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# Enhancing the nutritional profile of regular wheat bread while maintaining technological quality and adequate sensory attributes†

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Plant proteins, and legume proteins in particular, have become the centre of attention moving towards a more sustainable and, therefore, more plant-based human diet. Especially hybrid products, containing wheat and legume proteins, promise a balanced amino acid composition and an upgraded nutritional value of both protein sources. This study investigates a high-protein hybrid bread (HPHB) formulation, where wheat flour was partially replaced by high-protein ingredients from faba bean, carob and gluten. In addition to a detailed characterisation of technological quality and sensory profile, also the formulation's nutritional value was examined in comparison to regular wheat bread. Therefore, macronutrient composition, antioxidant potential, amino acid profile and contents of antinutritional compounds were analysed. Furthermore, protein digestibility was determined in an *in vitro* model and *in vivo*. Dough analysis revealed significant differences of the HPHB formulation compared to regular wheat dough. However, results obtained for bread quality characteristics prove HPHB to be equal to regular wheat bread and sensory results and the determined sensory attributes suggest high consumer acceptance. Nutritional analyses of HPHB showed a more favourable macronutrient composition in comparison to regular wheat bread; as well as low contents of antinutritional compounds and high antioxidant potential linked to high levels of phenolics. Also an improved amino acid profile, increased nitrogen utilisation rate (by 69 %) and higher protein efficiency ratio were determined, which are associated with enhanced protein quality. This suggests HPHB, and similar formulations of its kind, as a valuable and healthy food choice, which can contribute to adequate protein supply in predominantly plant-based diets.

## 1 Introduction

Protein from plant sources, next to other trends like digestive health and good carbs/bad carbs, is currently one of the most popular and important trends in the food sector.<sup>1</sup> One of the reasons for that is an increasing awareness amongst consumers, authorities and industry of the need to create a more sustainable food system considering planetary boundaries.<sup>2,3</sup> According to many

recent reports, this requires a shift to a predominantly plant-based human diet.<sup>2,4</sup> Since we are also facing a growing world population, with a prospect of about 10 billion by 2050,<sup>2</sup> research plays a key role in finding ways to provide high-quality protein from plant sources to cover future protein needs. Even though it is known that current protein consumption exceeds the average daily requirement in many parts of the world, this is usually linked to high intakes of animal protein and necessary changes in the food system and human diet are likely to pose a challenge to sufficient protein supply in the future.<sup>2,4</sup> In many cases, the overconsumption of protein is associated with a general overconsumption of food and energy intakes exceeding recommended levels<sup>4</sup> and does not reflect an overconsumption of protein relative to other macronutrients. Furthermore, recommendations for daily protein intakes are based on high-quality protein. When large amounts of protein of lower quality are consumed, intakes might need to be increased in order to meet the body's amino acid requirements.<sup>5</sup> Apart from sustainability considerations, dietary

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27 recommendations advice a reduction of animal protein intake  
28 in favour of increased plant protein consumption for a healthy  
29 diet. Many reported adverse effects of high protein intake are  
30 largely related to proteins from animal sources and the co-intake  
31 of sodium, nitrate, nitrite and saturated fatty acids when red meat  
32 or dairy products are consumed.<sup>2,4,6</sup> Also an overconsumption of  
33 food carbohydrates, especially refined carbohydrates, has been  
34 reported for a large number of countries and associated with in-  
35 creased health risks.<sup>7</sup> Research concerning new alternative plant  
36 protein sources is mostly focused on legumes. Due to their abil-  
37 ity to grow in a variety of different climates and to fix nitrogen  
38 in the soil, they are particularly promising for a local crop culti-  
39 vation, a considerably reduced use of fertilisers and a food pro-  
40 duction with a lower carbon and water footprint.<sup>3,8,9</sup> Legumes  
41 are naturally rich in protein, which contains high amounts of  
42 the essential amino acid (AA) lysine but lacks sulphur-containing  
43 amino acids (SAAs).<sup>8,10,11</sup> This makes legumes particularly inter-  
44 esting for the complementation of cereal based diets, since cereals  
45 have little lysine and higher amounts of SAAs.<sup>12,13</sup> Efforts have  
46 been made to combine both protein sources in "hybrid products"  
47 containing cereals and legumes and especially wheat bread has  
48 proven a suitable cereal matrix for the incorporation of legume  
49 protein ingredients.<sup>14</sup> Ideal bread should have a lower glycaemic  
50 index than regular white bread, be an important source of pro-  
51 teins, and contain tolerated dietary fibre, vitamins, magnesium,  
52 trace elements and antioxidants.<sup>15,16</sup> Jenkins *et al.*<sup>7</sup> state that,  
53 in the context of decreased physical activity in our population,  
54 foods should possess nutritional density rather than nutrient den-  
55 sity. This means that the intake of essential nutrients (macro and  
56 micro) per calorie will need to increase in order to meet require-  
57 ments at lower caloric intake levels. Legumes are rich in micronu-  
58 trients and compounds with antioxidant activity, which could help  
59 to enhance the nutritional value of wheat bread.<sup>14,17,18</sup> Also a  
60 lowered glycaemic load, increased protein content and improved  
61 protein quality could be achieved by the fortification of wheat  
62 bread with legume proteins. Numerous research articles have in-  
63 vestigated the effects of legume ingredients, from faba bean (*Vicia faba*)  
64 and carob (*Ceratonia siliqua*) seeds in particular, on both  
65 the technological as well as nutritional quality of breads.<sup>14,19-23</sup>  
66 However, many of these publications report inferior technologi-  
67 cal and sensory characteristics in favour of increased nutritional  
68 quality. Additionally, there are concerns regarding antinutritional  
69 compounds (ANCs) originating from legumes such as trypsin in-  
70 hibitors, tannins, lectins and the pyrimidine glycosides vicine and  
71 convicine. Trypsin inhibitors, which can negatively impact pro-  
72 tein digestibility, are present in many plants but are particularly  
73 important in legumes.<sup>24,25</sup> Vicine and convicine are mainly found  
74 in faba beans and can trigger adverse physical conditions like fav-  
75 ism.<sup>26-28</sup> This leads to a low popularity of legume ingredients  
76 and cereal/legume hybrid products.<sup>29</sup> Next to an enhanced nutri-  
77 tional value, adequate technological quality and sensory proper-  
78 ties are essential for a high consumer acceptance of such products  
79 and for an acceleration of the protein transition in our diet. This  
80 is why this study proposes and fully characterises a new bread  
81 formulation, which was designed to match the technological qual-  
82 ity of regular wheat bread, but promises an improved nutritional

83 profile with a higher protein content and higher protein quality in  
84 particular. Therefore, plant-based high-protein ingredients (HPIs)  
85 from faba beans, carob and wheat, selected based on findings by  
86 Hoehnel *et al.*<sup>30</sup>, were incorporated in a regular wheat bread.  
87 This high-protein hybrid bread (HPHB) formulation was evalu-  
88 ated regarding technological, nutritional and sensory characteris-  
89 tics using regular wheat bread as a reference (RWB). The HPHB  
90 formulation, containing a dry-processed faba bean HPI as its main  
91 source of non-wheat protein, also promises improved sustain-  
92 ability;<sup>31</sup> especially when compared to other high-protein bread  
93 formulations that are commercially available. These often con-  
94 tain dairy ingredients as non-wheat protein source. Vogelsang-  
95 O'Dwyer *et al.*<sup>32</sup> reported a life cycle assessment (LCA) of the  
96 dry-processed faba bean HPI used in this study, which confirmed  
97 reduced use of land and water resources as well as lower impact  
98 on climate change (carbon footprint) and aquatic eutrophication  
99 in comparison to cow's milk powder. This makes HPHB and for-  
100 mulations of its kind even more promising to partially cover pro-  
101 tein needs of future predominantly plant-based diets.

## 102 2 Materials and Methods

### 103 2.1 Materials

104 Three high-protein ingredients (HPIs) were applied in the high-  
105 protein hybrid bread (HPHB) formulation. Faba bean flour (pro-  
106 tein content 61.25 %DM, fat 3.81 %DM, ash 5.43 %DM, fibre  
107 0.35 %DM, carbohydrates by difference 29.17 %DM, total  
108 starch 7.77 %DM;<sup>30</sup> obtained by dry fractionation), which  
109 was experimentally produced and provided by Fraunhofer Insti-  
110 tute IVV, Freising, Germany; carob germ flour (protein content  
111 55.04 %DM, fat 0.20 %DM, ash 7.04 %DM, fibre 17.67 %DM, car-  
112 bohydrates by difference 20.05 %DM, total starch < 0.2 %DM;<sup>30</sup>  
113 GRINDSTED VEG PRO S1) from Danisco, UK and vital gluten  
114 (protein content 72.38 %DM, fat 0.72 %DM, ash 0.87 %DM, fibre  
115 < 0.1 %DM, carbohydrates by difference 15.31 %DM, total starch  
116 4.95 %DM;<sup>30</sup> NUTRALYS W) from Roquette, France. Wheat flour  
117 was supplied by Whitworth Bros Ltd, UK; dry yeast by Puratos,  
118 Belgium; salt by Glacia British Salt Ltd, UK; sugar (granulated  
119 Irish sugar) by Nordzucker (Ireland) Ltd, Ireland; psyllium (VITA-  
120 CEL P95) by J. Rettenmaier & Söhne, Germany; vegetable oil by  
121 Musgrave, Ireland; and xylanase (Biobake 715) by Kerry Group,  
122 Ireland. For *in vivo* digestibility trials, the following ingredients  
123 were used for the preparation of diets: casein (C) from Lacpol  
124 Co., Poland; soya protein isolate (SPI) ISOPRO 900 HI charac-  
125 terised as non-GMO protein isolate from EDMIR-POL Co., Poland;  
126 soya flour (SF) SOPRO TB 200 from EDMIR-POL Co., Poland;  $\alpha$ -  
127 cellulose (C8002) from Sigma-Aldrich, Missouri, USA; soya oil  
128 from ZPT Co., Poland; choline chloride from SIGMA, Poland;  
129 cholesterol from PPH Standard Co., Poland; sucrose from POCH  
130 SA Co., Poland; and corn starch from Avebe, The Netherlands.  
131 Enzymes for *in vitro* digestion trials were purchased from Sigma-  
132 Aldrich, Missouri, USA: Pepsin from porcine gastric mucosa; EC  
133 3.4.23.1; P7000; 727 U/mg and pancreatin from porcine pan-  
134 creas; 4 x USP; P1750. All other chemicals were also purchased  
135 from Sigma-Aldrich, Missouri, USA unless stated otherwise.

## 136 2.2 Technological Analysis

### 137 2.2.1 Flour Analysis

138 The properties of wheat flour (used for reference wheat bread)  
139 and the high-protein (HP) flour mix (used for HPHB) were anal-  
140 ysed. The HP flour mix contained wheat flour, the three HPIs  
141 (faba bean flour, carob germ flour, gluten) and psyllium in ra-  
142 tios according to HPHB formulation (Table 1). The moisture con-  
143 tent of the HP flour mix was calculated considering the mois-  
144 ture determined for each single ingredient. GlutoPeak test -  
145 Gluten-aggregation properties of wheat flour and the HP flour mix  
146 were investigated following the method previously described by  
147 Hoehnel *et al.*<sup>30</sup> using the GlutoPeak device (Brabender GmbH  
148 and Co KG, Duisburg, Germany). In brief, high shear was ap-  
149 plied to a flour/water slurry (50:50 ratio, adjusted when mois-  
150 ture of flour differed from 14 %). The device was operated at  
151 a paddle speed of 2750 rpm and temperature of 36 °C; torque  
152 was recorded over time. Variables Torque maximum (TM, ex-  
153 pressed in Brabender units BU) and Peak Maximum Time (PMT,  
154 expressed in s) were obtained from the curves. Rapid visco analy-  
155 sis - Examination of pasting behaviour using Rapid Visco Analysis  
156 (RVA Super 3, Newport Scientific, Warriewood, Australia) was  
157 performed according to AACC 76-21.02. The following heating  
158 profile was applied: equilibration at 50 °C for 1 min, heating to  
159 95 °C at 0.2 °C/s, holding at 95 °C for 162 s, cooling to 50 °C at  
160 0.2 °C/s, maintaining at 50 °C for 120 s. The variables peak vis-  
161 cosity (PV), setback and final viscosity (FV) were obtained from  
162 the viscograms.

### 163 2.2.2 Recipe Adaptation and Bread Production

164 Bread samples were prepared according to the formulations in  
165 Table 1. The HPHB formulation contains HPIs (faba bean flour,  
166 carob germ flour, gluten) and was designed to match the tech-  
167 nological quality of the reference wheat bread (RWB). A series  
168 of preliminary trials (data not shown) based on the results pre-  
169 sented by Hoehnel *et al.*<sup>30</sup> led to the establishment of the HPHB  
170 formulation. A total of 28 different recipes were screened to se-  
171 lect a combination of HPIs and to optimise their relative ratios  
172 for favourable technological characteristics. Furthermore, the in-  
173 troduction and optimal addition levels of the functional ingredi-  
174 ents psyllium, sugar and xylanase were investigated as part of the  
175 screening to achieve adequate dough handling characteristics and  
176 quality of the end product. For both formulations, the straight  
177 dough method was applied. Yeast was activated by dissolving in  
178 30 °C tap water for 10 min. The obtained yeast suspension was  
179 added to the remaining, previously weighed ingredients. A total  
180 amount of 3600 g dough was prepared. Mixing conditions were  
181 the following: RWB - MACPAN MX 10 spiral mixer (MACPAN  
182 SNC, Italy) at speed 1 for 6.5 min and speed 2 for 5 min; HPHB -  
183 Hobart A200N mixer (Hobart Manufacturing, UK), equipped with  
184 hook attachment, at speed 1 for 2 min and speed 2 for 7.5 min.  
185 After covering the dough and leaving it to rest for 5 min, it was  
186 divided into 7 pieces of 450 g ± 1 g. The pieces were moulded,  
187 put into baking tins and proofed for 90 min at 75 % humidity and  
188 35 °C (KOMA BV Sunrises, Reormond, the Netherlands). Baking  
189 was performed in deck ovens (MIWE Condo, Arnstein, Germany)  
190 at 220/230 °C top/bottom temperature for 35 min with open

191 draft throughout the whole baking process. The baking chamber  
192 was steamed with 400 mL prior to loading. After baking, breads  
193 were removed from tins and left to cool down for 2 h at ambient  
194 temperature. The results were obtained from three independently  
195 performed baking trials.

### 196 2.2.3 Dough Analysis

197 Doughs for determination of dough properties were prepared as  
198 described in section 2.2.2. Rheofermentometer - Formation and  
199 retention of gas in the fermenting doughs was analysed using a  
200 Rheofermentometer F3 (Chopin, France). A dough piece (300 g)  
201 was placed into the sample container and a weight constraint of  
202 1.5 kg was applied. The dough fermentation was monitored for  
203 3 h at a temperature of 35 °C (matching the proofing temperature  
204 used during bread production). The fermentation performance  
205 of the doughs was evaluated by the following variables obtained  
206 from the generated curves: Total gas volume produced ( $V_{total}$ ),  
207 volume of CO<sub>2</sub> lost ( $V_{lost}$ ) and volume of gas retained ( $V_{ret}$ ) from  
208 gaseous release curves; and maximum height of dough devel-  
209 opment ( $H_M$ ) from dough development curves. Large deforma-  
210 tion properties - Extensibility (expressed in mm) and resistance  
211 to extension (expressed in g) of the doughs were measured by a  
212 texture analyser (TA-XT plus, Stable Micro Systems, Surrey, UK)  
213 equipped with a 5 kg load cell and a Kieffer dough and gluten  
214 extensibility rig (test settings: pre-test speed 2 mm/s, test speed  
215 3.3 mm/s, post-test speed 10.0 mm/s, trigger force 5 g). The uni-  
216 axial extension test was performed after a dough resting time of  
217 20 min (room temperature) inside the dough strip mould. Ten  
218 intact strips of dough were measured from each of three batches  
219 per formulation.

### 220 2.2.4 Bread Quality Analysis

221 Specific volume (SV) was measured with a Volscan Profiler (Sta-  
222 ble Micro Systems, Surrey, UK) of 6 loaves per batch. For analysis  
223 of crumb structure and hardness, three slices (20 mm) were cut  
224 out of the middle of each of 2 bread loaves. A C-Cell Imaging Sys-  
225 tem (Calibre Control International Ltd, UK) was used to capture  
226 images of slices and to determine the variables: number of cells,  
227 area of cells and slice brightness. Crumb hardness was analysed  
228 with a texture analyser (TA-XT2i, Stable Micro Systems, Surrey,  
229 UK) equipped with a 25 kg load cell. A 35 mm cylindrical probe  
230 was used to compress the centre of the slice to 40 % of its height  
231 as part of a texture profile analysis (TPA): test speed 5 mm/s,  
232 post-test speed 10 mm/s, trigger force 0.05 N, waiting time be-  
233 tween compressions 5 s. TPA of bread slices was repeated on day  
234 2 and day 5 after baking to monitor bread staling (whole loaves  
235 were stored in plastic bags at ambient temperature in the bak-  
236 ery and sliced immediately before the measurement). Lightness  
237 of crust ( $L^*$ crust) and crumb ( $L^*$ crumb) was measured by a Col-  
238 orimeter CR-400 (Konica Minolta, Japan) using the CIE  $L^*a^*b^*$   
239 colour space.

### 240 2.2.5 Scanning Electron Microscopy

241 Bread crumb was separated from crust, cut into small cubes,  
242 frozen at -80 °C and freeze-dried. The dry crumb was further  
243 crushed into small fragments which were mounted onto plain  
244 aluminium stubs with double-sided carbon adhesive tape. After

**Table 1** Recipe for RWB and HPHB

Ingredient	Reference wheat bread		High-protein hybrid bread	
	% based on flour	% based on recipe	% based on flour	% based on recipe
Wheat flour	100.0	59.70	82.5 <sup>×</sup>	47.22 <sup>×</sup>
Faba bean flour	-	-	10.0 <sup>×</sup>	5.72 <sup>×</sup>
Carob germ flour	-	-	5.0 <sup>×</sup>	2.86 <sup>×</sup>
Gluten	-	-	2.5 <sup>×</sup>	1.43 <sup>×</sup>
Psyllium	-	-	2.0 <sup>×</sup>	1.14 <sup>×</sup>
Sugar	-	-	1.0	0.57
Baker's yeast	2.0	1.19	2.0	1.14
NaCl	2.0	1.19	2.0	1.14
Oil	1.0	0.60	1.0	0.57
Xylanase	-	-	0.0060	0.0034
Water	62.5	37.31	66.70	38.18
<b>Total</b>	<b>167.5</b>	<b>100.00</b>	<b>174.7</b>	<b>100.00</b>

<sup>×</sup> Ingredients are included in HP flour mix

245 coating with a 5 nm gold-palladium (80:20) layer using a Gold  
 246 Sputter Coater (BIO-RAD Polaron Division, SEMcoating system,  
 247 England), they were examined under high vacuum with a JOEL  
 248 scanning electron microscope (SEM) type 5510 (JOEL Technics  
 249 Ltd., Tokyo, Japan). Images were acquired at a constant acceler-  
 250 ating voltage of 5 kV.

### 251 2.3 Nutritional Analysis

252 Analysis of nutritional characteristics of the bread formulations  
 253 was performed on freeze-dried (according to the procedure de-  
 254 scribed in section 2.2.5) and subsequently milled (laboratory disc  
 255 mill; Bühler, Braunschweig, Germany) samples of bread crumb.  
 256 Results are expressed as contents in fresh bread considering the  
 257 moisture of freeze-dried and fresh bread crumb unless stated oth-  
 258 erwise.

#### 259 2.3.1 Compositional Analysis

260 The analysis of the following compositional data was performed  
 261 by Concept Life Science Ltd., UK based on the indicated validated  
 262 methods: energy (calculated considering protein, fat, available  
 263 carbohydrates and fibre), protein (Dumas method, modified after  
 264 AOAC 1977.992.15; nitrogen-to-protein conversion factor 6.25),  
 265 ash (removal of organic matter by oxidation at 550 °C, based on  
 266 ISO 936:1998), fat (low resolution proton nuclear magnetic reso-  
 267 nance (NMR), based on MQC-23-35 Oxford Instruments applica-  
 268 tion note), fatty acid profile (GC-FID of fatty acid methyl esters;  
 269 triglyceride conversion factor 0.956), total dietary fibre (TDF)  
 270 (gravimetric method, based on AOAC 991.43), sodium (flame  
 271 photometry after removal of organic matter). Moisture was de-  
 272 termined by air-oven method at 130 °C until constant mass was  
 273 reached. Total starch content was analysed using the enzyme kit  
 274 K-TSTA supplied by Megazyme, Ireland. Mono-, di- and oligosac-  
 275 charides were extracted from the freeze-dried product powders  
 276 as follows: 15 mL of 80/20 (v/v) ethanol/ultrapure water (80%  
 277 EtOH), which was heated to 55 ± 5 °C, were added to 2 g of sam-  
 278 ple. The mixture was vortexed until the powder was suspended  
 279 and then subjected to sonication (extraction step 1) utilising a  
 280 BANDELIN Sonoplus HD 3100 homogenizer (Berlin, Germany)  
 281 equipped with an MS73 microtip, operated twice for 15 s at  
 282 75 °amplitude. Hereupon, the sample was centrifuged at 1800 g

283 for 10 min and the supernatant transferred to another test tube  
 284 for further processing. Sonication and centrifugation were re-  
 285 peated (extraction step 2) after adding another 15 mL 80% EtOH  
 286 (at 55 ± 5 °C) to the pellet. The supernatants of both extraction  
 287 steps were pooled and concentrated using a vacuum centrifuge  
 288 system (Scanvac Scan Speed 32 with Scanvac VacSafe 15, Labo-  
 289 gene ApS, Lyngø, Denmark) with the following settings: 2 h at  
 290 1500 rpm and 45 °C, followed by 1 h at 2000 rpm and 50 °C;  
 291 average pressure 15 mbar. The concentrated extract was trans-  
 292 ferred into a 10 mL volumetric flask, which was filled up with  
 293 ultrapure water containing 50 mg/L NaN<sub>3</sub>, and filtered through  
 294 syringe driven polyamide filters (Chro-mafil AO-20/25, pore size:  
 295 0.20 µm, Machery-Nagel, Düren, Germany). Samples were ex-  
 296 tracted in duplicates and quantification of the sugars was per-  
 297 formed according to the method described by Ispiryan *et al.*<sup>33</sup>  
 298 using a Dionex ICS-5000<sup>+</sup> system (Sunnyvale, CA) equipped with  
 299 an electrochemical detector.

#### 300 2.3.2 Amino Acid Analysis

301 Determination of protein amino acid composition was carried out  
 302 by Mérieux NutriSciences CHELAB S.r.l., Italy based on ionic chro-  
 303 matography with postcolumn ninhydrin derivatisation (fluores-  
 304 cence detection; UV detection for tryptophan) after adequate ex-  
 305 traction and protein hydrolysis (separate hydrolysis procedures  
 306 for the determination of tryptophan, sulphur-containing AA and  
 307 remaining AA).

#### 308 2.3.3 *In vitro* Protein Digestion

309 A previously described static multi-step method for *in vitro*  
 310 protein digestibility (IVPD)<sup>34,35</sup> was used to simulate gastro-  
 311 pancreatic protein digestion. In short, sample amounts containing  
 312 50 ± 1 mg protein were weighed in and enzymatic hydrolysis was  
 313 started: pepsin digestion at 37 °C and pH 1-2 (1 h) followed by  
 314 sequential pancreatin digestion at 37 °C and pH 7-8 (short-term:  
 315 +1 h; medium-term: +3 h; long-term: +24 h). Ratios between  
 316 enzyme and substrate (w/w) were kept constant at 1:50 (pepsin  
 317 stage) and 1:10 (pancreatin stages). IVPD in % was determined  
 318 using a trinitrobenzenesulfonic acid (TNBS) assay. Results are ex-  
 319 pressed as the concentration of free α-amino groups in samples  
 320 in relation to an alanine standard solution representing 100 %  
 321 protein digestibility.

### 2.3.4 *In vivo* Nitrogen Balance

The animal protocol used in this study was approved by the local institutional Animal Care and Use Committee (Olsztyn, Poland) and the study was performed in accordance with EU Directive 2010/63/EU for animal experiments. The assessment was conducted on growing male Wistar rats weighing 173.2 g. The rats were randomly divided into groups of seven animals. All animals were housed individually over 14 days in metabolic cages with free access to water and the experimental diets (Table 2). The selection of the animals and their maintenance over the 14-day experiment followed common regulations. The environment was controlled with a 12 h light-dark cycle, a temperature of  $22 \pm 1$  °C, relative humidity of 45-65% and 20 air changes per hour. For experimental feeding the following diets were used: a standard control diet based on casein (C) as the main protein source (supplemented with 0.2% DL-methionine), a second control diet based on soya protein isolate (SPI, without any supplementation), a third control diet based on soya flour (SF, without any supplementation) and the experimental diets containing RWB and HPHB. All experimental diets were a modification of the AIN-93G diet for laboratory rodents recommended by the American Institute of Nutrition;<sup>36</sup> the dietary protein level was lowered to approx. 11% to measure the protein digestibility and utilisation rate. During the study, nitrogen (N) digestibility and utilisation tests (balance tests) were carried out. After a 9-day preliminary period, faeces and urine were thoroughly collected for 5 d from all rats (kept in balance cages; Tecniplast Spa, Buguggiate, Italy). The total N content of each diet as well as each faecal and urinal sample (collected in the balance period) was analysed in duplicate (AOAC 979.09). The rats from each diet group were additionally monitored for body-weight (BW) gains (recording BWs at the beginning and end of the study) and diet intake (daily record), which enabled calculation of the protein efficiency ratio (PER). All physiological measurements were carried out for each animal separately (n = 7 per diet group).

### 2.3.5 Antinutritional Compounds

Trypsin inhibitors were extracted from the lyophilised product powders by adding 2.5 mL sodium acetate buffer (0.1 M, pH4.9) to 350 mg sample and homogenising the mixture for 2 min using an Ultra Turrax. After centrifugation for 5 min at 3000 g (EBA 12 Centrifuge; Hettich Zentrifugen, Tuttlingen, DE), the supernatant was transferred to a new test tube and the extraction procedure was repeated with the same conditions with the pellet. Both supernatants were pooled, stored in the fridge overnight and centrifuged again (5 min, 3000 g) immediately before trypsin inhibitor activity (TIA) analysis. TIA was determined following the method described by Joehnke *et al.*<sup>34</sup> with some modifications. In brief, TIA levels were measured against a trypsin solution (stock concentration 0.1 mg/mL). A solution of N- $\alpha$ -benzoyl-L-arginine-4-nitroanilide (L-BAPA) with 0.22 mg/mL was used as substrate. Spectrometric quantification was performed at 410 nm and based on a molar extinction coefficient of the reaction product (4-nitroaniline) of  $8800 \text{ M}^{-1} \text{ cm}^{-1}$ . One trypsin inhibitor unit (TIU) is defined as the amount of inhibitor required to reduce the trypsin activity by one unit. One trypsin activity unit (TAU)

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

Component of diet	C	SPI	SF	RWB	HPHB
Casein	11.15				
DL-Methionine	0.20				
Soya protein isolate		10.80			
Soya flour			19.69		
Reference wheat bread				67.79	
High-protein hybrid bread					43.85
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 <sup>1</sup>	3.50	3.50	3.50	3.50	3.50
Vitamin mix <sup>2</sup>	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	6.21	30.15

<sup>1</sup> AIN-93G-MX: mineral mixture as specified by Reeves<sup>36</sup> (1997)

<sup>2</sup> AIN-93G-VX: vitamin mixture as specified Reeves<sup>36</sup> (1997)

is defined as the amount of enzyme that catalyses the hydrolysis of 1  $\mu\text{mol}$  L-BAPA into 4-nitroaniline within 1 min at pH 8.2 and 37 °C. Contents of vicine and convicine were determined after an extraction of 500 mg of sample with boiling methanol as described by Petersen *et al.*<sup>37</sup>. Quantification was achieved using micellar electrokinetic capillary chromatography as reported by Bjerregaard *et al.*<sup>38</sup> and with vicine as external standard.

### 2.3.6 Antioxidant Potential

Extraction - Phenolic compounds were extracted from the product powders using 80/20 (v/v) methanol/water (80% MeOH), at a solid to solvent ratio of 1:10 (w/v), for 15 min at 50 °C as described by Amarowicz *et al.*<sup>39</sup>. The extraction was repeated twice, the supernatants were filtered and pooled, and the methanol was evaporated under vacuum with a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). The remaining aqueous extract was lyophilised. Total phenolic content (TPC) - TPC of phenolic extracts was determined using Folin-Ciocalteu's phenol reagent following a method described by Amarowicz and Raab<sup>40</sup>. The results were expressed as mg catechin equivalent. Trolox equivalent antioxidant capacity (TEAC) - TEAC was determined according to the method reported by Re *et al.*<sup>41</sup>. In brief, a ABTS<sup>•+</sup> (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) solution was prepared by mixing an aqueous ABTS stock solution with 2.45 mM (final concentration) sodium persulfate. This mixture was shaken for 12-16 h at room temperature in the dark until a stable oxidative state was reached. The ABTS<sup>•+</sup> stock solution was diluted with methanol to an absorbance of 0.720 at 734 nm for subsequent analysis. For the spectrophotometric assay, 2 mL of the diluted ABTS<sup>•+</sup> solution were mixed with 20  $\mu\text{l}$  of reconstituted phenolic extract (10 mg/mL in methanol); absorbance was determined at 734 nm at 37 °C for 10 min. A calibration curve was generated using a Trolox standard and the results were expressed as  $\mu\text{mol}$  Trolox equivalent. Ferric-reducing antioxidant power (FRAP) - FRAP assay was performed as described by Benzie and Strain<sup>42</sup>. The FRAP value was calculated and expressed as  $\mu\text{mol Fe}^{2+}$  using a  $\text{Fe}^{2+}$  calibration curve. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay - The radical scavenging effect of the phenolic extracts was measured as described in Amarowicz *et al.*<sup>43</sup>. A



**Table 3** Sensory attributes and extremes of intensity scales used for QDA of breads

Attribute	Definition	Extremes
<b>Odour</b>		
Sweet	Odour characteristic of sweet buns produced from wheat flour	None - very intense
Acidulous	Odour characteristic of fermented products (e.g. vinegar, yoghurt)	None - very intense
<b>Appearance</b>		
Beige colour	Crumb colour intensity	Light - dark
Pore size	Visual impression of bread crumb porosity	Small - big
Pore distribution	Regularity of pore distribution in the crumb	Irregular - regular
<b>Texture (manual)</b>		
Elasticity	The extent to which bread crumb returns to its original shape when stretched	Low - high
<b>Texture (oral)</b>		
Chewiness	Extent of chewing necessary to prepare food for swallowing	Low - high
Adhesiveness	Degree of adhesiveness when chewing the food 10 times	Low - high
Moisture	Moisture released by the food after 10 chews	Low - high
<b>Taste</b>		
Rye-wheat bread	Aroma characteristics of commercial rye-wheat bread (retronasal)	None - very intense
Salty	Taste characteristic of NaCl (1 % in water)	None - very intense
Acidulous	Taste characteristics of citric acid (1 % in water)	None - very intense
Aftertaste	Lingering sensation after swallowing the sample	None - very intense
<b>Overall quality</b>	Conclusive evaluation of all attributes and their harmonic balance	Bad - very good

ing analysis of variance. Before the sensory analysis, a 28-hour panel training was conducted on various bread samples, including bread from the local supermarkets, with the aim to familiarise the sensory panel with innovative bread samples and their features. A list of sensory attributes was created. Initially, panellists chose characteristics describing the samples individually, followed by a joint agreement on distinguishing attributes and their descriptions (see Table 3). A continuous scale (10 cm long) with the extremes specified in Table 3 was used. Sensory evaluation was carried out in three independent sessions.

## 2.5 Statistical Analysis

All measurements were performed in triplicate unless stated otherwise. Data analysis was carried out 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 show significant differences ( $p < 0.05$ ). When available, values are given as the mean  $\pm$  standard deviation or uncertainty (amino acid profile).

## 3 Results and Discussion

### 3.1 Technological Characteristics

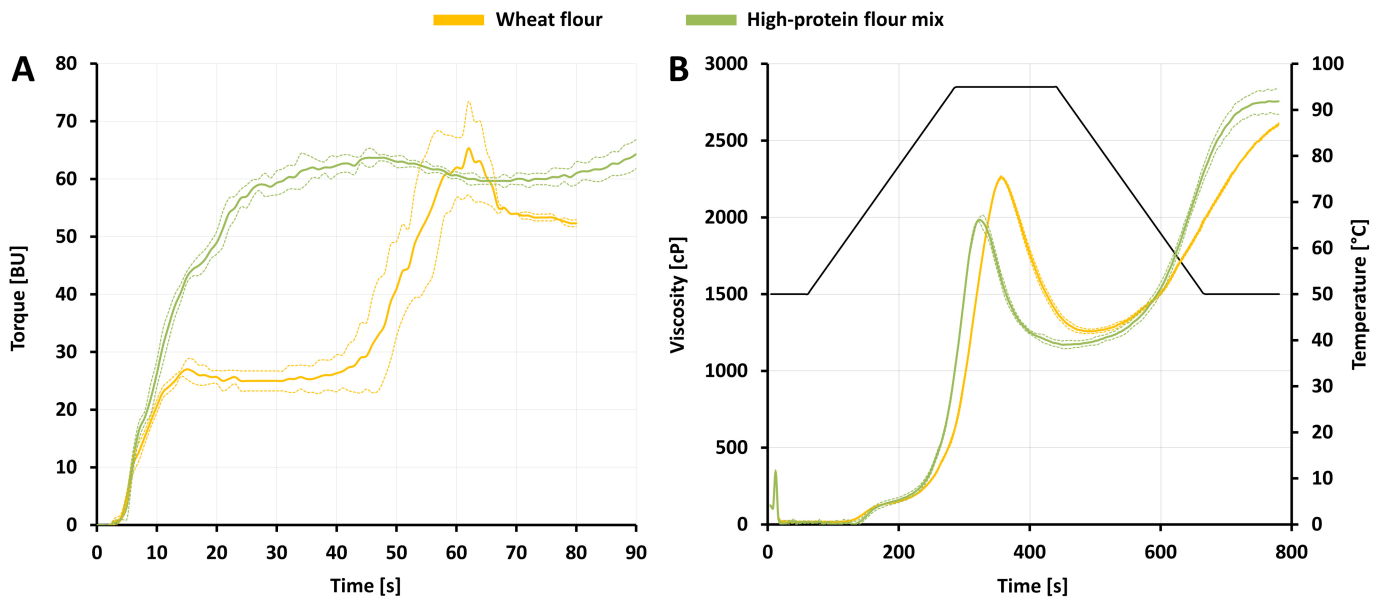
#### 3.1.1 Flour and Dough Properties

The properties of flours and doughs used for breadmaking have a high impact on the quality of bread products. In addition to the ability to form a stable gluten-network, rheological characteristics such as pasting behaviour, dough extensibility and the dough's proofing performance determine flour and dough quality. Gluten-aggregation and pasting behaviour were evaluated for RWB based on wheat flour and for HPHB based on HP flour mix (Table 1). The aim was to compare measurements, which are commonly performed to determine baking quality of flours, for the two formulations in this study. It was decided to include not only the HPIs in the HP flour mix for flour analyses, but also psyllium, which was expected to have a high impact on rheological properties. Sugar and xylanase were shown to have no significant effect on the performance of the HP flour mix in these tests (preliminary trials, data not shown) and were left out. The GlutoPeak test revealed striking differences in gluten-aggregation properties of the two flours. The variables obtained from the curves are presented in Table 4. Wheat flour exhibits with 68 BU a significantly higher TM than HP flour mix (64 BU), but PMT was detected 14 s earlier for HP flour mix (46 s) than for wheat flour (60 s). When pure wheat flours are measured, a general trend towards earlier and higher gluten peaks for stronger flours with higher gluten contents and/or higher gluten quality has been reported in literature.<sup>44–46</sup> The gluten content in HP flour mix (calculated based on composition of ingredients and an average gluten content in wheat flour protein of 80 %) is about 0.5 % lower than in wheat flour, which could explain the slightly lower TM detected for HP flour mix. However, Hoehnel *et al.*<sup>30</sup> showed that the partial replacement of wheat flour by HPIs leads to complex changes in the gluten-aggregation profiles, which do not follow this general trend. Therefore, a comparison of gluten-aggregation profiles in addition to TM and PMT (or other variables obtained from the

methanolic solution (0.1 mL), containing 0.02–0.10 mg of extract, was mixed with 2 mL of deionised water, and was then added to a methanolic solution of DPPH $\cdot$  (1 mM, 0.25 mL). The mixture was vortexed for 1 min and left to stand at room temperature for 20 min. the absorbance of the solution was measured at 517 nm. The results were expressed as half maximal effective concentration (EC<sub>50</sub>) of the phenolic extract that scavenged 50% of DPPH radicals.

### 2.4 Sensory Analysis

Descriptive sensory profiling (quantitative descriptive analysis – QDA) was carried out in order to characterise the bread samples using an expert panel (n=8). The QDA procedure used in the study was in accordance with the standard ISO 13299:2016. Panellists with appropriate methodological preparation and experience in sensory profiling were selected, trained and monitored following ISO 13299:2016. Before the sensory analysis, the panellists' performance was evaluated using three parameters – repeatability, discrimination ability and homogeneity by apply-



**Fig. 1** Flour properties of wheat flour and HP flour mix: (A) Gluten-aggregation profiles obtained by GlutoPeak test; (B) Viscograms obtained from rapid visco analysis describing pasting behaviour of RWB and HPHB with black line representing the applied temperature profile. Dashed curves represent standard deviation.

486 curves) is required (see Figure 1). The profile of wheat flour follows the typical sequence of initial torque increase, equilibrium plateau, rapid torque increase, peak maximum and torque decrease due to breakdown of gluten-network. The HP flour mix shows no pronounced equilibrium plateau and the torque increases rapidly towards its maximum right in the beginning of the measurement. Instead of a sharp peak with a rapid gluten breakdown, the peak is broad and torque remains high after its maximum. According to Goldstein *et al.*<sup>47</sup>, a fast build-up of gluten-network followed by a sharp peak and rapid breakdown is associated with weak flours. The profile of HP flour mix indicates a strong and stable gluten-network due to the broad gluten-peak and delayed gluten breakdown. This could be caused by a co-networking of gluten with non-wheat proteins from faba bean and carob as suggested by Hoehnel *et al.*<sup>30</sup>. The lack of equilibrium plateau and rapid torque increase at the start can be explained by the high water absorption of psyllium and gluten<sup>48,49</sup> resulting in a higher initial viscosity of the sample slurry. Table 4 shows variables describing the pasting behaviour of the flours.

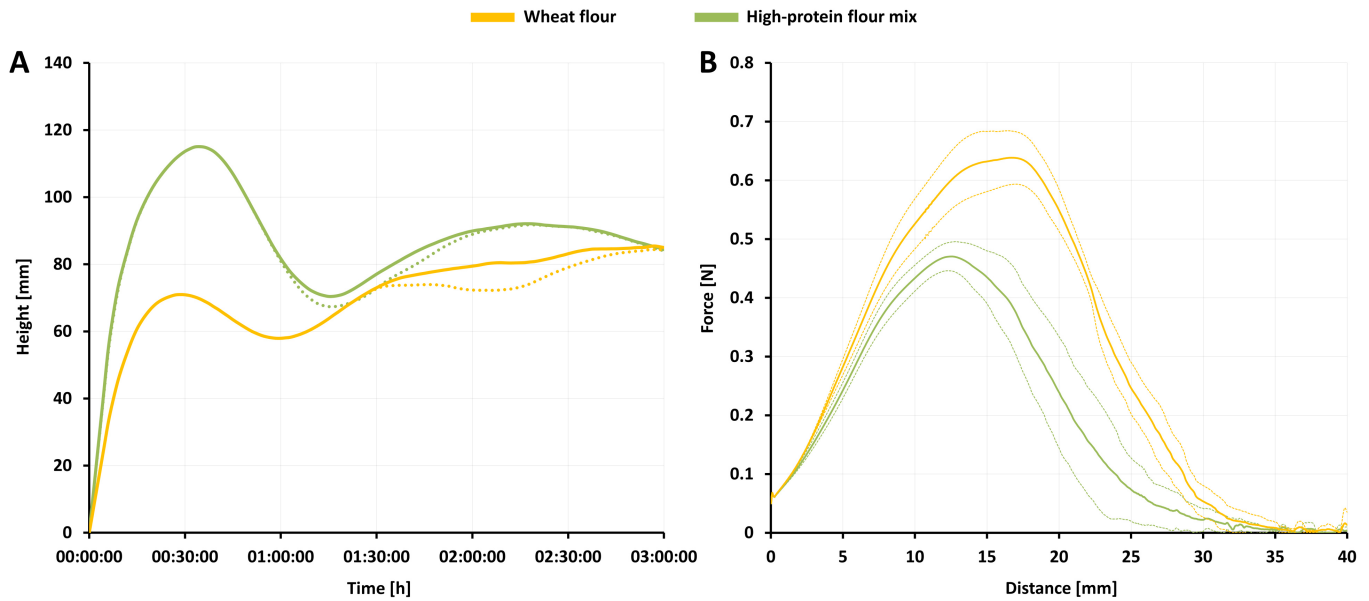
505 The corresponding viscograms are displayed in Figure 1. The viscograms suggest a generally similar pasting behaviour of wheat flour and HP flour mix with only small discrepancies. However, significant differences have been detected for PV, setback and FV. The PV of HP flour mix is with 1989 cP lower than for wheat flour with 2261 cP. This can be attributed to the lower starch content in HP flour mix and, thus, less gelatinising starch, which has been previously observed in systems based on wheat flour<sup>50</sup> as well as systems based on rice flour.<sup>51</sup> The presence of psyllium in the HP flour mix is expected to increase viscosity of the sample due to its well-known high water absorption and gelling properties (at low temperatures as well as upon heating).<sup>52,53</sup> This might have partly compensated for the reduced viscosity owing to less starch. Hence, only a small difference in PV has been found. In contrast to a lower PV, HP flour mix exhibits higher FV and setback compared to wheat flour. Especially the setback expressed in relation to PV is remarkably high for HP flour mix (wheat flour: 59.7 %, HP flour mix: 79.8 %). A similar pattern was observed by Hoehnel *et al.*<sup>30</sup> in a flour blend containing 15 % faba bean flour. Since this ingredient contains a considerable amount of non-wheat starch, high setback and FV could be related to the retrogradation properties of faba bean starch. Dough analyses provide information on rheological and expansion properties of the formulations during proofing. Large deformation properties (Table 5) reveal a reduced extensibility and resistance to extension for the HPHB dough (13.04 mm and 0.475 N, respectively) compared to RWB (16.76 mm and 0.647 N, respectively). According to literature,<sup>54</sup> reduced resistance to extension as well as area under the curve are indicative of weaker doughs. However, also the shape of the curve (see Figure 2), and the ratio of resistance to extension and extensibility (R/E) in particular, seems im-

**Table 4** Flour properties of wheat flour (used for reference wheat bread) and HP flour mix (used for high-protein hybrid bread)

Variable	Wheat flour	HP flour mix
<b>GlutoPeak</b>		
Peak maximum time (PMT) [s]	60 ± 4 <sup>a</sup>	46 ± 2 <sup>b</sup>
Torque maximum (TM) [BU]	68 ± 1 <sup>a</sup>	64 ± 1 <sup>b</sup>
<b>Rapid Visco Analyser</b>		
Peak viscosity (PV) [cP]	2261 ± 9 <sup>a</sup>	1989 ± 17 <sup>b</sup>
Setback [cP]	1350 ± 19 <sup>b</sup>	1587 ± 59 <sup>a</sup>
Final viscosity (FV) [cP]	2607 ± 10 <sup>b</sup>	2756 ± 84 <sup>a</sup>

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





**Fig. 2** Dough properties of RWB and HPHB: **(A)** Gas release curves obtained by Rheofermentometer measurements (dotted line represents gas retained in the dough); **(B)** Extensibility plots obtained Kieffer rig microextension tests (dashed curves represent standard deviation).

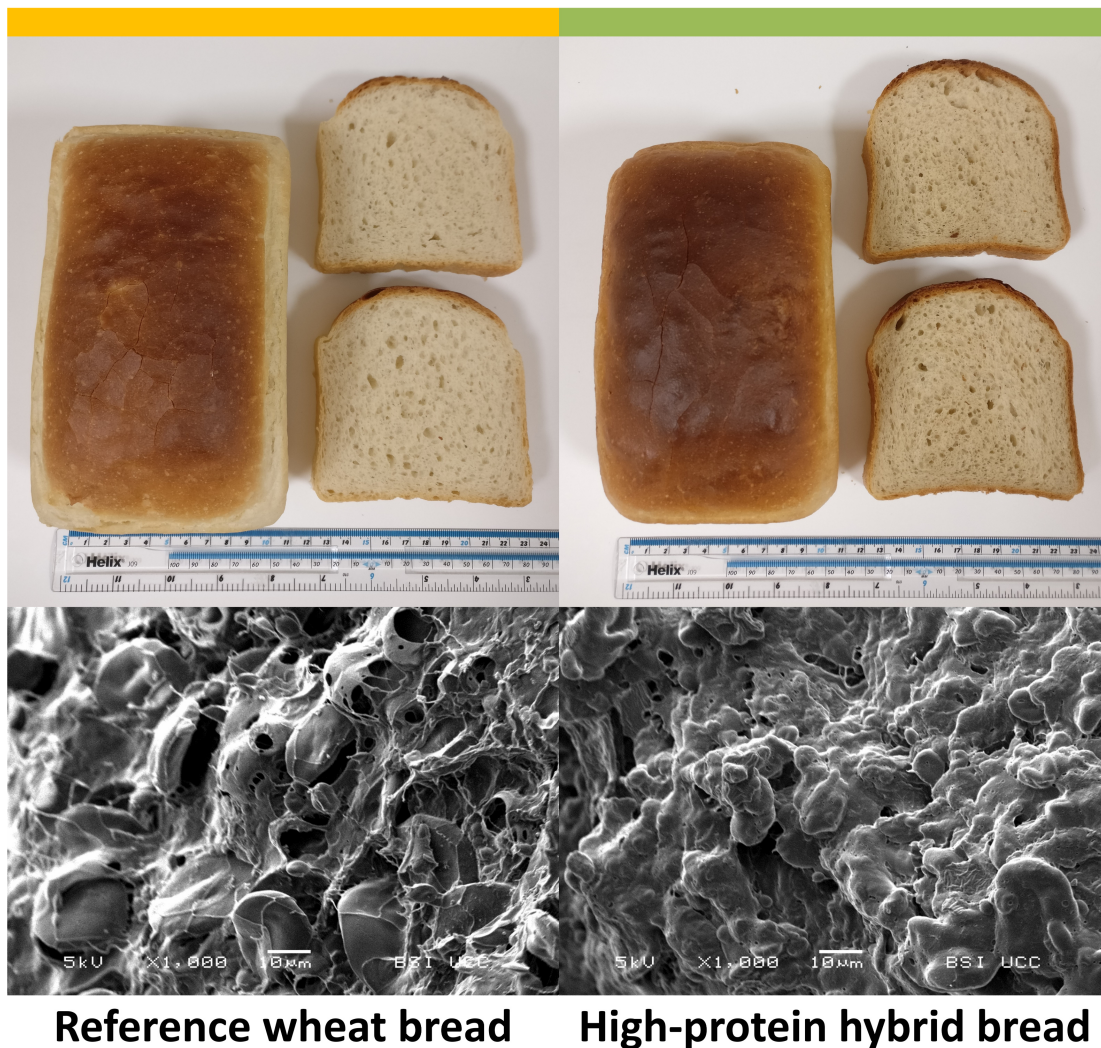
portant and provides information on the doughs' viscoelastic balance.<sup>54</sup> This ratio is with 0.039 N/mm for RWB and 0.036 N/mm for HPHB very similar for both formulations and suggests similar expansion properties. Variables describing the proofing performance of the doughs were obtained by Rheofermentometer measurements and are shown in Table 5. No significant difference was detected for dough development expressed as maximum height ( $H_M$ ) with 67.3 mm for RWB and 61.6 mm for HPHB, which is in line with the similar expansion properties suggested by microextension tests. HPHB shows significantly higher total gas volume ( $V_{total}$ ; 2449.7 mL) and retained gas volume ( $V_{ret}$ ; 2416.3 mL) than RWB (1982.7 mL and 1924.3 mL, respectively). Also a tendency towards a lower lost gas volume ( $V_{lost}$ ) for HP flour mix was observed. The gas release curves from Rheofermentometer measurements are displayed in Figure 2. The initial gas release is much more pronounced for HPHB than for RWB. This can be explained by the small amount (0.57 %) of added sugar in HPHB, which leads to higher initial yeast activity and gas production. The initial peak is followed by a temporary decline in gas release

in both formulations. This represents the point where easily accessible sugars have been consumed by the yeast and further sugars are made available by enzymatic breakdown of starch and other polysaccharides present in the samples. Gas production at the start is the only remarkable difference in an otherwise very similar gas release profile throughout the measurement. Hence, the added sugar represents the main factor for the increased  $V_{total}$  of HPHB. Even though the difference observed in  $V_{lost}$  is not significant, also the curves suggest a tendency towards better gas retention of HPHB dough. This is in accordance with the findings of Courtin and Delcour.<sup>55</sup> They explained a positive effect of water-extractable arabinoxylans (AX) on gas retention of doughs related to a strengthening of liquid films surrounding CO<sub>2</sub> bubbles, thereby limiting gas diffusion. The psyllium in HPHB contains a considerable amount of AX, of which a small percentage is water-extractable.<sup>56,57</sup> Additionally, xylanase, which degrades water-unextractable AX (in HPHB from wheat flour<sup>58</sup> and psyllium<sup>57</sup>), increases the amount of water-extractable or solubilised AX present in the dough and their effect on gas retention properties.<sup>55</sup> Xylanase degradation of water-unextractable AX has also been reported to lead to a lowered water-binding capacity of AX and redistribution of water in favour of gluten, therefore facilitating gluten-network formation.<sup>49,59</sup> Wang *et al.*<sup>60</sup> discussed the formation of a secondary network based on AX with the ability to strengthen the gluten-network by entanglement and possibly the creation of diferulic bridges. This is in line with the stability of the gluten-network in HPHB and delayed breakdown indicated by GlutoPeak test results and represents an additional stabilising effect besides potential co-networking of gluten with non-wheat proteins.

**Table 5** Dough properties of reference wheat bread formulation and high-protein hybrid bread formulation

Variable	RWB	HPHB
<b>Kieffer rig extensibility</b>		
Resistance to extension [N]	0.647 ± 0.059 <sup>a</sup>	0.475 ± 0.045 <sup>b</sup>
Extensibility [mm]	16.76 ± 1.25 <sup>a</sup>	13.04 ± 1.44 <sup>b</sup>
<b>Rheofermentometer</b>		
Dough development ( $H_M$ ) [mm]	67.3 ± 5 <sup>a</sup>	61.6 ± 1 <sup>a</sup>
Total gas volume ( $V_{total}$ ) [mL]	1982.7 ± 171.1 <sup>b</sup>	2449.7 ± 102.3 <sup>a</sup>
Volume of CO <sub>2</sub> lost ( $V_{lost}$ ) [mL]	58.0 ± 30.5 <sup>a</sup>	33.0 ± 19.2 <sup>a</sup>
Volume of gas retained ( $V_{ret}$ ) [mL]	1924.3 ± 192.1 <sup>b</sup>	2416.3 ± 103.0 <sup>a</sup>

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



**Fig. 3** Photographs and micrographs (obtained by SEM) of RWB and HPHB.

### 3.1.2 Bread Quality Characteristics

The breads produced from both formulations examined in this study are presented in Figure 3. A visual evaluation reveals little differences in loaf size and crumb structure between RWB and HPHB, but a considerably darker crust and crumb colour for HPHB. The results obtained for bread quality characteristics confirm this general observation and are reported in Table 6. No significant differences between RWB and HPHB have been detected regarding bake loss and SV. The initial crumb hardness on day 0 is with 6.98 N for HPHB slightly higher than for RWB (5.13 N). However, this can only be considered a small difference, especially when compared to previously reported increases in crumb hardness caused by the incorporation of legume ingredients in wheat bread.<sup>22,30,61,62</sup> Additionally, the crumb hardness measured on day 2 and day 5 does not show significant differences between RWB and HPHB. This indicates similar staling properties of both formulations, with a tendency towards less staling for HPHB. Staling rates calculated for day 2 are 1.42 for RWB and 0.78 for HPHB, which represents a by 45 % lower staling rate of HPHB. Staling rates calculated for day 5 are 2.08 and 1.31 for RWB and

HPHB, respectively. Also here, HPHB shows a by 37 % lower staling rate. Recrystallising starch is considered to be the main factor for staling of bread crumb.<sup>63,64</sup> Therefore, the decreased crumb staling in HPHB could be related to its lower starch content and, supposedly, a lower amount of gelatinised starch which

**Table 6** Bread quality characteristics of reference wheat bread and high-protein hybrid bread

Variable	RWB	HPHB
Bake loss [%]	12.3 ± 0.6 <sup>a</sup>	11.9 ± 0.8 <sup>a</sup>
Specific volume (SV) [ml/g]	3.73 ± 0.07 <sup>a</sup>	3.75 ± 0.13 <sup>a</sup>
Hardness day 0 [N]	5.13 ± 0.43 <sup>b</sup>	6.98 ± 0.60 <sup>a</sup>
Hardness day 2 [N]	12.41 ± 1.43 <sup>a</sup>	12.41 ± 1.23 <sup>a</sup>
Hardness day 5 [N]	15.81 ± 0.85 <sup>a</sup>	16.15 ± 2.06 <sup>a</sup>
Number of cells	5009 ± 245 <sup>b</sup>	5563 ± 575 <sup>a</sup>
Cell area [%]	52.4 ± 0.3 <sup>a</sup>	51.7 ± 0.5 <sup>b</sup>
Slice brightness	137 ± 4 <sup>a</sup>	108 ± 3 <sup>b</sup>
Lightness of crumb (L*crumb)	63.6 ± 2.2 <sup>a</sup>	60.4 ± 3.9 <sup>a</sup>
Lightness of crust (L*crust)	41.9 ± 5.0 <sup>a</sup>	34.6 ± 2.9 <sup>b</sup>

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

610 can recrystallise. Also AX and xylanases have been reported to  
 611 decrease staling of wheat-based bread formulations.<sup>65,66</sup> The ef-  
 612 fect has been attributed to a competition for water and therefore  
 613 a reduced swelling and gelatinisation of starch.<sup>65,66</sup> Maeda and  
 614 Morita<sup>67</sup> observed reduced staling up to 3 days caused by both  
 615 water-extractable and water-unextractable AX. While their study  
 616 was focused on wheat AX, Czuchajowska *et al.*<sup>68</sup> also reported  
 617 a reduced crumb hardness after 72 h when psyllium was incor-  
 618 porated in wheat bread. Specific volume and crumb hardness  
 619 are generally accepted as the main indicators of bread quality.  
 620 Therefore, the presented results confirm a technological quality  
 621 of HPHB similar to RWB. The evaluation of crumb structure re-  
 622 veals small differences between the formulations. A slightly finer  
 623 crumb structure was observed for HPHB indicated by a higher  
 624 number of cells (5563) and smaller cell area (51.7 %) compared  
 625 to RWB with a number of cells of 5009 and a cell area of 52.4 %.  
 626 This can be related to the higher initial yeast activity and gas  
 627 production in HPHB. Moulding of dough, in addition to shap-  
 628 ing the dough pieces, leads to a division of gas cells produced  
 629 prior to moulding (during mixing and dough rest).<sup>69</sup> In HPHB,  
 630 more gas is produced before moulding and a higher number of  
 631 small gas cells can be generated. Additionally, these gas cells are  
 632 stabilised by water-extractable and solubilised AX as explained  
 633 above, which can minimise the coalescence of gas cells as they  
 634 expand during proofing and lead to a high number of cells in  
 635 the final product. The higher number of cells and smaller cell  
 636 area measured for HPHB could also be partially responsible for its  
 637 slightly higher initial crumb hardness. Values obtained for crumb  
 638 and crust colour (Table 5) confirm the visually perceivable dif-  
 639 ferences between RWB and HPHB. Slice brightness (obtained by  
 640 C-Cell imaging) is significantly lower for HPHB with 108 than for  
 641 RWB with 137. This is in line with the lower lightness of crumb  
 642 measured for HPHB. A big difference was observed in lightness  
 643 of crust, which was significantly lower for HPHB (34.6) than  
 644 for RWB (41.9). The darker crust of HPHB is likely related to  
 645 its higher protein content and higher presence of reducing sug-  
 646 ars (see Table 7), thus, an increased potential for Maillard reac-  
 647 tion.<sup>30,70</sup> The micro-structure of the bread crumb of both for-  
 648 mulations was captured by scanning electron microscopy (SEM).  
 649 The resulting micrographs are displayed in Figure 3. While RWB  
 650 shows a rather porous layer of gluten covering partly intact starch  
 651 granules, HPHB has a thicker and more continuous layer. This  
 652 might be due to the presence of non-wheat proteins from faba  
 653 bean and carob on one hand and psyllium on the other hand. The  
 654 fact that a very homogenous and continuous layer was formed,  
 655 further supports the theory of a co-networking of gluten with non-  
 656 wheat proteins and psyllium AX.

### 657 3.2 Nutritional Characteristics

#### 658 3.2.1 Macronutrient Composition and Sugar Profile

659 Compositional analysis of both formulations was performed in  
 660 order to evaluate changes in macronutrient composition caused  
 661 by the partial replacement of wheat flour by plant-based HPIs in  
 662 HPHB and addition of psyllium to the formulation (Table 7). The  
 663 determined bread constituents include all items that are manda-

**Table 7** Composition of reference wheat bread and high-protein hybrid bread, contents expressed in % of the fresh bread unless stated otherwise

Component	RWB	HPHB
Moisture	45.74 ± 0.06	45.91 ± 0.29
Energy [kcal/100 g]	211.6	209.0
Protein	8.2	13.0
proteinE * [%E]	15.5	24.8
Ash	1.6	2.0
Fat	0.91	1.25
SFA	0.11	0.17
MUFA	0.26	0.36
PUFA	0.50	0.67
Total carbohydrates**	43.5	37.9
Total dietary fibre (TDF)	1.8	2.8
Available carbohydrates**	41.7	35.1
Total starch	36.1 ± 1.2	28.5 ± 0.6
Sodium	0.466	0.440
Sodium expressed as salt (NaCl)	1.16	1.10
Sum of mono- and disaccharides	1.21 ± 0.00	1.13 ± 0.02
Arabinose	<0.01	<0.01
Xylose	<0.01	<0.01
Galactose	0.01 ± 0.00	0.03 ± 0.00
Glucose	0.02 ± 0.00	0.03 ± 0.00
Fructose	0.02 ± 0.00	0.04 ± 0.00
Sucrose	0.01 ± 0.00	0.01 ± 0.00
Maltose	1.16 ± 0.00	1.02 ± 0.02
Maltotriose	0.03 ± 0.00	0.02 ± 0.00
Raffinose/Stachyose	<0.01	0.01 ± 0.00
Verbascose	<0.01	0.02 ± 0.00

Moisture, total starch and sugar profile: means ± standard deviation

\* calculated based on energy content, protein content and 4 kcal/g protein

\*\* calculated by difference

664 tory for nutritional food product labelling according to European  
 665 food legislation (regulation (EU) No 1169/2011<sup>71</sup>). In addition,  
 666 the sugar profile, total starch content and other important com-  
 667 ponents of the samples were measured or calculated. Protein con-  
 668 tent and content of available carbohydrates represent the main  
 669 differences in the macronutrient profile of RWB and HPHB. This  
 670 is essentially caused by the replacement of wheat flour, which is  
 671 high in starch (72.38 %DM), by HPIs with protein contents of  
 672 61.25 %DM (faba bean flour), 55.04 %DM (carob germ flour)  
 673 and 83.11 %DM (gluten) and starch contents below 10 %DM  
 674 (protein and starch contents of wheat flour and HPIs previously  
 675 reported by Hoehnel *et al.*<sup>30</sup>). While the total energy level of the  
 676 formulations is similar (RWB 211.6; HPHB 209.0), a shift from  
 677 wheat starch to non-wheat protein characterises the macronutri-  
 678 ent profile of HPHB. This shift is also evident when proteinE val-  
 679 ues (percentage of calories provided by protein) are compared.  
 680 In contrast to RWB with 15.5 %E, the HPHB formulation reaches  
 681 a proteinE of 24.8 %E and therefore qualifies for a "high in pro-  
 682 tein" nutritional claim in accordance with European food legis-  
 683 lation (regulation (EC) No 1924/2006<sup>72</sup>), where a proteinE of  
 684 20 % is set as requirement. Bread is a staple food with global  
 685 importance as source of dietary carbohydrates, protein and fi-  
 686 bre.<sup>15</sup> However, within the past 200 years, the consumption of  
 687 refined-carbohydrate products, including bakery products from  
 688 refined wheat flour (white bread, white bagels, white buns), has  
 689 substantially increased. At the same time, significantly less regu-  
 690 lar starchy foods like beans, lentils and wholegrain bakery prod-  
 691 ucts are consumed.<sup>7</sup> This is largely associated with generally bet-



692 ter sensory characteristics of refined-carbohydrate products and  
693 potentially higher consumer acceptance due to their sweet taste  
694 when starch is rapidly digested by salivary amylase.<sup>7,15</sup> The main  
695 concern regarding this development is related to high glycaemic  
696 indices due to rapidly digestible starch.<sup>2,7,15,73</sup> High-glycaemic-  
697 load and high-glycaemic-index diets have been associated with  
698 elevated risk for diabetes, heart disease and certain types of can-  
699 cer.<sup>74–78</sup> Due to its reduced content of available carbohydrates,  
700 HPHB is expected to have a lowered glycaemic load in compari-  
701 son to RWB. Even a decreased glycaemic index could be expected,  
702 since psyllium has been reported to lower the glycaemic index of  
703 foods when added to conventional diets.<sup>79,80</sup> Holt *et al.*<sup>81</sup> found  
704 a significantly lowered blood glucose response of high-protein  
705 bread when they compared equal-energy portions of high-protein  
706 bread and regular white bread. Furthermore, an isocaloric re-  
707 placement of refined starch or sugar by protein, like it is the case  
708 for HPHB compared to RWB, has been reported to reduce blood  
709 pressure and blood lipid concentrations.<sup>2,82</sup> Also the lack of fibre  
710 in refined-carbohydrate foods compared to wholegrain alterna-  
711 tives and legumes has been critically discussed.<sup>7,15</sup> Dietary fibre  
712 is associated with many health benefits and dietary recommen-  
713 dations advice a daily intake of 25 g or more for adults.<sup>2,83</sup> In  
714 the present study, HPHB contains with 2.8 % considerably more  
715 dietary fibre than RWB with 1.8 %. This is related to the in-  
716 corporation of faba bean flour, carob germ flour and psyllium  
717 in HPHB, which represent ingredients with notable contents of  
718 both soluble and insoluble fibre.<sup>30,57</sup> Especially psyllium has been  
719 reported in literature as dietary fibre with beneficial effects re-  
720 garding the risk of diabetes, obesity, high blood pressure and  
721 heart disease.<sup>52</sup> Apart from refined carbohydrates, also fat and  
722 salt (sodium chloride) are dietary components which are often  
723 critically discussed.<sup>84–86</sup> While HPHB contains with 0.440 % an  
724 amount of sodium similar to RWB (0.466 %), it has a slightly  
725 elevated fat content. However, this increase is mainly caused  
726 by higher contents of MUFA and PUFA, which are nutritionally  
727 more favourable than saturated fats.<sup>2,84</sup> Both formulations con-  
728 tain similar amounts of sugar (mono- and disaccharides) and their  
729 sugar profiles reveal little differences. They confirm that sucrose  
730 added in the recipe of HPHB is fully consumed during yeast fer-  
731 mentation, which was also evident in the results obtained from  
732 dough analyses. Slightly increased galactose and the presence of  
733 oligosaccharides like raffinose, stachyose and verbascose can be  
734 associated with high contents of galactooligosaccharides (GOS)  
735 reported for faba beans.<sup>87</sup> Slightly lower maltose and maltotriose  
736 levels in HPHB are potentially related to its lower starch content.

### 737 3.2.2 Amino Acid Profile

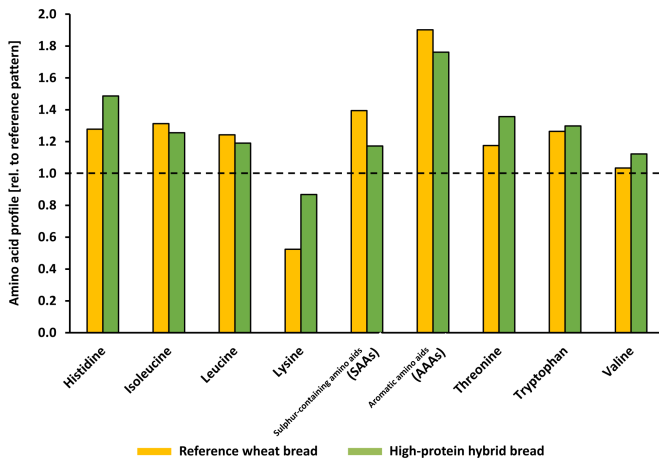
738 Many dietary recommendations advice a substantial decrease in  
739 the consumption of animal protein and a shift towards protein  
740 from plant sources.<sup>2,4</sup> Even though bread can be considered an  
741 important source of plant protein, the poor protein quality of  
742 wheat makes regular wheat bread (from both wholegrain or re-  
743 fined wheat flour) an inadequate choice to partially compensate  
744 future plant-protein requirements; especially when a substantial  
745 decrease in high-quality animal protein consumption is taken into  
746 account. The poor protein quality of wheat is mainly linked to

**Table 8** Amino acid composition of reference wheat bread and high-protein hybrid bread

Content [%Protein]	RWB	HPHB
<b>Indispensable and conditionally indispensable AAs</b>		
Histidine	1.92 ± 0.23	2.23 ± 0.27
Isoleucine	3.94 ± 0.48	3.77 ± 0.46
Leucine	7.33 ± 0.89	7.03 ± 0.85
Lysine	2.36 ± 0.29	3.90 ± 0.48
Cystine	1.99 ± 0.24	1.61 ± 0.19
Methionine	1.08 ± 0.13	0.97 ± 0.12
Cystine + Methionine (SAAs)	3.07 ± 0.37	2.58 ± 0.31
Phenylalanine	4.82 ± 0.59	4.46 ± 0.55
Tyrosine	2.41 ± 0.30	2.23 ± 0.27
Phenylalanine + Tyrosine (AAAs)	7.23 ± 0.88	6.69 ± 0.82
Threonine	2.70 ± 0.33	3.12 ± 0.38
Tryptophan	0.76 ± 0.48	0.78 ± 0.30
Valine	4.03 ± 0.49	4.38 ± 0.53
Total indispensable AAs	43.63 ± 5.71	43.75 ± 5.54
<b>Dispensable AAs</b>		
Asparagine/aspartic acid	4.13 ± 0.50	6.08 ± 0.74
Glutamine/glutamic acid	30.39 ± 3.69	25.71 ± 3.12
Glycine	3.94 ± 0.48	4.10 ± 0.50
Alanine	3.05 ± 0.37	3.40 ± 0.42
Serine	4.97 ± 0.61	4.63 ± 0.56
Proline	10.48 ± 1.27	8.11 ± 0.99
Arginine	3.74 ± 0.46	6.50 ± 0.79
Total dispensable AAs	60.69 ± 7.38	58.53 ± 7.10

Amino acid contents ± uncertainty values

747 an unbalanced amino acid composition, and to its lack of the  
748 indispensable amino acid lysine in particular.<sup>5,12,15</sup> The amino  
749 acid profile of RWB and HPHB was determined and is reported  
750 in Table 8. The results show that the proportions of indispens-  
751 able and dispensable amino acids are very similar in both for-  
752 mulations. Amongst the dispensable amino acids, only the lev-  
753 els of glutamine/glutamic acid, proline and arginine differ sub-  
754 stantially between RWB and HPHB. While wheat is particularly  
755 rich in glutamine, glutamic acid and proline but contains little  
756 arginine,<sup>12</sup> faba bean and carob show a complementary pattern  
757 for these AA.<sup>32,88</sup> Especially faba bean protein contains relatively  
758 small amounts of glutamine/glutamic acid and is high in argi-  
759 nine. This causes a decreased level of glutamine/glutamic acid  
760 and proline but an increased level of arginine in HPHB. Regarding  
761 the profile of indispensable AA in RWB and HPHB, many minor  
762 differences were observed. However, the lysine level is approxi-  
763 mately 65 % higher in HPHB than in RWB. Also this change can  
764 be attributed to faba bean and carob proteins which are naturally  
765 richer in lysine than wheat.<sup>12,32,88</sup> Even though the difference  
766 of lysine contents expressed in %Protein might seem small, this  
767 difference has a big impact on the breads' overall amino acid bal-  
768 ance and, thus, their protein quality. Especially when compared  
769 to a reference pattern of indispensable amino acids (for adults)  
770 recommended by WHO<sup>89</sup> and EFSA<sup>90</sup>, the significance becomes  
771 evident. The quantity of indispensable amino acids in RWB and  
772 HPHB relative to the amino acids in the reference pattern is pre-  
773 sented in Figure 4. The comparison with the reference pattern re-  
774 veals that in both formulations lysine is the only AA, which does  
775 not reach the quantity specified as recommended intake (= 1).  
776 Therefore, lysine represents the limiting AA of the protein in RWB



**Fig. 4** Profile of indispensable amino acids of reference wheat bread and high-protein wheat bread expressed relative to the requirement pattern (WHO 2007<sup>89</sup>) and based on an average intake of 0.66 g protein/kg

777 and HPHB. The increased lysine content in HPHB (87 % of lysine  
778 in reference pattern) compared to RWB (52 % of reference  
779 pattern) leads to a much more balanced AA profile that almost covers  
780 the recommended intake of all indispensable AA. The expression  
781 of AA levels in a food protein relative to the levels in a reference  
782 protein is referred to as amino acid score (AAS). Table 9 shows an  
783 overview of AAS and limiting AAs of RWB and HPHB and the ingredi-  
784 ents used for their production (wheat flour and HPIs). The

**Table 9** Amino acid scores (AASs) for breads and their raw materials

Protein source	AAS	Limiting AAs
RWB	0.52	Lysine
HPHB	0.87	Lysine
Wheat flour*	0.57	Lysine
Faba bean flour**	0.66	SAAs
Carob germ flour*	- (1.02)***	- (Valine)***
Gluten*	0.37	Lysine

\* calculated from amino acid composition; determined as for RWB and HPHB (data not shown)

\*\* calculated from amino acid composition; determined as for RWB and HPHB and reported by Vogelsang-O'Dwyer *et al.*<sup>32</sup>

\*\*\* not strictly limiting ( $\geq 1$ ), but represents AA with lowest level relative to reference pattern

784

785 HPHB formulation does not only have an improved AAS compared to RWB, but also in comparison to wheat flour and HPIs.  
786 The combination of the ingredients in HPHB leads to an upgrade  
787 in nutritional value of most raw materials when AAS is used to  
788 evaluate nutritional protein quality. The exception is the protein  
789 from carob germ flour, which has a nutritionally favourable AA  
790 pattern itself. Since the calculation of AAS is based on a recom-  
791 mended amino acid reference pattern, which considers an average  
792 intake of 0.66 g protein/kg bodyweight, this evaluation assumes  
793 that RWB or HPHB (or the ingredients) are the sole source  
794 of protein in the diet. In a real diet, proteins from other foods can  
795 potentially compensate for AA deficiencies. However, the ability  
796 of a dietary protein source to fulfil amino acid requirements on its  
797 own is regarded as an adequate approach to compare nutritional  
798 quality of proteins.  
799

### 800 3.2.3 Protein Digestibility and Utilisation

801 The informative value of AASs is also limited because they do not  
802 reflect the protein's digestibility, absorption and utilisation.<sup>91</sup> In  
803 the present study, protein digestibility was evaluated in an *in vitro*  
804 model as well as in an *in vivo* trial with rats (Table 10). *In vitro*  
805 protein digestibility (IVPD) of RWB and HPHB was monitored  
806 after 1 h of pepsin digestion and, subsequently, 1 h of pancrea-  
807 tin digestion, which is indicative of the digestibility in the hu-  
808 man digestive system. Additionally, IVPDs were measured after  
809 a medium term (3 h) and a long term (24 h) pancreatin di-  
810 gestion to evaluate the maximum achievable degradation of the  
811 proteins. Both the digestion mimicking gastric conditions (1 h  
812 pepsin) as well as the simulated intestinal digestion (1 h pan-  
813 creatin) yielded higher ratios of degraded protein for HPHB than  
814 for RWB, indicated by significantly higher IVPD values. This sug-  
815 gests a slightly improved protein digestibility of HPHB, which is  
816 remarkable since legumes, in HPHB specifically faba bean and  
817 carob, are often critically discussed regarding their contents of  
818 trypsin inhibitors and an associated decrease in protein digestibil-  
819 ity.<sup>92</sup> However, due to the incorporation of only 5.72 % of faba  
820 bean flour in the whole HPHB formulation (see Table 1), a sub-  
821 stantially reduced content of trypsin inhibitors, as compared to  
822 the faba bean raw material, is expected. A detailed discussion  
823 of the trypsin inhibitor activity (TIA) in HPHB follows in chapter  
824 3.2.4. A higher degree of protein degradation in HPHB could be  
825 explained by the higher abundance of lysine and arginine in this  
826 formulation. Trypsin, which is a predominant proteolytic enzyme  
827 in pancreatin, cleaves protein and peptide chains at the carboxyl  
828 side of these positively charged AA. Pancreatin also contains chy-  
829 motrypsin, which cleaves after hydrophobic AA with bulky side  
830 chains like phenylalanine, tryptophan and tyrosine. The contents  
831 of these AA are very similar in HPHB and RWB. However, abun-  
832 dance of target AA for trypsin and chymotrypsin proteolysis is  
833 not the only relevant factor. Also accessibility of such AA in the  
834 three-dimensional protein structure is of high importance. This  
835 suggests that HPHB contains a higher number of AA accessible  
836 for tryptic/chymotryptic digestion. The *in vivo* protein digestibil-

**Table 10** *In vitro* protein digestibility and *in vivo* nitrogen balance

Variable	RWB	HPHB
<b><i>In vitro</i> protein digestibility (IVPD) [%]</b>		
Pepsin 1 h	1.1 ± 0.4 <sup>b</sup>	2.0 ± 0.3 <sup>a</sup>
Pancreatin 1 h (short term)	14.2 ± 0.6 <sup>b</sup>	17.2 ± 0.3 <sup>a</sup>
<b><i>In vivo</i> nitrogen balance</b>		
N intake [g/5 d]	1203 <sup>b</sup> ± 359	1556 ± 94 <sup>a</sup>
N in faeces [mg/5 d]	138 ± 47 <sup>b</sup>	183 ± 12 <sup>a</sup>
N faecal [% N intake]	11.4 ± 1.0 <sup>a</sup>	11.8 ± 1.0 <sup>a</sup>
N in urine [mg/5 d]	766 ± 206 <sup>a</sup>	733 ± 35 <sup>a</sup>
N urinary [% N intake]	64.4 ± 3.1 <sup>a</sup>	47.1 ± 1.8 <sup>b</sup>
N digestibility [%]	88.6 ± 1.0 <sup>a</sup>	88.2 ± 1.0 <sup>a</sup>
N utilisation [%]	24.2 ± 2.7 <sup>b</sup>	41.0 ± 2.7 <sup>a</sup>
PER [g/g]	1.13 ± 0.39 <sup>b</sup>	2.13 ± 0.17 <sup>a</sup>

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



**Table 11** Contents of antinutritional compounds of reference wheat bread and high-protein hybrid bread, contents refer to fresh bread or dry matter as indicated

Antinutritional compound	RWB based on fresh bread	HPHB	RWB based on dry matter*	HPHB
Trypsin inhibitor activity (TIA) [TIU/mg]	n.d.	0.21 ± 0.01	n.d.	0.39 ± 0.02
Vicine [%]	n.d.	0.056 ± 0.005	n.d.	0.103 ± 0.009
Convicine [%]	n.d.	0.044 ± 0.001	n.d.	0.081 ± 0.002

Means ± standard deviation

\* calculated based on moisture of fresh bread given in Table 7; for comparison purposes

ity trials performed with rats yielded several variables indicative of the breads' nutritional value (Table 10). The most important are N intake, N digestibility, N utilisation and protein efficiency ratio (PER). N intake was monitored as a reference value to calculate relative faecal and urinary N losses. N intake was significantly higher for rats which were fed the diet containing HPHB (1556 g/5 d) compared to rats with RWB diet (1203 g/5 d). Since diets were adjusted to contain the same amount of protein, this means that rats consumed significantly more of their whole diet with HPHB. It is remarkable that N intake with HPHB diet even exceeded that of rats with the control casein diet (1262 g/5 d, data not shown). This could be associated with a higher palatability of HPHB diet compared to diets containing RWB or casein. N digestibility (according to faecal N loss) was similar between the two bread formulations in this study and no significant differences were found. Although literature reports good correlations between *in vitro* and *in vivo* digestibility data,<sup>93</sup> some legumes have been found to reach higher digestibility in *in vitro* experiments than *in vivo*.<sup>91</sup> This is in agreement with the slightly higher IVPD observed for HPHB in comparison to RWB in this study. N digestibility is also used to calculate the protein digestibility corrected amino acid score (PDCAAS), which is the most commonly used indicator of nutritional protein quality. Since N digestibility of RWB and HPHB is similar, PDCAAS values follow the same trend as AAS values discussed in the previous section. Related to its higher lysine content, HPHB reaches a PDCAAS of 0.77, which is 67 % higher than PDCAAS of RWB with 0.46. N utilisation considers N loss in both faeces and urine. Caused by a significantly lower urinary N loss of rats fed with HPHB diet, a by 69 % increased N utilisation was observed for HPHB compared to RWB. This is mainly linked to the improved AA pattern and higher content of lysine in HPHB. It has been shown that the lack of one or more essential AAs (provided by the diet and absorbed after digestion) leads to a plateau in AA retention. Other absorbed essential AA, which are present in excess of the limiting AA according to the required AA pattern, are oxidised in the blood and excreted with the urine.<sup>89,94</sup> In both animal and human studies, a correlation was found between level of imbalance of indispensable AA in the diet and inefficient AA utilisation leading to limited protein synthesis.<sup>95,96</sup> Corresponding to the higher N utilisation, also the determined PER was with 2.13 g/g significantly higher for rats with HPHB diet than for rats with RWB diet (1.13 g/g). Protein efficiency ratio is a widely used indicator of protein quality and reflects the protein's ability to fulfil AA requirements for growth (experiment performed with growing rats). An influence

of overall calorie and protein intake on N utilisation and PER has been discussed.<sup>91,94</sup> Therefore, differences in N utilisation and PER between HPHB and RWB in this study might be partially related to the higher N intake (hence, higher calorie intake) that was observed for HPHB. While both *in vitro* and *in vivo* models have their limitations, especially regarding transferability of results to the human digestive and metabolic system, they offer a valid comparison of proteins and their nutritional quality.<sup>93,97</sup> Protein digestibility is a matter of the degree of hydrolysis and release of amino acids for absorption. True protein quality is considered a measure of the balance of AA which are absorbed and utilised in the human body to achieve defined metabolic actions (e.g., growth).<sup>5,94</sup> Even though it is unknown, which AA in particular are absorbed and utilised in which ratios, the presented results (including AA profile, IVPD, N digestibility, N utilisation and PER) conclusively suggest improved protein quality of HPHB compared to RWB.

### 3.2.4 Antinutritional Compounds

Trypsin inhibitors and the pyrimidine glycosides vicine and convicine are considered antinutritional compounds and their activity/contents have been determined for HPHB and RWB in this study (Table 11). It is well known that trypsin inhibitors have the ability to form a complex with the proteolytic enzyme trypsin leading to its inactivation. While this can cause adverse effects like increased pancreatic secretory activity and pancreatic hypertrophy,<sup>24</sup> it is often responsible for substantially reduced protein digestibility.<sup>25</sup> No trypsin inhibitor activity (TIA) was detected for RWB. The TIA of 0.21 TIU/mg measured for HPHB can be considered very low compared to the approximately 10 fold higher TIA in the faba bean raw material used for HPHB reported by Vogelsang-O'Dwyer *et al.*<sup>32</sup>. However, this reduction of TIA is mainly related to the dilution effect in the bread matrix. While heat treatment is an efficient way to inactivate trypsin inhibitors (changes in active site conformation), baking seems to be considerably less efficient than other thermal processing techniques.<sup>92</sup> In addition to faba bean, also carob germ flour could be a source of trypsin inhibitors in HPHB.<sup>98,99</sup> According to the determined IVPD of HPHB and RWB, the remaining TIA in HPHB from faba beans or carob seeds did not lead to a decreased protein digestibility of HPHB compared to RWB. The results do not allow for an interpretation whether this is due to a negligible TIA in the bread matrix or due to the overall improved protein quality compensating for TIA. The ANCs vicine and convicine are particularly relevant in foods containing faba beans.<sup>100</sup>

When ingested by individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, these compounds can trigger favism, which leads to acute haemolytic anaemia.<sup>28</sup> On average, the sum of vicine and convicine accounts for about 1 %DM in faba beans.<sup>27,100</sup> However, efforts in plant breeding have led to cultivars with contents of the pyrimidine glycosides as low as 0.01 - 0.02 %DM.<sup>27</sup> Vogelsang-O'Dwyer *et al.*<sup>32</sup> reported a content (vicine + convicine) of 1.25 %DM in the faba bean flour used for HPHB. While vicine and convicine were, expectedly, not detected in RWB, HPHB contains 0.056 % vicine and 0.044 % convicine (contents referring to fresh bread). In a recent study by Gallo *et al.*<sup>101</sup>, G6PD deficient men consumed large quantities (500 g) of faba beans from a low vicine/convicine variety (0.016 % based on wet weight as ingested). It was confirmed that this level of intake was safe and favism was not triggered. Based on the outcomes from Gallo *et al.*<sup>101</sup> and the results of the present study, the consumption of at least 80 g of HPHB (equivalent to 2 slices of bread with a typical weight of 38 g per slice<sup>102</sup>) can be considered safe for individuals with G6PD deficiency. The incorporation of faba bean flour in HPHB leads to a substantial dilution of ANCs as compared to the raw material. This underlines the value of HPHB, and formulations of its kind, with regard to nutritional aspects. In theory, the separate consumption of legumes and cereals as part of a balanced diet can guarantee a balanced AA intake similar to the pattern of HPHB. But the presence of higher amounts of ANCs, which affect protein digestibility and AA bioavailability, might substantially reduce the capacity of legumes to compensate for the lack of lysine in cereals, when consumed separately.

### 3.2.5 Antioxidant Potential

Phenolic compounds, and specifically phenolic acids and flavonoids, exhibit many biological activities. They are well known for their antioxidant activity through which they prevent oxidative damage of biomolecules like lipids, proteins and DNA.<sup>103</sup> Amongst many other factors, such oxidative damages have been associated with the occurrence of both degenerative and neurodegenerative diseases such as cancer, inflammatory and cardiovascular conditions and Alzheimer's disease.<sup>104</sup> It has been demonstrated in epidemiological studies that high intake of foods containing high levels of compounds with antioxidant activity (e.g., whole-grain foods and legumes) can help to prevent the development of these diseases.<sup>105-108</sup> The total content of phenolics of RWB and HPHB was determined. Additionally, the antioxidant

potential of the phenolic extracts of the breads was evaluated using ABTS, FRAP and DPPH assays. The results are presented in Table 12. The total content of phenolics is with 66.1 mg/100 g substantially higher in HPHB than in RWB with only 15 mg/100 g. Also the assays performed to determine antioxidant activity of the phenolic extracts conclusively suggest an increased antioxidant potential of HPHB than RWB. High levels of antioxidant compounds have been reported for legumes<sup>18</sup> and faba bean and carob in particular.<sup>17,43,109</sup> Therefore, they are expected to be the main contributors to the enhanced antioxidant potential of HPHB. The same trend was observed by Turfani *et al.*<sup>23</sup> when they evaluated antioxidant potential of breads enriched with carob flours. Also wheat is naturally rich in phenolics. But since these compounds are mainly found in the bran fraction, the antioxidant potential of breads produced from refined wheat flour is usually low.<sup>110</sup> Ragaee *et al.*<sup>111</sup> investigated the content of phenolics and antioxidant potential of refined wheat bread when wheat flour was partially replaced (30 %) by wholegrain flours from different cereals (wheat, rye, oats, barley). The incorporation of all whole-grain cereals flours increased the breads' antioxidant potential. The highest content of phenolics of approximately 70 mg/100 g was observed when wholegrain rye flour was added, which is similar to the content of phenolics reached by HPHB in the present study. Since the phenolics in a food matrix are present either free or bound to polysaccharides, a prediction whether they can exert antioxidant activity *in vivo* is difficult. Digestibility of the food, which determines bioavailability of the phenolics, is an important factor and *in vivo* antioxidant activity does not always correlate with *in vitro* data.<sup>112</sup> However, the results in this study clearly show higher antioxidant potential for HPHB than RWB.

### 3.3 Sensory Characteristics

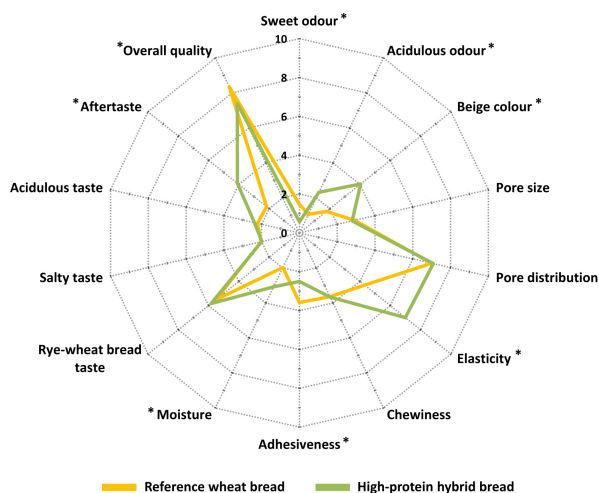
Consumer acceptance of food products is highly depending on sensory characteristics, which are in turn related to the products' technological quality. Due to its enhanced nutritional profile and qualification for the nutritional claim "high in protein",<sup>72</sup> HPHB can be considered a functional food. According to consumer surveys reported in literature, consumers evaluate functional foods the same way they evaluate conventional foods. Functional benefits are perceived merely as added value and cannot outweigh inferior sensory properties.<sup>113</sup> Sensory analysis for the two formulations in this study was performed with a trained panel using selected descriptors for bread quality (Figure 5). Reference wheat bread and HPHB reached similar scores for attributes describing taste and porosity of the crumb. Interestingly, the differences in crumb structure, which were observed in technological analyses of the breads, were not perceived by the panellists. The results for HPHB further indicate an improved crumb texture, which is often perceived as an indicator of freshness amongst consumers.<sup>114,115</sup> Compared to RWB, it scored significantly higher in elasticity and lower in adhesiveness. While elasticity of bread crumb is recognised as a favourable attribute, adhesiveness is often associated with stickiness and an unpleasant mouthfeel.<sup>115</sup> Both formulations reached similar scores in chewiness. This shows that the slightly increased initial crumb hardness for HPHB, which was

**Table 12** Antioxidant potential of reference wheat bread and high-protein hybrid bread, contents refer to fresh bread unless stated otherwise

Antioxidant potential	RWB	HPHB
Total phenolics [mg/100 g]	15.8 ± 0.3 <sup>b</sup>	66.1 ± 0.3 <sup>a</sup>
ABTS [mmol Trolox/100 g]	0.08 ± 0.01 <sup>b</sup>	1.02 ± 0.03 <sup>a</sup>
FRAP [mmol Fe <sup>2+</sup> /100 g]	0.23 ± 0.01 <sup>b</sup>	0.77 ± 0.01 <sup>a</sup>
<b>Antiradical activity (DPPH)</b>		
EC <sub>50</sub> [mg extract/mL] <sup>×</sup>	6.22 ± 0.18 <sup>a</sup>	1.15 ± 0.03 <sup>b</sup>

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

<sup>×</sup> Concentration of phenolic extract of breads able to scavenge 50 % of DPPH radicals



**Fig. 5** Sensory characteristics of reference wheat bread and high-protein hybrid bread; asterisks \* indicate attributes which showed significant differences between RWB and HPHB ( $p < 0.05$ )

1057 placement of refined wheat-starch by plant protein. The protein  
 1058 quality was improved, judging by a better AA profile, increased N  
 1059 utilisation and higher protein efficiency ratio. Mainly due to the  
 1060 dilution effect in the bread matrix, only low levels of ANCs origi-  
 1061 nating from faba bean and carob were measured. Furthermore,  
 1062 determination of phenolics and antioxidant activity indicate high  
 1063 antioxidant potential for HPHB. Apart from favourable technologi-  
 1064 cal and nutritional characteristics, the proposed formulation also  
 1065 has high sensory quality which suggests high consumer accep-  
 1066 tance. In a time in which we are looking for ways to adequately  
 1067 and sustainably provide enough high-quality plant protein for a  
 1068 future human diet, we cannot afford to focus only on meat and  
 1069 dairy replacement products; especially considering that these ap-  
 1070 plications often require highly purified or additionally function-  
 1071 alised plant proteins obtained by wet-processing. In the proposed  
 1072 high-protein hybrid bread formulation, dry-processed protein in-  
 1073 gredients from faba bean and carob were applied and provide a  
 1074 substantial amount of non-wheat protein. The increased content  
 1075 of plant protein with higher protein quality in HPHB and formu-  
 1076 lations of its kind, could improve the capacity of the staple food  
 1077 bread to cover protein needs in future plant-based diets. The re-  
 1078 sults also suggest that a replacement of regular wheat bread by  
 1079 high-protein hybrid breads could be beneficial in currently con-  
 1080 sumed diets.

1022 detected in texture profile analysis (TPA), had no perceptible  
 1023 negative impact on the mouthfeel of the bread crumb. High-  
 1024 protein hybrid bread scored higher than RWB in moisture of  
 1025 crumb, which is considered another indicator for bread fresh-  
 1026 ness and quality.<sup>115</sup> Significant differences have been found re-  
 1027 garding the odour profile of the formulations. While for RWB a  
 1028 slight sweet and almost no acidulous odour was perceived, HPHB  
 1029 had no perceivable sweet odour and slightly stronger acidulous  
 1030 odour than RWB. In accordance with the results of instrumen-  
 1031 tal crumb colour measurements, a darker/more beige colour was  
 1032 observed for HPHB. Also a moderate increase in aftertaste was  
 1033 identified in HPHB. However, the overall sensory quality was  
 1034 rated only slightly lower for HPHB than for RWB. This identifies  
 1035 HPHB as a bread formulation with adequate sensory quality when  
 1036 compared to RWB, suggesting high consumer acceptance. The  
 1037 scores of HPHB for sensory attributes like acidulous odour and  
 1038 beige colour suggest similarities to the typical sensory profile of  
 1039 sourdough bread.<sup>116,117</sup> Because of the popularity of sourdough  
 1040 bread amongst consumers, this could further contribute to a high  
 1041 consumer acceptance of HPHB.

## 1042 4 Conclusion

1043 A mixture of HPIs was used to partially replace wheat flour in regu-  
 1044 lar wheat bread to produce a high-protein bread. The HPIs and  
 1045 their ratios were selected based on previous results by Hoehnel  
 1046 *et al.*<sup>30</sup> to represent both beneficial expected nutritional prop-  
 1047 erties as well as adequate baking properties. In order to match  
 1048 the technological quality of a regular wheat bread, which was  
 1049 used as a reference, also three functional ingredients (psyllium,  
 1050 sugar, xylanase) were added. Dough and bread quality compar-  
 1051 able to the reference wheat bread were observed for high-protein  
 1052 hybrid bread (HPHB); mainly mediated by the functional proper-  
 1053 ties of carob and gluten protein as well as psyllium and xylanase.  
 1054 Additionally, a substantially enhanced nutritional profile of the  
 1055 proposed HPHB compared to regular wheat bread was achieved.  
 1056 The macronutrient composition was improved by an isocaloric re-

## 1081 Conflicts of interest

1082 There are no conflicts to declare.

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## 1092 Abbreviations

1093 The following abbreviations are used in this manuscript:

AA	Amino acid
SAA	Sulphur-containing amino acids
ANC	Antinutritional compound
HPI	High-protein ingredient
HPHB	High-protein hybrid bread
RWB	Reference wheat bread
LCA	Life cycle assessment
HP	High-protein
TM	Torque maximum
PMT	Peak maximum time
PV	Peak viscosity
FV	Final viscosity
$V_{total}$	Total gas volume produced
$V_{lost}$	Volume of CO <sub>2</sub> lost
$V_{ret}$	Volume of gas retained
$H_M$	Maximum height of dough development
SV	Specific volume
L*crust	Lightness of crust

L* crumb	Lightness of crumb	1124
IVPD	<i>In vitro</i> protein digestibility	1125
TNBS	Trinitrobenzenesulfonic acid	1126
C	Casein	1127
SF	Soya flour	1128
SPI	Soya protein isolate	1129
BW	Body weight	1130
PER	Protein efficiency ratio	1131
L-BAPA	N- $\alpha$ -benzoyl-L-arginine-4-nitroanilide	1132
TIU	Trypsin inhibitor unit	1133
TAU	Trypsin activity unit	1134
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid	1135
TPC	Total phenolic content	1136
TEAC	Trolox equivalent antioxidant capacity	1137
FRAP	Ferric-reducing antioxidant power	1138
DPPH	2,2-diphenyl-1-picrylhydrazyl	1139
QDA	Quantitative descriptive analysis	1140
ANOVA	Analysis of variance	1141
AX	Arabinoxylans	1142
%DM	Percentage based on dry matter	1143
proteinE	Percentage of calories provided by protein	1144
%E	Percentage based on energy	1145
SFA	Saturated fatty acids	1146
MUFA	Mono unsaturated fatty acids	1147
PUFA	Poly unsaturated fatty acids	1148
%Protein	Percentage based on protein	1149
AAA	Aromatic amino acids	1150
AAS	Amino acid score	1151
PDCAAS	Protein digestibility corrected amino acid score	1152
TIA	Trypsin inhibitor activity	1153
EC <sub>50</sub>	Half maximal effective concentration	1154

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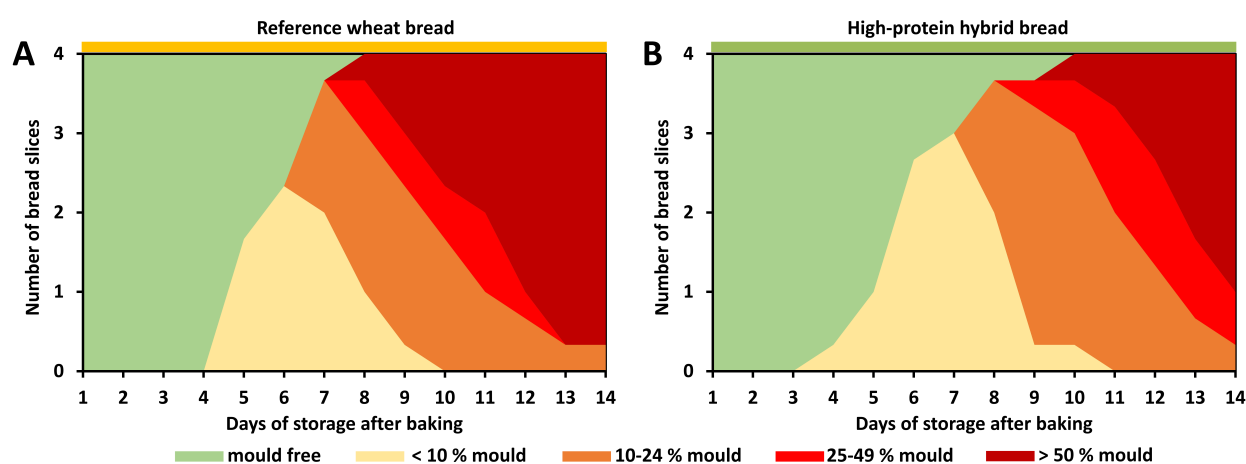
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## ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

for Food & Function article "Enhancing the nutritional profile of regular wheat bread while maintaining technological quality and adequate sensory attributes"

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### Microbiological Shelf Life and Water Activity of Reference Wheat Bread (RWB) and High-Protein Hybrid Bread (HPHB)



**Fig. 1** Microbiological shelf life of (A) RWB and (B) HPHB as indicated by ambient air challenge test. Results represent the mean of three independently performed challenge tests.

### Results and Discussion

In addition to crumb staling, the shelf life of bread is affected by microbial deterioration. While also bacteria and yeast can cause bread spoilage, a contamination with fungal spores from the bakery environment after baking is considered the most common reason.<sup>1</sup> Mold growth typically shows a positive correlation with water availability in the food product; the critical water activity, however, varies with fungal species, temperature and substrate.<sup>2</sup> Apart from an unpleasant visual experience for consumers, mould

spoilage can cause the formation of off-flavours, allergenic compounds and mycotoxins, potentially even before visibility of fungal growth.<sup>3</sup> It also leads to a substantial amount of food waste - in UK households an estimated 20 % of bread goes to waste due to mould growth.<sup>4,5</sup> Therefore, susceptibility to mould deterioration represents a food safety hazard and indicator for economic loss and should be considered when bread quality is evaluated. The microbial shelf life of both bread formulations was monitored in an ambient air challenge test. The results are presented in Figure 1. A slight tendency towards earlier onset of mould growth for HPHB was observed. The results also suggest a deceleration of mould growth in HPHB represented by later onset of stages 3 - 5 (10 to > 50 % of slices covered in mould). However, these tendencies cannot be considered significant differences and the experiment generally indicated a similar microbial shelf life of HPHB and RWB. This observation can be supported by very similar water activities measured for both formulations (RWB  $0.945 \pm 0.003$ , HPHB  $0.943 \pm 0.003$ ).

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## Materials and Methods used for the Determination of Microbiological Shelf Life and Water Activity

Microbiological shelf life of the breads was evaluated using an ambient mould challenge test as described by Dal Bello *et al.*<sup>6</sup> with some modifications. Bread loaves were sliced in a sterile manner to obtain four slices of 20 mm thickness per loaf. Instead of a treatment with conidial solutions of fungi, each slice was microbiologically challenged by exposure to the bakery ambient air for 5 min on each side. The slices were separately packed in sterile plastic bags which were heat sealed. To guarantee comparable aerobic conditions in all bags, a filter pipette tip was inserted. During a storage period of 14 days (at room temperature), mould growth was visually evaluated. Based on the percentage of slice area covered with fungal growth, slices were sorted into five categories as follows: 0 % - mould free, <10 % mould, 10-24 % mould, 25-49 % mould, >50 % mould. Four slices were monitored from each of three batches per formulation. Water activity of the fresh bread crumb was measured using a water activity meter (HygroLab, Rotronic, Basserdorf, Switzerland).

## Abbreviations

The following abbreviations were used:

HPHB	High-protein hybrid bread
RWB	Reference wheat bread

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