dried. 106 Freeze-dried pasta was milled prior to nutritional analysis. Results are ex-107 pressed as contents per dry matter considering the moisture of the freeze-108 dried pasta powders unless stated otherwise.

109 *2.3. Compositional Analysis*

110 Compositional analysis was performed as previously described by Hoehnel et al.²¹. In brief, analysis of the following compositional data was 111 112 performed by Concept Life Science Ltd., UK based on the indicated vali-113 dated methods: energy (calculated considering protein, lipids, available car-114 bohydrates and fibre), protein (Dumas method, modified after AOAC 1977.992.15; nitrogen-to-protein conversion factor 6.25), ash (removal of 115 organic matter by oxidation at 550 °C, based on ISO 936:1998), lipids (low 116 resolution proton nuclear magnetic resonance (NMR), based on MQC-23-117 118 35 Oxford Instruments application note), fatty acid profile (GC-FID of 119 fatty acid methyl esters; triglyceride conversion factor 0.956), total dietary fibre (gravimetric method, based on AOAC 991.43), sodium (flame pho-120 121 tometry 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).

125 2.4. In Vitro Starch Digestion

A starch digestion was performed *in vitro* to obtain the hydrolysis index 126 127 (HI) value of HPHP by monitoring the release of reducing sugars through-128 out 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) 129 by the area under the sugar release curve of RWP (30 to 240 min). The 130 131 digestion (pepsin treatment at pH 1.5 followed by incubation with pancre-132 atic α -amylase at pH 6.9 for 5 hours) was carried out following the procedure reported by Brennan and Tudorica²² with some modifications. During 133 α -amylase incubation, samples were collected every 30 minutes and their 134 contents of reducing sugars were determined spectrophotometrically with 135 136 3,5-dinitrosalicylic acid (DNS; 10 g/L) as colouring reagent (100 μ L of both DNS and sample solution were mixed and then further diluted with 1 mL 137 138 buffer; absorbance read at 546 nm after 15 min incubation at 110 °C). The 139 level of released reducing sugars was expressed relative to the level digest-140 ible starch in the sample.

141 2.5. Amino Acid Analysis

142 Determination of protein amino acid composition was performed by Mé143 rieux NutriSciences CHELAB S.r.l., Italy based on ionic chromatography
144 with postcolumn ninhydrin derivatisation (fluorescence detection; UV)

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

148 2.6. Antinutritional Compounds

149 Antinutritional compounds in pasta samples were analysed as described by Hoehnel et al.²¹ for bread samples. In brief, trypsin inhibitors were ex-150 151 tracted from the freeze-dried pasta samples (raw and cooked) with a sodium acetate buffer (0.1 M, pH4.9). Trypsin inhibitor activity (TIA) was deter-152 mined following the method described by Joehnke et al.²³ with some modi-153 fications described by Hoehnel et al.²¹. For analysis of vicine and convicine, 154 155 freeze-dried pasta (raw and cooked) were extracted with boiling methanol according to procedure reported by Petersen et al.²⁴. Quantification was 156 achieved by micellar electrokinetic capillary chromatography (MCEC) with 157 vicine as external standard as described by Bjergegaard et al.²⁵. 158

159 2.7. In Vitro Protein Digestion

In vitro simulation of gastro-pancreatic protein digestion was performed 160 as described by Hoehnel et al.²¹ based on a previously reported static multi-161 step method to determine *in vitro* protein digestibility (IVPD)^{23,26}. In short, 162 163 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 164 165 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 166 at 1:50 (pepsin stage) and 1:10 (pancreatin stages). IVPD in % was 167

determined using a trinitrobenzenesulfonic acid (TNBS) assay. Results are
expressed as the concentration of free α-amino groups in the digested samples in relation to an alanine standard solution representing 100 % protein
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 experiments. In vivo nitrogen balance was investigated according to the 176 procedure described by Hoehnel et al.²¹. The experiment was performed 177 178 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 main protein source (supplemented with 0.2% DL-methionine), a second 181 182 control diet based on soya protein isolate (without any supplementation), a third control diet based on soya flour (without any supplementation); and 183 184 the experimental diets containing RWP and HPHP (Table 2). All experimental diets represent modifications of the AIN-93G diet for laboratory 185 186 rodents recommended by the American Institute of Nutrition²⁷ (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).

195 *2.9. Statistical Analysis*

196 All measurements were performed in triplicate unless stated otherwise. 197 Data analysis was performed using RStudio, version 1.2.1335 with R version 198 3.6.1 (RStudio Inc, USA; R Core Team, r-project). One-way analysis of 199 variance (ANOVA) with post-hoc pairwise Tukey's test was used to identify 200 significant differences (p < 0.05 unless stated otherwise). When available, 201 values are given as the mean \pm standard deviation or uncertainty (amino 202 acid profile).

203 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.

210 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

214 decreased carbohydrate content, amongst other minor differences to RWP, characterise the macronutrient profile of HPHP. The HPHP formulation 215 216 contains with 23.2 %DM approximately 6 %DM more protein than RWP 217 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 218 219 semolina by HPIs primarily causes a substitution of wheat starch by non-220 wheat protein. The determined energy contents are with 221 393.0 kcal/100 g DM (RWP) and 396.8 kcal/100 g DM (HPHP) very simi-222 lar in both formulations. Therefore, the compositional changes in HPHP 223 can additionally be considered an isocaloric replacement of wheat starch by 224 non-wheat protein. It has been reported that the isocaloric replacement of 225 dietary carbohydrate by protein in diets reduces blood lipid concentrations and blood pressure.²⁸ This effect was attributed to the reduced intake of 226 carbohydrate rather than the increased intake of protein.²⁸ However, the 227 metabolic effects of dietary carbohydrate are influenced by its glycaemic 228 index (GI).²⁹ Consequently, the isocaloric replacement of dietary carbohy-229 drate by protein might be more or less effective (with regard to blood lipid 230 231 concentrations, blood glucose or other metabolic conditions) depending on the GI of the replaced carbohydrate. Appel et al.²⁸ observed a positive in-232 fluence on blood lipid concentrations and blood pressure when medium GI 233 234 (between 68 and 75) carbohydrate was replaced by protein. Even though 235 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 236 its unique and dense structure, which seems to prevent or delay enzymatic 237 degradation of starch.^{13,14} While in other carbohydrate foods, like bread, the 238

239 incorporation of plant-based protein ingredients has been reported to significantly lower GI,³⁰ only small effects or no significant changes have been 240 reported for pasta.^{8,13,31} The HI values determined in this study (Table 3) 241 242 suggest a decreased starch digestibility of HPHP (86.0 %) compared to 243 RWP (100 %). This trend is also evident with regard to the sugar release 244 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 245 246 additionally a lower GL due to the significantly lower level of digestible 247 starch in HPHP than in RWP (Table 3). This suggests HPHP as favourable 248 pasta formulation compared to RWP, since diets with high GI and/or GL 249 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-250 wheat proteins in HPHP also brings the ratio of calories provided by protein 251 up to 23.4 % compared to the lower ratio of 17.6 % in RWP. This is par-252 ticularly important with regard to European legislation (regulation (EC)) 253 No $1924/2006^{35}$), where a protein level of at least 20 % of calories provided 254 255 by protein is specified as requirement for a 'high in protein' nutritional 256 claim made on foods. Besides non-wheat proteins, also a considerable 257 amount of non-wheat starch is brought into the HPHP formulation by 258 buckwheat flour; accounting for approx. 9.4 %DM (calculated based on 259 composition of raw materials and HPHP recipe). Buckwheat is known to 260 contain substantial amounts of resistant starch (RS), which has been linked to several health benefits and could further improve HPHP's nutritional 261 value.^{36,37} However, the contents of RS determined for RWP and HPHP are 262 263 both relatively low and HPHP contains with 0.56 %DM even slightly less

264 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 265 buckwheat groats.³⁶ Other minor compositional changes include the con-266 267 tents 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 268 considered a small difference and both formulations are low in fat (threshold 269 for 'low fat' nutritional claim in European legislation³⁸: 3 g of fat per 100 g 270 of solids). Furthermore, studies have shown that the fatty acid balance of 271 a diet is more critical than the total fat intake.³⁹ Although dietary recom-272 mendations refer to the whole diet, RWP's and HPHP's contribution to a 273 balanced fatty acid profile can be compared. A desired ratio of 274 SFA:MUFA:PUFA in a diet seems to approximate 1:1.3:1.³⁹ Both formula-275 tions contribute with 1:1.3:2.6 (RWP) and 1:1.4:2.3 (HPHP) similarly ele-276 vated amounts of PUFA to complement other low-PUFA diet components 277 (e.g. milk fat). The increased ash content of 1.4 % in HPHP (1.0 %DM in 278 RWP) is likely related to a higher concentration of minerals caused by the 279 incorporation of HPIs. Vogelsang-O'Dwyer et al.⁴⁰ determined the mineral 280 composition of the lupin protein isolate used in the present study and re-281 ported high levels of the nutritionally valuable minerals Ca, Fe and Zn. 282 Tazrart et al.⁸ and Petitot et al.¹² found substantially elevated levels of Ca, 283 284 Fe and Zn in pasta fortified with faba bean flour. The determined fibre 285 content for HPHP is with 3.8 %DM slightly lower than for RWP with 286 4.8 %DM. However, the analytical standard method applied in this study (AOAC 991.43) has been reported to not sufficiently quantitate food com-287 288 ponents 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.

296 *3.2. Amino Acid Profile*

297 Wheat based staple foods, like regular wheat pasta, represent an im-298 portant source of plant-based dietary protein. However, wheat protein has 299 low quality which is related to its unbalanced amino acid (AA) profile and 300 a lack of indispensable AA; specifically lysine.⁴³ Besides changes in the mac-301 ronutrient profile, the incorporation of pseudocereal and legume HPIs in 302 HPHP also introduces a shift in the AA pattern. The AA contents of RWP 303 and HPHP are presented in Table 4 and expressed in percent relative to 304 the formulations' protein content in order to allow for a direct comparison 305 of the AA profiles. Amongst the dispensable AAs, the contents of aspara-306 gine/aspartic acid, glutamine/glutamic acid, proline and arginine differ sub-307 stantially between RWP and HPHP. Wheat is rich in glutamine/glutamic 308 acid and proline but contains relatively small amounts of asparagine/aspartic acid and arginine.⁴³ Buckwheat, faba bean and lupin represent a com-309 plementary pattern with respect to these AAs.^{37,40,44} Therefore, reduced lev-310 els of glutamine/glutamic acid and proline and raised levels of aspara-311 312 gine/aspartic acid and arginine are present in HPHP. The profiles of

313 indispensable AAs in RWP and HPHP also exhibit several differences, spe-314 cifically with regard to threenine, tryptophan, lysine and sulphur-contain-315 ing AAs (SAAs). The incorporation of HPIs leads to a small increase in the 316 level of threenine 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} 317 The tryptophan level was below the limit of quantification (LOQ; equals 318 0.29 %Protein for RWP) and above the limit of determination (LOD: equals 319 0.58 %Protein for RWP) for RWP. For HPHP, a tryptophan content of 320 321 0.84 %Protein was determined. While wheat semolina, faba bean and lupin 322 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 323 324 HPHP due to the use of buckwheat HPI in addition to legume HPIs. Lysine 325 represents the indispensable AA with the biggest discrepancy between RWP and HPHP. The HPHP formulation contains 3.90 %Protein which 326 represents a by 34 % increased lysine level in comparison to RWP with 327 2.58 %Protein. In contrast to this increase observed for lysine, the results 328 329 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 330 HPHP. These differences in indispensable AAs might seem small when lev-331 332 els expressed in %Protein are compared. But some of these differences represent a significant improvement of the pasta's amino acid balance. This 333 334 becomes apparent when AA levels are expressed relative to a reference pattern of AA intake for adults recommended by WHO⁴⁶ (Figure 3). The com-335 parison of the AAs in RWP and HPHP with the reference pattern shows 336 337 that lysine does not reach the recommended level (= 1). Thus, lysine

338 represents the limiting AA in the protein from both formulations. However, the increased lysine content in HPHP reaches 87~% of the recommended 339 level as opposed to 57 % in RWP. Furthermore, the incorporation of buck-340 341 wheat flour secures an adequate level of tryptophan in HPHP by raising its 342 content to 140 % of the required amount. This makes the HPHP AA profile, which nearly covers the recommended intake of all indispensable AAs, much 343 344 more balanced. Amino acid scores (AASs) represent the content of the limiting AA of a protein calculated relative to the reference pattern and are 345 346 commonly used to interpret protein quality. Table 5 summarises the AASs 347 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 348 349 protein isolate for partial wheat semolina substitution leads to an upgrade 350 of the protein quality of all protein sources. Only buckwheat flour represents an exception and possesses a desirable AA profile on its own. The 351 partial substitution of wheat semolina by legumes has previously been re-352 353 ported to improve the AA profile of pasta protein; specifically through increased lysine levels.⁹ However, an optimal substitution ratio (leg-354 ume:wheat) has been discussed with regard to SAAs.⁹ While lysine levels 355 rise with increased legumes: wheat ratios, SAA contents drop; due to low 356 357 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 358 SAAs in buckwheat which even exceeds that of wheat semolina.^{43,45} The use 359 360 of AASs to evaluate protein quality is based on the assumption that the 361 considered daily intake of 0.66 g protein per kg bodyweight is entirely cov-362 ered 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.

367 3.3. Antinutritional Compounds

The activity/contents of trypsin inhibitors and the pyrimidine glycosides 368 369 vicine and convicine have been determined for both raw and cooked RWP 370 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 371 372 complexes with the intestinal enzyme trypsin (or chymotrypsin) thereby 373 inhibiting their proteolytic activity and potentially causing a decrease in 374 protein digestibility.¹⁵ They have also been reported to cause other adverse 375 physical conditions like pancreatic hypertrophy and increased pancreatic secretory activity.⁴⁷ The TIAs determined for raw and cooked HPHP were 376 377 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,⁴⁰ 378 the HPIs from faba bean and buckwheat are likely to cause the higher TIA 379 in HPHP. Vogelsang-O'Dwyer et al.⁴⁴ found a TIA of 2.34 TIU/mg (based 380 on dry matter) in the faba bean flour applied in HPHP. Also the low di-381 382 gestibility of buckwheat protein has been associated with a high trypsin inhibitor activity.^{5,48} The results in the present study additionally show that 383 384 the cooking process leads to a substantial decrease in TIA in both RWP 385 and HPHP formulation. A very low TIA of 0.38 TIU/mg was measured for 386 raw RWP and no TIA was detected in cooked RWP. For HPHP, the

387 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 ar-388 ticles have addressed the impact of various processing techniques on TIA 389 in foods.^{16,47,49} Thermal treatments such as boiling, cooking and roasting 390 391 have been mentioned as the most efficient procedures to inactivate legume 392 trypsin inhibitors. The mechanism of this inactivation primarily relies on conformational changes of their active site which prevents complex for-393 394 mation with and inhibition of trypsin. Soybean trypsin inhibitors, for ex-395 ample, 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. 396 397 The achieved reduction of TIA is in agreement with results reported in 398 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 spa-399 400 ghetti fortified with 15-20 % pea and lentil flour. The small remaining TIA 401 in this study could be related to the lack of a heat drying process (which is 402 performed in most studies investigating pasta quality) after extrusion of 403 RWP and HPHP which could contribute to trypsin inhibitor inactivation. 404 Furthermore, trypsin inhibitors originating from buckwheat seeds were 405 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 406 contain the thermostable ANCs vicine and convicine.⁵¹ These pyrimidine 407 408 glycosides can trigger an adverse physical condition called favism in human individuals that are deficient in glucose-6-phosphate dehydrogenase 409 (G6PD).¹⁸ Since triggered favism leads to acute haemolytic anaemia in 410 G6PD deficient consumers,¹⁸ it is important to monitor vicine and convicine 411

412 levels in faba bean containing foods. Recent efforts in plant breeding with 413 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 414 415 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 de-416 417 tected, 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 418 findings of Vogelsang-O'Dwyer et al.⁴⁴ who reported a VC content of 419 420 1.25 %DM in the faba bean flour applied in HPHP (expected amount of 421 VC in HPHP approximates 0.50 %DM based on calculation considering recipe and ingredient composition). Cooked HPHP contains only 422 423 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 424 425 procedure. Besides enzyme treatment, also aqueous extraction at elevated temperatures, has been mentioned as efficient technique to eliminate or 426 reduce vicine and convicine contents in faba beans.^{17,51,52} A recent study by 427 Gallo et al.⁵³ investigated the consumption of large quantities of faba beans 428 (500 g) with a low VC content of 0.016 % (in wet weight as ingested) by 429 G6PD deficient men. Favism was not triggered and the results suggested 430 431 that this level of VC intake is safe for G6PD deficient individuals. In the 432 context of these findings, also the consumption of approx. 300 g cooked 433 HPHP (exceeds the daily intake of cooked pasta recommended by dietary guidelines of European countries⁵⁴) can be considered safe. While the results 434 435 in the present study confirm a substantial reduction of TIA and VC content 436 due to cooking of pasta, also the dilution of both ANCs due to incorporation

437 of the HPIs in a complex pasta formulation should be addressed. The sep438 arate consumption of protein from sources with complementary AA pat439 terns (cereals, pseudocereals, legumes) can in theory provide a balanced AA
440 intake. However, higher ANC levels in raw materials, as opposed to hybrid
441 formulations like HPHP, can impair protein digestibility and, therefore,
442 bioavailability of AA from some of these sources.

443 3.4. Protein Digestibility and Utilisation

In vitro digestion was performed with raw and cooked pasta in order to 444 evaluate the effect of the cooking procedure on the samples' susceptibility 445 446 to degradation by pepsin and pancreatin. The results are presented in Ta-447 ble 7. The pepsin treatment and subsequent short term treatment with 448 pancreatin mimics the protein digestion of RWP and HPHP in the human 449 gastrointestinal tract. Medium and long term treatment with pancreatin were carried out in order to obtain an indication of the maximum achievable 450 451 protein degradation under the same conditions. Two major trends were observed. For pepsin treatment as well as short and medium term pancreatin 452 453 treatment of both formulations, raw pasta reached significantly (except 454 RWP after medium term pancreatin treatment) higher IVPDs than cooked 455 pasta. This trend was clearly detected even for the HPHP formulation, 456 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 457 protein digestibility. Petitot et al.¹⁴ who reviewed the impact of pasta pro-458 459 cessing on starch and protein digestibility concluded that thermally induced 460 protein aggregation could increase resistance to pepsin/pancreatin

461 degradation of protein in cooked pasta. It is also possible that, similar to 462 observations reported for *in vitro* starch digestion^{13,14}, the pasta structure created during the cooking process (coagulated protein network entrapping 463 464 starch granules) slows down enzymatic degradation of proteins. The fact 465 that IVPDs of cooked pasta after long term pancreatin treatment are not 466 smaller than for raw pasta further supports this theory. According to these results, changes in structure and substrate accessibility induced by the 467 468 cooking procedure seem to have a larger impact on protein digestibility in 469 HPHP than TIA. Furthermore, the difference in IVPD between raw and cooked pasta seems to be more pronounced for HPHP than for RWP. This 470 is in line with the previous findings of Hoehnel et al.¹⁹. Their results of 471 472 technological quality analysis of RWP and HPHP suggested a thicker protein network in HPHP than RWP; and a strengthened protein network in 473 474 HPHP through interactions between wheat and non-wheat proteins. The second clear trend with regard to IVPDs concerns the difference between 475 476 RWP and HPHP. Both raw and cooked HPHP reach higher IVPDs for all 477 digestive treatments (except long term pancreatin treatment) than their 478 RWP equivalent. Again, higher IVPDs were observed despite higher de-479 tected TIAs for these samples. This indicates that TIA levels might have been too low to affect enzymatic protein degradation. Also Laleg et al.⁵⁵ 480 and Tazrart et al.⁸ found slightly increased protein digestibility of pasta 481 482 formulations fortified with legume ingredients when compared to wheat 483 semolina controls. A higher protein digestibility in formulations containing 484 pseudocereal and legume ingredients could be related to a higher abundance 485 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 486 487 in vivo nitrogen balance trials performed with rats are presented in Table 7. 488 The most important variables are N intake (which is used to calculate rel-489 ative N losses in urine and faeces) as well as N digestibility, N utilisation and PER, which are indicative of the pastas' nutritional value. No signifi-490 491 cant 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 492 493 RWP and HPHP in the experimental diets was adjusted so they contained 494 the same amount of protein, this indicates that similar amounts of total 495 diet were consumed by RWP and HPHP rats. The values determined for N digestibility were not significantly different between RWP (88.0%) and 496 497 HPHP (88.4 %). In vitro digestion, however, indicated higher digestibility of HPHP. According to literature, in vitro and in vivo digestibility data 498 usually show good correlations; but it has also been reported that legumes 499 tend to reach higher digestibility in vitro than in vivo.⁵⁶ The protein digest-500 501 ibility corrected amino acid score (PDCAAS) represents another commonly 502 used indicator of protein quality and is calculated considering faecal N di-503 gestibility and AASs (discussed in amino acid section above). Due to the similar N digestibility of RWP and HPHP, PDCAAS values follow the same 504 trend as AAS values. While RWP has a PDCAAS of only 0.50, HPHP 505 reaches a value of 0.79 owing to its higher lysine level. The significantly 506 507 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 %). 508 This is likely related to the improved AA balance of HPHP. The lack of 509 510 one or more indispensable AAs, amongst absorbed AAs, has been reported

511 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 512 urine after oxidisation.^{46,57} Furthermore, a positive correlation between bal-513 514 ance of dietary AAs and AA utilisation was found in both animal and hu-515 man 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 516 determined for rats with HPHP diet (2.13 g/g) was significantly higher 517 than that determined for RWP rats (1.37 g/g). The PER reflects a protein's 518 519 value with respect to AA requirements for growth. Both in vitro and in vivo models to determine protein quality for human nutrition have their 520 521 limitations and true protein quality depends on the AA profile which is absorbed and utilised to achieve specific metabolic actions in the human 522 body.^{57,59} However, AA profile and values like IVPD, N digestibility, N uti-523 lisation rate and PER provide a valid comparison of protein from RWP and 524 525 HPHP and conclusively indicate improved protein quality in HPHP.

526 4. CONCLUSION

527 The present study evaluates the nutritional profile of a high-protein hybrid pasta (HPHP) formulation. In this formulation, a combination of three 528 529 HPIs from buckwheat, faba bean and lupin was used to partially substitute 530 wheat semolina. The nutritional value of the pasta was assessed in compar-531 ison to regular wheat pasta as a reference with a focus on protein quality. 532 The results confirm that HPHP represents a pasta formulation with an 533 overall enhanced nutritional profile. The improved macronutrient composi-534 tion is primarily characterised by an isocaloric replacement of dietary

535 carbohydrate by plant-based protein. With regard to ANCs brought into 536 the pasta formulation by pseudocereal and legume HPIs, the results show 537 that the cooking procedure realises a substantial reduction of trypsin inhib-538 itor activity and contents of vicine and convicine. The small remaining 539 activity/contents seem to not adversely affect nutritional quality of HPHP. 540 All measures indicative of protein quality determined in this study (AA composition, IVPD, N digestibility, N utilisation, PER) conclusively sug-541 542 gest improved protein quality of HPHP compared to RWP. The results also 543 indicate that specifically the combination of pseudocereal and legume HPIs 544 to replace wheat semolina is beneficial to achieve a balanced AA profile. In 545 addition to its enhanced nutritional profile, HPHP has been shown to pos-546 sess technological and sensory quality similar to RWP by Hoehnel et al.¹⁹ which identifies HPHP as an attractive alternative to regular wheat pasta 547 in currently consumed diets. Furthermore, HPHP and formulations of its 548 kind represent an increased potential of wheat based staple foods to con-549 550 tribute to a sufficient intake of high-quality protein in future predominantly 551 plant-based diets.

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559 ABBREVIATIONS

560 5612 5623 5664 5667 5667 5575 5775 5776 5778 5778 5780 5781 5780 5781 5780 5782 5780 5782 5785 5780 5782 5782 5785 5782 5785 5782 5785 5782 5785 5785	ANC HPI HPHP RWP %DM C SPI SF %E OCT HI DNS TIA MCEC IVPD TNBS BW PER ANOVA proteinE GI GL SFA	Antinutritional compound High-protein ingredient High-protein hybrid pasta Reference wheat pasta Percentage based on dry matter Casein Soya protein isolate Soya flour Percentage based on energy Optimal cooking time Hydrolysis index 3,5-Dinitrosalicylic acid Trypsin inhibitor activity Micellar electrokinetic capillary chromatography In vitro protein digestibility Trinitrobenzenesulfonic acid Body weight Protein efficiency ratio Analysis of variance Percentage of calories provided by protein Glycaemic index Glycaemic load Saturated fatty acids
578	ANOVA	Analysis of variance
579	proteinE	Percentage of calories provided by protein
580	GI	Glycaemic index
581	GL	Glycaemic load
582	SFA	Saturated fatty acids
583	MUFA	Monounsaturated fatty acids
584	PUFA	Polyunsaturated fatty acids
585	GOS	Galactooligosaccharides
586	FODMAP	Fermentable oligo-, di-, mono-saccharides and polyols
587	%Protein	Percentage based on protein
	AA	Amino acid
509	SAA	Sulphur containing amino acids
501	AAA	Aromatic amino acids
502	LOQ	Limit of datamaination
592		Amino acid score
594	TIU	Trypsin inhibitor unit
595	G6PD	Chicose-6-phosphate dehydrogenase
596	VC	Sum of vicine and convicine
597	PDCAAS	Protein digestibility corrected amino acid score
551	I DOARD	i rotoni digestionity corrected annio acid score

598 REFERENCES

- 599 1 Cecchini C, Menesatti P, Antonucci F, Costa C. Trends in research on durum wheat and pasta, a bibliometric mapping approach. Cereal Chem. 97(3):581–8 (2020).
- Jenkins DJA, Kendall CWC, Marchie A, Augustin LSA. Too Much Sugar, Too Much Carbohydrate, or
 just Too Much? Am J Clin Nutr. 79(5):711–2 (2004).
- 603 3 Mercier S, Moresoli C, Mondor M, Villeneuve S, Marcos B. A Meta-Analysis of Enriched Pasta: What
 604 Are the Effects of Enrichment and Process Specifications on the Quality Attributes of Pasta? Compr Rev
 605 Food Sci Food Saf. 15(4):685–704 (2016).
 - 27

- 606 4 Bustos MC, Perez GT, Leon AE. Structure and quality of pasta enriched with functional ingredients.
 607 RSC Adv. 5:30780–92 (2015).
- Mir NA, Riar CS, Singh S. Nutritional constituents of pseudo cereals and their potential use in food
 systems: A review. *Trends Food Sci Technol.* **75**:170–80 (2018).
- 6 Ranganathan J, Vennard D, Waite R, Searchinger T, Dumas P, Lipinski B. Shifting Diets: Toward a
 611 Sustainable Food Future. In: 2016 Global Food Policy Report. Washington, D.C.: International Food
 612 Policy Research Institute (IFPRI); p. 66–792016.
- 613 7 Biney K, Beta T. Phenolic profile and carbohydrate digestibility of durum spaghetti enriched with buck614 wheat flour and bran. LWT Food Sci Technol. 57:569–79 (2014).
- 615 8 Tazrart K, Lamacchia C, Zaidi F, Haros M. Nutrient composition and *in vitro* digestibility of fresh pasta
 616 enriched with Vicia faba. J Food Compos Anal. 47:8–15 (2016).
- 617 9 Giménez MA, Drago SR, De Greef D, Gonzalez RJ, Lobo MO, Samman NC. Rheological, functional and nutritional properties of wheat/broad bean (*Vicia faba*) flour blends for pasta formulation. *Food Chem.*619 134:200-6 (2012).
- Laleg K, Cassan D, Barron C, Prabhasankar P, Micard V. Structural, Culinary, Nutritional and Anti nutritional Properties of High Protein, Gluten Free, 100% Legume Pasta. *PLOS One.* 11(9):1–19 (2016).
- by Green and Yellow Pea, Lentil, and Chickpea Flours. J Food Sci. 70(6):S371-6 (2005).
- Petitot M, Boyer L, Minier C, Micard V. Fortification of pasta with split pea and faba bean flours: Pasta processing and quality evaluation. *Food Res Int.* 43:634–41 (2010).
- 626 13 Petitot M, Barron C, Morel MH, Micard V. Impact of Legume Flour Addition on Pasta Structure:
 627 Consequences on Its *In Vitro* Starch Digestibility. *Food Biophys.* 5:284–99 (2010).
- 628 14 Petitot M, Abecassis J, Micard V. Structuring of Pasta Components during Processing: Impact on Starch
 629 and Protein Digestibility and Allergenicity. *Trends Food Sci Technol.* 20(11–12):521–32 (2009).
- 630 15 Gilani GS, Xiao CW, Cockell KA. Impact of Antinutritional Factors in Food Proteins on the Digestibility
 631 of Protein and the Bioavailability of Amino Acids and on Protein Quality. *Br J Nutr.* 108:S315–32
 632 (2012).
- 633 16 Patterson CA, Curran J, Der T. Effect of Processing on Antinutrient Compounds in Pulses. *Cereal Chem.*634 94(1):1–10 (2017).
- 635 17 Khamassi K, Ben Jeddi F, Hobbs D, Irigoyen J, Stoddard F, O'Sullivan DM, et al. A baseline study of
 636 vicine-convicine levels in faba bean (*Vicia faba* L.) germplasm. *Plant Genet Resour.* 11(3):250–7 (2013).
 - 28

- 637 18 Luzzatto L, Arese P. Favism and glucose-6-phosphate dehydrogenase deficiency. N Engl J Med.
 638 378(1):60-71 (2018).
- 639 19 Hoehnel A, Bez J, Amarowicz R, Arendt EK, Zannini E. Combining high-protein ingredients from pseudocereals and legumes for the dedvelopment of high-protein hybrid pasta: Maintained technological quality and adequate sensory attributes; accepted for publication in. J Sci Food Agric. (2020).
- 642 20 Hoehnel A, Axel C, Bez J, Arendt EK, Zannini E. Comparative Analysis of Plant-Based High-Protein
 643 Ingredients and Their Impact on Quality of High-Protein Bread. J Cereal Sci. 89 (2019).
- 644 21 Hoehnel A, Bez J, Petersen IL, Amarowicz R, Juśkiewicz J, Arendt EK, et al. Enhancing the nutritional
 645 profile of regular wheat bread while maintaining technological quality and adequate sensory attributes.
 646 Food Funct. 11:4732–51 (2020).
- 647 22 Brennan CS, Tudorica CM. Evaluation of Potential Mechanisms by Which Dietary Fibre Additions
 648 Reduce the Predicted Glycaemic Index of Fresh Pastas. Int J Food Sci Technol. 43(12):2151–62 (2008).
- 649 23 Joehnke MS, Rehder A, Sørensen S, Bjergegaard C, Sørensen JC, Markedal KE. In Vitro Digestibility of
 650 Rapeseed and Bovine Whey Protein Mixtures. J Agric Food Chem. 66(3):711–9 (2018).
- 651 24 Petersen IL, Hansen HCB, Ravn HW, Sørensen JC, Sørensen H. Metabolic effects in rapeseed (*Brassica napus* L.) seedlings after root exposure to glyphosate. *Pestic Biochem Physiol.* 89(3):220–9 (2007).
- 653 25 Bjergegaard C, Simonsen H, Sørensen H. Determination of heterocyclic compounds by micellar electro654 kinetic capillary chromatography. J Chromatogr A. 680(2):561–9 (1994).
- 655 26 Joehnke MS, Lametsch R, Sørensen JC. Improved in vitro digestibility of rapeseed napin proteins in mixtures with bovine beta-lactoglobulin. *Food Res Int.* 123:346–54 (2019).
- 657 27 Reeves PG. Components of the AIN-93 Diets as Improvements in the AIN-76A Diet. Symposium: Animal
 658 Diets for Nutritional and Toxicological Research. 127:838S-841S (1997).
- Appel LJ, Sacks FM, Carey VJ, Obarzanek E, Swain JF, Miller ER, et al. Effects of protein, monoun-saturated fat, and carbohydrate intake on blood pressure and serum lipids: Results of the OmniHeart randomized trial. J Am Med Assoc. 294(19):2455–64 (2005).
- 29 Ludwig DS. The Glycemic Index: Physiological Mechanisms Relating to Obesity, Diabetes, and Cardiovascular Disease. J Am Med Assoc. 287(18):2414–23 (2002).
- 664 30 Hall RS, Thomas SJ, Johnson SK. Australian sweet lupin flour addition reduces the glycaemic index of
 665 a white bread breakfast without affecting palatability in healthy human volunteers. Asia Pac J Clin
 666 Nutr. 14(1):91–7 (2005).
- 667 31 Greffeuille V, Marsset-Baglieri A, Molinari N, Cassan D, Sutra T, Avignon A, et al. Enrichment of pasta
 - 29

- with faba bean does not impact glycemic or insulin response but can enhance satiety feeling and digestive
 comfort when dried at very high temperature. *Food Funct.* 6(9):2996–3005 (2015).
- 670 32 Salméron J, Manson JE, Stampfer MJ, Colditz GA, Wing AL, Willett WC. Dietary Fiber, Glycemic
 671 Load, and Risk of Non-insulin-Dependent Diabetes Mellitus in Women. J Am Med Assoc. 277(6):472–7
 672 (1997).
- 673 33 Liu S, Willett WC, Stampfer MJ, Hu FB, Franz M, Sampson L, et al. A Prospective Study of Dietary
 674 Glycemic Load, Carbohydrate Intake, and Risk of Coronary Heart Disease in US Women. Am J Clin
 675 Nutr. 71:1455–61 (2000).
- 676 34 Franceschi S, Dal Maso L, Augustin L, Negri E, Parpinel M, Boyle P, et al. Dietary Glycemic Load and
 677 Colorectal Cancer Risk. Ann Oncol. 12:173–8 (2001).
- 678 35 European Parliament & Council. Regulation (EC) No 1924/2006 of the European Parliament and of the
 679 Council on Nutrition and Health Claims Made on Foods. 2006.
- 680 36 Skrabanja V, Laerke HN, Kreft I. Effects of hydrothermal processing of buckwheat (*Fagopyrum esculen-tum* Moench) groats on starch enzymatic availability in vitro and in vivo in rats. *J Cereal Sci.* 28(2):209–
 682 14 (1998).
- 683 37 Christa K, Soral-Śmietana M. Buckwheat grains and buckwheat products Nutritional and prophylactic
 684 value of their components A review. *Czech J Food Sci.* 26(3):153–62 (2008).
- 585 38 European Parliament & Council. Regulation (EC) No 1924/2006 on nutrition and health claims made on foods. Off J Eur Communities. L404(1924):1–15 (2006).
- 687 39 Hayes KC. Dietary fat and heart health: in search of the ideal fat. Asia Pac J Clin Nutr. 11 Suppl
 688 7:394-400 (2002).
- 689 40 Vogelsang-O'Dwyer M, Bez J, Petersen IL, Joehnke MS, Detzel A, Busch M, et al. Techno-Functional,
 690 Nutritional and Environmental Performance of Protein Isolates from Blue Lupin and White Lupin. *Foods.*691 9(230):1–24 (2020).
- 41 McCleary B V., DeVries JW, Rader JI, Cohen G, Prosky L, Mugford DC, et al. Determination of insoluble, soluble, and total dietary fiber (CODEX definition) by enzymatic-gravimetric method and liquid
 chromatography: Collaborative study. J AOAC Int. 95(3):824–44 (2012).
- 42 Ispiryan L, Zannini E, Arendt EK. Characterization of the FODMAP-profile in cereal-product ingredi696 ents. J Cereal Sci. 92:1–10 (2020).

^{697 43} Sosulski FW, Imafidon GI. Amino Acid Composition and Nitrogen-to-Protein Conversion Factors for
698 Animal and Plant Foods. J Agric Food Chem. 38(6):1351–6 (1990).

- 44 Vogelsang-O'Dwyer M, Bez J, Petersen IL, Joehnke MS, Sørensen JC, Detzel A, et al. Comparison of
 Faba Bean Protein Ingredients Produced Using Dry Fractionation and Isoelectric Precipitation: TechnoFunctional, Nutritional and Environmental Performance. *Foods.* 9:1–25 (2020).
- 702 45 Tömösközi S, Langó B. Buckwheat: Its Unique Nutritional and Health-Promoting Attributes. In: Taylor
 703 JRN, Awika JM, editors. Gluten-Free Ancient Grains: Cereals, Pseudocereals, and Legumes: Sustainable,
 704 Nutritious, and Health-Promoting Foods for the 21st Century. Woodhead Publishing, Elsevier Ltd; p.
 705 161–772017.
- 46 Joint FAO/WHO/UNU Expert Consultation. Protein and Amino Acid Requirements in Human Nutri tion. WHO Technical Report Series. 935:1–265 (2007).
- 708 47 Vidal-Valverde C, Frias J, Diaz-Pollan C, Fernandez M, Lopez-Jurado M, Urbano G. Influence of Processing on Trypsin Inhibitor Activity of Faba Beans and Its Physiological Effect. J Agric Food Chem.
 710 45(9):3559–64 (1997).
- 711 48 Ikeda K, Sakaguchi T, Kusano T, Yasumoto K. Endogenous factors affecting protein digestibility in buckwheat. Cereal Chem. 68(4):424–7 (1991).
- Avilés-Gaxiola S, Chuck-Hernández C, Serna Saldívar SO. Inactivation Methods of Trypsin Inhibitor in Legumes: A Review. J Food Sci. 83(1):17–29 (2018).
- 715 50 Tsybina TA, Dunaevsky YE, Musolyamov AK, Egorov TA, Belozersky MA. Cationic inhibitors of serine
 716 proteinases from buckwheat seeds. *Biokhimiya*. 66(9):1157–64 (2001).
- 51 Khazaei H, Purves RW, Hughes J, Link W, O'Sullivan DM, Schulman AH, et al. Eliminating vicine and convicine, the main anti-nutritional factors restricting faba bean usage. *Trends Food Sci Technol.* 91:549–56 (2019).
- 52 Marquardt RR, Muduuli DS, Frohlich AA. Purification and Some Properties of Vicine and Convicine
 T21 Isolated from Faba Bean (*Vicia faba* L.) Protein Concentrate. J Agric Food Chem. 31(4):839–44 (1983).
- 722 53 Gallo V, Skorokhod OA, Simula LF, Marrocco T, Tambini E, Schwarzer E, et al. G6PD-deficient subjects
 723 after ingestion of low No red blood cell damage and no hemolysis in vicine/convicine Vicia faba seeds.
 724 Blood. 131(14):1617-21 (2018).
- Food-Based Dietary Guidelines in Europe [Internet]. Official website of the European Union. p. last
 updated: 01/02/2020 [cited 2020 Jun 5]. Available from: https://ec.europa.eu/jrc/en/health-knowledge gateway/promotion-prevention/nutrition/food-based-dietary-guidelines
- 55 Laleg K, Barron C, Cordelle S, Schlich P, Walrand S, Micard V. How the structure, nutritional and
 sensory attributes of pasta made from legume flour is affected by the proportion of legume protein. *LWT Food Sci Technol.* 79:471–8 (2017).
 - 31

- 731 56 Boye J, Wijesinha-Bettoni R, Burlingame B. Protein quality evaluation twenty years after the introduction of the protein digestibility corrected amino acid score method. *Br J Nutr.* 108(SUPPL. 2):S183–211
 733 (2012).
- 734 57 Millward DJ, Layman DK, Tomé D, Schaafsma G. Protein quality assessment: Impact of expanding
 735 understanding of protein and amino acid needs for optimal health. Am J Clin Nutr. 87(suppl)(5):1576S736 1581S (2008).
- 737 58 Ha E, Zemel MB. Functional properties of whey, whey components, and essential amino acids: Mechanisms underlying health benefits for active people (Review). J Nutr Biochem. 14(5):251–8 (2003).
- 59 Butts CA, Monro JA, Moughan PJ. *In vitro* determination of dietary protein and amino acid digestibility
 for humans. *Br J Nutr.* 108(SUPPL. 2):282–7 (2012).

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Figure Legends

Figure 1: Photographs of RWP and HPHP (as reported by Hoehnel et al.¹⁹): \mathbf{A} – raw pasta; \mathbf{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.

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

Component of diet	С	SPI	\mathbf{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

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

 1 AIN-93G-MX: mineral mixture as specified by $\rm Reeves^{27}$

² AIN-93G-VX: vitamin mixture as specified by Reeves²⁷

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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
$\text{proteinE}^{\dagger}$ [%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

 Table 3: Composition of reference wheat pasta (cooked) and high-protein hybrid pasta (cooked);

 contents expressed in %DM unless stated otherwise

Moisture and total, digestible and resistant starch: means \pm 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)

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	$7.36~\pm~0.89$
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	$3.10~\pm~0.38$	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

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

Amino acid contents \pm 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)

37

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
$\operatorname{Semolina}^{\dagger}$	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 a mino acid composition; determined as for RWP and HPHP and reported by Vogels ang-O'Dwyer et al. 40,44

 $^{\$}$ Not strictly limiting (\geq 1), but represents AA with lowest level relative to reference pattern

38

Table 6: Contents of antinutritional compounds of reference wheat pasta and high-protein hybrid

 bread, contents refer to dry matter as indicated

	RWP		HP	HP
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 \pm standard deviation with different letters in the same row were significantly different at p < 0.05 n.d. – not detected

39

Table 7	7: In	vitro	digestibility	and in	vivo	nitrogen	balance
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	RWP		HF	PHP		
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)	$22.0 \pm 1.4^{\circ}$	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 \pm standard deviation with different letters in the same row were significantly different at p < 0.05

Figures



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

742



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.