TRANSLATING PLANT PROTEIN BENEFITS TO CONSUMER PRODUCTS

Physicochemical, Technological and Nutritional Aspects of Utilising Plant Protein Ingredients for Cereal Food Applications

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LIST OF ABBREVIATIONS

a*	Colour value defining the space between magenta (positive) and green (negative) in the CIELAB colour space colour space
AA	Amino acid
AAA	Aromatic amino acids
AAS	Amino acid score
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANC	Antinutritional compound
ANOVA	Analysis of variance
L-BAPA	N-α-benzoyl-*L*-arginine-4-nitroanilide
AX	Arabinoxylans
b*	Colour value defining the space between yellow (positive) and blue (negative) in the CIELAB colour space colour space
BP	High-protein buckwheat pasta
BU	Brabender units
B-3U	Betamyl-3 units as defined in K-BETA test kit to express β-amylase activity
BW	Bodyweight
C	Casein
CA	Correlation analysis
CaEDTA	Calcium disodium ethylene diamine tetraacetate
Carrez I	15 g per 100 mL of ultrapure water, potassium hexacyanoferrate(II) trihydrate
Carrez II	23 g per 100 mL of ultrapure water, zinc acetate dihydrate
cc	Correlation coefficient
CFU	Colony forming units
CU	Ceralpha units as defined in K-CERA test kit to express α-amylase activity
CWB	Control wheat bread
CT	Concentration treatment
DampF	damping factor
DF	Faba bean dehulled flour
DFB	Wheat bread containing DF
DFSD	DF fermented with *Leuconostoc citreum* TR116 starting from inoculation (0 h)
DFSDB	Wheat bread containing DFSD
DIAAS	Digestible indispensable amino acid score

DMSO	Dimethylsulfoxide
DNS	3,5-Dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
%DM	Percentage based on dry matter
EC_{50}	Half maximal effective concentration
EPS	Exopolysaccharides
EtOH	Ethanol
80% EtOH	80/20 (v/v) ethanol/ultrapure water
ΔE	Differential colour index
%E	Percentage based on energy
DY	Dough yield
FAO	Food and Agriculture Organization of the United Nations
FB	Faba bean
FODMAPs	Fermentable oligo-, di-, mono-saccharides and polyols
FP	High-protein faba bean pasta
FQ	Fermentation quotient
FU	Farinograph units
FV	Final viscosity
FRAP	Ferric-reducing antioxidant power
FWA	Farinograph water absorption
GDD	Global Dietary Database
GI	Glycaemic index
GL	Glycaemic load
GMO	Genetically modified organism
GOS	Galactooligosaccharides
G6PD	Glucose-6-phosphate dehydrogenase
HI	Hydrolysis index
H_M	Maximum height of dough development
HP	High-protein
HPHB	High-protein hybrid bread
HPHP	High-protein hybrid pasta
HPC	High performance carbohydrate column
HPI	High-protein ingredient
HT	Heat treatment
IAA	Indispensable amino acid
IEP	Isoelectric precipitation
IVPD	*In vitro* protein digestibility
L*	Lightness value in the CIELAB colour space
LAB	Lactic acid bacteria

LCA	Life cycle assessment
LOD	Limit of determination
LOQ	Limit of quantification
L*crumb	Lightness of crumb in the CIELAB colour space
L*crust	Lightness of crust in the CIELAB colour space
LP	High-protein lupin pasta
MCEC	Micellar electrokinetic capillary chromatograph
MeCN	Acetonitrile
MeOH	Methanol
MRS	deMan-Rogosa-Sharpe
MUFA	Mono unsaturated fatty acids
NaN_3	Sodium azide
OCT	Optimal cooking time
PCA	Principal component analysis
PDCAAS	Protein digestibility corrected amino acid score
PER	Protein efficiency ratio
PI	Protein ingredient
PMT	Peak maximum time
PP	Polypropylene
PR	Air-classified faba bean high-protein flour
PRB	Wheat bread containing PR
PRSD	PR fermented with *Leuconostoc citreum* TR116 starting from inoculation (0 h)
PRSDB	Wheat bread containing PRSD
proteinE	Percentage of calories provided by protein
PUFA	Poly unsaturated fatty acids
PV	Peak viscosity
%Protein	Percentage based on protein
QDA	Quantitative descriptive analysis
QuEChERS	Quick, easy, cheap, effective, rugged and safe
RFO	Raffinose family oligosaccharides
RID	Refractive index detector
RDS	Rapidly digestible starch
RSM	Response surface methodology
RWB	Reference wheat bread
RWP	Reference wheat pasta
SAA	Sulphur-containing amino acids
SDG	Sustainable Development Goals
SEM	Scanning electron microscopy

SF	Soy flour
SFA	Saturated fatty acids
SP	Sugar-Pak I column
SPE	Solid phase extraction
SPI	Soy protein isolate
SV	Specific volume
TEAC	Trolox equivalent antioxidant capacity
TAU	Trypsin activity unit
TDF	Total dietary fibre
TIA	Trypsin inhibitor activity
TIU	Trypsin inhibitor unit
TM	Torque maximum
TNBS	Trinitrobenzenesulfonic acid
TPC	Total phenolic content
TTA	Total titratable acidity
TVP	Texturised vegetable protein
UF	Ultrafiltration
UPW	Ultrapure water
VC	Sum of vicine and convicine
V_{lost}	Volume of CO_2 lost
V_{ret}	Volume of gas retained
V_{total}	Total gas volume produced
WAI	Water absorption index
WHO	World Health Organization
WRI	World Resources Institute

ABSTRACT

The way we currently produce and consume food puts both environmental sustainability and human health at great risk. A global shift to predominantly plant-based diets as part of a food system transformation promises remedies for environmental implications as well as rising diet-related disease risks. Nonetheless, a decreased consumption of animal-based foods and increased inclusion of plant-based protein in our diets entails challenges with regard to the development of plant-based protein-foods and their convenience, technological quality and nutritional quality, which were addressed in this thesis. A thorough physicochemical characterisation of plant-based high-protein ingredients (HPIs) is important to explore their full potential for human nutrition and to identify appropriate processing techniques. For example, the quantification of short-chain carbohydrates in plant-based HPIs is required when fermentation technology is applied. In a study comparing commonly used aqueous extraction methods for short-chain carbohydrates with alternative ethanolic extraction methods, ethanolic extraction was found to be superior. Due to high water absorption capacity and gelation, aqueous extraction is often not applicable to plant-based HPIs from a practical perspective. Furthermore, enzymatic activity causing conversion or degradation of short-chain carbohydrates can be minimised with ethanolic extraction. Cereal-based staple foods, like bread and pasta, represent promising matrices for the incorporation of plant-based HPIs (e.g. partial replacement of wheat flour or wheat semolina) and offer the opportunity to provide plant-based protein-foods in convenient formats with high consumer acceptance. In a fundamental bread application study, several plant-based HPIs (derived from cereals, potato, legumes) were screened for their baking performance in high-protein breads (> 20% of calories provided by protein). Based on the findings of this study, an optimised high-protein bread formulation (containing faba bean and carob HPIs) with technological quality and sensory attributes similar to regular wheat bread and improved nutritional profile was proposed. This high-protein hybrid bread was characterised by an isocaloric replacement of wheat starch by non-cereal protein (starch content – 21%, which indicates reduced glycaemic load) and an improved amino acid balance (lysine content based on protein + 65%). Also, a substantially improved nitrogen utilisation (+ 69%) and protein efficiency ratio (+ 88%) compared to regular wheat bread were found in *in vivo* nitrogen balance tests. Fermentation (utilising the strain *Leuconostoc citreum* TR116) was investigated as a tool to further improve HPIs' baking performance and proven to successfully functionalise faba bean ingredients for bread applications, for example, by improving gluten-aggregation, bread volume (+ 19–22%) and crumb hardness (– 38–49%) in the presence of fermented (instead of unfermented) faba bean ingredients. For pasta applications, the combination of HPIs derived from pseudocereals (buckwheat) and legumes (lupin, faba bean) was particularly beneficial to achieve good technological quality since the HPIs were shown to compensate each others' (positive and negative) effects on quality characteristics like cooking loss, stickiness and tensile strength. The improvement of the high-protein hybrid pasta's nutritional quality (compared to regular wheat pasta) was also quantified and found to be similar to that observed for high-protein hybrid bread (compared to regular wheat bread).

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CHAPTER 1

Introduction

1.1 Introduction

The food system, in its current state, is responsible for a large proportion of environmental changes, such as biodiversity loss, nitrogen cycle disruption and climate change [1, 2]. For example, food production has been linked to approx. 30% of anthropogenic greenhouse gas emissions [3]. Moreover, unhealthy diets have been identified as the greatest global burden of disease (Global Panel on Agriculture and Food Systems for Nutrition, 2016 [4]) and the incidence rate of diet-related non-communicable diseases like obesity, diabetes and cardiovascular disease is on the rise. This shows that the way we currently produce and consume food severely affects both environmental sustainability and human health. It also demonstrates that a reconfiguration of the food system is urgently required, which has been discussed in many recent publications [2, 3, 5–12]. A global shift to predominantly plant-based diets is regarded as one of the most effective strategies to achieve fundamental food system transformation [2, 13–15]. A comparison of vegetarian and non-vegetarian diets by Marlow *et al.* [16] concluded that vegetarian diets require approx. 13 times less fertiliser than non-vegetarian diets (which promises reduced disruption of the nitrogen cycle), mainly due to the exclusion of beef. van Dooren *et al.* [17] assigned sustainability scores (based on associated greenhouse gas emissions and land use; the higher the score, the more sustainable) to vegan, vegetarian and non-vegetarian diets and found that vegan diets scored highest (130), followed by vegetarian (109) and other diets (e.g. Mediterranean (102), average Dutch diet (68)), particularly due to the exclusion of meat and dairy. van Dooren *et al.* [17] also determined health scores (based on contents of foods/compounds positively (e.g. vegetables, fruits, fibre) and negatively (e.g. saturated fat, free sugars, salt) associated with human health) and reported similar trends as for sustainability scores (with the exception that Mediterranean scored highest): vegan diet – 118, vegetarian diet – 100, Mediterranean diet – 122, average Dutch diet – 75.

In the shift to predominantly plant-based diets, dietary protein plays a pivotal role for several reasons [18]. Most animal foods are high in protein and represent an important source of dietary protein for human nutrition. Therefore, environmental implications related to our current food system are strongly interlinked with protein production [11]. However, insufficient protein intake (rather than insufficient calorie intake) can lead to malnutrition [18, 19]. Thus, it is vital that adequate protein intakes can be provided by plant-based protein sources when predominantly plant-based diets are consumed. The often reported inferior protein digestibility of plant-based protein compared to animal-based protein and the lack of some indispensable amino acids (IAAs) in individual plant-based protein sources have raised concerns

about the adequacy of plant-based protein supply [20–25]. Nonetheless, several publications have highlighted that the key to providing adequate dietary protein from predominantly plant-based diets is a large diversity in consumed plant-based protein-foods [3, 21, 23, 26, 27].

Chapter 2 of this thesis provides an overview on current protein intake levels (in comparison to recommended protein intakes), the diversity of current plant-based protein supply and protein-food related consumer trends. Based on these considerations, principles for the development of plant-based protein-foods in support of food system transformation are proposed and promising plant-based protein sources, ingredient types and food applications are reviewed. Furthermore, the importance of the availability of plant-based high-protein ingredients (HPIs) for the development of plant-based protein-foods is highlighted.

Cereals, pseudocereals and legumes are considered as some of the most promising sources for plant-based HPIs. The increased utilisation of such plant-based protein sources for human nutrition bears several challenges, including their fundamental physicochemical characterisation, which builds the foundation for research in this area. For example, the reliable quantification of short-chain carbohydrates in plant-based protein sources and HPIs helps to identify appropriate processing techniques (e.g. fermentation) and food applications. Since plant-based HPIs often have high water absorption and gelling capacity, aqueous extraction procedures with high solid/liquid ratios are difficult to apply. **Chapter 3** compares commonly used aqueous extraction procedures with ethanolic extractions and assesses their suitability for the extraction of short-chain carbohydrates from plant-based HPIs for subsequent quantification with HPLC-RID.

There are different food product groups with great potential to incorporate a large diversity of plant-based proteins in our diets. In addition to plant-based substitutes of traditional animal-based foods (e.g. dairy, meat, fish), also cereal/non-cereal staple food hybrids are promising. These are cereal staple foods that contain non-cereal HPIs. Bread and pasta, which enjoy high consumer acceptance worldwide due to their perceived high level of convenience and usually high sensory quality, are popular examples of such cereal staple foods. However, many studies have reported detrimental effects of plant-based HPI incorporation on the products' quality characteristics and sensory attributes [28–40]. Inferior technological and sensory quality of such high-protein hybrid products compared to regular bread and pasta needs to be addressed since it can lead to poor consumer acceptance.

Chapter 4 presents a thorough investigation of selected plant-based HPIs from cereals (wheat gluten, zein), potato and legumes (carob, pea, lupin, faba bean) and their incorporation in simple wheat bread formulations. Fifteen percent of wheat flour were replaced by the HPIs to ascertain that at least 20% of calories were

provided by protein in the final bread products (requirement for 'high in protein' nutrition claim according to European legislation [41]). For each formulation, the impact of HPIs on dough characteristics (e.g. water absorption, gluten-aggregation profile, starch gelatinisation, dough rheology) and bread quality characteristics (e.g. loaf volume, crumb texture, crumb structure) was analysed to assess the HPIs' baking performance.

Based on results presented in Chapter 4, an optimised high-protein hybrid bread (HPHB) formulation containing HPIs from faba bean, carob and gluten is proposed in **Chapter 5**. This HPHB formulation was designed to possess technological quality and sensory attributes similar to regular wheat bread. In addition to technological and sensory quality, the HPHB formulation was thoroughly characterised regarding nutritional aspects. This characterisation is focused on protein quality, including the analysis of amino acid composition, *in vitro* protein digestibility and *in vivo* nitrogen balance. Many scientists have urged for a more holistic nutritional evaluation of plant-based protein-foods, rather than a sole focus on protein quality [26, 42, 43]. Therefore, also HPHB's macronutrient profile and antioxidant potential were determined.

Fermentation technology is one of the oldest biotechnological processes in food production, specifically, sourdough technology in bread production [44]. It has also been reported as a powerful tool to improve the nutritional value of legumes (by reducing levels of antinutritional compounds) and to functionalise legume ingredients for bakery applications. **Chapter 6** investigates single strain fermentation of faba bean ingredients with the multifunctional lactic acid bacteria (LAB) strain *Leuconostoc citreum* TR116 and its impact on the faba bean ingredients' performance in bread formulations.

Pseudocereal and legume HPIs were also used to formulate a high-protein hybrid pasta (HPHP). **Chapter 7** explores the technological quality and sensory attributes of this HPHP (containing a combination of pseudocereal and legume HPIs) in comparison to regular wheat pasta as well as pastas containing single pseudocereal and legume HPIs. The nutritional value of HPHP is addressed in **Chapter 8** by examining protein quality (amino acid profile, *in vitro* protein digestibility, *in vivo* nitrogen balance), macronutrient profile and *in vitro* starch digestibility in comparison to regular wheat pasta.

The objective of this thesis is the exploration of ways to translate plant protein benefits to consumer products with a focus on cereal food applications. Figure 1.1 provides a structural overview of this thesis.

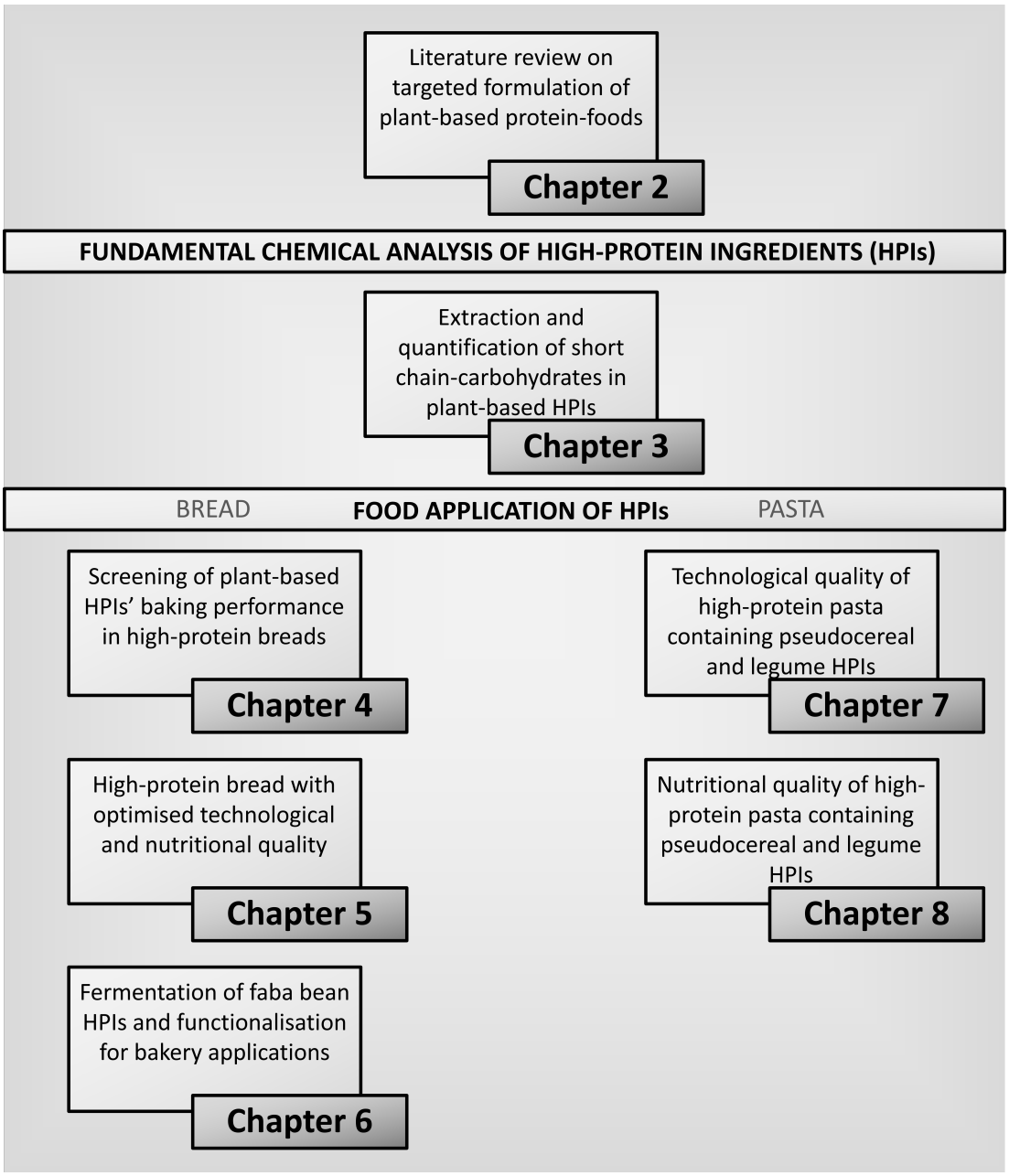


Figure 1.1: Structural overview of the thesis.

1.2 References

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CHAPTER 2

Targeted Formulation of Plant-Based Protein-Foods: Supporting the Food System's Transformation in the Context of Human Health, Environmental Sustainability and Food Trends

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2.1 Abstract

With food production being a key contributor to nitrogen cycle disruption, biodiversity loss and climate change, the lacking sustainability of our food systems is a global issue. At the same time, unhealthy diets have been identified as the greatest global burden of disease. Both aspects underline the urgency of food system transformation and call for immediate action. A global shift to predominantly plant-based diets has been described as an effective strategy to make the food system more sustainable. While concerns regarding adequate protein supply from predominantly plant-based diets have been raised, many publications state that adequate protein can be provided when sufficiently diverse plant-based diets are consumed. In particular, a more holistic approach in evaluating food and diet quality (supply of dietary fibre, micronutrients and moderate levels of glycaemic load, salt, saturated fats) has been advocated. This review article provides an overview on current protein intake levels, the diversity of current protein supply and consumer trends and proposes principles for the targeted formulation of plant-based protein-foods that support and accelerate food system transformation. Vegetal protein intake seems to lack diversity and is mainly covered by cereal foods worldwide. Therefore, an increased inclusion of non-cereal proteins in plant-based protein-foods is desirable.

2.2 Introduction and Environmental Perspective

Food production has been identified as one of the strongest drivers for global environmental change and three of the most serious implications are nitrogen cycle disruption, biodiversity loss and climate change [1–3]. Recent estimates associate approx. 30% of anthropogenic greenhouse gas emissions with food production, as well as 70% of freshwater use and the occupation of 37% of ice-free land surface [2, 4]. Multiple publications have pointed out that fundamental changes in the food system are needed to preserve environmental health [2–11]. Furthermore, our chances to reach the Sustainable Development Goals (SDGs) established by the UN [5] and to meet targets set in the Paris Agreement depend greatly on a transformation of the food system [8]. The increasing prevalence of diet-related disease risks indicates that also the preservation of human health, in addition to environmental health, requires food system transformation [4, 12–15]. According to the 2016 report of the Global Panel on Agriculture and Food Systems for Nutrition [14], unhealthy diets represent the greatest global burden of disease. In fact, six out of the top eleven disease risk factors are diet-related and rank amongst other serious risk factors like alcohol and drug use, unsafe sex, air pollution and tobacco smoking [14, 15]. Inadequate nourishment, which refers to overnutrition, undernutrition and malnutrition, is on the rise, and so are obesity, diabetes and other diet-related non-communicable diseases [16]. For example, the increasing consumption of high glycaemic load (GL) diets based on a large amount of foods high in rapidly digestible starch and sugar has been associated with obesity and increased risk for diabetes, cardiovascular disease and colorectal and breast cancer [17–19].

The growing world population adds further strain to the global food system. In order to feed roughly 10 billion mouths by 2050, an overall rise in food demand is expected if we do not change the way we produce, process, consume and dispose of food [20–23]. Besides, an adequate distribution of food resources across high-, medium- and low-income countries will become even more challenging. Also, the increasing urbanisation worldwide, but particularly in developing countries, bears difficulties for adequate and sustainable food supply [24]. There is a consensus amongst researchers, economists and politicians that all of these aspects are to be accommodated during food system transformation and multiple strategies to achieve this have been proposed [2, 25]. The global adoption of primarily plant-based diets (with flexibility to include necessary regional and cultural adjustments) has been mentioned as the most effective strategy and scientists urge for action to promote this diet shift [2, 26–28]. In the light of recent events, this approach has become even more attractive since some studies suggest a link between animal-

based food systems and emerging zoonoses such as SARS-CoV-2 [29–31]. Two other important strategies to change the food system are a substantial reduction of food waste and a decrease of food losses by improving technologies, particularly agricultural processes (e.g. irrigation systems) [2]. However, it has been argued that, in order to ascertain the food system transformation, a combination of various strategies, including those mentioned above, is necessary. Essentially, an international "multi-sector" and "multi-level" approach is needed to change the food we consume as well as the way it is produced [2, 31–33]. Moreover, all strategies need to be accompanied by science-based high-level policy interventions [2, 6, 10, 25, 32, 34]. Such policies might include the stopping of the expansion of agricultural land, the introduction of a meat tax and the development of other tax schemes or incentive schemes that support sustainable consumer products [33–36].

While collaborative action along the food chain is crucial to achieve food system transformation, a detailed evaluation of the possible contributions of each link of the chain is also needed. This review focuses on consumer product development and aspects related to formulating healthy plant-based protein-foods in support of the food system's transformation, specifically in support of the shift to predominantly plant-based diets. Plant-based protein-foods have been amongst the top 10 key trends in the food sector for many years and the market is expected to keep growing [37–39]. Since food product development represents a link between food industry, food research and consumers, it is bound to play a key role in amalgamating consumer food trends and food system changes required to preserve human and environmental health.

2.3 The Matter of Protein Adequacy

The advocacy of a shift to predominantly plant-based diets has sparked a debate concerning the macronutrient protein and its "pivotal role" in food system transformation [31]. Since animal-based foods provide significant amounts of high-quality protein in many human diets, it has been discussed whether a shift to mainly plant-based diets might lead to inadequate protein intake [40–42]. But many publications have reported that sufficient, and largely even supersufficient, amounts of protein are being consumed in most world regions and that a reduction of animal-based protein intake would not cause insufficient protein supply [4, 26, 31]. Uncertainties about required and recommended intakes of dietary protein as well as contradicting scientific evidence regarding the effects of high protein intakes (larger than currently recommended intakes [43]) have additionally fuelled these discussions [40].

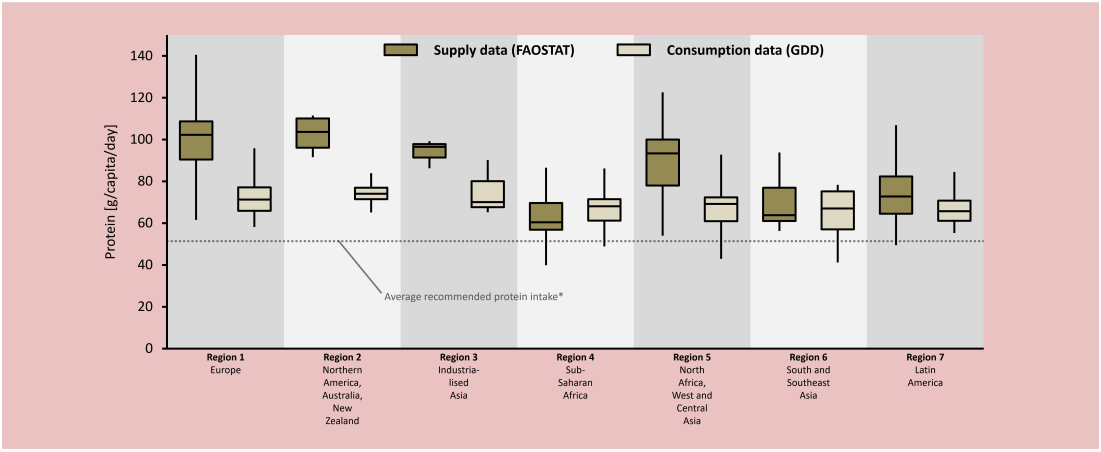


Figure 2.1: Protein supply/consumption across seven world regions (displaying range, 1st quartile, median, 3rd quartile): protein **supply** in 2015 according to FAOSTAT data and protein **consumption** in 2015 according to Global Dietary Database (GDD) data. * Average recommended protein intake based on 0.83 g/kg/day and average bodyweight of 62 kg [43, 45].

2.3.1 Protein Quantity

The first step this article takes in reviewing this matter is to look at the current state of protein supply and consumption globally. Figure 2.1 displays protein supply (FAOSTAT) and protein consumption (Global Dietary Database; GDD) data for the year 2015 and for seven world regions. The grouping of world regions was based on the classification used by FAO (2011) [44] with some modifications due to missing data in either FAOSTAT or GDD for the year 2015. An exhaustive list of all included countries for each region is available in Table A.A1 in Appendix A. The presented data show that the supply of dietary protein seems more than sufficient in most world regions when compared to the average recommended intake (0.83 g/kg/day and an average bodyweight of 62 kg [43, 45]). However, it has repeatedly been reported that calculated food supply data do not adequately reflect and, in many cases, overestimate food consumption because factors like food waste in households are not considered [31, 46–50]. While the presented protein consumption data confirm this trend (for the year 2015), they also suggest that not only protein supply but also protein consumption levels are above average recommended intakes in many countries, but primarily in world regions 1-3, which represent high- and medium-income countries [44]. The same conclusion was drawn in previous articles from the World Resources Insitute (WRI) based on consumption data that were obtained by applying the GlobAgri model to FAOSTAT supply data for the year 2009 (three year average 2008-2010) [4, 51]. Nevertheless, according to the 2015 data presented in Figure 2.1, the population in some countries in regions 4, 5 and 6 seem to consume insufficient amounts of dietary protein. The apparent widespread protein overconsumption should be discussed in the context of widespread overconsumption of dietary energy, which has also

been mentioned in the literature [51]. This is why recommendations to reduce protein overconsumption are usually preceded by recommendations to reduce energy overconsumption [31]. Furthermore, the fact that protein requirements mostly refer to intakes per bodyweight can lead to a flawed interpretation of the data presented in Figure 2.1. The recommended intake for adults included in the graph was calculated based on values established by FAO/WHO in 2007 (0.83 g/kg/day) [43] and the global average bodyweight of 62 kg [45] which equals 51.5 g/capita/day. However, the average bodyweight in high-income areas is considerably higher than the global average (e.g. 70.8 kg in Europe, 80.7 kg in Northern America and 74.1 kg in Oceania). Therefore, the recommended protein intake for adults would amount to 58.8 g/capita/day, 67.0 g/capita/day and 61.5 g/capita/day in Europe, Northern America and Oceania, respectively. Additionally, some consumer groups require more dietary protein than adults. This concerns children as well as individuals with higher levels of physical activity [43, 52, 53]. Recent research advances have also suggested that the elderly (individuals aged > 65) might have higher protein requirements than previously assumed and that elevated levels of dietary protein consumption might help to protect bone health and prevent sarcopenia [54–57]. This aspect should not be underestimated, especially in the context of the advancing world population ageing [58]. Another important consideration when discussing sufficient protein supply within a sustainable future food system are world population prospects, which project a world population of 9.7 billion in 2050 [12]. Thus, it can be expected that, even if current protein overconsumption is going to be reduced, there will be a growing demand for dietary protein [3, 22, 23]. In the light of recommended diet shifts, this demand should be covered primarily by plant-based protein, which requires sufficient availability of plant-based protein-foods. Population growth is also expected to further increase the globally uneven distribution of protein resources, which is an issue that needs to be addressed as part of food system transformation [31].

The main purpose of ingesting dietary protein is to provide the human body with sufficient substrate for biosynthesis pathways, specifically for the synthesis of proteins. Dietary protein is a source of nitrogen and indispensable amino acids as well as signal amino acids (e.g. leucine) that regulate metabolism and stimulate anabolism [59]. When assessing required and recommended dietary protein intake, the majority of methods is based on the maintenance of N balance [43, 54, 60]. Some articles highlight that this concept does not consider the relevance of protein beyond maintaining N balance and that the importance of dietary protein for optimal health is underestimated [54, 59–61]. Many studies have found a positive impact of protein intakes above the average recommended level on overall health, blood pressure, blood lipid levels, weight management, satiety and the prevalence

of obesity and diabetes type 2 [61–64]. One of the most interesting aspects is the improved satiety reported for high-protein diets, which could contribute to a reduction of energy overconsumption [65]. Other publications have reported that a low-protein diet increased life expectancy in mice and was associated with decreased mortality and reduced cancer incidence in humans [66–68]. The presence of this conflicting evidence, which has been referred to as "protein paradox", is likely associated with the fact that the protein level of diets cannot be evaluated in isolation and that study results always also depend on food matrix and other diet components (macro- and micronutrients) [65]. Some studies have found that high intakes of plant-derived proteins were associated with either positive effects or less negative effects than high protein intakes from animal sources [65, 69, 70]. This is encouraging with regard to a shift to primarily plant-based diets.

2.3.2 Protein Quality

The quality of dietary protein largely depends on the bioavailability of indispensable amino acids (IAAs) from this protein when it is ingested [59, 71]. IAA bioavailability is mainly affected by the protein's amino acid composition (compared to the human amino acid requirement pattern of indispensable amino acids [43]) and digestibility [59]. Based on both aspects, protein quality is often described by the protein digestibility corrected amino acid score (PDCAAS) or, more recently, the digestible indispensable amino acid score (DIAAS), which considers the digestibility of each single indispensable amino acid rather than that of the whole protein (used for PDCAAS) [71, 72]. Many studies report that plant proteins have a lower protein quality than animal proteins [40, 71, 73, 74]. This aspect is also known as "protein trade-off" in the context of the shift towards predominantly plant-based diets [73]. The lower protein quality of many plant sources is associated with a lack of certain IAAs [40]. Animal proteins, on the other hand, often possess an amino acid profile that represents a better match with the human requirement pattern [43, 65, 71, 75]. The lower quality of plant proteins has also been attributed to their often inferior digestibility when compared to animal proteins [40]. Antinutritional compounds (ANCs), such as phytates and trypsin inhibitors, which naturally occur in many plants, have been found to substantially reduce protein digestibility when present in the ingested food [40]. However, both ANC contents and protein digestibility greatly depend on food matrix, food structure and applied processing techniques [76–78]. Figure 2.2A shows the proportions of animal and plant protein contributing to the total protein supply across seven world regions. While these are protein supply data which do not accurately reflect consumption, they still provide an indication of the extent to which different protein sources contribute to total protein

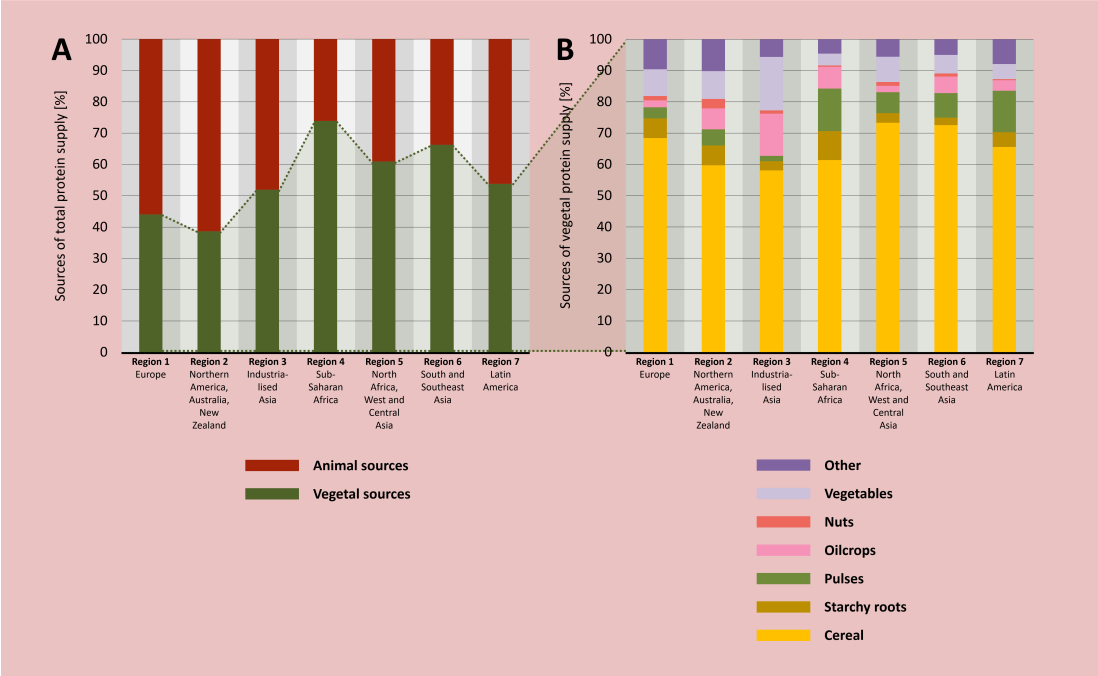


Figure 2.2: Sources of protein supply across seven world regions in 2015 according to FAO food balance data (FAOSTAT): (**A**) Proportions of protein from animal and vegetal sources of total protein supply (based on mean animal and vegetal protein supply across the countries of each region); (**B**) Proportions of different protein sources of vegetal protein supply (based on mean protein supply from listed vegetal sources across the countries of each region).

consumption. In regions 1, 2, 3 and 7, approx. 45-60% of total protein is provided by animal sources. In regions 4, 5 and 6, less than 40% is covered by animal sources. Considering a substantial reduction of animal food consumption as part of the food system's transformation, the remaining small amount of animal protein together with the current supply of vegetal protein could be insufficient to reach recommended protein intakes. Moreover, it has been argued that, when dietary protein is mainly provided by plant sources, recommended protein intake levels should be raised by approximately 5-10% to account for lower protein quality [40, 79]. While this is a very theoretical approach and it can only provide a limited representation of the real protein adequacy of future predominantly plant-based diets, it suggests the need for an increased plant-based protein supply. Notions that plant-based protein is not fit to satisfy human protein requirements at all have been frequently addressed within the past few years. The WRI "debunk protein myths" in their World Resources Report from 2019 [4] by stating that a diverse plant-based diet is capable of providing adequate protein as well as micronutrients for human nutrition. Many other publications also mention that this diversity in plant-based protein is crucial for adequate protein supply from predominantly plant-based diets and the key to overcoming the lack of certain IAAs in individual plant proteins [34, 40, 71, 80]. Figure 2.2B displays the proportions of different plant sources contributing to total vegetal protein supply. These data show that

in all seven world regions, the majority of vegetal protein supply is covered by cereals. Furthermore, non-cereal protein supply accounts for as little as 30% in four (including Europe) out of seven regions. The data clearly suggest that plant-based protein supply is lacking diversity in many regions. A predominantly plant-based diet based on these proportions of plant sources could lead to insufficient supply of the IAAs lysine, threonine and tryptophan, which are potentially limiting in cereal protein [81]. Mariotti and Gardner [82] evaluated the diet composition of different consumer groups in Europe: regular meat-eaters, low meat-eaters, vegetarians and vegans, amongst others. They reported that vegetarians and vegans consume considerably more legumes, vegetarian alternatives and nuts than regular meat-eaters but also slightly more cereal products. They concluded that there was sufficient diversity in plant-based protein intake for vegetarians and vegans and no or very low risk for lysine deficiency. But they also estimated the expected lysine inadequacy of European diets when intake of animal protein is reduced and isocalorically replaced by plant-based foods. Their simulations showed that when animal protein was entirely replaced by the mixture of plant foods that is currently consumed by animal protein consuming individuals, the prevalence of lysine inadequacy could be as high as 80% (at plant protein intake of approximately 85% of total protein intake). This is in agreement with the data presented in Figure 2.2, which suggests that this might not only be true for European diets but also for diets in regions 2, 3, 6 and 7. When Mariotti and Gardner [82] considered a partial replacement of animal protein by a mixture of legumes, seeds and nuts as opposed to the already consumed mixture of plant foods in their simulations, they predicted drastically decreased prevalence of lysine inadequacy.

2.3.3 Overall Diet Quality is Paramount

Another aspect associated with a high proportion of plant-based protein intake coming from cereals is the glycaemic index (GI) and GL of such diets [83]. Cereal products often contain large amounts of rapidly digestible starch (RDS) [84–86]. This is even more pronounced for refined cereal products (as opposed to whole-grain products), which have been increasingly consumed within the last decades [2, 48, 83]. Such diets have been associated with several health risks such as obesity and diabetes [17], as mentioned above. Thus, a diet shift should not only target a substantial reduction of the consumption of animal foods and a diversification of plant-based protein supply but also consider a moderation of diet-GL [2, 17, 83]. The EAT-Lancet commission has proposed a reference diet which was established considering both human health and environmental sustainability based on the planetary boundaries concept [1, 87]. Table 2.1 summarises supply

and consumption data from 2015 (both expressed as g food/capita/day in contrast to g protein/capita/day as in the two figures above) for two plant-based food groups (cereals and the sum of legumes, beans, nuts and seeds) in comparison to the intakes recommended in the EAT-Lancet reference diet [2]. While Del Gobbo

Table 2.1: Supply/consumption of specific food groups across seven world regions.

Supply/consumption [g/capita/day]	Region 1	Region 2	Region 3	Region 4	Region 5	Region 6	Region 7
Supply cereal	362.2	288.2	479.7	390.3	488.6	588.8	385.1
Consumption cereal	— (no data available)						
EAT-Lancet reference diet	232 (0-60% of calories) as wholegrain products (rice, wheat, corn and other)						
Supply legumes/oilcrops/nuts	22.1	34.3	34.9	50.0	32.8	58.1	43.2
Consumption legumes/beans/nuts/seeds	18.0	23.1	32.4	26.7	28.7	36.8	27.5
EAT-Lancet reference diet	125 (25-250) legumes, soy foods, peanuts and treenuts						

et al. [49] found that food balance data from FAOSTAT (for the years 1980-2009) potentially underestimate legume consumption and overestimate cereal consumption, the data for 2015 provided in Table 2.1 show that consumption of legumes, beans, nuts and seeds was slightly lower than the supply data suggest. When compared to the EAT-Lancet reference diet, it appears that supply and consumption of legumes, beans, nuts and seeds is much lower than the recommended intake. For cereals, a comparison is more difficult since there were no GDD consumption data available for this food group and the EAT-Lancet reference diet only refers to an intake of wholegrain products (refined cereal products are not part of the reference diet). However, the data suggest that the supply of cereal products (refined and wholegrain) is considerably higher than the recommended intake (wholegrain only). This means that an increased consumption of legumes, beans, nuts and seeds is not only beneficial from a nutritional perspective to diversify plant-based (protein-) food intake but also from an environmental sustainability point of view.

Protein undoubtedly is an important macronutrient, but many scientists urge for a more holistic evaluation of the nutritional value of foods and the whole diet instead of a mere focus on protein quantity and quality [34, 82, 88]. The coingestion of dietary fibre and important micronutrients as well as compounds with potentially adverse health impacts with protein-foods should be taken into account when their nutritional quality is evaluated.

2.4 Consumer Trends

A major diet shift will require changes in consumers' food choices, which depend on many different factors: availability, culture, social status, affordability, sensory attributes, convenience of preparation, consumer beliefs, consumer knowledge, individual perceptions, price, brands and advertising are only a few from a long

list of aspects associated with multiple scientific disciplines [34, 89, 90]. In recent years, consumers' awareness of the impact of food production and diets on the environment and on human health/wellbeing has increased substantially [38, 91]. While this might influence the food choices of some, the majority of consumers still underestimates the environmental impact of meat consumption, for instance [92]. In a systematic review from 2017, Hartmann and Siegrist [92] conclude that consumers generally show low willingness to change their behaviour in relation to meat consumption (referring to both reduction as well as substitution by alternative protein-foods). Research has clearly shown that to prevent substantial environmental damage caused by the food system in the future and to protect human health, a global shift to predominantly plant-based diets is a key strategy [2]. But De Boer and Aiking [93] mentioned that this science-based reasoning seems to be not adequately available to the consumer or is too difficult to comprehend. The gap between consumer behaviour and required change of the food system needs to be addressed at multiple levels (diet, dish, dish ingredients and bites) by the relevant food chain entities (policy-makers, farmers, retailers, food processors, restaurant owners) [31, 89, 94]. For example, the inclusion of environmental health and sustainability considerations in dietary guidelines by policy-makers might not flip a switch and bring about food system transformation on its own, but it could certainly instigate increased acceptance of predominantly plant-based diets and advocate associated health benefits [31, 89]. Such dietary guidelines have already been established on a national level in several countries, for example, in the UK, the Netherlands, Germany and Sweden [31, 95, 96]. A dietary intervention study published by Micheelsen et al. [97] in 2014 investigates the acceptability of the "New Nordic Diet", which was established to improve public health in Denmark. This diet is characterised by reduced meat intake (by 35%) and increased intake of plant-based foods (whole-grain foods, fruit, vegetables, nuts) compared to an "Average Danish Diet". Regional and seasonal aspects are also considered to determine the diet's food basket. In an evaluation of the diets' environmental impact, it was found that specifically the reduced meat consumption and avoiding long-distance imports contributed to reduced environmental impact of the "New Nordic Diet" in comparison to the "Average Danish Diet" [98]. A key finding of the intervention study was that while it scored high in eating acceptance (tastiness), it lacked practical acceptability [97]. This seemed to be primarily related to tedious/time-consuming food preparation as well as the perception of high price and limited availability of the required food products. Therefore, practical acceptability was identified as the main barrier to consumers adopting this diet at the time of the intervention study (2010-2011). Macdiarmid *et al.* [99] also showed that many consumers seem to have the impression healthy (and environmentally

sustainable [31]) diets are not compatible with their fast-paced lives. This is where other food system sectors like food processing, retail and food service, which have great economic and cultural influence on the food system [2, 100], can help to induce change in consumer food choice and to promote diet shifts. Also, the increasingly important role of innovation and entrepreneurship in the food sector has great potential to drive change in consumer behaviour and the food system in general [101]. What all of these sectors have in common is the need to develop new products. This is why product development should aim to increase diversity of plant-based protein supply and to improve practicability of consuming predominantly plant-based diets by providing healthy plant-based protein-foods in more convenient formats. However, more convenient formats also imply the need for food processing which certainly represents a trade-off regarding the sustainability of these foods; whole legumes, for instance, can be expected to have a lower environmental footprint than processed foods containing legumes. Furthermore, processed foods have an increasingly negative reputation amongst consumers, which is also closely associated with the 'clean-label' trend [102, 103]. It is true that diets with high intakes of ultra-processed foods, which are commonly characterised by high fat, sugar and salt contents and low levels of dietary fibre, protein, micronutrients and bioactive compounds, have been associated with an elevated risk of non-communicable diseases and obesity [104–106]. However, some extent of food processing is indispensable and has assured sufficient and safe food supply for centuries; for example, by increasing shelf life, microbiological quality and digestibility and by reducing ANCs and potentially toxins [14, 104–106]. While the consumption of plant-based whole foods is to be encouraged, moderate and targeted processing can help to produce healthy plant-based protein-foods with improved nutritional quality [104, 107]. Aschemann-Witzel *et al.* [108] conclude in their review from 2020 that consumers' "scepticism toward processing has to be overcome through more plant-based foods meeting both consumer interests": health and 'clean-label'.

2.5 Targeted Product Development

Based on the considerations presented above, the development of plant-based protein-foods should follow five key principles, which are summarised in Figure 2.3. *(1) Selecting the right protein sources - Principle 1* aims at intentionally choosing those protein sources that, based on scientific evidence, promise both environmental and nutritional benefits. In favour of diversifying plant-based protein supply, mainly non-cereal raw materials should be in the focus (which does not mean that cereal products are not an important and essential part of healthy di-

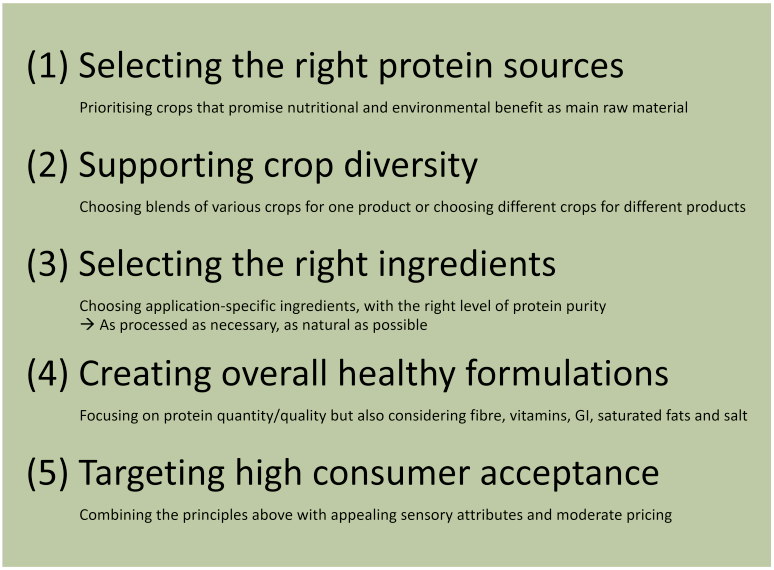


Figure 2.3: Principles of targeted plant-based protein-food development.

ets). Also, locally grown crops should be prioritised since the use of local resources is known to considerably lower the environmental footprint of foods [28, 73, 98]. *(2) Supporting crop diversity - Principle 2* targets the selection of various different crops as raw materials either in combination for the same product or individually for a range of different products. Crop diversity has been mentioned to be important for soil fertility and in the battle against declining yields and nutritional quality of crops caused by climate change [2, 109, 110]. Actively choosing various crops for food formulation creates increased demand and could promote their cultivation by farmers, as postulated by Tsolakis *et al.* [111] for faba beans. Furthermore, it has been reported that blending different plant-based raw materials is the key to improving nutritional and technological quality of the final food products [39, 80]. *(3) Using the right ingredients - Principle 3* is directed at choosing the ingredient types (derived from the previously selected protein sources) that are best suited for the application in question. This concerns those ingredients contributing to the protein content (protein ingredients; PIs) of the food as well as other ingredients that are part of the formulation. For PIs, it means choosing the level of protein purification and other types of processing (e.g. heat treatment, enzyme treatment, high-pressure treatment, fermentation, specific chemical modification) that are required. Both protein purification and other processing steps affect physicochemical, techno-functional and nutritional properties of the ingredients [112–114] such as protein content, solubility, dispersibility, protein digestibility, and content of ANCs [76, 78, 115]. In support of environmental sustainability, processing should be kept at the necessary level, and raw materials should be used as efficiently as possible. In this context, the use of side-streams from plant-based food production is highly encouraged. Both PIs as well as other ingredients that are often

required for the formulation of plant-based protein-foods can be represented by food processing side-streams [116, 117]. Especially side-streams high in starch or fibre have been successfully applied as functional ingredients in food product development [118]. *(4) Creating healthy formulations - Principle 4* addresses the need to develop products with adequate nutritional value. The combination of ingredients and further processing steps should be chosen to obtain a final food product with good overall nutritional quality. Protein digestibility is an important factor with impact on nutritional quality and should be considered and monitored in food formulation since it has been shown to greatly depend on food processing and food matrix [76]. With regard to protein quality, the focus lies on sufficient lysine intake from predominantly plant-based diets [81]. In this regard, also the bioavailability of lysine is crucial and depressed bioavailability due to processing related chemical modification of lysine should be taken into account [40, 119]. In general, the developed protein-foods should either provide balanced amino acid profiles in accordance with the human requirement pattern [43] or possess an amino acid profile that complements that of cereal proteins in widely consumed staple foods. While protein quality is of great importance in protein-foods, also the presence of other micro- and macronutrients as well as levels of compounds with potentially adverse health effects are to be considered. Specifically, salt, saturated fats and the GL of the foods, which are factors that have been associated with adverse health effects and increased risk of non-communicable diseases, need to be kept at low or moderate levels [17–19, 104–106]. Many plant-based protein sources (e.g. legumes, nuts and seeds) are also known to be rich in dietary fibre, essential minerals like iron and zinc, B vitamins, phenolic compounds and prebiotic oligosaccharides [78], which have been identified as the main contributors to health benefits associated with the consumption of plant foods [34, 78]. Therefore, these valuable food constituents should be preserved as much as possible during ingredient and food processing, and adequate levels should remain in the developed plant-based protein-foods. While high levels of these compounds could also be achieved by supplementation with purified food additives, this inevitably requires extensive processing and generally opposes sustainability considerations [104]. Some of the beneficial plant constituents mentioned above (e.g. phenolic compounds, prebiotic oligosaccharides) are also considered as antinutrients [120]. Therefore, the contents of these and other ANCs should be monitored during raw material and ingredient selection, but also during food formulation and final processing. *(5) Targeting high consumer acceptance - Principle 5* advocates the combination of the previously discussed principles in a product that, additionally, promises high consumer acceptance. While it would be detrimental for achieving food system transformation to drop any of the principles above in favour of high

consumer acceptance, it would be equally disadvantageous to develop expensive products with low sensory quality that decelerate diet shifts by making plant-based protein-foods less appealing to consumers. Research shows that factors like affordability, availability and tastiness still rank above health and sustainability considerations in consumers' food choices [46, 97, 106, 121]. Providing a wide range of tasty and convenient plant-based protein-foods with a healthy nutritional pattern could help steer consumer food choices towards healthier and more sustainable products. A more detailed view on which protein sources, ingredient types and food applications are the most promising for the development of plant-based protein-foods according to these five principles is given in the following sections.

One question that remains is how plant-based protein-foods will be identified by consumers as what they are, specifically because consumers seem to lack knowledge about good sources of dietary protein and adequate quantities [42, 122, 123]. Nutrition and health related information given as part of food labelling is legally regulated in many countries and attached to specific requirements concerning the food product's composition and nutritional quality. The requirements for protein related statements vary between countries and refer to different variables such as weight-based protein content per serving, energy-based protein content per serving or protein quality determined *in vivo* [124]. In order to be able to make consumers aware of the nature of plant-based protein-foods, the respective requirements should be considered during product development. For instance, according to European legislation, a food qualifies for the claim 'source of protein' when at least 12% of calories are provided by protein and for the claim 'high in protein' when at least 20% of calories are provided by protein [125]. The identification of protein-foods based on the percentage of calories that is provided by protein seems desirable in the light of widespread calorie overconsumption. This is particularly important for plant-based substitutes of animal foods since animal foods typically have a high protein-energy/total-energy ratio [61].

2.5.1 Promising Protein Sources

Plant-based dietary protein can be obtained from a great variety of sources, including the following: cereals, pseudocereals, legumes, oilseeds, nuts, starchy roots/tubers, vegetables/leaves/shoots, fruit and algae [75, 126, 127]. The suitability of these plant sources for protein supply in human nutrition, in terms of environmental sustainability and human health, depends on many aspects, including their natural protein content, cost for harvesting, availability of processing techniques, extent of required processing, geographical location and climate [75].

Cereals have been an important food source for centuries. With an annual production of nearly 3000 million tonnes (mt), cereals also are the most cultivated crop category worldwide in comparison to sugar crops with approx. 2200 mt, oilcrops with 1100 mt and pulses with only 88 mt (FAOSTAT 2019). Even though cereals, with an average protein content of 10–12%, do not count as the plant sources with the highest protein levels, they still represent a substantial source of dietary protein since they are consumed in large quantities (for example, in the form of staple foods bread and pasta). As demonstrated above, the majority of plant-based protein supply is covered by cereals and cereal products in currently consumed diets. Due to a low abundance of IAAs such as lysine and potentially threonine and tryptophan (relative to human AA requirement pattern [43]) [75, 128, 129], a diversification of plant-based protein intake has been advocated to ascertain adequate protein supply from predominantly plant-based diets [34, 80, 82]. Therefore, cereals are not in the focus as main protein source for formulating new plant-based protein-foods. Besides, from an environmental point of view, it has been argued that an increased cultivation of food crops other than maize, wheat and rice (e.g. less common cereals such as oat, sorghum, millet or non-cereal crops) for food production is desirable for improved crop rotation systems and agricultural biodiversity [2, 107]. However, cereal proteins possess unique techno-functional characteristics [130] and their utilisation for plant-based protein-foods as functional ingredients or as PIs to complement other protein sources is to be encouraged. *Pseudocereals* such as buckwheat, quinoa and amaranth, which are also referred to as edible seeds or ancient grains, contain with 12–19% slightly more protein than cereals [131, 132]. Pseudocereals additionally have a more balanced profile of IAAs than cereals. Especially the lysine level is higher than in most cereal sources [128, 131, 132]. Potentially limiting (amino acid scores (AASs) between 0.8 and 1.0) amino acids in comparison to the human requirement pattern [43] are isoleucine, leucine, lysine and valine [132]. Due to an overall excellent nutritional profile, considering contents of minerals, vitamins and phytochemicals in addition to the balanced amino acid profile, pseudocereals have been mentioned as "the grains of the twenty-first century" [131, 133]. This statement is further supported by the environmental benefits of these crops. Ancient grains are known as sustainable crops that produce adequate and consistent yields with minimal agricultural inputs [134]. Moreover, they are valued with regard to their genetic diversity and their potential to grow under harsh conditions [134]. This latter aspect is particularly important with respect to increasingly difficult climatic conditions due to climate change [2]. Manners *et al.* [135] evaluated the suitability of various protein-rich crops (pseudocereals and legumes) considering the current and future climate in Europe and identified quinoa as the pseudocereal

with the most significant potential. But in spite of all their positive traits, crops like quinoa, amaranth and buckwheat remain largely underutilised as food (protein) sources in many parts of the world [134] (global annual production of approx. 1.61 mt for buckwheat and 0.16 mt for quinoa; FAOSTAT 2019). Chia is another type of pseudocereal, which is best-known for its high content of dietary fibre (34.0–53.8%) [136, 137]. But apart from being a valuable source of dietary fibre, chia contains between 18 and 24% protein [138]. However, the relatively low lysine content compared to the other pseudocereals makes it slightly less promising to complement cereal-based protein intake. Furthermore, due to its status as novel food, food applications for chia are more restricted than for other raw materials in the European Union [139]. A very promising group of plant-based protein sources is the *legume family* with protein contents of 20–40% [75, 140, 141]. This group includes pulses, which are defined as legumes that are harvested exclusively to obtain dry seeds [127, 142]. Many legumes possess an amino acid profile that complements that of cereals. Specifically, high lysine contents (higher than in cereals and often even higher than in human requirement pattern [43]) have been reported [75, 128, 143]. This makes legumes and oilseeds the most favourable plant-based protein sources to include in cereal-rich diets from a nutritional point of view [82]. The environmental benefits associated with growing legumes are another reason for their importance in transforming the food system. The disruption of nitrogen cycles, which is mainly caused by the excessive application of synthetically produced fertilisers, is one of the most severe environmental impacts of food production [2]. Cropping systems that include legumes require less fertiliser due to their ability to produce nitrogen and improve soil fertility [107, 109]. Soy, which belongs to the legume family but is often considered as oilcrop (also the case for peanuts), is one of the most popular plant-based protein sources, and its global production amounts to 333 mt per year (FAOSTAT 2019) [144]. However, it has gained increasingly negative reputation during the last decade due to its allergenicity and the cultivation of genetically modified soy [122, 145]. The use of genetically modified organisms (GMOs) is surrounded by concerns regarding human health and environmental implications. While there is very little scientific evidence indicating adverse effects caused by approved GMO food crops and the net environmental impact of GMOs is probably positive [146], it remains a controversial topic, and no international consensus has been reached in the scientific (and political) community [147, 148]. This review does not cover a detailed discussion of advantages and disadvantages of genetic engineering of food crops. However, the persisting consumer scepticism towards GMO foods needs to be mentioned [149, 150]. This has led to a growing replacement of soy in food applications by alternative plant-based protein sources like pea and lupin [151, 152]. Also, other

legumes have moved into the focus for plant-based protein supply, including faba bean, chickpea, lentil and mung bean [75]. However, more research is needed since cold-weather legumes, such as faba bean, lentil, lupin, pea and chickpea, seem more susceptible to abiotic stresses (compared to warm-weather legumes like soy) and yield instabilities have been observed [135, 153]. Similar to pseudo-cereals, legumes continue to be drastically underutilised for human nutrition and plant-based protein supply [107]. As mentioned above, according to the EAT-Lancet reference diet (which considers human and environmental health), a much higher consumption of legumes is recommended [2]. *Non-legume oilseeds and nuts* represent another group of promising plant-based protein sources [154, 155]. Interesting members of this group are rapeseed/canola, sunflower seed, flax seed, hempseed, pumpkin seed, sesame seed, sacha inchi seed, almonds and cashew nuts, which contain approx. 16–36% protein [75, 155, 156]. While the amino acid profiles of these protein sources vary greatly, some examples stand out due to high levels of lysine (e.g. rapeseed/canola, baru almond) [75, 155]. Apart from being a source of dietary protein, oilseeds and nuts provide high levels of mono- and polyunsaturated fatty acids, which makes their nutritional profile even more valuable for human diets [157]. Oilseed proteins can be obtained from oilseed press cakes as a side-stream of vegetable oil production, which suggests oilseeds as a sustainable source of dietary protein with low carbon footprint [154]. Nevertheless, processing oily raw materials continues to be challenging, and further research and technological advances are needed [154]. *Other plants like starchy roots/tubers, vegetables/leaves/shoots and fruits* generally have lower protein contents (exceptions are bamboo leaves and ora-pro-nobis leaves with 13 and 28% protein, respectively) [75, 127]. Therefore, the relevance of these plants as protein sources (without processing to concentrate protein) strongly depends on the quantities that are consumed. Furthermore, the utilisation of these protein sources for the formulation of plant-based protein-foods would require protein concentration. With respect to environmental sustainability, the value of these protein sources depends on the extent of required processing and whether processing side-streams can be valorised (e.g. potato starch) [158]. Aquatic plants like *algae* (e.g. seaweed, Chlorella and Spirulina) *and duckweed* also qualify as plant-based protein sources with protein contents as high as 40–60% (algae) or 20–35% (duckweed) based on dry matter [159, 160]. Most algae and duckweed species possess relatively balanced amino acid profiles in comparison to the human requirement pattern [43] [159, 160]. Some algae, like *Chlorella vulgaris*, have been reported to be particularly rich in lysine (exceeding the lysine content in the human requirement pattern by nearly 100%) [159]. Both algae and duckweed have been mentioned as promising alternative proteins with respect to nutritional and

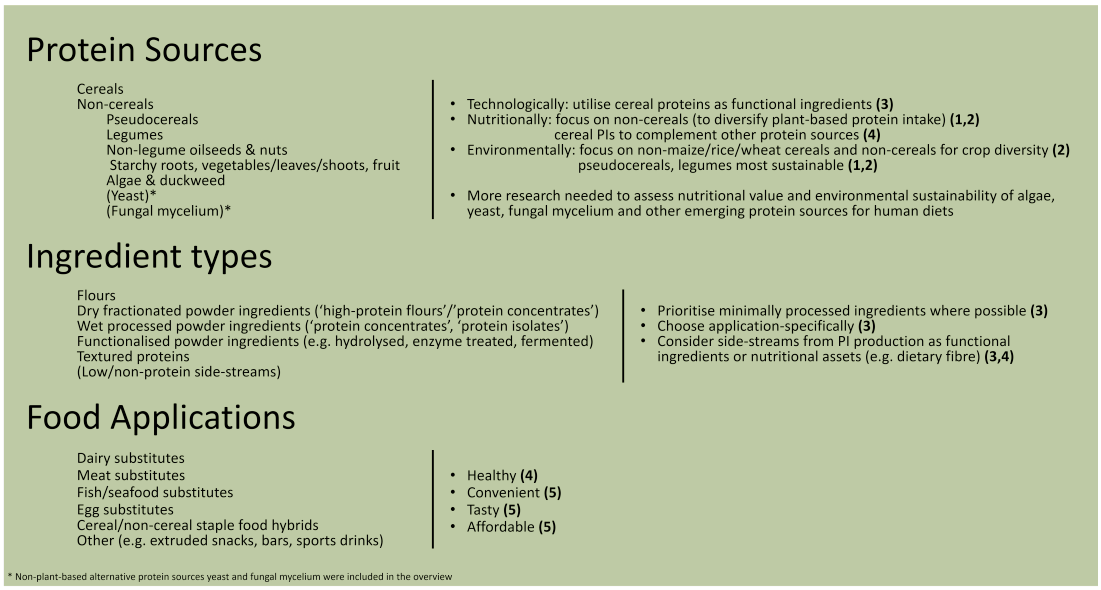


Figure 2.4: Overview of plant-based* protein sources, ingredient types and food applications relevant for the development of plant-based protein-foods. Bold numbers in brackets refer to the principles of targeted product development introduced above.

environmental considerations. However, further research is needed with regard to nutritional characteristics, sustainability and yields in large-scale production [160, 161]. This is also the case for *yeast and fungal mycelium* [162]. While these protein sources are not plant-based, they represent emerging non-animal protein sources with great potential for the formulation of sustainable protein-foods. Figure 2.4 summarises how the principles of targeted product development can be applied at the level of protein sources.

2.5.2 Promising Ingredient Types

Raw material processing to produce ingredients is usually associated with at least one (or multiple) of the following objectives: improving techno-functional characteristics (e.g. facilitating application in food formulations), improving nutritional quality (e.g. protein concentration and removal of ANCs and allergens), improving food safety (e.g. extending shelf life), improving sensory qualities (e.g. removal of undesirable aroma compounds) [104]. *Flours* are typically obtained by grinding dry raw materials (for raw materials with a high water content potentially preceded by a drying step), in some cases after removal of inedible/undesirable parts (e.g. dehulling) [104]. Flours, especially when derived from raw materials with a naturally high protein content (e.g. pseudocereals, pulses), can represent valuable and sustainable ingredients for the formulation of plant-based protein-foods. However, various food applications (e.g. plant-based beverages such as milk substitutes) require ingredients with a higher degree of protein purification [112]. Protein puri-

fication can be achieved with either dry fractionation or wet processing, and the produced ingredients are usually referred to as protein concentrates (in the case of dry fractionation sometimes high-protein flour) or protein isolates (protein content usually > 80% based on dry matter) [163]. *Dry fractionation* has been described as sustainable processing option to produce plant-based PIs (especially for pulses) and requires far less energy and water than wet processing [113, 118, 164]. Due to the mild processing conditions, proteins can retain their native and sometimes better functionality [112, 113, 118]. *Wet processing* can be applied to a variety of starting materials, including flours, previously dry fractionated high-protein flours or previously defatted raw materials (e.g. oilseed press cakes) [112, 154]. Commonly used wet processing methods are isoelectric precipitation, ultrafiltration and salt extraction/micellisation [112]. Protein isolates derived from the same raw material but produced with different wet processing methods can vary greatly in their techno-functional and nutritional properties [165, 166]. While wet processing requires higher energy and water input than dry fractionation, life cycle assessment (LCA) has shown that wet processed plant-based PIs are still a sustainable ingredient option, for example, when compared to milk protein [113, 114, 165]. Additional processing can be applied to either raw materials or ingredients in order to produce functionalised ingredients. Such treatments include (partial) protein hydrolysis, enzymatic treatments (other than hydrolysis), heat treatment or fermentation [112, 167–172]. Another way of functionalising plant-based protein is the production of texturised vegetable protein (TVP) [173]. Texturisation is commonly achieved by applying a combination of thermal and mechanical energy (extrusion technology), and a large variety of different TVP types can be produced representing either textured PIs (e.g. for application in meat substitute systems) or final food products (e.g. meat substitutes) [173]. Side-streams from the above-mentioned processing techniques or from the production of other plant-based foods can be used in the formulation of plant-based protein foods [116, 117, 174–177]. Depending on the nature of the respective side-stream, they may be applied as PIs, as sources of valuable macro- and micronutrients (e.g. dietary fibre and minerals) or as functional ingredients to optimise sensory and nutritional properties of the protein-foods. Figure 2.4 summarises how the principles of targeted product development can be applied at the level of ingredient types.

2.5.3 Promising Food Applications

This section deals with consumer product options (types of protein-foods) that have high potential to diversify plant-based protein intake. The first product group comprises plant-based substitutes of traditional animal-based foods, which

play an essential role in the shift to predominantly plant-based diets due to their increasing consumer acceptance and market share [88, 178–180]. This group includes *dairy substitutes* (milk, yoghurt, cheese, cream and ice cream), *meat substitutes* (sausages, chicken nuggets, burger patties, meatballs, whole cuts of meat), *fish/seafood substitutes* and *egg substitutes* [35, 112, 179, 181, 182]. However, the 2020 report on plant-based ingredients and their integration across food and drink categories (by Food Ingredients Europe) states: "Plant-based is about more than meat alternatives. Consumers also are looking for more fruits, vegetables, grains and legumes in convenient formats" [39]. Another product group that offers an excellent opportunity to diversify plant-based protein intake is comprised of cereal/non-cereal staple food hybrids, which refers to cereal-based staple foods (e.g. bread and pasta) that contain non-cereal PIs [183–185]. These cereal/non-cereal staple food hybrids represent a direct diversification of plant-based protein supply within the very product group, which currently contributes to the high proportion of cereal protein in overall vegetal protein supply. The perceived high level of convenience of cereal staple foods amongst consumers further highlights their potential. Moreover, bread and pasta applications require less protein purity than some other protein-foods and allow for the utilisation of minimally processed PIs such as flours and high-protein flours [186–189]. It has been shown that the incorporation of legume and pseudocereal PIs also improves the overall nutritional profile of bread and pasta [187, 189]. The partial replacement of wheat flour or wheat semolina by non-cereal PIs in the recipes leads to an isocaloric replacement of starch by non-cereal protein in the food matrix (assuming the non-cereal PI contains more protein than wheat flour or wheat semolina). Therefore, a lowered GL and potentially improved satiating effect (due to increased total protein content) of such protein-foods can be expected [187, 189]. Other protein-foods, which can contribute to a diversification of plant-based protein supply when produced according to the principles of targeted product development, are the following: extruded snacks, sauces and dressings, bars, smoothies, porridge, sports drinks/sports nutrition products [179]. Figure 2.4 summarises how the principles of targeted product development can be applied at the level of food applications.

2.5.4 Currently Commercially Available Plant-Based Protein Ingredients

For the formulation of plant-based protein-foods, product developers rely heavily on the availability of suitable ingredients. This refers to all plant-based ingredients needed for the recipe but primarily to the main ingredients, PIs, that deliver a

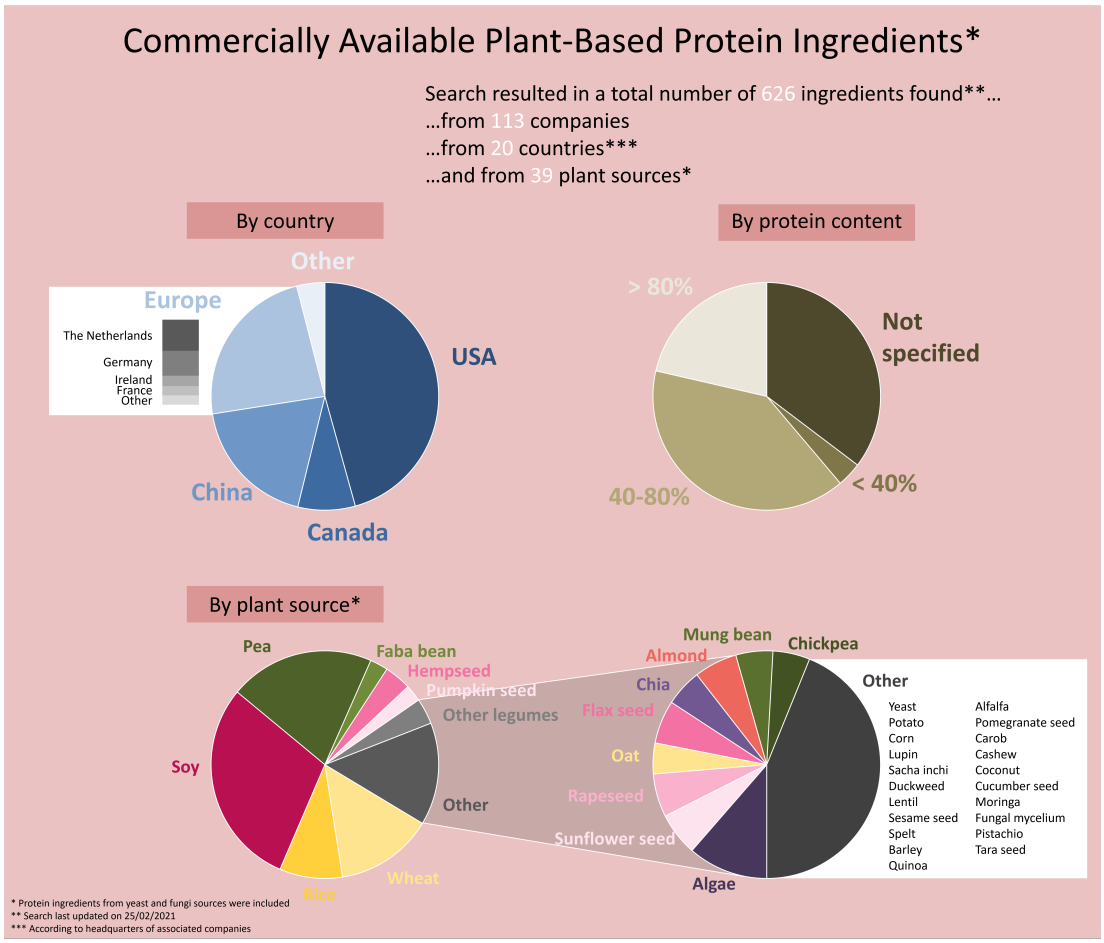


Figure 2.5: Overview of commercially available plant-based* protein ingredients (last updated on 25/02/2021), including ingredients from the non-animal protein sources yeast and fungi.

substantial amount of protein. For the purpose of this review, a web search was performed to obtain an overview of currently commercially available plant-based PIs. The search was focused on plant-based PIs in powder form. However, also textured PIs and non-plant-based alternative protein sources like yeast and fungi were considered. Minimally processed grains and pulses available as simple flours, which can represent promising PIs for certain applications (especially flours of high-protein plant sources), were not the focal point of the search due to their vast abundance (at least for some plant sources). The plant protein market is rapidly growing and constantly changing. The results presented in Figure 2.5 on currently commercially available plant-based PIs are based on a web search which was last updated on February 25th, 2021. Seventy-two (11.5%) of the 626 evaluated ingredients were marketed as textured proteins, while the remaining 554 were described as PIs in powder form (labelled "protein", "protein concentrate" or "protein isolate"). The full list of PIs is available in Table A.A2 in Appendix A. Figure 2.5 provides an overview of the evaluated PIs sorted by country, by protein content and by plant source. The categorisation by country is based on the headquar-

ters of the company associated with each ingredient. A substantial proportion of ingredients is associated with US (45.7%), Chinese (18.7%) or Canadian (8.1%) companies. Also, European companies provide a large number of plant-based PIs, accounting for 23.5% of all evaluated ingredients. Other countries with significant contributions include India (1.4%), Switzerland (1.3%), Japan (0.6%), Thailand (0.6%) and Argentina (0.5%). The ingredients were also classified with regard to their protein contents. For 35.3% of the evaluated ingredients, the protein content was not specified on the suppliers' webpage and only available upon request. Two out five ingredients were advertised with protein contents between 40 and 80%, and for approx. one out of five, a protein content of over 80% was declared. A protein level of less than 40% was only specified for a small proportion of ingredients. This can be attributed to the exclusion of grain and pulse flours from the search, which would be expected to be the main contributors to this protein content category. The 626 evaluated ingredients are derived from 39 different sources: 37 plant sources, yeast and fungi. The four plant sources that stand out are soy, pea, wheat and rice, which account for 29.6%, 20.6%, 13.9% and 8.9% of all ingredients, respectively. One reason for the large proportion of PIs from these well-established plant sources is related to the more extensive (research) knowledge and experience with these raw materials [144]. Therefore, these PIs are often available as a range of specialised ingredient types with optimised techno-functional characteristics for specific applications. Other PIs with significant market share are derived from faba bean (2.6%), hemp (3.8%) and pumpkin seed (2.4%). Many promising and emerging plant-based protein sources such as mung bean, chickpea, lentil, quinoa and algae (as well as yeast and fungi) still seem underrepresented amongst commercially available PIs. It is unlikely that the presented data include every plant-based PI on the market. Additionally, overlapping and conflicting information from suppliers and manufacturers can lead to double entries in some cases where product names are not clear. However, the presented data are suitable to identify trends regarding commercially available plant-based PIs. While the plant-based sector is growing and more and more PIs have become available in recent years, the majority (approx. 73.0%) of PIs is derived from only four different plant sources (according to the internet search performed for this review): soy, pea, wheat and rice. In order to improve the diversity of plant-based protein intake by consumers, an improved availability of PIs from a larger range of promising plant sources is required. This will help to provide a diverse range of healthy plant-based protein-foods in convenient formats.

2.6 Conclusion

There is sufficient evidence to show that protein is consumed in larger amounts than currently recommended in many high-/medium-income countries. However, many uncertainties about adequate protein intake for optimal health remain. Furthermore, between 45 and 60% of the protein supply in high-/medium-income is covered by animal foods, and the majority of vegetal protein supply in diets worldwide is covered by cereal protein. A shift to predominantly plant-based diets to protect human and environmental health should include a diversification of plant-based protein intake as well as a reduction of calorie overconsumption and a moderation of diet GL. Formulating plant-based protein-foods with these targets in mind can help to advance the food system's transformation. However, consumer food choice is a complex process and represents a potentially limiting factor for a shift towards predominantly plant-based diets. Targeted product development of tasty, convenient and healthy plant-based protein-foods has great potential to guide consumers' participation in transforming the food system.

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CHAPTER 3

Comparative Study of Sugar Extraction Procedures for HPLC Analysis and Proposal of an Ethanolic Extraction Method For Plant-Based High-Protein Ingredients

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3.1 Abstract

The increasing importance of plant-based proteins in the food sector makes a reliable compositional analysis of plant-based high-protein ingredients a necessity. Specifically, the quantification of short-chain carbohydrates is relevant for multiple areas, including food product development, food labelling and fundamental food chemistry and food technology research. Commonly used extraction procedures for subsequent HPLC separation and quantification of short-chain carbohydrates have been discussed controversially regarding a range of complications that can potentially lead to inaccurate sugar determination. The present study compares the sugar levels in wheat flour and wholemeal wheat flour determined with different aqueous and ethanolic extraction procedures. These procedures included measures to prevent enzyme activity and microbial growth, which represent two of the most relevant challenges in sugar extraction from food samples. Differences in sugar levels (sum of sucrose/maltose, glucose and fructose) as high as 1.8 %DM (wheat flour) were observed between the employed extraction procedures. Ethanolic extraction (80% ethanol in ultrapure water) with the use of the antimicrobial agent sodium azide but without Carrez clarification was identified as most promising for sugar determination in plant-based high-protein ingredients. A screening of high-protein ingredients derived from cereals (wheat gluten), pseudocereals (quinoa, amaranth, buckwheat) and legumes (soy, pea, lupin, lentil, carob, chickpea, faba bean) concerning their levels of sucrose, maltose, glucose and fructose confirmed the applicability of the chosen extraction procedure.

3.2 Introduction

Plant-based foods, and plant-based proteins in particular, are a growing sector in the food industry and one of the key trends in food, nutrition and health [1]. Research in this area focuses on nutritional and sensory characteristics but also on processing techniques to functionalise and optimise plant-based high-protein ingredients (HPIs) for multiple food applications. Promising raw materials that have received major research attention are cereals, pseudocereals and legumes. A reliable analysis of these ingredients' composition (and derived food products) builds the foundation for this research and becomes, thus, increasingly important. Specifically, the quantification of short-chain carbohydrates is relevant with regard to a range of different aspects. This includes the nutritional evaluation of foods, food labelling, the assessment of an ingredient's applicability to specific processing techniques (such as fermentation) and the understanding of compositional, techno-functional and structural changes during processing (such as protein isolation or incorporation in complex foods). Sugar analysis in plant-based HPIs with common aqueous extraction methods for subsequent HPLC separation, such as AACC 80-04.01, can bear challenges related to gelation and high water absorption of these samples. While an increase of the liquid/solid ratio during extraction promises improvement in this regard, it also results in less concentrated extracts. This negatively impacts the achievable limit of quantification (LOQ) and the ability to obtain meaningful results, especially when measuring with low sensitivity detectors such as refractive index detectors (RIDs). Moreover, a range of other complications with aqueous extractions has been reported and addressed in the literature. The primary concern refers to di-, oligo- and polysaccharide degrading enzymes present in the sample when extractions are carried out under conditions that allow their activity [2]. These can be endogenous plant-derived enzymes or microbial enzymes (from the natural microbial population of the raw materials or microbial contaminations). Additionally, enzymes originating from food additives, processing aids or other food ingredients (e.g. added malt in wheat flour) might be relevant. Further issues in sugar analysis can be related to microbial growth in the samples during or after extraction, as well as polysaccharide co-extraction and the presence of proteins in the extracts [3, 4]. Potential measures that aim to prevent enzyme activity, microbial growth, starch co-extraction or to remove co-extracted proteins are the following: sample pre-treatment with alcohol (methanol or ethanol), heat treatment, extraction with alcoholic solutions, use of antimicrobial agents (e.g. sodium azide; NaN_3) and performance of sample clean-up (e.g. Carrez clarification) [2–4]. The present study's objective is the comparative investigation of selected aqueous and ethanolic extraction procedures with and without such

measures. These are applied to wheat flour, representing a regular plant-based food ingredient, and to wholemeal wheat flour, representing a less homogenous and more complex food ingredient to challenge the extraction procedures. This will help identify a suitable method for the extraction and quantification of short-chain carbohydrates from plant-based HPIs derived from the following raw materials: wheat (gluten), quinoa, buckwheat, amaranth, soy, pea, lupin, lentil, carob, chickpea and faba bean.

3.3 Experimental

3.3.1 Materials

Wheat flour (baker's flour) was supplied by Whitworth Bros Ltd, UK and wholemeal wheat flour by Odlums, Valeo Foods, Ireland. The following commercial high-protein ingredients (HPIs) were used: soy protein isolate (Clarisoy 100; further referred to as 'soy') supplied by ADM, Illinois, US; pea protein isolate (NUTRALYS PEA BF; further referred to as 'pea') from Roquette, France; vital gluten (NUTRALYS W; further referred to as 'gluten'); carob germ flour (GRINDSTED VEG PRO S1; further referred to as 'carob') from Danisco, UK; and chickpea flour (GF gram flour; further referred to as 'chickpea') from Doves Farm Foods Ltd, UK. Eight experimentally produced HPIs were provided by Fraunhofer Institute IVV, Germany: one regular flour – dehulled lentil flour (further referred to as 'lentil'); five high-protein flours (fine fractions; obtained by air-classification) – quinoa high-protein flour (further referred to as 'quinoa'), amaranth high-protein flour (further referred to as 'amaranth'), buckwheat high-protein flour (further referred to as 'buckwheat'), and faba bean high-protein flour (further referred to as 'faba bean'); and three protein isolates – lentil protein isolate I (isolated by isoelectric precipitation; IEP; further referred to as 'lentil IEP'), lentil protein isolate II (isolated by ultrafiltration; UF; further referred to as 'lentil UF') and lupin protein isolate (from blue lupin; further referred to as 'lupin'). The compositional data (analyses performed as indicated below) of all these ingredients are presented in Table 3.1. Ultrapure water, with a resistivity of 18.2 MΩ · cm and a total organic carbon (TOC) content of < 5 ppb, was used for all extraction procedures and the preparation of eluents and reference standards. Sodium azide (NaN_3) was obtained from Thermo Fisher Scientific (Alfa Aesar, UK). HPLC-grade acetonitrile (MeCN), methanol (MeOH) and ethanol (EtOH) were purchased from Sigma-Aldrich (Missouri, USA). All other chemicals were also purchased from Sigma-Aldrich (Missouri, USA) unless stated otherwise.

Table 3.1: Composition of wheat flour, wholemeal wheat flour and HPIs.

Ingredient	Moisture [%]	Protein [%DM]	Fat [%DM]	Ash [%DM]	TDF*** [%DM]	Carbohydrates** [%DM]
Wheat flour	12.6	14.8	1.0	0.8	2.3	81.2
Wholemeal wheat flour	12.8	12.6	1.5	1.4	2.8	81.7
Soy*	5.7	96.8	0.3	0.2	< 0.1	2.7
Pea*	9.7	80.2	6.5	5.9	2.9	4.6
Lupin*	6.5	94.5	2.9	5.6	< 0.1	–
Gluten*	8.2	83.1	0.7	0.9	< 0.1	15.3
Lentil IEP****	4.9	86.9	4.7	5.7	1.9	2.6
Lentil UF****	5.6	99.3	0.7	3.7	< 0.1	–
Carob*	6.1	55.0	0.2	7.0	17.7	20.0
Chickpea	10.2	23.6	6.2	2.6	6.2	61.4
Quinoa	10.4	37.4	18.1	4.3	3.3	36.9
Amaranth	11.7	40.9	18.1	7.4	14.6	18.9
Buckwheat	10.5	22.5	2.8	3.2	1.6	69.9
Faba bean*	13.1	61.2	3.8	5.4	0.3	29.2
Lentil	11.2	28.3	1.3	3.2	4.5	62.7

* Compositional data previously reported by Hoehnel *et al.* [5]
** Calculated by subtraction.
*** Total dietary fibre.
**** Lentil protein isolates produced by isoelectric precipitation (IEP) and ultrafiltration (UF).

3.3.2 Compositional Analysis

Results from compositional analysis were expressed as the percentage based on dry matter (%DM). Compositional analysis for HPIs was performed as follows: moisture was determined according to the air-oven method AACC 44-15.02; fat based on AACC 30–25.01 (Soxhlet method using SoxCap and Soxtec (Foss UK Ltd, UK), including a digestion step with 4 M HCl and a subsequent extraction step); ash based on AOAC 923.03 (removal of organic matter in a muffle furnace at 550 °C for 5 h); protein was based on MEBAK 1.5.2.1 (nitrogen-to-protein conversion factor 6.25). The analysis of total dietary fibre (TDF) was performed based on AOAC 991.43 (gravimetric method) by Concept Life Science Ltd, UK. Carbohydrate values were obtained by calculation (100%−[protein%+moisture%+ash%+fat%+TDF%]). Compositional analysis for wheat flour and wholemeal wheat flour was performed by Concept Life Sciences, UK, as previously specified by Hoehnel *et al.* [6].

3.3.3 Comparison of Extraction Procedures

Short-chain carbohydrates were extracted from wheat flour, wholemeal wheat flour and HPIs using nine different extraction procedures. Six of the procedures are based on aqueous extraction (A1 to A6); the other three extraction procedures are based on ethanolic extraction (E1 to E3). A2 to A6 are based on A1 and include small adaptions and additional treatments as indicated in the following sections. Similarly, E2 and E3 are based on E1 and include small adaptions and additional treatments, which are also specified below. Figure 3.1 provides an overview of all

extraction procedures employed in this study (for comparison reasons, this figure also includes AACC 80-04.01, which was not used in the present study).

3.3.3.1 A1 Extraction

This aqueous extraction procedure is based on AACC 80-04.01 and was adapted according to laboratory equipment and practicability as follows: 8 mL of UPW (room temperature) were added to 2 g of sample in a 15 mL polypropylene (PP) test tube (conical bottom). The mixture was vortexed and shaken by hand (if necessary) until the powder was fully suspended. This was followed by 20 min of shaking at room temperature using an orbital shaker (UNI MAX 1010, Schwabach, Germany). After shaking, the sample was centrifuged at 1800 g for 10 min (20 °C). The supernatant was quantitatively transferred into a 10 mL volumetric flask, and the volume adjusted to 10 mL with UPW. The extract was filtered through a syringe-driven polyamide (PA) filter with a pore size of 0.20–0.45 μm (Machery-Nagel, Germany) and analysed immediately (HPLC) or stored frozen in 2 mL screw-cap tubes with sealing ring until further analysis.

3.3.3.2 A2 Extraction

The extraction was performed according to the procedure specified for A1 with one modification. A dilute NaN_3 solution (50 ppm in UPW) was used instead of UPW in A1.

	AACC 80-04.01	A1 (Aqueous extraction) Adapted after AACC	A2 (A1 + NaN_3)	A3 (A1 + MeOH + HT)	A4 (A3 + HT)	A5 (A4 + NaN_3)	A6 (A1 + Carrez)	E1 (Ethanolic extraction)	E2 (E1 + NaN_3)	E3 (E1 + Carrez)
Weight [g]	10	2	2	2	2	2	2	2	2	2
Use of NaN_3 50 ppm in UPW	No	No	Yes	No	No	Yes	No	No	Yes	No
Solvent	Distilled water 100 mL; RT	UPW 8 mL; RT	NaN_3 8 mL; RT	MeOH + UPW 1 mL + 7 mL; RT	MeOH + UPW 1 mL + 7 mL; RT	MeOH + UPW 1 mL + 7 mL; RT	UPW 8 mL; RT	80% EtOH 2x 15 mL; 55°C	80% EtOH 2x 15 mL; 55°C	80% EtOH 2x 15 mL; 55°C
Extraction Stirring / Shaking / Sonication	Stirring + Sonication 20 min; RT	Shaking 20 min; RT	Shaking 20 min; RT	Shaking Combined with HT 20 min; RT	Shaking Combined with HT 20 min; RT	Shaking Combined with HT 20 min; RT	Shaking 20 min; RT	2x Sonication 2x 15 s	2x Sonication 2x 15 s	2x Sonication 2x 15 s
Treatment Heat treatment (HT) Carrez Concentration treatment (CT)	No	No	No	HT	HT	HT	Carrez	CT	CT	CT + Carrez
Centrifugation	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Dilution Final solvent and volume	ACN 25 mL; incl. 10 mL sample aliquot	UPW 10 mL	NaN_3 10 mL	UPW 10 mL	UPW 10 mL	NaN_3 10 mL	UPW 10 mL	UPW 10 mL	NaN_3 10 mL	UPW 10 mL
Filtration	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Figure 3.1: Overview of extraction methods employed in this study (highlighted in orange) and comparison to AACC method 80-04.01.

3.3.3.3 A3 Extraction

This extraction procedure includes two modifications of A1. Instead of 8 mL of UPW, the following solvents were added to the solid sample: 1 mL MeOH and 7 mL UPW. MeOH was added first to ensure the sample was soaked in MeOH before adding the UPW. Furthermore, instead of 20 min of shaking at room temperature (as specified in A1), the samples were subjected to a heat treatment at 80 °C for 10 min using a shaking incubator.

3.3.3.4 A4 Extraction

This extraction procedure was performed according to A3 but with extended heat treatment for 20 min instead of 10 min.

3.3.3.5 A5 Extraction

The extraction was performed according to procedure A4, but instead of UPW, a dilute NaN_3 solution (50 ppm) was used.

3.3.3.6 A6 Extraction

The extraction was performed according to A1, with an additional Carrez clarification step. The Carrez solutions (Carrez I and II) were added after transferring the extract into the 10 mL volumetric flask and before adjusting the volume to 10 mL with UPW. First, 250 μL of Carrez I (15 g potassium hexacyanoferrate(II) trihydrate per 100 mL UPW) were added to the sample, followed by 250 μL Carrez II (23 g zinc acetate dihydrate per 100 mL UPW). The volume was then adjusted to 10 mL with UPW, the contents of the volumetric flask were transferred into a new 15 mL test tube and centrifuged at 1800 g for 10 min (20 °C).

3.3.3.7 E1 Extraction

For the following extractions, alcohol/water mixtures with a high proportion of alcohol were used as extraction solvent. The alcohol is evaporated by vacuum centrifugation as part of the extraction procedures. EtOH was chosen due to the fact that it is less hazardous than MeOH. E1 was carried out as follows: 15 mL of 80/20 (v/v) ethanol/UPW (80% EtOH), which was heated to 55 $\pm$ 5 °C, were added to 2 g of sample in a 50 mL PP test tube. The mixture was vortexed to make sure the powder was fully suspended. The sample was then sonicated (BANDELIN Sonoplus HD 3100 homogenizer with MS73 microtip) twice for 15 s with a 5 s break (75°amplitude) and centrifuged at 1800 g for 10 min (20 °C). The supernatant was transferred to a new 50 mL PP tube and the remaining pellet reextracted by adding

another 15 mL 80% EtOH (55 ± 5 °C). The mixture was vortexed until the pellet was resuspended and the sonication with subsequent centrifugation repeated as described above. The second supernatant was pooled with the first, and the entire sample extract was concentrated by vacuum centrifugation (Scanvac Scan Speed 32 with Scanvac VacSafe 15, Labogene ApS, Denmark) with the following conditions: run 1 – 1500 rpm, 45 °C, 2 h; followed by run 2 (after replacement of ice in the cold trap and cold water in the pump) – 2000 rpm, 50 °C. The concentrated extract (around 6 mL) was transferred into a 10 mL volumetric flask; filled up with UPW, filtered through a syringe-driven PA filter with a pore size of 0.20–0.45 μm (Machery-Nagel, Germany) and analysed immediately (HPLC) or stored frozen in 2 mL screw-cap tubes with sealing ring until further analysis.

3.3.3.8 E2 Extraction

E2 represents an adaptation of E1. It was carried out as described above, but a dilute NaN_3 solution (50 ppm) was used instead of UPW to fill up the volumetric flask to 10 mL after concentration of the sample extract. This procedure was previously used and described by Hoehnel *et al.* [6] for the extraction of freeze-dried bread samples.

3.3.3.9 E3 Extraction

The extraction was performed according to the procedure described for E1 with one modification. After concentration of the sample extract and its transfer to the 10 mL volumetric flask, a Carrez clarification was realised by adding 250 μL of both Carrez I and Carrez II (as specified above for A6). The volumetric flask was then filled up with UPW, and the contents were transferred to a 15 mL test tube for centrifugation (1800 g for 10 min; 20 °C) before filtration.

3.3.4 Determination of α-Amylase and β-Amylase Activity

The activity of both α-amylase (K-CERA) and β-amylase (K-BETA3) were determined using the indicated enzyme kits from Megazyme, Ireland. The enzyme activities were assessed to interpret differences observed between wheat flour and wholemeal wheat flour due to the different extraction procedures. The assays were carried out according to the manuals provided with the kits and values expressed in Ceralpha units (CU) and Betamyl-3 units (B-3U) for α-amylase and β-amylase, respectively.

3.3.5 Determination of Sugar Contents of Plant-Based High-Protein Ingredients

A range of thirteen HPIs was screened for their contents of sucrose/maltose, glucose and fructose. The ethanolic extraction procedure E2 was chosen for the screening. This decision was based on the results obtained from comparing the different extraction procedures above and the fact that extracting HPIs is facilitated by using ethanolic solutions and higher volumes to minimise gelation related issues. Two different columns were used for the separation and quantification of the sugars, as specified below.

3.3.6 Separation and Quantification of Mono- and Disaccharides

Separation and quantification of mono- and disaccharides were achieved with an Agilent Infinity 1260 equipped with an RID. A dilute solution of calcium disodium ethylene diamine tetraacetate (CaEDTA; 0.0001 M) in UPW was used as eluent. The separation of sugars was achieved with a Sugar-Pak I (SP) column (300 × 6.5 mm, 10 μm; packed with a microparticulate cation-exchange gel in calcium form; Waters Corporation, MA, USA) and the following conditions: column temperature 80 °C; flow rate 0.5 mL/min; isocratic elution with 0.0001 M CaEDTA. A separation of sucrose and maltose was not achieved with this column. Therefore, a second column was used to allow for an individual quantification of sucrose and maltose for the screening of HPI sugar levels: High Performance Carbohydrate (HPC) column (4.6 × 250 mm, 4 μm spherical silica bonded with trifunctional amino propyl silane; Waters Corporation, MA, USA) with the following conditions: column temperature 40 °C; flow rate 1.0 mL/min; isocratic elution with 78/22 (v/v) MeCN/UPW; sample extracts were mixed with MeCN in a ratio of 40/60 (v/v) extract/MeCN. Mixtures of reference standards (sucrose, maltose, glucose and fructose; all > 98% purity) were used to quantify (standard concentrations ranging from 0.01–10 g/L for the SP column and from 0.1–3.5 g/L for the HPC column). Results of sugar levels in wheat flour, wholemeal wheat flour and HPIs are expressed in grams of analyte per 100 g of dry matter of the sample (%DM).

3.3.7 Statistical Analysis

Extractions for the comparison of extraction procedures were carried out in duplicate and extractions for screening HPI sugar levels in triplicate. Data analysis was carried out with RStudio, v.1.2.1335 with R v.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 utilised to determine significant differences ($p < 0.05$).

3.4 Results and Discussion

Figure 3.1 presents a condensed overview of the different extraction procedures (and their conditions) employed in this study to support the results' interpretation. The outcomes of both the comparison of extraction procedures and the screening of sugar levels in HPIs are visualised in Figures 3.2 and 3.3. The exact values (means, standard deviations), including results of one-way ANOVA, are enclosed in Tables A.B1 and A.B2 in Appendix B.

3.4.1 Comparison of Extraction Procedures

Figure 3.2 reflects the large discrepancies in determined sugar levels that were observed using the different extraction procedures. Specifically, differences in sugar contents determined for wheat flour and wholemeal wheat flour, which were subjected to all nine extraction procedures, were found to be as high as 1.3–1.8 %DM (sum of sucrose/maltose, glucose and fructose). Soy could only be used to assess differences among the three ethanolic extraction procedures E1–E3 since the aqueous extractions were not applicable to this ingredient (high water absorption/gelation).

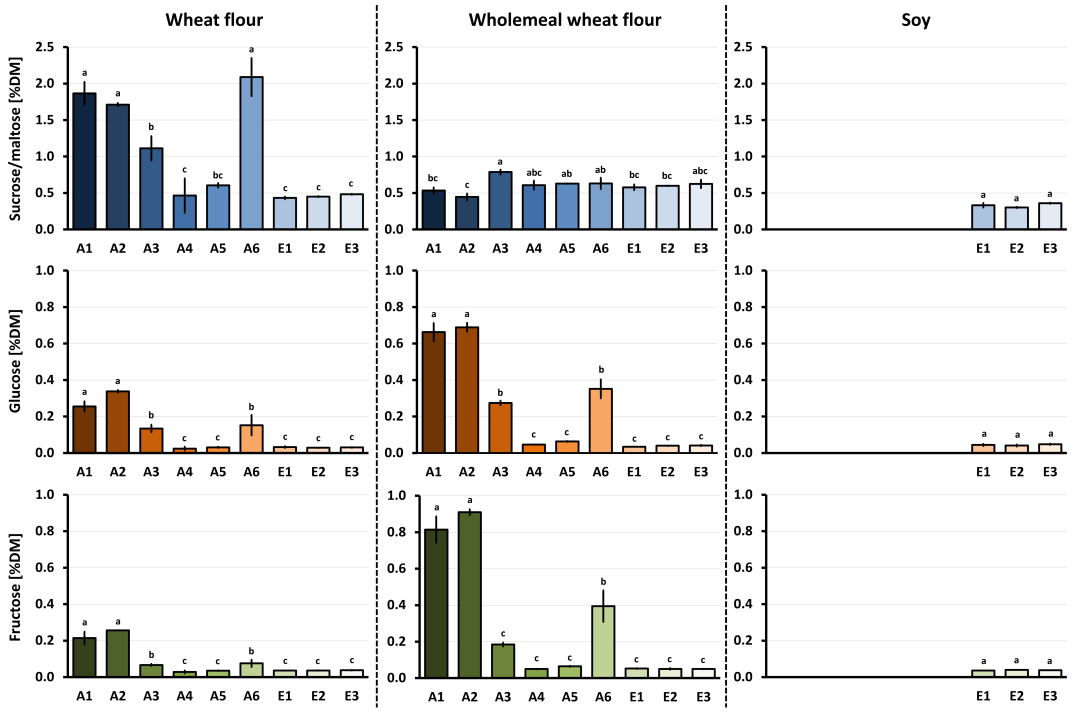


Figure 3.2: Sucrose/maltose (blue), glucose (orange) and fructose (green) levels determined for wheat flour, wholemeal wheat flour and soy protein isolate with the different extraction procedures A1–A6 and E1–E3. Bars within the same individual charts and with different letters represent significantly different sugar levels ($p < 0.05$).

3.4.1.1 Use of Methanol and Heat Treatment

Extraction procedures A3 and A4 include the replacement of 1 mL of the extraction solvent UPW by MeOH and a heat treatment. While shaking at 80 °C was carried out for only 10 min in A3, it was prolonged to a total of 20 min in A4. Significantly lower levels of sucrose/maltose, glucose and fructose were observed with A3 compared to A1 (except for sucrose/maltose in wholemeal wheat flour: slightly but significantly higher level with A3). Even smaller values than with A3 were obtained with A4 for all evaluated sugars. Sugar contents can be influenced by the degradation of di-, oligo- and polysaccharides present in the samples. Specifically, enzymatic degradation can occur. These enzymes can be endogenous plant-derived enzymes or microbial enzymes originating from the natural microbial population of the raw material or from microbial contamination during sample storage and sample preparation (if no antimicrobial agents used). A few examples of such enzymes that are relevant for carbohydrate degradation in cereal and legume matrices are the following: amylases (α- and β-amylases [7]), β-glucanases [8], invertases [9] and fructan exohydrolases [10, 11] as well as α-galactosidases [12] and levansucrases [13]. While, for example, for fructan degrading enzymes, it has been reported that their remaining activity is relatively small in mature wheat grains [10, 11], it is also known that carbohydrate levels can change during storage of food ingredients and products, which was associated with potential enzyme activity or physicochemical processes [14–17]. Several different measures have been used to inactivate enzymes. Pre-treatment with alcohol (MeOH or EtOH) and heat treatment at 80 °C, similar to procedures A3 and A4, were applied during aqueous extraction of multiple different sample matrices [2, 4, 18] and has been shown to substantially reduce amylase activity in wholemeal wheat flour and to decrease measured levels of glucose [4]. Also, an increase of the determined sucrose level and a decrease in fructose were reported, which was attributed to the inactivation of potentially present invertase by the treatment [4]. This is in line with the lower levels of glucose and fructose determined in both wheat flour and wholemeal wheat flour when extracted with procedures A3 and A4 (compared to A1). The sucrose/maltose contents, however, were lower (wheat flour) or similar (wholemeal wheat flour) to A1 instead of higher (except A3 in wholemeal wheat flour: sucrose/maltose significantly higher than with A1). This observation might be caused by the lack of separation between sucrose and maltose and the determination of the sum of both sugars. In A1, where no measures against enzyme activity were taken, both a conversion of sucrose into glucose and fructose (potentially by invertases) as well as a degradation of starch into maltose and small amounts of glucose (amylases) is possible. The enzymatically released maltose would lead to

high values for sucrose/maltose. An inhibition of both enzymes would prevent the degradation of sucrose but also the accumulation of maltose and thereby cause a reduction in the sum value for sucrose/maltose, which was observed for A3 and A4 in wheat flour (the exception of seemingly stable sucrose/maltose values in wholemeal wheat flour will be addressed separately below). Instead of an inactivation of enzymes, the generally lower sugar levels observed with A3 (compared to A1) could have been attributed to an incomplete extraction due to the shorter extraction time (but in spite of the increased temperature, which generally accelerates extraction). However, the fact that A4 resulted in even lower values for sucrose/maltose, glucose and fructose than A3 contradicts this theory and rather indicates that a more efficient enzyme inactivation was achieved with the prolonged heat treatment (20 min instead of 10 min in A3). A further extension of the heat treatment to 30 min did not lead to an additional decrease in sugar levels (data not shown). In aqueous extractions without or with insufficient measures to inhibit enzymes, their activity cannot only lead to carbohydrate degradation during extraction but also at any time after extraction (during sample handling at room temperature or above). This is particularly relevant for samples containing large amounts of polysaccharides (e.g. starch), which are partially co-extracted when using UPW as extraction solvent.

3.4.1.2 Aqueous vs Ethanolic Extraction

Besides alcohol (MeOH or EtOH) pre-treatment and heat treatment, alcohol/water mixtures with varying alcohol contents (30–80%) have been described to minimise complications related to enzyme activity when used as extraction solvents for short-chain carbohydrates and are commonly used [2, 19]. However, higher alcohol contents in the extraction solvent have been discussed controversially with regard to extractability of short-chain carbohydrates. Johansen *et al.* [2] and Xiaoli *et al.* [19] found that, while low levels of alcohol do not seem to reduce extraction yields, MeOH or EtOH levels of 50% or higher cause a drastically reduced extractability of short-chain carbohydrates like galactooligosaccharides (GOS) and sucrose (unless extraction was performed at boiling point). On the other hand, other publications report no negative impact of the use of 80% MeOH or EtOH (often in combination with elevated temperatures) on the extraction yield of short-chain carbohydrates [14, 20–24]. In the present study, generally lower sugar levels were determined with the ethanolic extraction procedures than with the aqueous extractions (except for sucrose/maltose in wholemeal wheat flour). This might, at first, suggest that these methods do not provide satisfying extraction efficacy. However, a comparison of E1 with A4, which is not expected to show incomplete sugar extraction, reveals no significant differences in determined sugar levels. This

indicates efficient enzyme inhibition and adequate sugar extractability rather than incomplete sugar extraction for E1. The fact that the ethanolic extractions in this study were performed with two consecutive extraction steps (re-extraction of the pellet obtained after first extraction and centrifugation) potentially contributes to this outcome. Furthermore, instead of the commonly used shaking (in aqueous extractions in this study) or stirring, sonication was applied to facilitate extraction. According to Machado *et al.* [25] and Karki *et al.* [26], sonication results in significantly more efficient sugar extraction than stirring. Therefore, the combination of 80% EtOH with elevated temperature (55 °C), re-extraction of the pellet and sonication seems to achieve adequate sugar extraction. A comparison of determined sugar levels with values reported in the literature is difficult and bears limited validity due to natural variations in raw material composition (different cultivars, growing, processing and storage conditions) and differences in extraction procedures. However, the sugar contents in wheat flours determined by Ispiryan *et al.* [27] (glucose, fructose), Ziegler *et al.* [18] (glucose, fructose) and MacArthur and D'Appolonia [28] (sucrose, maltose, glucose, fructose) are generally better reflected by values obtained with the extraction procedures A4 and E1 in the present study. Slightly increased glucose values in the literature in comparison to values obtained with E1 could be associated with the expected starch co-extraction and its possible degradation when using UPW as extraction solvent [18, 27] (or the lack of using antimicrobial agents [18]). Starch from various plant materials has been described to be insoluble in 80% EtOH which makes this a promising solvent to minimise starch co-extraction [29].

3.4.1.3 Differences between Wheat Flour and Wholemeal Wheat Flour

Wheat flour and wholemeal wheat flour show slightly different trends regarding the sugar levels obtained with the investigated extraction procedures. For wheat flour, A1 resulted in much higher sucrose/maltose values than A3, A4 and E1. For wholemeal wheat flour, on the other hand, A1 led to similar or even slightly lower sucrose/maltose values than A3, A4 and E1. In the case of glucose and fructose, the observed trends do not differ between wheat flour and wholemeal wheat flour. However, much higher glucose and fructose levels were determined with A1 in wholemeal wheat flour than in wheat flour. Also, the glucose/fructose ratio (values below 1 represent excess fructose) determined with A1 is much lower in wholemeal wheat flour (approx. 1.24; A1) than in wheat flour (approx. 0.76; A1). The fact that wholemeal wheat flour contains the outer parts of the wheat grain to a much higher degree than wheat flour consequently results in both a different pattern of present endogenous and microbial (inclusion of outer layers causes a larger microbial population [30]) enzymes as well as a higher

content of non-starch oligo- and polysaccharides (such as fructans) which can act as substrates for these enzymes [31]. The activities of α- and β-amylase were determined in wheat flour and wholemeal wheat flour (Table 3.2) to support the results' interpretation. The activity of α-amylase accounted for 0.119 AAU/g in wheat flour and 0.122 AAU/g in wholemeal wheat flour. The determined values for β-amylase activity were 34.8 BAU/g (wheat flour) and 35.4 BAU/g (wholemeal wheat flour). While the determined activities of α- and β-amylase for wheat flour and wholemeal wheat flour were very similar (no significant differences), possibly invertase or other fructan degrading enzymes were more active in wholemeal wheat flour. Comparing A1 results of wheat flour and wholemeal wheat flour, this would explain the generally higher glucose and fructose levels, the lower glucose/fructose ratio (fructan degradation releases primarily fructose) and the relatively low sucrose/maltose levels in wholemeal wheat flour due to sucrose breakdown.

3.4.1.4 Use of Sodium Azide

The use of NaN_3 was investigated due to the potential impact of microbes present in the raw material or introduced by microbial contamination during sample storage or extraction. Growth of these microbes can lead to degradation (enzymatic; as discussed above) or consumption (metabolism) of sugars during sample preparation, storage and analysis at room temperature (e.g. HPLC autosampler without thermostat). This can negatively impact the accuracy and reproducibility of determined sugar levels, and the use of NaN_3 can, therefore, be seen as a preventative measure. The methods A2, A5 and E2 represent extraction procedures where a dilute solution of NaN_3 (50 ppm) in UPW was used as extraction solvent (A2, A5) or final solvent (E2). They were performed exactly like their counterparts without NaN_3, which are A1, A4 and E1, respectively. Therefore, a comparison of the results obtained for the following pairs allows for an assessment of the effect of NaN_3 on sugar extraction: A1/A2, A4/A5, E1/E2. Figure 3.2 reveals that no significant differences were found within these pairs for levels of sucrose/maltose, glucose and fructose. This implies that no microbial growth interfered in the samples without NaN_3. It also represents encouragement for the use of NaN_3 in sugar extractions

Table 3.2: Activity of α- and β-amylase determined for wheat flour and wholemeal wheat flour.

Enzyme activity	Wheat flour	Wholemeal Wheat Flour
α-Amylase activity [CU/g]*	0.119 ± 0.006^a	0.122 ± 0.016^a
β-Amylase activity [B-3U/g]**	34.8 ± 0.6^a	35.4 ± 0.4^a

Means $\pm$ standard deviation with different letters in the same row indicate significant differences at $p < 0.05$.

* α-Amylase activity expressed in Ceralpha units (CU).

** β-Amylase activity expressed in Betamyl-3 units (B-3U).

since no negative impact on sugar determination was observed.

3.4.1.5 Use of Carrez Clarification

The methods A6 and E3 represent extraction procedures where a Carrez clarification was applied to the extracts just before adjusting the final volume. They were otherwise performed exactly like their counterparts without Carrez clarification, which are A1 and E1, respectively. No significant differences were detected between the sugar levels obtained with the ethanolic extractions E1 and E3. However, amongst the aqueous extractions, significantly smaller values were determined with A6 (compared to A1) for glucose and fructose in both wheat flour and wholemeal wheat flour. The sucrose/maltose levels observed with A6 were not significantly different from those of A1 in both wheat flour and wholemeal wheat flour (despite a small tendency towards higher values). The slightly lower glucose and fructose contents determined with A6 could indicate a small decrease in enzyme activity due to co-precipitation of proteins (potentially including some carbohydrate degrading enzymes) and polysaccharides (potentially including enzyme substrates) with zinc hexacyanoferrate(II) [3]. However, since the Carrez clarification was performed only at the end of the extraction procedure, determined glucose and fructose contents were still higher than with A4 and E1. Overall, no clear benefit of Carrez clarification concerning the determined sugar levels was observed in the present study. On the contrary, it was found that the abundance/height of matrix peaks (specifically at early retention times) in chromatograms (data not shown) of the separation with the cation-exchange column was largely increased when Carrez clarification was applied. This complicates peak integration of early eluting compounds (e.g. sucrose/maltose) and is probably related to the higher content of ions in these samples [3].

3.4.2 Screening of Sugar Contents in High-Protein Ingredients

Based on the results presented above, extraction procedure E2 was selected to screen sugar contents in a range of HPIs. The sugar levels in E2 extracts of 7 HPIs with protein contents < 80 %DM (flours) and 6 HPIs with protein contents > 80 %DM (isolates) were determined with two different HPLC separation methods using two different columns (Figure 3.3). This allowed for both a direct comparison of these values with the results of the different extraction procedures and an individual quantification of sucrose and maltose. Generally, higher values for sucrose/maltose were determined in the extracts of HPIs with protein contents < 80 %DM. However, there are exceptions to this trend. Chickpea, buckwheat, faba bean and lentil contain with approx. 1–2 %DM relatively low

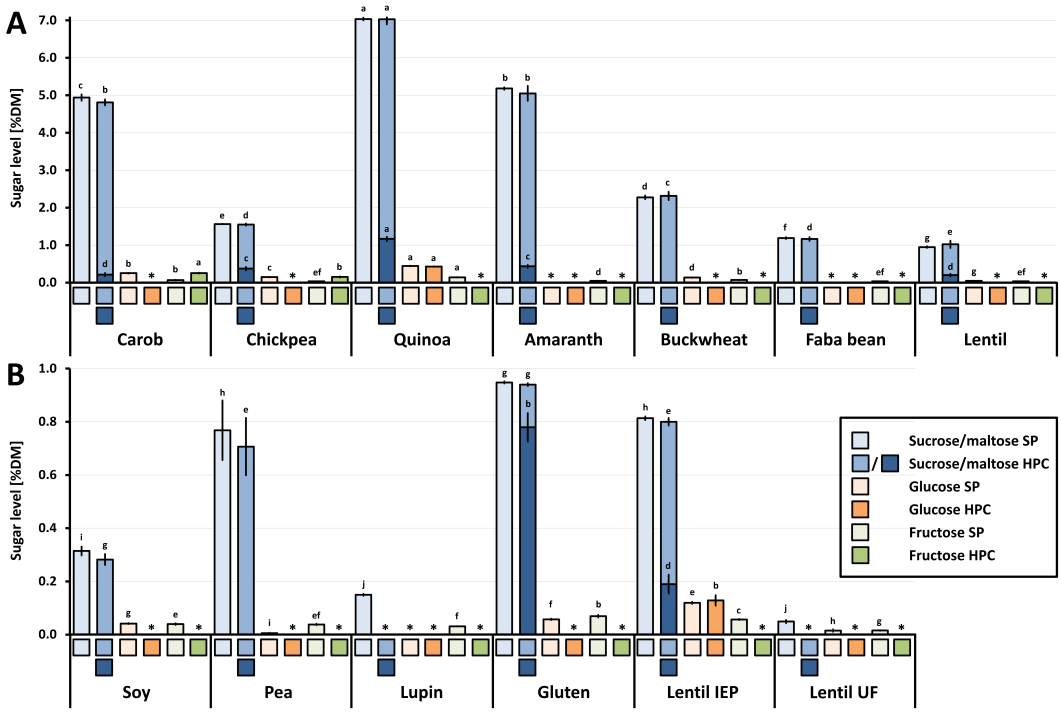


Figure 3.3: Sugar levels determined in HPIs using extraction procedure E2 and HPLC separation with two different columns (SP: Sugar-Pak I column, Waters Corporation, MA, USA; HPC: High Performance Carbohydrate column, Waters Corporation, MA, USA) to obtain individual values for sucrose and maltose. * Asterisks indicate where sugars were not detected or below 0.01 %DM (SP) or 0.15 %DM (HPC). Bars with the same colour (across **A** and **B**) and different letters represent significantly different sugar levels ($p < 0.05$). (**A**) HPIs with protein contents < 80 %DM; (**B**) HPIs with protein contents > 80 %DM.

amounts of sucrose/maltose when compared to the other HPIs with proteins contents < 80 %DM. Pea, gluten and lentil IEP represent HPIs with proteins contents > 80 %DM but exhibit similar levels of sucrose/maltose just below 1 %DM. The majority of the tested HPIs contains only sucrose and no maltose (not detected or in levels below the LOQ of 0.15 %DM) or sucrose in excess of maltose, which is the case for carob, chickpea, quinoa, amaranth, lentil and lentil IEP. Only gluten was found to contain with 0.78 %DM more maltose than sucrose (0.16 %DM), which might have been released from starch during gluten extraction from wheat. Glucose and fructose levels were below or approx. at 0.15 %DM for most of the HPIs. In quinoa, a slightly higher glucose level was found, which was indicated by both separation methods (SP and HPC column). The results obtained by separation on the HPC column largely confirm the results determined with the SP column. This provides reassurance for the identity of the quantified sugars since these columns represent decisively different separation principles. However, discrepancies were observed for the glucose and fructose values in carob and chickpea. The glucose levels for carob (0.25 %DM) and chickpea (0.15 %DM) determined with the SP column were not confirmed by the values obtained with the HPC column, even

though these levels exceed the HPC-LOQ of 0.15 %DM. Interestingly, fructose levels of 0.26 %DM (carob) and 0.15 %DM (chickpea) were determined with the HPC column as opposed to the much smaller levels of 0.07 %DM (carob) and 0.03 %DM (chickpea) with SP separation. These discrepancies could be explained by the presence of an unidentified compound that coelutes with glucose on the SP column but with fructose on the HPC column. The fact that similarly elevated levels for glucose on the one hand (SP column) and fructose on the other hand (HPC column) were measured in these two ingredients of related botanical origin supports this theory. The investigated samples lentil, lentil IEP and lentil UF are differently processed ingredients derived from the same lentil raw material. While the protein extraction procedure (isoelectric precipitation) applied to produce lentil IEP does not appear to have a big impact on the sugar content and sugar profile, the ultrafiltration used to produce lentil UF leads to a removal of the majority of short-chain carbohydrates quantified in this study. This was previously postulated by Alonso-Miravalles *et al.* [32], who compared lentil IEP and lentil UF with regard to their physicochemical and techno-functional characteristics. Berrios *et al.* [23] applied sugar extraction procedures similar to E2 to determine mono-, di- and oligosaccharides in chickpea flour and lentil flour. The results reported in their study are largely in agreement with the findings of the present study. The sugar contents determined for carob, quinoa, amaranth, buckwheat and faba bean also correspond to previously reported results in the literature [33–36]. However, the high-protein flours of quinoa, amaranth and buckwheat analysed in this study appear to contain slightly higher levels of sucrose/maltose than reported for regular flours. While this might be related to natural variations, it could also indicate an accumulation of these sugars in the high-protein fraction during dry fractionation.

3.5 Conclusion

The comparison of several aqueous and ethanolic extraction procedures revealed big differences in determined sugar levels for wheat flour and wholemeal wheat flour. These differences are likely associated with the activity of carbohydrate degrading enzymes (endogenous plant-derived or microbial), polysaccharide co-extraction and the inhibition of both in some of the investigated extraction procedures. Ethanolic extraction procedures with a high volume of extraction solvent, which can minimise problems related to gelation and water absorption during the extraction of plant-based high-protein ingredients, generally resulted in lower determined sugar levels than aqueous extractions. While this could have suggested incomplete sugar extraction, the results of ethanolic extractions also

resembled (no significant differences) those of aqueous extraction procedures where other measures for enzyme inhibition were applied (MeOH pre-treatment, heat treatment). Moreover, the presented results indicate incomplete enzyme inhibition in aqueous extractions with MeOH pre-treatment combined with a 10 min heat treatment as opposed to longer heat treatments (20 or 30 min). Ethanolic extraction with the application of NaN_3 but without Carrez clarification was found to be the most suitable (amongst the investigated extraction procedures in this study) to determine sugar contents in HPIs. For wheat flour and wholemeal wheat flour, and potentially other samples with low gelation capacity and water absorption in comparison to most plant-based high-protein ingredients, both ethanolic extraction and aqueous extraction with MeOH pre-treatment and a heat treatment of 20 min or longer allow for reliable sugar extraction.Aqueous extractions without sufficient inhibition of enzymes can lead to an overestimation of short-chain carbohydrates (and potentially related underestimation of carbohydrates with a higher degree of polymerisation acting as substrates for these enzymes) by several grams per 100 grams of sample dry matter (in this study approx. 1.8 %DM for wheat flour and 1.3 %DM for wholemeal wheat flour). This can have implications for the assessment of suitable processing techniques for food ingredients (such as fermentation) as well as for food labelling and evaluation of the nutritional value of food products.

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CHAPTER 4

Comparative Analysis of Plant-Based High-Protein Ingredients and Their Impact on Quality of High-Protein Bread

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4.1 Abstract

The orientation of consumers and industry towards plant-based foods and high-protein products is persistently increasing. Bread, as a staple food, is a promising matrix for the incorporation of plant-based high-protein ingredients to combine both trends. This study aims to provide a better understanding of techno-functional changes and impacts of plant proteins during bread production, which could advance the development of high-quality products with high levels of plant protein. A selection of high-protein ingredients from wheat, maize, potato, carob, pea, lupin and faba bean were subjected to compositional analysis and applied in wheat bread formulations, replacing 15% of wheat flour. Their impact on dough properties (gluten-aggregation, pasting behaviour, rheology) as well as bread quality (volume, crumb structure, crumb hardness) was analysed. The high-protein ingredients were found to affect gluten-aggregation, pasting and bread characteristics. Results indicated a weakened gluten-network in doughs containing potato and pea protein. Also pasting behaviour was mostly affected by the potato protein suggesting a heat induced improvement of its baking performance. Good bread quality, represented by high specific volumes and low crumb hardness, was observed for gluten, zein and carob. Breads with pea, lupin and faba bean showed only slightly inferior quality characteristics.

4.2 Introduction

Plant proteins are a growing market in the food sector and represent an opportunity to meet nutritional needs of the growing world population, while at the same time realising the transition to a more sustainable food production. Thus, the demand from consumers, industry and governmental institutions for high-quality food with high contents of plant protein is substantial. Bread plays an important role in the human diet and relatively large amounts are consumed worldwide [1]. It is therefore a promising matrix for the incorporation of plant protein ingredients in order to supply a broad range of customers with plant-based high-protein food. Definitions and requirements for the attribute 'high-protein' can be found in many regulations all over the world and they vary greatly. According to regulation (EC) No 1924/2006, a product qualifies for the claim 'high in protein' if 20% of its calories are provided by proteins. In order to reach this protein content while maintaining adequate product quality, plant-based ingredients with high protein contents can be utilised. Protein ingredients from plant sources are usually classified by their protein content: as flours (protein < 65 %DM), concentrates (protein > 65 %DM) and isolates (protein > 90 %DM) [2]. However, the terminology is sometimes misleading since especially isolated legume protein products often do not reach a protein level of 90 %DM but are still considered isolates regarding their production procedure [3]. Hence, this study will focus on the generalised term high protein ingredients (HPIs). The partial substitution of wheat flour for bread production by HPIs from numerous sources has been previously investigated, including dairy proteins [4]; cereals [5], pseudocereals [6] and legumes [7–9]. However, these studies are usually focused on one type of plant protein. This study aims for a comparison of HPIs from different plant sources and investigates their impact on wheat bread formulations and their suitability to produce high-protein quality breads. The selected HPIs are the cereal proteins gluten (wheat) and zein (maize), potato protein, and proteins from the following legumes: carob (*Ceratonia siliqua*), pea (*Pisum sativum*), lupin (*Lupinus angustifolius*) and faba bean (*Vicia faba*). While some of these ingredients are commercially available and well characterised, others are produced from emerging protein sources and their properties and potential for bakery applications need to be explored. Wheat gluten can be described as a group of proteins, which are mainly responsible for the structure-forming ability of wheat flour during dough production and baking. Gluten has unique viscoelastic properties, protein-protein interactions, water holding capacity and thermosetting characteristics [10]. It is widely used as bread improver in the baking industry. Zein is the prolamine fraction originating from maize proteins. Amongst other cereal prolamines, it exhibits the highest content of hydrophobic amino acids and thus a

very high protein hydrophobicity and low water solubility [11]. Potato proteins can be obtained from the side-product potato juice from potato starch production and therefore represent an economically promising ingredient for plant-based high-protein foods. Previous studies report high water solubility and indicate outstanding techno-functional properties including heat induced gelling [12]. Legumes are generally accepted to be beneficial with regard to environmental aspects, due to their ability to fix nitrogen, and from a nutritional perspective [1]. While cereal proteins exhibit high contents of sulphur containing amino acids and are low in other essential amino acids (e.g., lysine), legumes represent the opposite and are therefore favourable for incorporation in cereal products to balance the amino acid profile and increase protein quality [1]. Important protein ingredients from legumes are high-protein flours (obtained by dry-processing) and isolates (produced by wet processing). Dry-processed (i.e., air-classified) products exhibit native protein characteristics and lower protein contents, whereas protein isolates have a higher protein purity and altered properties conditioned by the isolation procedure [13]. Furthermore, isolates mainly consist of the protein fractions albumins and globulins. An exceptional legume protein is carob germ protein, which contains a high glutelin fraction (about 68%) called caroubin. This protein has been reported to have gluten-like properties [14, 15]. The objective of this study is to characterise protein-rich fractions of a wide range of plant sources and to evaluate their impact on dough and bread quality characteristics.

4.3 Experimental

4.3.1 Materials

Five commercially available high-protein ingredients (HPIs) were used in this study. Potato protein isolate (Patissionate 306 P) was obtained from Avebe, the Netherlands; pea protein isolate (NUTRALYS PEA BF) from Roquette, France; carob germ flour (GRINDSTED VEG PRO S1) from Danisco, UK; vital gluten (NUTRALYS W) from Roquette, France; and corn protein (Zein) from Flo Chemical Corporation, Massachusetts, US. Additionally, two HPIs: blue lupin protein isolate and faba bean flour (fine fraction, protein-rich) were experimentally produced and provided by Fraunhofer Institute IVV, Freising, Germany. Wheat flour was supplied by Whitworth Bros Ltd, UK; dry yeast (4% moisture, 50% protein, 5% fat, 40% carbohydrates) by Puratos, Belgium; salt by Glacia British Salt Ltd, UK; and vegetable oil by Musgrave, Ireland. Chemicals were purchased from Sigma-Aldrich (Missouri, USA) unless stated otherwise.

4.3.2 Compositional Analysis

HPIs and wheat flour where subjected to compositional analysis including determination of protein (nitrogen-to-protein conversion factor 6.25, based on MEBAK 1.5.2.1), dry matter/moisture (air-oven method at 130 °C until constant mass reached), ash (incineration in muffle furnace at 550 °C for 5 h, charred prior to muffling using open flame, based on AOAC 923.03) and fat (Soxhlet method using SoxCap and Soxtec (Foss UK Ltd, UK), digestion with 4 M HCl prior to extraction, based on AACC 30-25.01). The analysis of total dietary fibre (TDF) was performed by Concept Life Science Ltd in accordance with AOAC 991.43. The carbohydrate value was obtained by subtraction (i.e., 100%−[protein%+moisture%+ash%+fat%+TDF%]). Total starch content was analysed using the enzyme kit K-TSTA supplied by Megazyme, Ireland. All values, except the moisture content, are expressed as percentage based on dry matter (%DM).

4.3.3 Empirical Dough Analysis

Properties of wheat flour and mixtures of wheat flour and HPIs (HPI/flour blends) in a ratio of 85% to 15%, respectively, were analysed. Moisture contents of blends were calculated considering the determined moisture contents of wheat flour and HPIs according to their ratios.

4.3.3.1 Farinograph

Water absorption (FWA) was determined by Farinograph (Brabender GmbH and Co KG, Duisburg, Germany) measurements following AACC 54-21.02. Titration trials were performed adjusting doughs from wheat flour and HPI/flour blends to a consistency of (500 ± 20) Farinograph units (FU).

4.3.3.2 GlutoPeak Test

Gluten-aggregation properties of wheat flour and the HPI/flour blends were investigated using the GlutoPeak (Brabender GmbH and Co KG, Duisburg, Germany). This device applies high shear to a flour/water slurry. 9 g flour (based on 14% moisture; adjustments according to AACC 82-23.01) were added to 9 g of deionised water (36 °C) weighed into the sample cup (adjusted to reach a slurry weight of 18 g). The measurement was started immediately after a brief premixing step. The paddle speed was set to 2750 rpm and water circulating through the jacketed sample cup maintained the sample temperature at 36 °C. The torque reading was recorded over time. The maximum test time was set to 10 min and measurements were stopped approx. 30 to 50 s after detection of the major peak. The curve

was evaluated by the software provided with the instrument regarding Torque Maximum (TM, expressed in Brabender units BU) and Peak Maximum Time (PMT, expressed in s).

4.3.3.3 Rapid Visco Analysis

The pasting behaviour was examined using Rapid Visco Analysis (RVA Super 3, Newport Scientific, Warriewood, Australia) following AACC 76-21.02. A heating profile was applied: equilibration at 50 °C for 1 min, heating to 95 °C at 0.2 °C/s, holding at 95 °C for 162 s, cooling to 50 °C at 0.2 °C/s, maintaining at 50 °C for 120 s. The pasting variables peak viscosity (PV), setback and final viscosity (FV) were determined from the viscogram using manufacturer-supplied software.

4.3.4 Recipe Adaptation and Bread Production

Bread samples were prepared according to the formulation in Table 4.1. For incorporation of HPIs, 15% of wheat flour were replaced. Water levels where applied as determined by Farinograph trials - FWAs (Table 4.3). Baking properties of the HPIs were evaluated by preparation and examination of micro-scale bread loafs (based on 65 g of dough). The straight dough method was applied. After activating the yeast by dissolving in 30 °C tap water for 10 min, the yeast suspension was added to the remaining, previously weighed ingredients. A total dough amount of 630 g was prepared by mixing with a Kenwood Chef (Kenwood Manufacturing Co. Ltd., UK) kitchen machine at speed 1 and 2 for 1 and 7 min, respectively. After dividing the dough into nine pieces of 65 g $\pm$ 1 g, the pieces where moulded (very sticky doughs were shaped with a dough scraper), put into baking tins and proofed for 75 min at 85% humidity and 30 °C (KOMA BV Sunriser, Reormond, the Netherlands). Baking was performed in a deck oven (MIWE Condo, Arnstein, Germany) at 210 °C top and bottom temperature for 14 min with steaming the baking chamber prior to loading with 700 mL and open draft throughout the whole baking process. Breads where removed from tins and left on a grid at ambient

Table 4.1: Recipe for control and high-protein ingredient (HPI) formulations.

Ingredient	% based on flour	% based on recipe
Wheat flour	100.0 (85.0**)	59.70
HPI	0.0 (15.0**)	0.00
Baker's yeast	2.0	1.19
NaCl	2.0	1.19
Oil	1.0	0.60
Water	62.5 (FWA*/**)	37.31
Total	167.5	100.00

* FWA - Farinograph water absorption according to Table 4.3.
** For HPI formulations.

temperature for 1 h to cool down. The presented values represent the mean of three independently performed baking trials.

4.3.5 Dough Rheology

Dough was produced according to the procedure reported in Section 4.3.4, but yeast was omitted. Dough was allowed to rest for about 10 min before measurements. Viscoelastic properties of doughs were examined by using a stress/strain-controlled rotational rheometer (MCR 301 Anton Paar, GmbH, Germany) equipped with a PP50 parallel-plate measuring system (serrated surface to avoid slippage). The lower plate was set to 30 °C. After positioning 10 g of dough between the plates, the upper plate was lowered to 1.025 mm, the sample trimmed and a layer of mineral oil applied to prevent desiccation. The final gap was set to 1 mm prior to starting the test. The rheological variables storage modulus (G'), loss modulus (G'') and damping factor (DampF $= tan\delta = G''/G'$), describing viscoelastic dough properties, were obtained from frequency sweeps ($\omega = 100–0.1$ Hz; data obtained at angular frequency of 2.54 Hz for control and 2.58 Hz for HPI doughs, respectively). A constant strain of 0.01% (linear viscoelastic range previously determined by amplitude sweeps) was applied.

4.3.6 Bread Characteristics

Specific volume (SV) was measured with a Volscan Profiler (Stable Micro Systems, Surrey, UK). To analyse crumb structure and hardness, three slices (20 mm) were cut out of the middle of each of 3 loaves. A C-Cell Imaging System (Calibre Control International Ltd, UK) was used to capture images of the slices and to determine the variables: number of cells and area of cells. Crumb hardness was analysed with a TA-XT2i Texture Analyser (Stable Micro Systems, Surrey, UK) equipped with a 25 kg load cell. A 20 mm cylindrical probe was used to compress the centre of the slice to 40% of its height as part of a texture profile analysis (TPA): test speed 5 mm/s, post-test speed 10 mm/s, trigger force 0.05 N, waiting time between compressions 5 s. Lightness of crust (L*crust) was measured by a Colorimeter CR-400 (Konica Minolta, Japan) using the CIELAB colour space.

4.3.7 Statistical Analysis

All measurements were performed in triplicate. Data analysis was carried out using R (version 3.5.1). One-way ANOVA with post-hoc pairwise Tukey test was used to show significant differences ($p < 0.05$). A correlation analysis (CA, significance level $p < 0.05$) determining correlation coefficients (cc) as well as a principal component

analysis (PCA) was conducted using the main technological properties of doughs and breads to evaluate and visualise correlations and identify groups among control and HPI formulations.

4.4 Results

4.4.1 Composition

The protein content determined for wheat flour is 14.09 %DM (Table 4.2). High-protein ingredients show values at least 3.5 times higher. The lowest levels were obtained for carob (55.04 %DM) and faba bean (61.25 %DM). Pea and gluten contain 80.19 %DM and 83.11 %DM protein, respectively, representing the medium-protein-level HPIs. Zein, potato and lupin show protein values over 90 %DM ranging from 91.79 %DM for zein to 94.51 %DM for lupin. In spite of different protein levels, the authors decided for a constant replacement of 15% wheat flour in order to reach a comparable dilution of wheat gluten and starch. The determined fat content of gluten, potato and carob was <1 %DM. Wheat flour (1.97 %DM), zein (2.66 %DM), lupin (2.94 %DM), faba bean (3.81 %DM) and pea (6.45 %DM) are slightly higher in fat. Potato, gluten and zein show low ash contents between 0.05 %DM (potato) and 1.16 %DM (zein), similar to wheat flour with 1.09 %DM. In contrast to this, ash levels of faba bean, lupin, pea and carob range from 5.43 %DM (faba bean) up to 7.04 %DM (carob). Total dietary fibre accounts for 2.9 %DM in zein, 2.3 %DM in wheat flour and less in the other HPIs. Carob however contains 17.7 %DM of fibre. A total starch content of 72.38 %DM was found for wheat flour. HPIs contain less than 0.2 %DM of starch; except gluten and faba bean, which show starch values of 4.95 %DM and 7.77 %DM, respectively. In order to interpret the protein levels of control and HPI formulations, percentages of calories provided by protein (proteinE) were calculated. This was achieved by taking into consideration the compositional data of all ingredients (Table 4.2) as well as the ingredient ratios in the formulations (Table 4.1). Protein accounts only for 14.5% of calories in the control formulation. All HPIs formulations show values higher than 20% ranging from 21.2% (carob) to 26.6% (lupin).

4.4.2 Empirical Dough Analysis

4.4.2.1 Farinograph

In wheat dough production, the amount of water added and the resulting dough consistency are important parameters to allow sufficient distribution and hydration of dough materials and gluten-network development. The obtained FWAs

Table 4.2: Composition of wheat flour and HPIs.

Component	Wheat flour/control	Gluten	Zein	Potato	Carob	Pea	Lupin	Faba bean
Moisture [%]	13.01 ± 0.05^{f}	8.20 ± 0.02^{c}	6.44 ± 0.08^{b}	10.87 ± 0.02^{e}	6.06 ± 0.02^{a}	9.73 ± 0.02^{d}	6.45 ± 0.06^{b}	13.07 ± 0.12^{f}
Protein [%DM]	14.09 ± 0.00^{a}	83.11 ± 0.42^{e}	91.79 ± 0.38^{f}	94.06 ± 2.13^{fg}	55.04 ± 0.28^{b}	80.19 ± 1.43^{d}	94.51 ± 1.50^{g}	61.25 ± 0.66^{c}
Fat [%DM]	1.97 ± 0.10^{bcd}	0.72 ± 0.02^{ab}	2.66 ± 0.12^{cde}	0.12 ± 0.07^{a}	0.20 ± 0.02^{a}	6.45 ± 0.52^{f}	2.94 ± 0.13^{de}	3.81 ± 0.13^{e}
Ash [%DM]	1.09 ± 0.01^{c}	0.87 ± 0.01^{b}	1.16 ± 0.09^{d}	0.05 ± 0.04^{a}	7.04 ± 0.03^{h}	5.90 ± 0.01^{g}	5.62 ± 0.01^{f}	5.43 ± 0.03^{e}
Total dietary fibre (TDF) [%DM]	2.3	< 0.1	0.7	< 0.1	17.7	2.9	< 0.1	0.3
Carbohydrates* [%DM]	80.6	15.3	3.2	5.8	20.0	4.6	–	29.2
Total starch [%DM]	72.38 ± 0.10^{c}	4.95 ± 0.27^{a}	< 0.20	< 0.20	< 0.20	< 0.20	< 0.20	7.77 ± 0.02^{b}
proteinE (formulation)** [%]	14.5	24.8	26.2	26.1	21.2	24.2	26.6	21.2

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

* Calculated by subtraction, required for calculation of proteinE of formulation.

** Percentage of calories provided by protein (proteinE) calculated based on compositional analysis of ingredients.

Table 4.3: Properties of wheat flour and HPI/flour blends and quality characteristics of control and HPI bread formulations.

Variable	Wheat flour/control	Gluten	Zein	Potato	Carob	Pea	Lupin	Faba bean
Farinograph								
Farinograph water absorption (FWA) [%]	63.0 ± 0.5^{b}	70.2 ± 0.2^{d}	60.8 ± 0.2^{a}	65.2 ± 0.3^{c}	69.8 ± 0.6^{d}	71.7 ± 0.6^{e}	66.2 ± 0.3^{c}	62.2 ± 0.3^{b}
GlutoPeak								
Peak maximum time (PMT) [s]	53 ± 2^{e}	n.d.	105 ± 4^{f}	17 ± 3^{a}	41 ± 1^{d}	25 ± 1^{b}	33 ± 1^{c}	34 ± 1^{c}
Torque maximum (TM) [BE]	74 ± 1^{e}	n.d.	48 ± 1^{a}	54 ± 0^{b}	58 ± 1^{c}	72 ± 3^{e}	59 ± 1^{c}	51 ± 2^{ab}
Rapid Visco Analyser								
Peak viscosity (PV) [cP]	2390 ± 13^{e}	1683 ± 12^{c}	1415 ± 39^{a}	2365 ± 44^{e}	1540 ± 14^{b}	1518 ± 12^{b}	1500 ± 15^{b}	1852 ± 28^{d}
Setback [cP]	1282 ± 7^{f}	848 ± 9^{c}	877 ± 30^{c}	1058 ± 10^{d}	791 ± 17^{b}	680 ± 6^{a}	1161 ± 4^{e}	1158 ± 3^{e}
Final viscosity (FV) [cP]	2544 ± 10^{e}	1783 ± 4^{c}	1689 ± 52^{b}	2192 ± 22^{d}	1665 ± 35^{b}	1589 ± 19^{a}	2162 ± 15^{d}	2200 ± 17^{d}
Rheometer								
Damping factor (DampF)	0.401 ± 0.004^{c}	0.385 ± 0.005^{c}	0.387 ± 0.004^{c}	0.437 ± 0.016^{d}	0.327 ± 0.005^{a}	0.325 ± 0.002^{a}	0.349 ± 0.001^{b}	0.329 ± 0.002^{a}
Bread analysis								
Specific volume (SV) [mL/g]	2.55 ± 0.20^{bc}	3.81 ± 0.05^{d}	2.63 ± 0.06^{bc}	2.81 ± 0.23^{c}	2.73 ± 0.34^{bc}	2.00 ± 0.08^{a}	1.98 ± 0.03^{a}	2.26 ± 0.08^{ab}
Number of cells	1671 ± 47^{ab}	1902 ± 57^{b}	1835 ± 73^{ab}	1811 ± 240^{ab}	1709 ± 105^{ab}	1708 ± 92^{ab}	1596 ± 91^{ab}	1543 ± 115^{a}
Cell area [%]	45.5 ± 0.8^{ab}	49.0 ± 0.2^{c}	46.3 ± 0.3^{ab}	46.6 ± 0.2^{b}	46.0 ± 1.5^{ab}	44.5 ± 0.7^{a}	45.0 ± 0.6^{ab}	46.6 ± 0.5^{b}
Hardness [N]	11.81 ± 1.62^{bc}	4.12 ± 0.45^{a}	15.10 ± 1.47^{cd}	19.02 ± 4.80^{d}	7.84 ± 1.08^{ab}	16.68 ± 1.24^{cd}	20.11 ± 1.14^{d}	16.30 ± 3.08^{cd}
Lightness of crust (L*crust)	71.8 ± 3.5^{de}	66.5 ± 0.2^{bc}	72.8 ± 1.3^{e}	70.6 ± 0.8^{cde}	62.3 ± 2.5^{ab}	66.8 ± 0.6^{bcd}	64.2 ± 0.8^{b}	57.1 ± 2.6^{a}

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

n.d., Not detectable (analysis could not be performed due to exceeded maximum torque of the device.

are presented in Table 4.3 and show significant differences and indicate different water absorptions of HPIs and different abilities to compete for water with wheat flour. For wheat flour a FWA of 63.0% was determined. Except for zein (60.8%) and faba bean (62.2%), all HPI/flour blends absorb significantly more water than wheat flour. A moderate increase was observed for potato (65.2%) and lupin (66.2%). Carob, gluten and pea caused the highest FWAs accounting for 69.8%, 70.2% and 71.7%, respectively.

4.4.2.2 GlutoPeak Test

During GlutoPeak measurements, a high speed rotating element subjects the sample slurry to an intense mechanical action. This allows gluten-aggregation and network-formation. The result is an increase in recorded torque, followed by a decline due to a destruction of the network by further mixing. Considering the small sample sizes and short measuring times, the GlutoPeak test is ideal to provide valuable information about rheological properties of HPI/flour blends. Measurements for the gluten formulation could not be performed with the reported method due to exceeded maximum torque of the device. Figure 4.1B schematically shows a typical GlutoPeak curve of wheat flour for comparison and explanation of variables. The torque curve observed for wheat flour (Figure 4.1A) slightly rises and reaches the equilibrium plateau in an initial phase. The following strong increase in torque reaches the TM of 74 BU at a PMT of 53 s. The replacement of 15% of wheat flour by HPIs leads to aggregation profiles entirely different to the typical curve shape. Significantly different PMTs and TMs were obtained. Pea shows an immediate increase in torque within the first few seconds of the test and is lacking the equilibrium plateau. The TM of 83 BU is reached after 16 s in form of a sharp peak. After the maximum, a small decrease of torque was observed. In contrast to this, zein exhibits a curve with a small equilibrium plateau at the start and two broad peaks. A TM of 48 BU was detected after 105 s. The overall curve shape of carob with two broad peaks and a slightly pronounced equilibrium plateau is similar to zein. However, the curve obtained for carob appears much more condensed, TM is higher (58 BU) and PMT lower (41 s). The aggregation profiles of lupin and faba bean both exhibit a slightly pronounced equilibrium plateau and a sharp peak, followed by a slight decrease in torque and a small shoulder. The TM determined for lupin is with 59 BU higher than for faba bean (51 BU). Peak maximum times accounting for 34 s (lupin) and 33 s (faba bean) and are not significantly different from each other. The GlutoPeak curve of potato is lacking the equilibrium plateau. Torque increases immediately and reaches the maximum of 54 BU after 17 s which is pronounced as a steady state rather than a sharp peak. It is followed by a very strong decrease in torque.

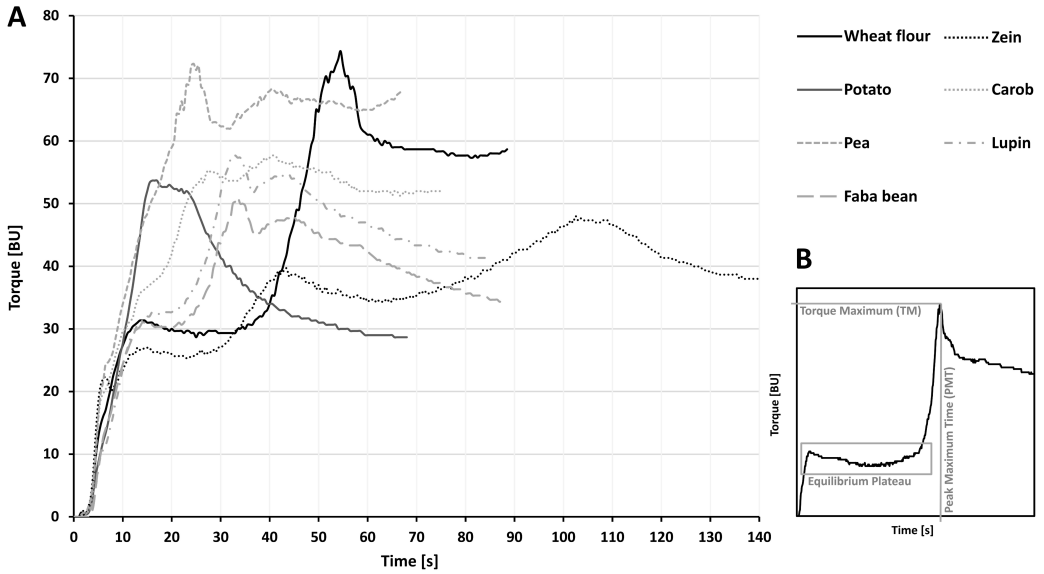


Figure 4.1: (**A**) GlutoPeak curves of wheat flour and HPI/flour blends; (**B**) schematic GlutoPeak curve of wheat flour with explanation of associated variables and terms.

4.4.2.3 Rapid Visco Analyis

Rapid visco analysis of wheat flour and HPI/flour blends was performed to investigate the impact of HPIs on pasting properties and their behaviour during heating. Significant differences were observed amongst the HPI/flour blends and compared to wheat flour (Table 4.3). Except for potato, all HPIs lead to a decrease in PV compared to wheat flour with 2390 cP. The lowest PVs from 1415 cP (zein) to 1540 cP (lupin) were obtained for zein, lupin, pea and carob. Slightly higher PVs (but smaller than wheat flour) were measured for gluten (1683 cP) and faba bean (1852 cP). Only the HPI/flour blend containing potato showed no significant difference in PV compared to wheat flour. Setbacks of all HPI/flour blends are lower than for wheat flour (1282 cP). However, potato (1058 cP), faba bean (1158 cP) and lupin (1161 cP) show setbacks in the same range as wheat flour. The other HPIs exhibit much smaller values between 680 cP (pea) and 877 cP (zein). Determined FVs range from 1589 cP (pea) up to 2544 cP (wheat flour). Final viscosities of all HPI/flour blends were lower than for wheat flour (2544 cP). While potato has a PV similar to wheat flour, it shows a significantly lower FV.

4.4.3 Dough Rheology

Control and HPI doughs (full recipe, yeast omitted) were subjected to oscillatory tests in order to obtain information about their viscoelastic properties. A DampF of 0.401 (Table 4.3) was obtained for the control. All doughs containing legume HPIs have a significantly lower DampF. The decline is more pronounced for pea (0.325),

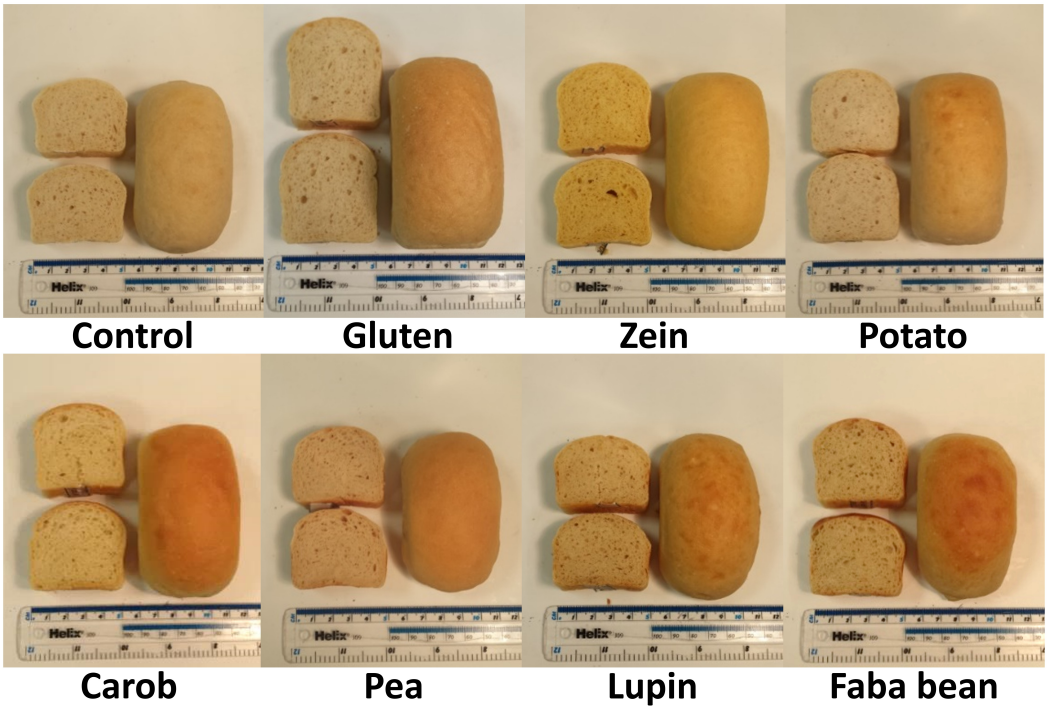


Figure 4.2: Appearance of loaves and slices of control and HPI breads.

carob (0.327) and faba bean (0.329) than for lupin (0.349). The lower DampFs represent a more elastic behaviour of these doughs compared to the control. The DampFs of zein (0.387) and gluten (0.385) are not significantly different from the control. The highest DampF was observed for potato, representing the sample with the most viscous behaviour.

4.4.4 Quality Characteristics of Breads

A visual evaluation of the breads (Figure 4.2) reveals striking differences in colour, size and crumb structure. This observation was confirmed by determination of bread quality characteristics. The SV of the control bread is 2.55 mL/g (Table 4.3). The gluten bread reaches a SV of 3.81 mL/g, which is higher than the SV of all other breads. Breads from lupin (1.98 mL/g) and pea (2.00 mL/g) are smaller than the control; breads containing faba bean (2.26 mL/g), zein (2.63 mL/g), carob (2.73 mL/g) and potato (2.81 mL/g) are not significantly different from the control. However, significant differences were observed within the latter group and between this group and the smaller breads with lupin and pea. The results for crumb structure variables show few significant differences. The number of cells ranges from 1543 (faba bean) to 1902 (gluten). The cell areas lie between 44.5% (pea) and 49.0% (gluten). Potato, faba bean and gluten exhibit larger cells than the pea bread. With a hardness of 4.12 N, the gluten bread is softer than the control (11.81 N). In contrast to this, potato (19.02 N) and lupin (20.11 N) lead to a harder

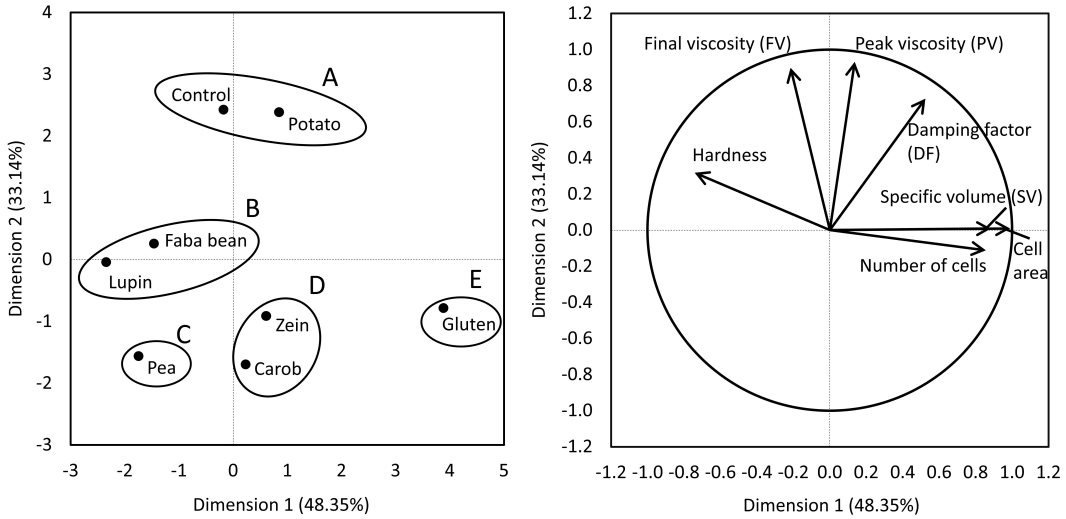


Figure 4.3: Principal component analysis of dough and bread characteristics: (**left**) Analysed observations with hierarchical classification, (**right**) Analysed variables.

crumb. Carob (7.84 N), zein (15.10 N), faba bean (20.11 N) and pea (16.68 N) breads are not significantly different in hardness compared to the control. The darkest crust was observed for faba bean (57.07) and the lightest for zein (72.80). Crusts of the control (71.84) and the potato (70.58) bread exhibit a lightness similar to the zein bread. Carob, lupin, gluten and pea breads represent moderate L*crust values ranging from 62.33 (carob) to 66.83 (pea).

4.4.5 Principal Component Analysis

A PCA was performed based on selected dough and bread characteristics (Figure 4.3). The first Dimension mainly represents hardness, cell area, number of cells and SV, whereas Dimension 2 models a measure of DampF, PV and FV. Hierarchical classification was used to divide the formulations into 5 groups. Cluster A in the upper half of the diagram represents the control and potato formulation and is characterised by high PV and FV. Clusters B and C in the bottom left comprise formulations containing legumes which exhibit low DampFs, SVs and higher hardness. Zein and carob formulations represent cluster D with low PV and FV but medium SV and hardness. The gluten formulation forms cluster E and is clearly separated from the other groups by its very high SV and area of cells as well as low hardness.

4.5 Discussion

The objective of this study is to evaluate and compare the performance of different HPIs in wheat bread formulations and their potential for the production of

high-protein breads. The proteinE was calculated based on compositional results and exceeds 20% for all HPI formulations in this study (Table 4.2). Compositional changes occurring during proofing and baking (i.e., starch degradation, sugar consumption by yeast, denaturation and degradation of proteins [16]) will slightly change the proteinE of breads compared to formulations. Nevertheless, the values suggest a great potential for these formulations to produce high-protein breads in accordance with regulatory requirements in Europe. Next to legal compliance, an understanding of the effects leading to different qualities of high-protein breads is important for further product prototyping. The proteins present in the HPIs are likely to affect the characteristics of the formulations depending on their structural and functional properties and by potential interactions with wheat flour components (protein impact). Additionally, the replacement of 15% of wheat flour leads to a dilution of the technologically most important flour components gluten and starch (dilution effect); and the presence of minor components in the HPIs can also influence dough and baking characteristics.

4.5.1 Water Absorption

One major impact of the HPIs on the formulations is a change in their water absorption to reach a certain consistency. In spite of the dilution effect on gluten and starch, an overall trend towards increased FWAs was observed because HPIs mainly contain proteins, which absorb water themselves. The partial substitution of wheat flour by legume protein isolates or flours, namely carob, pea and lupin, has been reported to increase FWAs [8, 9, 17]. An increase of protein content in wheat flour by 1% is expected to cause an average FWA increase of 1% [18]. This is in accordance with the FWA increase of 7% for the gluten/flour blend, whose protein content is 9.6% higher than for the wheat flour. However, a general correlation between total protein contents of HPI/flour blends and FWAs could not be observed (cc: 0.19). In contrast to the general trend, zein caused a FWA decrease. This can be explained by its high insolubility combined with a very high number of hydrophobic amino acids and overall protein hydrophobicity [11] leading to a low water absorption. No significant change in FWA was observed for faba bean. This difference, especially compared to the other legume HPIs, might be due to its relatively low protein content and its production procedure. Air-classification of legumes, compared to wet protein isolation procedures, usually produces ingredients with higher solubility [13], which could also lead to lower water absorption.

4.5.2 Impact of HPIs on Gluten-Aggregation

While GlutoPeak measurements have been utilised to investigate wheat flour quality and differences in wheat protein properties, they, to the best of our knowledge, have not been used to determine aggregation behaviour of wheat flour blends containing non-wheat HPIs. High-quality wheat flours with higher protein contents (i.e. higher gluten contents) usually exhibit earlier and higher peaks than weak flours in GlutoPeak curves due to a stronger gluten-network [19]. Based on this, measurements with HPI/flour blends are expected to show lower and later peaks compared to the wheat flour (dilution effect). Even though the results in this study do show an overall tendency towards lower TMs, the majority of the peaks exhibit earlier instead of later PMTs (except zein). This suggests that there are other factors affecting the gluten-aggregation in HPI/flour blends. According to Bouachra *et al.* [20], who reported an impact of lactic acid on GlutoPeak curves, the presence of (positive) charges in the sample influences gluten-aggregation. An increased number of charges leads to stronger intramolecular repulsive forces which promotes faster unfolding of gluten molecules and therefore accelerates gluten-aggregation. At the same time, the increased presence of charges in the sample causes a more rapid breakdown of gluten because of higher intermolecular repulsive forces and the hindrance of the formation of new bonds. The applied HPIs differ in their solubility and content of charged amino acids, which are both factors to increase the number of available charges in the sample slurry. Legumes and potato exhibit high contents of charged amino acids; additionally, potato proteins are highly water soluble [2, 3, 12, 21]. Zein on the other hand, contains only few charged amino acids and has a very high overall hydrophobicity [11]. In accordance with the present results, the content of charged amino acids in combination with protein solubility seems to be the major influence of HPIs on PMTs. A flour with a fast build-up of gluten-network, sharp gluten-peak and rapid breakdown is considered weak, whereas a longer PMT seems to allow for a fully developed and more stable gluten-network [22]. This implies a relatively weak gluten-network with pea and potato; medium strength with faba bean, lupin and carob; and high strength with zein. The TM appears to be mainly related to the water absorption of HPIs. A good correlation between FWA and TM was found (cc: 0.86) when comparing HPI/flour blends (wheat flour exempt because of different gluten content). Furthermore, interactions between non-wheat proteins and gluten can occur and affect gluten-aggregation. Curves of all HPI/flour blends (except potato) exhibit a more or less pronounced second peak. This could be caused by interactions between gluten and HPI proteins subsequent to gluten-aggregation and during breakdown, delaying a rapid torque decrease. In general, synergistic interactions

between different food proteins forming a stronger coaggregated network have been previously reported [23]. Bugusu *et al.* [5] showed interactions between zein proteins and gluten in wheat dough systems using confocal microscopy after fluorescence labelling of the proteins. Feillet and Roulland [15] described caroubin, the main protein fraction in carob, as a gluten-like protein with similar rheological properties and similar protein interactions. Since zein and carob are the HPIs which lead to the most pronounced second peak in the GlutoPeak curve, a synergistic network-formation of these proteins with gluten can be hypothesised.

4.5.3 Impact of Heat Treatment on HPI/Flour Blends

The behaviour of a dough and its constituents during and after heat treatment is of major importance with regard to the baking process. Rapid visco analysis provides information on heat induced changes in viscosity of flours suspended in excess water. In wheat flour samples, it is mainly a tool to investigate starch gelatinisation properties. In HPI/flour blends, the starch content is substantially reduced (dilution effect). Less available starch in the samples leads to lower viscosities. This is in accordance with the generally lower PVs observed for HPI/flour blends compared to wheat flour. Only for gluten and faba bean, which contain considerable amounts of starch (4.95% and 7.77%, respectively), the decrease in PV is less pronounced. A positive correlation between the calculated sample starch content and PV was detected (cc: 0.950; potato exempt). Also López [17] reported a decreased PV in RVA when partially replacing wheat flour by plant protein isolates. Marco and Rosell [24] observed the same trend in rice-flour systems. The results of the present study suggest a unique pasting behaviour of potato protein in mixture with wheat flour. In spite of a much lower starch content, a PV as high as for wheat flour and a relatively low Setback and FV were measured. Marco and Rosell [24] proposed an impact of heat induced gelation of non-wheat proteins on PV and setback of rice flour/plant protein isolate mixtures. Although literature on gelling behaviour of potato proteins is scarce, some of the protein fractions (e.g., patatin) have been reported to form strong gels with properties similar to ovalbumin and beta-lactoglobulin [12].

4.5.4 Viscoelastic Dough Properties as Affected by HPIs

The DampFs reveal a clear grouping of the samples by botanical source of the proteins. All legume proteins lead to lower DampF, doughs with cereal HPIs exhibit DampFs similar to the control, and the potato protein increases dough DampF representing a larger viscous proportion than the control. However, small-deformation properties, as measured by oscillatory tests, do not necessarily rep-

resent large-deformation properties and performance in baking [25]. The fact that the potato dough shows the highest DampF can be related to its rapid gluten-breakdown observed in GlutoPeak measurements. The constant mixing time applied for all recipes might have exceeded the mixing tolerance of gluten in the potato formulation causing a lack of elasticity and a higher viscous proportion.

4.5.5 Quality Characteristics of High-Protein Breads

Gluten is known to improve SV and used as an additional ingredient in wheat bread recipes [26]. The replacement of 15% of wheat flour by gluten leads to breads expectedly high in quality, characterised by the highest SV and the lowest hardness in comparison to all other formulations. The legume breads from pea, lupin and faba bean show tendencies towards lower SV and higher hardness compared to the control. The partial replacement of wheat flour by pea protein isolate [9] and lupin flour [7] has been previously reported to decrease SVs and increase crumb hardness. Specific volume and crumb hardness of faba bean were not significantly different from the control, which suggests an adequate quality. Carob and zein breads show better overall quality characteristics than the control. This is consistent with the findings from Turfani *et al.* [8] who reported higher bread volumes with composite wheat/carob flour; Bugusu *et al.* [5] observed the same trend for breads from composite wheat/zein flours. Interactions and potentially synergistic network-formation have been suggested by GlutoPeak results for carob and zein. Due to the rapid gluten-breakdown observed in the GlutoPeak test and the high viscous proportion of the dough (high DampF), potato was expected to show poor bread quality. However, a high SV but also a high hardness were measured. As suggested by RVA, the potato protein is substantially affected by heat treatment. This seems to have a positive impact on expansion properties of the dough during baking. The high hardness might be caused by a competition of gelling protein and gelatinising starch for water during heating. Thus, less starch would be gelatinised and a higher initial hardness of the product would be observed. A hydration depletion of starch has been previously reported to increase crumb hardness by Martínez *et al.* [27]. Crust browning in breads is the result of caramelisation of sugars and the Maillard reaction. Components involved in those reactions are carbohydrates, proteins and water [28]. No correlation between L*crust and protein content of the formulations was detected (cc: 0.04). This might be related to the fact that the HPIs contain varying amounts of carbohydrates and lysine, which is an important source of primary amines for Maillard reaction in proteins [28] and therefore substantially supports browning.

4.5.6 Effects of Minor Components

The values for carbohydrate contents of HPIs in this study were obtained by calculation. Considering the determined contents of total starch and preliminary results for Fructose, Glucose, Maltose and Glucose (data not shown), the values suggest considerable amounts of non-starch carbohydrates and fermentable sugars present in some of the HPIs; faba bean, carob, gluten and pea in particular. Additional to its effect on crust browning, this can have an impact on yeast activity and SV of the breads. Carob leads to breads with high quality characterised by a high SV and low crumb hardness. Next to its protein properties, also its high fibre content can be partly responsible for this result. Carob seeds are the raw material for the production of locust bean gum. This hydrocolloid has been reported to improve baking properties and loaf volume [29]. Even though germ and endosperm, which is the source of locust bean gum, are separated during the production procedure, small amounts of locust bean gum hydrocolloids might be present in the fibre fraction of the investigated carob HPI contributing to its high bread quality. Also lipids are known to play an important role in wheat flour doughs. Depending on their nature, they are believed to have a positive effect on crumb softness and to support a finer crumb structure [30]. The fine crumb structure (characterised by a low cell area) observed for the pea bread could therefore be related to the high fat content of this HPI.

4.6 Conclusion

This study provides a comparative evaluation of HPIs from different plant sources applied in wheat bread formulations. The baking performance of a formulation mainly depends on the rheological properties of the dough and its behaviour during heat treatment and subsequent cooling. In plant-based HPI formulations, the proteins were found to affect both. An accelerated and weakened gluten-aggregation was observed for pea and potato. GlutoPeak results further suggest secondary network-formation between non-wheat proteins, especially carob and zein, and gluten. Due to heat induced gelation of non-wheat proteins, dough characteristics can undergo major changes during the baking process and potentially improve baking performance as observed for the potato formulation. The overall quality of the potato formulation is very similar to the control as visualised by PCA. Zein and carob, with dough characteristics very different to the control, lead to improved bread quality. Amongst the remaining legume formulations (pea, lupin, faba bean), especially the final quality of the faba bean bread is remarkable, since this HPI was produced without a wet protein isolation step and therefore

promises better sustainability. The establishment of a generic rule as to how HPIs influence final bread quality is difficult. Many factors play an important role and might even oppose each other's impact on the bread formulation. Key properties of HPIs were found to be gelling behaviour upon heat treatment, the ability of co-networking with gluten, the degree of gluten-network impairment and the extent of the presence of other, potentially techno-functional, minor components. Particularly the legume formulations, which promise balanced amino acid profiles for human nutrition, should be in the focus of future studies.

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CHAPTER 5

Enhancing the Nutritional Profile of Regular Wheat Bread While Maintaining Technological Quality and Adequate Sensory Attributes

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5.1 Abstract

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.

5.2 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 [1]. 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 [2, 3]. According to many recent reports, this requires a shift to a predominantly plant-based human diet [2, 4]. Since we are also facing a growing world population, with a prospect of about 10 billion by 2050 [2], 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 [2, 4]. In many cases, the overconsumption of protein is associated with a general overconsumption of food and energy intakes exceeding recommended levels [4] 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 [5]. Apart from sustainability considerations, dietary recommendations advise a reduction of animal protein intake in favour of increased plant protein consumption for a healthy diet. Many reported adverse effects of high protein intake are largely related to proteins from animal sources and the co-intake of sodium, nitrate, nitrite and saturated fatty acids when red meat or dairy products are consumed [2, 4, 6]. Also an overconsumption of food carbohydrates, especially refined carbohydrates, has been reported for a large number of countries and associated with increased health risks [7]. Research concerning new alternative plant protein sources is mostly focused on legumes. Due to their ability to grow in a variety of different climates and to fix nitrogen in the soil, they are particularly promising for a local crop cultivation, a considerably reduced use of fertilisers and a food production with a lower carbon and water footprint [3, 8, 9]. Legumes are naturally rich in protein, which contains high amounts of the essential amino acid (AA) lysine but lacks sulphur-containing amino acids (SAAs) [8, 10, 11]. This makes legumes particularly interesting for the complementation of cereal-based diets, since cereals have little lysine and higher amounts of SAAs [12, 13]. Efforts have been made to combine both protein sources in 'hybrid products' containing cereals and legumes and especially wheat bread has proven a suitable cereal matrix for the incorporation of legume protein

ingredients [14]. Ideal bread should have a lower glycaemic index than regular white bread, be an important source of proteins, and contain tolerated dietary fibre, vitamins, magnesium, trace elements and antioxidants [15, 16]. Jenkins *et al.* [7] state that, in the context of decreased physical activity in our population, foods should possess nutritional density rather than nutrient density. This means that the intake of essential nutrients (macro and micro) per calorie will need to increase in order to meet requirements at lower caloric intake levels. Legumes are rich in micronutrients and compounds with antioxidant activity, which could help to enhance the nutritional value of wheat bread [14, 17, 18]. Also a lowered glycaemic load, increased protein content and improved protein quality could be achieved by the fortification of wheat bread with legume proteins. Numerous research articles have investigated the effects of legume ingredients, from faba bean (*Vicia faba*) and carob (*Ceratonia siliqua*) seeds in particular, on both the technological as well as nutritional quality of breads [14, 19–23]. However, many of these publications report inferior technological and sensory characteristics in favour of increased nutritional quality. Additionally, there are concerns regarding antinutritional compounds (ANCs) originating from legumes such as trypsin inhibitors, tannins, lectins and the pyrimidine glycosides vicine and convicine. Trypsin inhibitors, which can negatively impact protein digestibility, are present in many plants but are particularly important in legumes [24, 25]. Vicine and convicine are mainly found in faba beans and can trigger adverse physical conditions like favism [26–28]. This leads to a low popularity of legume ingredients and cereal/legume hybrid products [29]. Next to an enhanced nutritional value, adequate technological quality and sensory properties are essential for a high consumer acceptance of such products and for an acceleration of the protein transition in our diet. This is why this study proposes and fully characterises a new bread formulation, which was designed to match the technological quality of regular wheat bread, but promises an improved nutritional profile with a higher protein content and higher protein quality in particular. Therefore, plant-based high-protein ingredients (HPIs) from faba beans, carob and wheat, selected based on findings by Hoehnel *et al.* [30], were incorporated in a regular wheat bread. This high-protein hybrid bread (HPHB) formulation was evaluated regarding technological, nutritional and sensory characteristics using regular wheat bread as a reference (RWB). The HPHB formulation, containing a dry-processed faba bean HPI as its main source of non-wheat protein, also promises improved sustainability [31]; especially when compared to other high-protein bread formulations that are commercially available. These often contain dairy ingredients as non-wheat protein source. Vogelsang-O'Dwyer *et al.* [32] reported a life cycle assessment (LCA) of the dry-processed faba bean HPI used in this study, which confirmed reduced use of land and water resources as well

as lower impact on climate change (carbon footprint) and aquatic eutrophication in comparison to cow's milk powder. This makes HPHB and formulations of its kind even more promising to partially cover protein needs of future predominantly plant-based diets.

5.3 Materials and Methods

5.3.1 Materials

Three high-protein ingredients (HPIs) were applied in the high-protein hybrid bread (HPHB) formulation. Faba bean flour (protein content 61.25 %DM, fat 3.81 %DM, ash 5.43 %DM, fibre 0.3 %DM, carbohydrates by difference 29.17 %DM, total starch 7.77 %DM [30]; obtained by dry fractionation), which was experimentally produced and provided by Fraunhofer Institute IVV, Freising, Germany; carob germ flour (protein content 55.04 %DM, fat 0.20 %DM, ash 7.04 %DM, fibre 17.7 %DM, carbohydrates by difference 20.05 %DM, total starch < 0.20 %DM [30]; GRINDSTED VEG PRO S1) from Danisco, UK and vital gluten (protein content 72.38 %DM, fat 0.72 %DM, ash 0.87 %DM, fibre < 0.1 %DM, carbohydrates by difference 15.31 %DM, total starch 4.95 %DM [30]; NUTRALYS W) from Roquette, France. Wheat flour was supplied by Whitworth Bros Ltd, UK; dry yeast by Puratos, Belgium; salt by Glacia British Salt Ltd, UK; sugar (granulated Irish sugar) by Nordzucker (Ireland) Ltd, Ireland; psyllium (VITACEL P95) by J. Rettenmaier & Söhne, Germany; vegetable oil by Musgrave, Ireland; and xylanase (Biobake 715) by Kerry Group, Ireland. For *in vivo* digestibility trials, the following ingredients were used for the preparation of diets: casein (C) from Lacpol Co., Poland; soy protein isolate (SPI) ISOPRO 900 HI characterised as non-GMO protein isolate from EDMIR-POL Co., Poland; soy flour (SF) SOPRO TB 200 from EDMIR-POL Co., Poland; α-cellulose (C8002) from Sigma-Aldrich, Missouri, USA; soy oil from ZPT Co., Poland; choline chloride from SIGMA, Poland; cholesterol from PPH Standard Co., Poland; sucrose from POCH SA Co., Poland; and corn starch from Avebe, The Netherlands. Enzymes for *in vitro* digestion trials were purchased from Sigma-Aldrich, Missouri, USA: Pepsin from porcine gastric mucosa; EC 3.4.23.1; P7000; 727 U/mg and pancreatin from porcine pancreas; 4 x USP; P1750. All other chemicals were also purchased from Sigma-Aldrich, Missouri, USA unless stated otherwise.

5.3.2 Technological Analysis

5.3.2.1 Flour Analysis

The properties of wheat flour (used for reference wheat bread) and the high-protein (HP) flour mix (used for HPHB) were analysed. The HP flour mix contained wheat flour, the three HPIs (faba bean flour, carob germ flour, gluten) and psyllium in ratios according to HPHB formulation (Table 5.1). The moisture content of the HP flour mix was calculated considering the moisture determined for each single ingredient. *GlutoPeak test* – Gluten-aggregation properties of wheat flour and the HP flour mix were investigated following the method previously described by Hoehnel *et al.* [30] using the GlutoPeak device (Brabender GmbH and Co KG, Duisburg, Germany). In brief, high shear was applied to a flour/water slurry (50:50 ratio, adjusted when moisture of flour differed from 14%). The device was operated at a paddle speed of 2750 rpm and temperature of 36 °C; torque was recorded over time. Variables Torque maximum (TM, expressed in Brabender units BU) and Peak Maximum Time (PMT, expressed in s) were obtained from the curves. *Rapid visco analysis* – Examination of pasting behaviour using a Rapid Visco Analyser (RVA Super 3, Newport Scientific, Warriewood, Australia) was performed according to AACC 76-21.02. The following heating profile was applied: equilibration at 50 °C for 1 min, heating to 95 °C at 0.2 °C/s, holding at 95 °C for 162 s, cooling to 50 °C at 0.2 °C/s, maintaining at 50 °C for 120 s. The variables peak viscosity (PV), setback and final viscosity (FV) were obtained from the viscograms.

5.3.2.2 Recipe Adaptation and Bread Production

Bread samples were prepared according to the formulations in Table 5.1. The HPHB formulation contains HPIs (faba bean flour, carob germ flour, gluten) and was designed to match the technological quality of the reference wheat bread (RWB).

Table 5.1: Recipes for reference wheat bread (RWB) and high-protein hybrid bread (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×	47.22×
Faba bean flour	–	–	10.0×	5.72×
Carob germ flour	–	–	5.0×	2.86×
Gluten	–	–	2.5×	1.43×
Psyllium	–	–	2.0×	1.14×
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
Total	167.5	100.00	174.7	100.00

× Ingredients are included in HP flour mix.

A series of preliminary trials (data not shown) based on the results presented by Hoehnel *et al.* [30] led to the establishment of the HPHB formulation. A total of 28 different recipes were screened to select a combination of HPIs and to optimise their relative ratios for favourable technological characteristics. Furthermore, the introduction and optimal addition levels of the functional ingredients psyllium, sugar and xylanase were investigated as part of the screening to achieve adequate dough handling characteristics and quality of the end product. For both formulations, the straight dough method was applied. Yeast was activated by dissolving in 30 °C tap water for 10 min. The obtained yeast suspension was added to the remaining, previously weighed ingredients. A total amount of 3600 g dough was prepared. Mixing conditions were the following: RWB – MACPAN MX 10 spiral mixer (MACPAN SNC, Italy) at speed 1 for 6.5 min and speed 2 for 5 min; HPHB – Hobart A200N mixer (Hobart Manufacturing, UK), equipped with hook attachment, at speed 1 for 2 min and speed 2 for 7.5 min. After covering the dough and leaving it to rest for 5 min, it was divided into 7 pieces of 450 g $\pm$ 1 g. The pieces where moulded, put into baking tins and proofed for 90 min at 75% humidity and 35 °C (KOMA BV Sunriser, Reormond, the Netherlands). Baking was performed in deck ovens (MIWE Condo, Arnstein, Germany) at 220/230 °C top/bottom temperature for 35 min with open draft throughout the whole baking process. The baking chamber was steamed with 400 mL prior to loading. After baking, breads were removed from tins and left to cool down for 2 h at ambient temperature. The results were obtained from three independently performed baking trials.

5.3.2.3 Dough Analysis

Doughs for determination of dough properties were prepared as described in section 5.3.2.2. *Rheofermentometer* – Formation and retention of gas in the fermenting doughs was analysed using a Rheofermentometer F3 (Chopin, France). A dough piece (300 g) was placed into the sample container and a weight constraint of 1.5 kg was applied. The dough fermentation was monitored for 3 h at a temperature of 35 °C (matching the proofing temperature used during bread production). The fermentation performance of the doughs was evaluated by the following variables obtained from the generated curves: Total gas volume produced (V_{total}), volume of CO_2 lost (V_{lost}) and volume of gas retained (V_{ret}) from gaseous release curves; and maximum height of dough development (H_M) from dough development curves. *Large deformation properties* – Extensibility (expressed in mm) and resistance to extension (expressed in g) of the doughs were measured by a texture analyser (TA-XT plus, Stable Micro Systems, Surrey, UK) equipped with a 5 kg load cell and a Kieffer dough and gluten extensibility rig (test settings: pre-test speed 2 mm/s, test speed 3.3 mm/s, post-test speed 10.0 mm/s, trigger force 5

g). The uniaxial extension test was performed after a dough resting time of 20 min (room temperature) inside the dough strip mould. Ten intact strips of dough were measured from each of three batches per formulation.

5.3.2.4 Bread Quality Analysis

Specific volume (SV) was measured with a Volscan Profiler (Stable Micro Systems, Surrey, UK) of 6 loaves per batch. For analysis of crumb structure and hardness, three slices (20 mm) were cut out of the middle of each of 2 bread loaves. A C-Cell Imaging System (Calibre Control International Ltd, UK) was used to capture images of slices and to determine the variables: number of cells, area of cells and slice brightness. Crumb hardness was analysed with a texture analyser (TA-XT2i, Stable Micro Systems, Surrey, UK) equipped with a 25 kg load cell. A 35 mm cylindrical probe was used to compress the centre of the slice to 40% of its height as part of a texture profile analysis (TPA): test speed 5 mm/s, post-test speed 10 mm/s, trigger force 0.05 N, waiting time between compressions 5 s. TPA of bread slices was repeated on day 2 and day 5 after baking to monitor bread staling (whole loaves were stored in plastic bags at ambient temperature in the bakery and sliced immediately before the measurement). Lightness of crust (L*crust) and crumb (L*crumb) was measured by a Colorimeter CR-400 (Konica Minolta, Japan) using the CIELAB colour space.

5.3.2.5 Scanning Electron Microscopy

Bread crumb was separated from crust, cut into small cubes, frozen at -80 °C and freeze-dried. The dry crumb was further crushed into small fragments which were mounted onto plain aluminium stubs with double-sided carbon adhesive tape. After coating with a 5 nm gold-palladium (80:20) layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEMcoating system, England), they were examined under high vacuum with a JOEL scanning electron microscope (SEM) type 5510 (JOEL Technics Ltd., Tokyo, Japan). Images were acquired at a constant accelerating voltage of 5 kV.

5.3.2.6 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.* [33] 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).

5.3.3 Nutritional Analysis

Analysis of nutritional characteristics of the bread formulations was performed on freeze-dried (according to the procedure described in Section 5.3.2.5) and subsequently milled (laboratory disc mill; Bühler, Brauchschweig, Germany) samples of bread crumb. Results are expressed as contents in fresh bread considering the moisture of freeze-dried and fresh bread crumb unless stated otherwise.

5.3.3.1 Compositional Analysis

The analysis of the following compositional data was performed by Concept Life Science Ltd., UK based on the indicated validated methods: energy (calculated considering protein, fat, available carbohydrates and fibre), protein (Dumas method, modified after AOAC 1977.992.15; nitrogen-to-protein conversion factor 6.25), ash (removal of organic matter by oxidation at 550 °C, based on ISO 936:1998), fat (low resolution proton nuclear magnetic resonance (NMR), based on MQC-23-35 Oxford Instruments application note), fatty acid profile (GC-FID of fatty acid methyl esters; triglyceride conversion factor 0.956), total dietary fibre (TDF) (gravimetric method, based on AOAC 991.43), sodium (flame photometry after removal of organic matter). Moisture was determined by air-oven method at 130 °C until constant mass was reached. Total starch content was analysed using the enzyme kit K-TSTA supplied by Megazyme, Ireland. Mono-, di- and oligosaccharides were extracted from the freeze-dried product powders as follows: 15 mL of 80/20 (v/v) ethanol/ultrapure water (80% EtOH), which was heated to 55 ± 5 °C, was added to 2 g of sample. The mixture was vortexed until the powder was suspended and then subjected to sonication (extraction step 1) utilising a BANDELIN Sonoplus HD 3100 homogenizer (Berlin, Germany) equipped with an MS73 microtip, operated twice for 15 s at 75 °amplitude. Hereupon, the sample was centrifuged at 1800 g for 10 min and the supernatant transferred to another test tube for further processing. Sonication and centrifugation were repeated (extraction step 2) after adding another 15 mL 80% EtOH (at 55 ± 5 °C) to the pellet. The supernatants of

both extraction steps were pooled and concentrated using a vacuum centrifuge system (Scanvac Scan Speed 32 with Scanvac VacSafe 15, Labogene ApS, Lynge, Denmark) with the following settings: 2 h at 1500 rpm and 45 °C, followed by 1 h at 2000 rpm and 50 °C; average pressure 15 mbar. The concentrated extract was transferred into a 10 mL volumetric flask, which was filled up with ultrapure water containing 50 mg/L NaN_3, and filtered through syringe-driven polyamide filters (Chro-mafil AO-20/25, pore size: 0.20 μm, Machery-Nagel, Düren, Germany). Samples were extracted in duplicates and quantification of the sugars was performed according to the method described by Ispiryan *et al.* [34] using a Dionex ICS-5000$^+$ system (Sunnyvale, CA) equipped with an electrochemical detector.

5.3.3.2 Amino Acid Analysis

Determination of protein amino acid composition was carried out by Mérieux NutriSciences CHELAB S.r.l., Italy based on ionic chromatography with postcolumn ninhydrin derivatisation (fluorescence detection; UV detection for tryptophan) after adequate extraction and protein hydrolysis (separate hydrolysis procedures for the determination of tryptophan, sulphur-containing AA and remaining AA).

5.3.3.3 *In Vitro* Protein Digestion

A previously described static multi-step method for *in vitro* protein digestibility (IVPD) [35, 36] was used to simulate gastro-pancreatic protein digestion. In short, sample amounts containing 50 $\pm$ 1 mg protein were weighed in and enzymatic hydrolysis was started: pepsin digestion at 37 °C and pH 1-2 (1 h) followed by sequential pancreatin digestion at 37 °C and pH 7-8 (short-term: +1 h; medium-term: +3 h; long-term: +24 h). Ratios between enzyme and substrate (w/w) were kept constant at 1:50 (pepsin stage) and 1:10 (pancreatin stages). IVPD in % was determined using a trinitrobenzenesulfonic acid (TNBS) assay. Results are expressed as the concentration of free α-amino groups in samples in relation to an alanine standard solution representing 100% protein digestibility.

5.3.3.4 *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 5.2). The selection of the animals and their maintenance

Table 5.2: Composition of diets for *in vivo* nitrogen balance trials containing either casein (C), soy protein isolate (SPI), soy flour (SF), reference wheat bread (RWB) or high-protein hybrid bread (HPHB) as protein source, values given in % of diet.

Component of diet	C	SPI	SF	RWB	HPHB
Casein	**11.15**				
DL-Methionine	**0.20**				
Soy protein isolate		**10.80**			
Soy 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
Soy oil	8.00	8.00	8.00	8.00	8.00
Mineral mix[1]	3.50	3.50	3.50	3.50	3.50
Vitamin mix[2]	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20	0.20	0.20
Cholesterol	0.30	0.30	0.30	0.30	0.30
Sucrose	5.00	5.00	5.00	5.00	5.00
Corn starch	62.65	62.29	54.31	6.21	30.15

[1] AIN-93G-MX: Mineral mixture as specified by Reeves [37]
[2] AIN-93G-VX: Vitamin mixture as specified by Reeves [37]

over the 14-day experiment followed common regulations. The environment was controlled with a 12 h light-dark cycle, a temperature of 22 ± 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 soy protein isolate (SPI, without any supplementation), a third control diet based on soy 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 [37]; 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).

5.3.3.5 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.* [35] with some modifications. In brief, TIA levels were measured against a trypsin solution (stock concentration 0.1 mg/mL). A solution of N-α-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 $M^{-1}cm^{-1}$. 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) is defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol *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.* [38]. Quantification was achieved using micellar electrokinetic capillary chromatography as reported by Bjergegaard *et al.* [39] and with vicine as external standard.

5.3.3.6 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.* [40]. 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 [41]. 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.* [42]. In brief, a $ABTS^{\bullet+}$ (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 $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ solution were mixed with 20 μl 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 μmol Trolox equivalent. *Ferric-reducing antioxidant power (FRAP)* – FRAP assay was performed as described by Benzie and Strain [43]. The FRAP value was calculated and expressed as μmol Fe^{2+} using a 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.* [44]. A 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 $DPPH^{\bullet}$ (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_{50}) of the phenolic extract that scavenged 50% of DPPH radicals.

5.3.4 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. Panelists with appropriate methodological preparation and experience in sensory profiling were selected, trained and monitored following ISO 13299:2016. Before the sensory analysis, the panelists' performance was evaluated using three parameters – repeatability, discrimination ability and homogeneity by applying analysis of variance. Additionally, 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 prior to the sensory analysis. A list of sensory attributes was created. Initially, panelists chose characteristics describing the samples individually, followed by a joint agreement on distinguishing attributes and their descriptions (see Table 5.3). A continuous scale (10 cm long) with the extremes specified in Table 5.3 was used. Sensory evaluation was carried out in three independent sessions.

5.3.5 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 ± standard deviation or uncertainty (amino acid profile).

Table 5.3: Sensory attributes and extremes of intensity scales used for QDA of breads.

Attribute	Definition	Extremes
Odour		
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
Appearance		
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
Texture (manual)		
Elasticity	The extent to which bread crumb returns to its original shape when stretched	Low - high
Texture (oral)		
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
Taste		
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
Overall quality	Conclusive evaluation of all attributes and their harmonic balance	Bad - very good

5.4 Results and Discussion

5.4.1 Technological Characteristics

5.4.1.1 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 5.1). 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 5.4. 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 [45–47]. The gluten content in HP flour mix (calculated

Table 5.4: 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
GlutoPeak		
Peak maximum time (PMT) [s]	60 ± 4^a	46 ± 2^b
Torque maximum (TM) [BU]	68 ± 1^a	64 ± 1^b
Rapid Visco Analyser		
Peak viscosity (PV) [cP]	2261 ± 9^a	1989 ± 17^b
Setback [cP]	1350 ± 19^b	1587 ± 59^a
Final viscosity (FV) [cP]	2607 ± 10^b	2756 ± 84^a

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

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.* [30] 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 curves) is required (see Figure 5.1). 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.* [48], 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

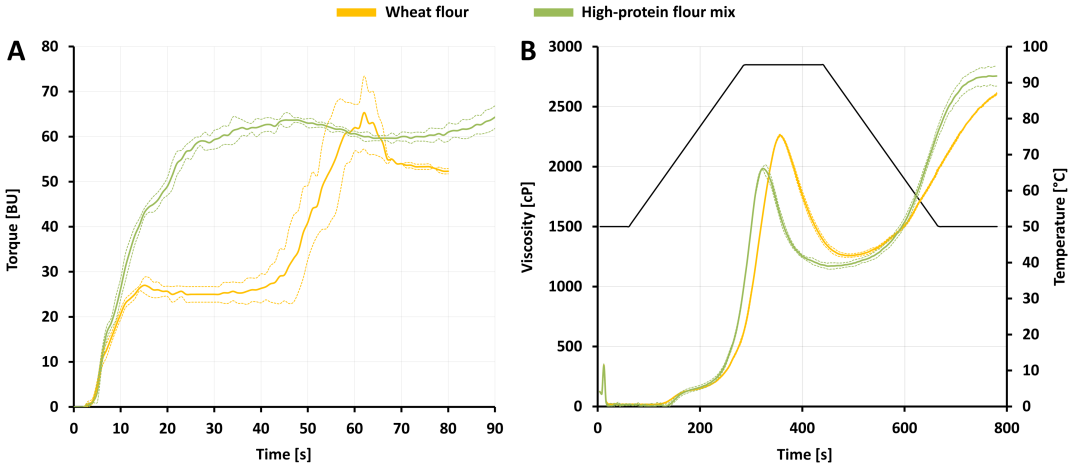


Figure 5.1: Flour properties of wheat flour and HP flour mix: (**A**) Gluten-aggregation profiles obtained by GlutoPeak test; (**B**) Viscograms obtained from rapid visco analysis describing pasting behaviour of RWB and HPHB with black line representing the applied temperature profile. Dashed curves represent standard deviation.

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.* [30]. The lack of equilibrium plateau and rapid torque increase at the start can be explained by the high water absorption of psyllium and gluten [49, 50] resulting in a higher initial viscosity of the sample slurry. Table 5.4 shows variables describing the pasting behaviour of the flours. The corresponding viscograms are displayed in Figure 5.1. 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 [51] as well as systems based on rice flour [52]. 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) [53, 54]. 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.* [30] 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 5.5) 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 [55], reduced resistance to extension as well as area under the curve are indicative of weaker doughs. However, also

Table 5.5: Dough properties of reference wheat bread (RWB) formulation and high-protein hybrid bread (HPHB) formulation.

Variable	RWB	HPHB
Kieffer rig extensibility		
Resistance to extension [N]	0.647 ± 0.059^a	0.475 ± 0.045^b
Extensibility [mm]	16.76 ± 1.25^a	13.04 ± 1.44^b
Rheofermentometer		
Dough development (H_M) [mm]	67.3 ± 5.0^a	61.6 ± 1.0^a
Total gas volume (V_{total}) [mL]	1983 ± 171^b	2450 ± 102^a
Volume of CO_2 lost (V_{lost}) [mL]	58 ± 31^a	33 ± 19^a
Volume of gas retained (V_{ret}) [mL]	1924 ± 192^b	2416 ± 103^a

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

the shape of the curve (see Figure 5.2B), and the ratio of resistance to extension and extensibility (R/E) in particular, seems important and provides information on the doughs' viscoelastic balance [55]. 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 5.5. 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 5.2A. 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

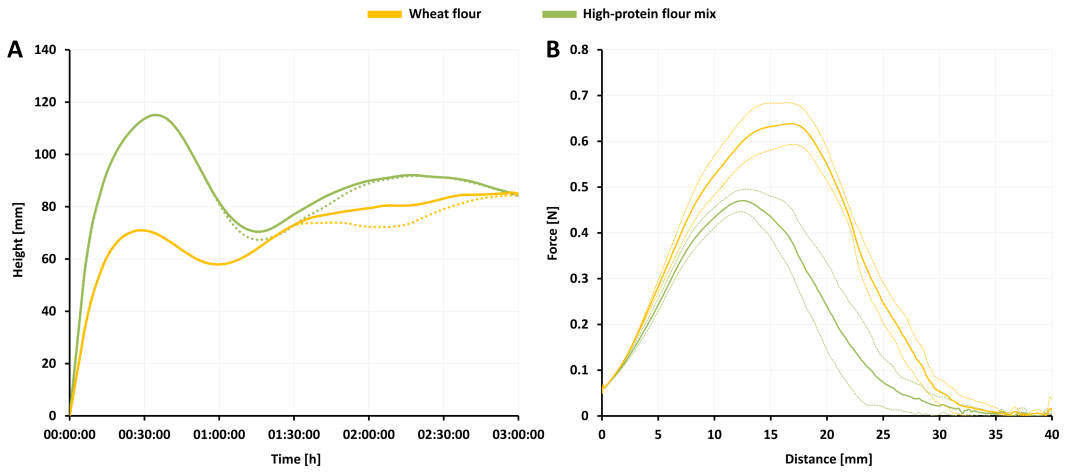


Figure 5.2: Dough properties of RWB and HPHB: (**A**) Gas release curves obtained by Rheofermentometer measurements (dotted line represents gas retained in the dough); (**B**) Extensibility plots obtained Kieffer rig microextension tests (dashed curves represent standard deviation).

[56]. They explained a positive effect of water-extractable arabinoxylans (AX) on gas retention of doughs related to a strengthening of liquid films surrounding CO_2 bubbles, thereby limiting gas diffusion. The psyllium in HPHB contains a considerable amount of AX, of which a small percentage is water-extractable [57, 58]. Additionally, xylanase, which degrades water-unextractable AX (in HPHB from wheat flour [59] and psyllium [58]), increases the amount of water-extractable or solubilised AX present in the dough and their effect on gas retention properties [56]. 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 [50, 60]. Wang *et al.* [61] 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.

5.4.1.2 Bread Quality Characteristics

The breads produced from both formulations examined in this study are presented in Figure 5.3. A visual evaluation reveals little differences in loaf size and crumb

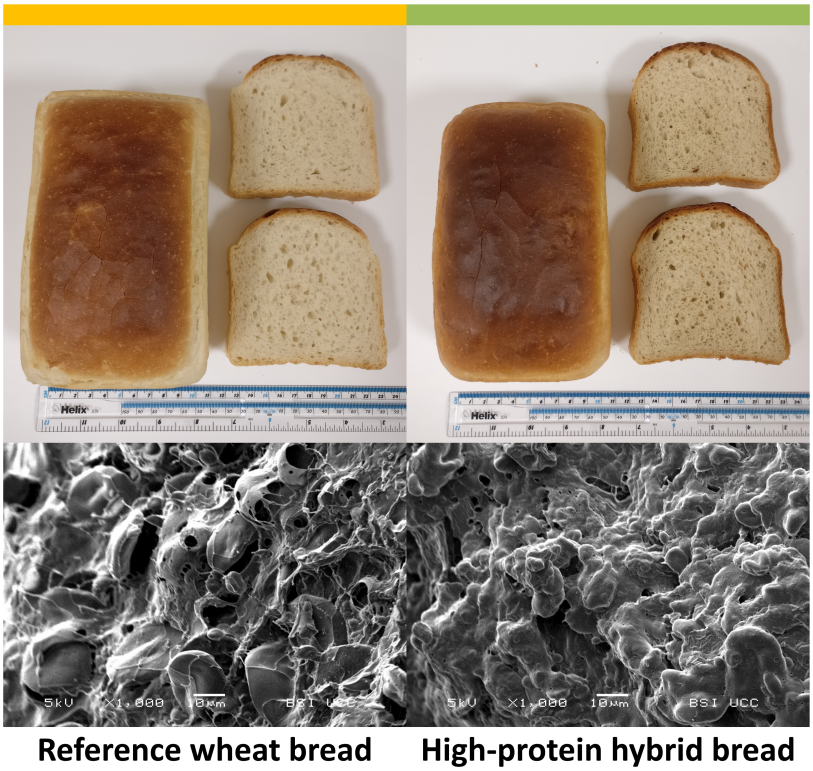


Figure 5.3: Photographs and micrographs (obtained by SEM) of RWB and HPHB.

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 5.6. 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 [22, 30, 62, 63]. 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. Recrystallysing starch is considered to be the main factor for staling of bread crumb [64, 65]. Therefore, the decreased crumb staling in HPHB could be related to its lower starch content and, supposedly, a lower amount of gelatinised starch which can recrystallise. Also AX and xylanases have been reported to decrease staling of wheat-based bread formulations [66, 67]. The effect has been attributed to a competition for water and therefore a reduced swelling and gelatinisation of starch [66, 67]. Maeda and Morita [68] observed reduced staling up to 3 days of storage caused by both water-extractable and water-unextractable AX. While their study was focused on wheat AX, Czuchajowska *et al.* [69] also reported a reduced crumb hardness after 72 h when psyllium was incorporated in wheat bread. Specific volume and crumb hardness are generally accepted as the main indicators of bread quality. Therefore, the presented results confirm a technological quality of HPHB similar to RWB. The evaluation of crumb structure reveals small differences between the formulations. A slightly finer crumb structure was observed for

Table 5.6: Bread quality characteristics of reference wheat bread (RWB) and high-protein hybrid bread(HPHB).

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

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

HPHB indicated by a higher number of cells (5563) and smaller cell area (51.7%) compared to RWB with a number of cells of 5009 and a cell area of 52.4%. This can be related to the higher initial yeast activity and gas production in HPHB. Moulding of dough, in addition to shaping the dough pieces, leads to a division of gas cells produced prior to moulding (during mixing and dough rest) [70]. In HPHB, more gas is produced before moulding and a higher number of small gas cells can be generated. Additionally, these gas cells are stabilised by water-extractable and solubilised AX as explained above, which can minimise the coalescence of gas cells as they expand during proofing and lead to a high number of cells in the final product. The higher number of cells and smaller cell area measured for HPHB could also be partially responsible for its slightly higher initial crumb hardness. Values obtained for crumb and crust colour (Table 5.5) confirm the visually perceivable differences between RWB and HPHB. Slice brightness (obtained by C-Cell imaging) is significantly lower for HPHB with 108 than for RWB with 137. This is in line with the lower lightness of crumb measured for HPHB. A big difference was observed in lightness of crust, which was significantly lower for HPHB (34.6) than for RWB (41.9). The darker crust of HPHB is likely related to its higher protein content and higher presence of reducing sugars (see Table 5.7), thus, an increased potential for Maillard reaction [30, 71]. The micro-structure of the bread crumb of both formulations was captured by scanning electron microscopy (SEM). The resulting micrographs are displayed in Figure 5.3. While RWB shows a rather porous layer of gluten covering partly intact starch granules, HPHB has a thicker and more continuous layer. This might be due to the presence of non-wheat proteins from faba bean and carob on one hand and psyllium on the other hand. The fact that a very homogenous and continuous layer was formed, further supports the theory of a co-networking of gluten with non-wheat proteins and psyllium AX.

5.4.1.3 Microbiological Shelf Life

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 [72]. 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 [73]. 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 [74]. 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 [75, 76]. Therefore, susceptibility to mould deterioration represents a food safety

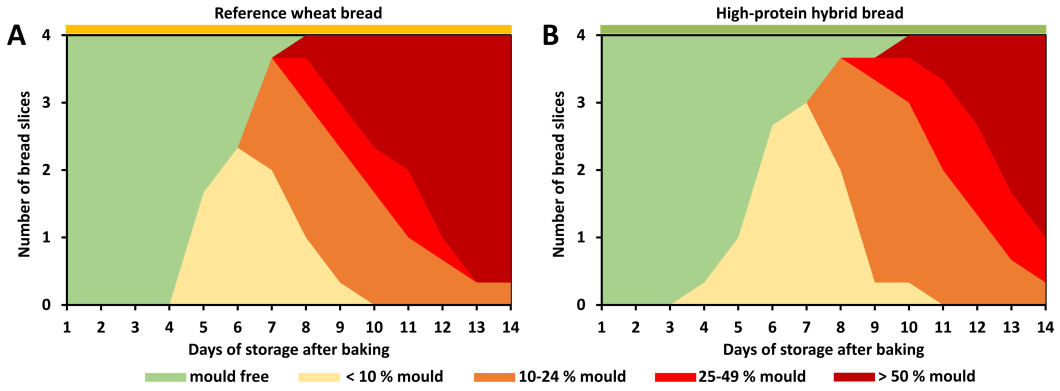


Figure 5.4: Microbiological shelf life of (**A**) RWB and (**B**) HPHB as indicated by ambient air challenge test. Results represent the mean of three independently performed challenge tests.

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 5.4. 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 ± 0.003, HPHB 0.943 ± 0.003).

5.4.2 Nutritional Characteristics

5.4.2.1 Macronutrient Composition and Sugar Profile

Compositional analysis of both formulations was performed in order to evaluate changes in macronutrient composition caused by the partial replacement of wheat flour by plant-based HPIs in HPHB and addition of psyllium to the formulation (Table 5.7). The determined bread constituents include all items that are mandatory for nutritional food product labelling according to European food legislation (regulation (EU) No 1169/2011 [77]). In addition, the sugar profile, total starch content and other important components of the samples were measured or calculated. Protein content and content of available carbohydrates represent the main differences in the macronutrient profile of RWB and HPHB. This is essentially caused by the replacement of wheat flour, which is high in starch (72.38 %DM), by HPIs with protein contents of 61.25 %DM (faba bean flour), 55.04 %DM (carob germ flour) and 83.11 %DM (gluten) and starch contents below 10 %DM (protein and starch contents of wheat flour and HPIs previously reported by Hoehnel *et al.* [30]). While

Table 5.7: Composition of reference wheat bread (RWB) and high-protein hybrid bread (HPHB), 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
Saturated fatty acids (SFA)	0.11	0.17
Mono unsaturated fatty acids (MUFA)	0.26	0.36
Poly unsaturated fatty acids (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.

the total energy level of the formulations is similar (RWB 211.6; HPHB 209.0), a shift from wheat starch to non-wheat protein characterises the macronutrient profile of HPHB. This shift is also evident when proteinE values (percentage of calories provided by protein) are compared. In contrast to RWB with 15.5 %E, the HPHB formulation reaches a proteinE of 24.8 %E and therefore qualifies for a 'high in protein' nutritional claim in accordance with European food legislation (regulation (EC) No 1924/2006 [78]), where a proteinE of 20% is set as requirement. Bread is a staple food with global importance as source of dietary carbohydrates, protein and fibre [15]. However, within the past 200 years, the consumption of refined-carbohydrate products, including bakery products from refined wheat flour (white bread, white bagels, white buns), has substantially increased. At the same time, significantly less regular starchy foods like beans, lentils and wholegrain bakery products are consumed Jenkins *et al.* [7]. This is largely associated with generally better sensory characteristics of refined-carbohydrate products and potentially higher consumer acceptance due to their sweet taste when starch is rapidly digested by salivary amylase [7, 15]. The main concern regarding this development is related to high glycaemic indices due to rapidly digestible starch [2, 7, 15, 79]. High-glycaemic-load and high-glycaemic-index diets have been associated with elevated risk for diabetes, heart disease and certain types of cancer

[80–84]. Due to its reduced content of available carbohydrates, HPHB is expected to have a lowered glycaemic load in comparison to RWB. Even a decreased glycaemic index could be expected, since psyllium has been reported to lower the glycaemic index of foods when added to conventional diets [85, 86]. Holt *et al.* [87] found a significantly lowered blood glucose response of high-protein bread when they compared equal-energy portions of high-protein bread and regular white bread. Furthermore, an isocaloric replacement of refined starch or sugar by protein, like it is the case for HPHB compared to RWB, has been reported to reduce blood pressure and blood lipid concentrations [2, 88]. Also the lack of fibre in refined-carbohydrate foods compared to wholegrain alternatives and legumes has been critically discussed [7, 15]. Dietary fibre is associated with many health benefits and dietary recommendations advice a daily intake of 25 g or more for adults [2, 89]. In the present study, HPHB contains with 2.8% considerably more dietary fibre than RWB with 1.8%. This is related to the incorporation of faba bean flour, carob germ flour and psyllium in HPHB, which represent ingredients with notable contents of both soluble and insoluble fibre [30, 58]. Especially psyllium has been reported in literature as dietary fibre with beneficial effects regarding the risk of diabetes, obesity, high blood pressure and heart disease [53]. Apart from refined carbohydrates, also fat and salt (sodium cloride) are dietary components which are often critically discussed [90–92]. While HPHB contains with 0.440% an amount of sodium similar to RWB (0.466%), it has a slightly elevated fat content. However, this increase is mainly caused by higher contents of MUFA and PUFA, which are nutritionally more favourable than saturated fats [2, 90]. Both formulations contain similar amounts of sugar (mono-and disaccharides) and their sugar profiles reveal little differences. They confirm that sucrose added in the recipe of HPHB is fully consumed during yeast fermentation, which was also evident in the results obtained from dough analyses. Slightly increased galactose and the presence of oligosaccharides like raffinose, stachyose and verbascose can be associated with high contents of galactooligosaccharides (GOS) reported for faba beans [93]. Slightly lower maltose and maltotriose levels in HPHB are potentially related to its lower starch content.

5.4.2.2 Amino Acid Profile

Many dietary recommendations advise a substantial decrease in the consumption of animal protein and a shift towards protein from plant sources [2, 4]. Even though bread can be considered an important source of plant protein, the poor protein quality of wheat makes regular wheat bread (from both wholegrain or refined wheat flour) an inadequate choice to partially compensate future plant protein requirements; especially when a substantial decrease in high-quality an-

Table 5.8: Amino acid (AA) composition of reference wheat bread (RWB) and high-protein hybrid bread (HPHB).

Content [%Protein]	RWB	HPHB
Indispensable and conditionally indispensable AAs		
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	33.34 ± 4.46	34.48 ± 4.41
Dispensable AAs		
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.
* Sum of sulphur containing amino acids (SAAs).
** Sum of aromatic amino acids (AAAs).

imal protein consumption is taken into account. The poor protein quality of wheat is mainly linked to an unbalanced amino acid composition, and to its lack of the indispensable amino acid lysine in particular [5, 12, 15]. The amino acid profile of RWB and HPHB was determined and is reported in Table 5.8. The results show that the proportions of indispensable and dispensable amino acids are very similar in both formulations. Amongst the dispensable amino acids, only the levels of glutamine/glutamic acid, proline and arginine differ substantially between RWB and HPHB. While wheat is particularly rich in glutamine, glutamic acid and proline but contains little arginine [12], faba bean and carob show a complementary pattern for these AA [32, 94]. Especially faba bean protein contains relatively small amounts of glutamine/glutamic acid and is high in arginine. This causes a decreased level of glutamine/glutamic acid and proline but an increased level of arginine in HPHB. Regarding the profile of indispensable AA in RWB and HPHB, many minor differences were observed. However, the lysine level is approximately 65% higher in HPHB than in RWB. Also this change can be attributed to faba bean and carob proteins which are naturally richer in lysine than wheat [12, 32, 94]. Even though the difference of lysine contents expressed in %Protein might seem small, this difference has a big impact on the breads' overall amino acid balance and, thus, their protein quality. Especially when compared to a reference pattern of

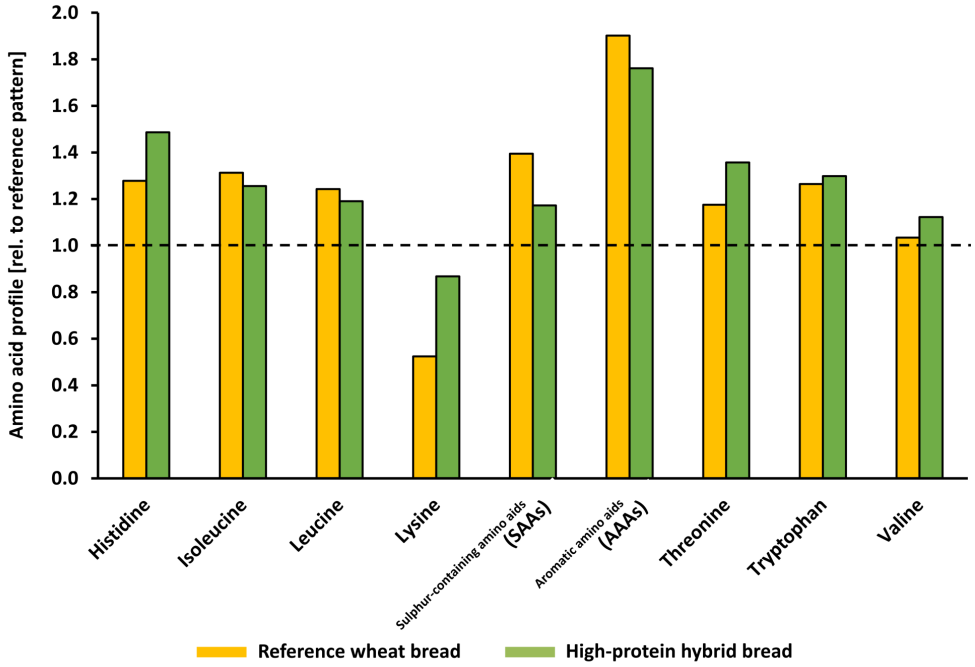


Figure 5.5: Profile of indispensable amino acids of reference wheat bread and high-protein wheat bread expressed relative to the requirement pattern (WHO 2007 [95]) and based on an average intake of 0.66 g protein/kg.

indispensable amino acids (for adults) recommended by WHO [95] and EFSA [96], the significance becomes evident. The quantity of indispensable amino acids in RWB and HPHB relative to the amino acids in the reference pattern is presented in Figure 5.5. The comparison with the reference pattern reveals that in both formulations lysine is the only AA, which does not reach the quantity specified as recommended intake (= 1). Therefore, lysine represents the limiting AA of the protein in RWB and HPHB. The increased lysine content in HPHB (87% of lysine in reference pattern) compared to RWB (52% of reference pattern) leads to a much more balanced AA profile that almost covers the recommended intake of all indispensable AA. The expression of AA levels in a food protein relative to the levels in a reference protein is referred to as amino acid score (AAS). Table 5.9 shows an overview of AAS and limiting AAs of RWB and HPHB and the ingredients used for their production (wheat flour and HPIs). The HPHB formulation does not only have an improved AAS compared to RWB, but also in comparison to wheat flour and HPIs. The combination of the ingredients in HPHB leads to an upgrade in nutritional value of most raw materials when AAS is used to evaluate nutritional protein quality. The exception is the protein from carob germ flour, which has a nutritionally favourable AA pattern itself. Since the calculation of AAS is based on a recommended amino acid reference pattern, which considers an average intake of 0.66 g protein/kg bodyweight, this evaluation assumes that RWB or HPHB (or

Table 5.9: Amino acid scores (AASs) for breads and their raw materials.

Protein source	AAS	Limiting Amino acids
Reference wheat bread (RWB)	0.52	Lysine
High-protein hybrid bread (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.* [32].
*** Not strictly limiting (≥ 1), but represents AA with lowest level relative to reference pattern.

the ingredients) are the sole source of protein in the diet. In a real diet, proteins from other foods can potentially compensate for AA deficiencies. However, the ability of a dietary protein source to fulfil amino acid requirements on its own is regarded as an adequate approach to compare nutritional quality of proteins.

5.4.2.3 Protein Digestibility and Utilisation

The informative value of AASs is also limited because they do not reflect the protein's digestibility, absorption and utilisation [97]. In the present study, protein digestibility was evaluated in an *in vitro* model as well as in an *in vivo* trial with rats (Table 5.10). *In vitro* protein digestibility (IVPD) of RWB and HPHB was monitored after 1 h of pepsin digestion and, subsequently, 1 h of pancreatin digestion, which is indicative of the digestibility in the human digestive system. Additionally, IVPDs were measured after a medium term (3 h) and a long term (24 h) pancreatin digestion to evaluate the maximum achievable degradation of the proteins. Both the digestion mimicking gastric conditions (1 h pepsin) as well as the simulated intestinal digestion (1 h pancreatin) yielded higher ratios of degraded protein for

Table 5.10: *In vitro* protein digestibility and *in vivo* nitrogen balance of reference wheat bread (RWB) and high-protein hybrid bread (HPHB).

Variable	RWB	HPHB
***In vitro* protein digestibility (IVPD) [%]**		
Pepsin 1 h	1.1 ± 0.4^b	2.0 ± 0.3^a
Pancreatin 1 h (short term)	14.2 ± 0.6^b	17.2 ± 0.3^a
Pancreatin 3 h (medium term)	18.4 ± 1.7^b	22.7 ± 1.2^a
Pancreatin 24 h (long term)	25.0 ± 0.0^b	31.1 ± 0.1^a
***In vivo* nitrogen balance**		
N intake [g/5 d]	1203 ± 359^b	1556 ± 94^a
N in faeces [mg/5 d]	138 ± 47^b	183 ± 12^a
N faecal [% N intake]	11.4 ± 1.0^a	11.8 ± 1.0^a
N in urine [mg/5 d]	766 ± 206^a	733 ± 35^a
N urinary [% N intake]	64.4 ± 3.1^a	47.1 ± 1.8^b
N digestibility [%]	88.6 ± 1.0^a	88.2 ± 1.0^a
N utilisation [%]	24.2 ± 2.7^b	41.0 ± 2.7^a
Protein efficiency ratio (PER) [g/g]	1.13 ± 0.39^b	2.13 ± 0.17^a

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

HPHB than for RWB, indicated by significantly higher IVPD values. This suggests a slightly improved protein digestibility of HPHB, which is remarkable since legumes, in HPHB specifically faba bean and carob, are often critically discussed regarding their contents of trypsin inhibitors and an associated decrease in protein digestibility Avilés-Gaxiola *et al.* [98]. However, due to the incorporation of only 5.72% of faba bean flour in the whole HPHB formulation (see Table 5.1), a substantially reduced content of trypsin inhibitors, as compared to the faba bean raw material, is expected. A detailed discussion of the trypsin inhibitor activity (TIA) in HPHB follows in Section 5.4.2.4. A higher degree of protein degradation in HPHB could be explained by the higher abundance of lysine and arginine in this formulation. Trypsin, which is a predominant proteolytic enzyme in pancreatin, cleaves protein and peptide chains at the carboxyl side of these positively charged AA. Pancreatin also contains chymotrypsin, which cleaves after hydrophobic AA with bulky side chains like phenylalanine, tryptophan and tyrosine. The contents of these AA are very similar in HPHB and RWB. However, abundance of target AA for trypsin and chymotrypsin proteolysis is not the only relevant factor. Also accessibility of such AA in the three-dimensional protein structure is of high importance. This suggests that HPHB contains a higher number of AA accessible for tryptic/chymotryptic digestion. The *in vivo* protein digestibility trials performed with rats yielded several variables indicative of the breads' nutritional value (Table 5.10). 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 [99], some legumes have been found to reach higher digestibility in *in vitro* experiments than *in vivo* [97]. 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 dicussed in the previous section (Section 5.4.2.2). 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 [95, 100]. 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 [101, 102]. 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 [97, 100]. 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 [99, 103]. 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) [5, 100]. 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.

5.4.2.4 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 5.11). 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 [24], it is often responsible for substantially reduced protein digestibility [25]. No trypsin inhibitor activity (TIA) was detected for RWB. The TIA of 0.21 TIU/mg measured for HPHB can be

Table 5.11: Contents of antinutritional compounds of reference wheat bread (RWB) and high-protein hybrid bread (HPHB), contents refer to fresh bread or dry matter as indicated.

Antinutritional compound	RWB	HPHB	RWB	HPHB
	based on fresh bread		based on dry matter*	
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 5.7; for comparison purposes.

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.* [32]. 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 [98]. In addition to faba bean, also carob germ flour could be a source of trypsin inhibitors in HPHB [104, 105]. 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 [106]. When ingested by individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, these compounds can trigger favism, which leads to acute haemolytic anaemia [28]. On average, the sum of vicine and convicine accounts for about 1 %DM in faba beans [27, 106]. However, efforts in plant breeding have led to cultivars with contents of the pyrimidine glycosides as low as 0.01 - 0.02 %DM [27]. Vogelsang-O'Dwyer *et al.* [32] 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.* [107], 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.* [107] 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 [108]) 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.

5.4.2.5 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 [109]. 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 [110]. 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 [111–114]. 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 5.12. 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 [18] and faba bean and carob in particular [17, 44, 115]. 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.* [23] 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 [116]. Ragaee *et al.* [117] investigated the content of phenolics and antioxidant

Table 5.12: Antioxidant potential of reference wheat bread (RWB) and high-protein hybrid bread (HPHB), contents refer to fresh bread unless stated otherwise.

Antioxidant potential	RWB	HPHB
Total phenolics [mg/100 g]	15.8 ± 0.3^{b}	66.1 ± 0.3^{a}
ABTS [mmol Trolox/100 g]	0.08 ± 0.01^{b}	1.02 ± 0.03^{a}
FRAP [mmol Fe^{2+}/100 g]	0.23 ± 0.01^{b}	0.77 ± 0.01^{a}
Antiradical activity (DPPH)		
EC_{50} [mg extract/mL]*	6.22 ± 0.18^{a}	1.15 ± 0.03^{b}

Means ± standard deviation with different letters in the same row were significantly different $p < 0.05$.
* Concentration of phenolic extract of breads able to scavenge 50% of DPPH radicals.

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 wholegrain 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 [118]. However, the results in this study clearly show higher antioxidant potential for HPHB than RWB.

5.4.3 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' [78], 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 [119]. Sensory analysis for the two formulations in this study was performed with a trained panel using selected descriptors for bread quality (Figure 5.6). 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 panelists. The results for HPHB further indicate an improved crumb texture, which is often perceived as an indicator of freshness amongst consumers [120, 121]. 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 [121]. Both formulations reached similar scores in chewiness. This shows that the slightly increased initial crumb hardness for HPHB, which was detected in texture profile analysis (TPA), had no perceptible negative impact on the mouthfeel of the bread crumb. High-protein hybrid bread scored higher than RWB in moisture of crumb, which is considered another indicator for bread freshness and quality [121]. Significant differences have been found regarding the odour profile of the formulations. While for RWB a slight sweet and almost no acidulous odour was perceived, HPHB had no perceivable sweet odour and slightly stronger acidulous odour than RWB. In accordance with

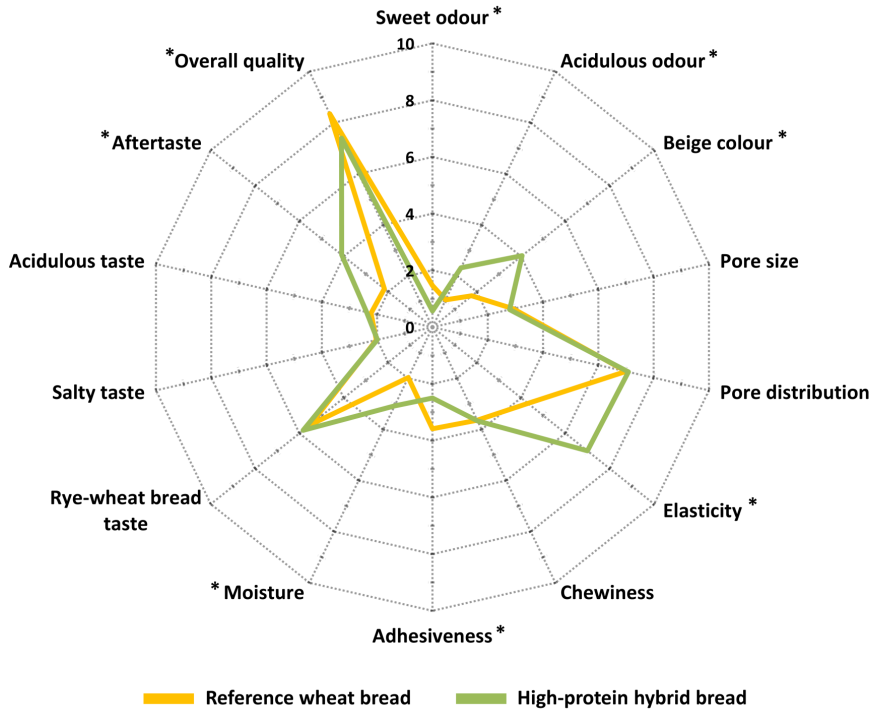


Figure 5.6: Sensory characteristics of reference wheat bread and high-protein hybrid bread; asterisks * indicate attributes which showed significant differences between RWB and HPHB ($p < 0.05$).

the results of instrumental crumb colour measurements, a darker/more beige colour was observed for HPHB. Also a moderate increase in aftertaste was identified in HPHB. However, the overall sensory quality was rated only slightly lower for HPHB than for RWB. This identifies HPHB as a bread formulation with adequate sensory quality when compared to RWB, suggesting high consumer acceptance. The scores of HPHB for sensory attributes like acidulous odour and beige colour suggest similarities to the typical sensory profile of sourdough bread [122, 123]. Because of the popularity of sourdough bread amongst consumers, this could further contribute to a high consumer acceptance of HPHB.

5.5 Conclusion

A mixture of HPIs was used to partially replace wheat flour in regular wheat bread to produce a high-protein bread. The HPIs and their ratios were selected based on previous results by Hoehnel *et al.* [30] to represent both beneficial expected nutritional properties as well as adequate baking properties. In order to match the technological quality of a regular wheat bread, which was used as a reference, also three functional ingredients (psyllium, sugar, xylanase) were added. Dough and bread quality comparable to the reference wheat bread were observed for high-protein hybrid bread (HPHB); mainly mediated by the functional properties

of carob and gluten protein as well as psyllium and xylanase. Additionally, a substantially enhanced nutritional profile of the proposed HPHB compared to regular wheat bread was achieved. The macronutrient composition was improved by an isocaloric replacement of refined wheat-starch by plant protein. The protein quality was improved, judging by a better AA profile, increased N utilisation and higher protein efficiency ratio. Mainly due to the dilution effect in the bread matrix, only low levels of ANCs originating from faba bean and carob were measured. Furthermore, determination of phenolics and antioxidant activity indicate high antioxidant potential for HPHB. Apart from favourable technological and nutritional characteristics, the proposed formulation also has high sensory quality which suggests high consumer acceptance. In a time in which we are looking for ways to adequately and sustainably provide enough high-quality plant protein for a future human diet, we cannot afford to focus only on meat and dairy replacement products; especially considering that these applications often require highly purified or additionally functionalised plant proteins obtained by wet processing. In the proposed high-protein hybrid bread formulation, dry-processed protein ingredients from faba bean and carob were applied and provide a substantial amount of non-wheat protein. The increased content of plant protein with higher protein quality in HPHB and formulations of its kind, could improve the capacity of the staple food bread to cover protein needs in future plant-based diets. The results also suggest that a replacement of regular wheat bread by high-protein hybrid breads could be beneficial in currently consumed diets.

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CHAPTER 6

Leuconostoc citreum TR116 as a Microbial Cell Factory to Functionalise High-Protein Faba Bean Ingredients for Bakery Applications

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6.1 Abstract

Grain legumes, such as faba beans, have been investigated as promising ingredients to enhance the nutritional value of wheat bread. However, a detrimental effect on technological bread quality was often reported. Furthermore, considerable amounts of antinutritional compounds present in faba beans are a subject of concern. Sourdough-like fermentation can positively affect baking performance and nutritional attributes of faba bean flours. The multifunctional lactic acid bacteria strain *Leuconostoc citreum* TR116 was employed to ferment two faba bean flours with different protein contents (dehulled flour (DF); high-protein flour (PR)). The strain's fermentation profile (growth, acidification, carbohydrate metabolism, antifungal phenolic acids) was monitored in both substrates. The fermentates were applied in regular wheat bread by replacing 15% of wheat flour. Water absorption, gluten-aggregation behaviour, bread quality characteristics and *in vitro* starch digestibility were compared to formulations containing unfermented DF and PR and to a control wheat bread. Similar microbial growth, carbohydrate consumption as well as production of lactic and acetic acid were observed in both faba bean ingredients. A less pronounced pH drop as well as a slightly higher amount of antifungal phenolic acids were measured in the PR fermentate. Fermentation caused a striking improvement of the ingredients' baking performance. GlutoPeak measurements allowed for an association of this observation with an improved gluten-aggregation. Given its higher potential to improve protein quality in cereal products, the PR fermentate seemed generally more promising as functional ingredient due to its positive impact on bread quality and only moderately increased starch digestibility in bread.

6.2 Introduction

Many dietary recommendations as well as guidelines to improve sustainability along the food chain advise a drastic decrease in animal proteins in our diets and an enhanced inclusion of plant-based protein sources [2–4]. Even though cereal products, such as the staple food bread, can be considered an important source of plant-based protein in human diets worldwide, its poor protein quality and relatively low protein content make it an inadequate choice to (at least partially) compensate for substantially decreased animal protein intakes in our diets. The poor quality of wheat protein is primarily related to an unbalanced amino acid profile and insufficient contents of indispensable amino acids, specifically lysine [5–7]. Also the high content of rapidly digestible starch causing a high glycaemic index (GI)/glycaemic load (GL) of white wheat bread has been mentioned as one of the rather disadvantageous traits [7–9]. Jenkins *et al.* [9] mentioned that foods should possess nutritional rather than nutrient density (referring to an increased intake of essential macro- and micronutrients per calorie); especially in the context of generally decreased physical activity in our modern society. The partial substitution of wheat flour in bread formulations by various plant-based protein ingredients (cereals, pseudocereals, potato, vegetable, legumes) with the objective to improve bread nutritional quality, and primarily protein content and quality, has been thoroughly investigated within the past two decades [10–16]. Boukid *et al.* [17] reported that specifically grain legumes, such as faba beans, are very promising for this approach. Besides their promising environmental foot print and beneficial nitrogen fixing properties during cultivation [18, 19], they also provide an amino acid profile that offers a matching complementation to that of wheat [13, 17, 20]. While a substantial improvement of the overall nutritional quality of the breads, with an emphasis on protein quality, was often achieved, many studies also reported severely decreased technological bread quality. Specifically higher levels of wheat flour replacement, which are chosen to enhance the potential nutritional improvement, seem to pose major challenges. Therefore, plant-based high-protein ingredients (HPIs) are particularly promising since they provide higher amounts of protein and a higher capacity to compensate for the lack of certain amino acids at lower levels of wheat flour substitution. Dry fractionated (air-classified) legume flours, for example, offer a substantially increased protein content while their production is less energy and resource-intensive [19, 21]. Another concern, besides drastically reduced technological bread quality, involves antinutritional compounds (ANCs) which occur in legumes and can, amongst other effects, negatively impact absorption of minerals and protein digestibility [22]. For the improvement of the baking performance of faba bean flour and to reduce its contents of ANCs fermentation

technology, and specifically sourdough-like single-strain fermentation, has been investigated and described to be a powerful tool [23–29]. While various lactic acid bacteria (LAB) strains promise a positive impact, specifically *Leuconostoc* species have been reported to produce or release a large range of compounds with biological activities and potentially beneficial techno-functional properties [30–33]. The strain *Leuconostoc citreum* TR116 is a multifunctional LAB strain and has, for example, been found to efficiently convert fructose present in the substrate into mannitol [34]. Fermentates produced with this strain have successfully been applied in beverages and bakery products to reduce contents of added sugar while maintaining adequate sensory characteristics [35–38]. In the present study, *Ln. citreum* TR116 is applied to ferment two faba bean (FB) ingredients: FB dehulled flour (DF) and air-classified FB high-protein flour (PR). The strain's fermentation profile in a low- and a high-protein environment originating from the same raw material is thereby compared. The produced sourdough-like fermentates are used to replace 15% (based on solids) of wheat flour in a regular wheat bread formulation. The results are compared to those of breads containing the same level of unfermented FB ingredients and to a control wheat bread. The objective of this study is to assess the potential of faba bean fermentation with *Ln. citreum* TR116 to create a functional fermented HPI for bakery applications. The study approach combining fundamental characterisation of faba bean fermentation with a detailed investigation of the application of the produced fermentates in breads promises high relevance of the results for advancing the protein transition.

6.3 Materials and Methods

6.3.1 Materials

Two different ingredients produced from faba beans (FB; *Vicia Faba*) were used in this study: faba bean dehulled flour (DF; protein content (N × 6.25) 33.94 %DM, fat 1.15 %DM, ash 3.28 %DM, fibre 10.2 %DM, carbohydrates by 62.00 %DM, total starch 47.37 %DM) and faba bean high-protein flour (PR; protein content (N × 6.25) 61.25 %DM, fat 3.81 %DM, ash 5.43 %DM, fibre 0.3 %DM, carbohydrates by difference 29.17 %DM, total starch 7.77 %DM [12]; obtained by dry fractionation), which were experimentally produced and provided by Fraunhofer Institute IVV, Freising, Germany. For the preparation of sourdough-like faba bean fermentates by controlled single-strain fermentation, the lactic acid bacteria (LAB) strain *Ln. citreum* TR116 was used, which was originally isolated from yellow pea sourdough. This strain belongs to the culture collection of the Department of Biological Sciences, Cork Institute of Technology. Conditions for the cultivation and storage

were according to those reported by Sahin *et al.* [36]. For baking trials, wheat flour was supplied by Whitworth Bros Ltd, Wellingborough, UK; dry yeast (4% moisture, 50% protein, 5% fat, 40% carbohydrates) by Puratos, Dilbeek, Belgium; salt by Glacia British Salt Ltd., Northwich, UK; and vegetable oil by Musgrave, Cork, Ireland. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

6.3.2 Production of Sourdough-Like Fermentates

To obtain a cell suspension of TR116, a single colony was transferred into 10 mL of deMan-Rogosa-Sharpe (MRS) broth and incubated at 30 °C for 24 h. This preinoculum was subcultured (100 μL in 10 mL MRS broth) and incubated at 30 °C for 16 h. The cells were harvested by centrifugation (4800 g, 10 min, 10 °C), washed twice with sterile tap water (addition of 10 mL tap water, resuspension and subsequent centrifugation with same conditions as above) and finally resuspended in 10 mL of sterile tap water and used as inoculum. The desired dough yield of 200 (dough yield; DY = 100 × (flour weight [g] + water weight [g])/(flour weight [g])) was achieved by mixing the respective faba bean ingredient and liquid (sterile tap water and cell suspension, volume calculated to reach an initial cell density of 7 log CFU (colony forming units)/g) in a ratio of 1:1 with a Kenwood Chef (Kenwood Manufacturing Co. Ltd., Havant, UK) kitchen machine equipped with a K-beater attachment for 1 min at speed 1, followed by 1 min at speed 2. Aliquots of the mixture were filled into separate sterile stomacher bags to enable analysis of the sourdough-like fermentates at different time points throughout the fermentation process. The stomacher bags were airtightly sealed and incubated at 30 °C for a maximum of 48 h. The presented results were obtained from three independently performed fermentation trials for each of the two faba bean ingredients. The sourdough-like fermentates produced from DF and PR are further referred to as DFSD and PRSD, respectively.

6.3.3 Characterisation of Fermentation Profile

To characterise and compare the fermentation profiles of *Ln. citreum* TR116 in the two different faba bean substrates, the fermentation process was monitored and fermentates were analysed every 6 hours (starting at 0 h) over a total fermentation time of 48 hours with respect to microbial growth, acidification, carbohydrate levels and contents of organic acids with antifungal activity. After sample collection at the respective time points, samples were either analysed fresh (cell count, pH, total titratable acidity (TTA)) or immediately frozen for further processing and extraction.

6.3.3.1 Determination of Cell Count, pH and Total Titratable Acidity

Microbial growth and acidification of the samples were monitored by measuring the cell count, pH and TTA. These analyses were performed with the fresh fermentates after sample collection from the incubator. Cell counts were determined by diluting 1 g of fermentate with 9 mL sterile Ringer's solution and preparing a decimal dilution series as described by Sahin *et al.* [36]. Dilutions were plated on MRS agar containing 0.05 g/L bromocresol green and incubated anaerobically for 48 h at 30 °C. The cell count was calculated based on the counted colonies using the weighted arithmetic average. The pH and TTA of the samples were measured according to AA 02-31.01 [39] in a duplicate for each time point of each of the three fermentation trials.

6.3.3.2 Preparation of Fermentates for Extraction

The frozen fermentate samples were mechanically crushed into smaller fragments and transferred into 50 mL test tubes. The samples were frozen at – 80 °C for a minimum of 30 minutes and subsequently freeze-dried. The dry fermentates were ground to a fine powder with a mortar and pestle and stored frozen in sealed 50 mL test tubes. For the calculation of results based on dry matter, the moisture of the freeze-dried fermentates was determined with the air-oven method [AACC method 44-15.02] [40]. The extractions described below were carried out in a duplicate for each time point of each of the three fermentation trials.

6.3.3.3 Levels of Organic Acids and Carbohydrates

For the quantification of organic acids and mono-, di- and oligosaccharides and polyols, the freeze-dried fermentates were extracted according to the procedure previously described by Hoehnel *et al.* [13] with some modifications. In brief, 1 g of sample was dispersed in 15 mL of 80/20 (v/v) ethanol/ultrapure water (80% EtOH), with a temperature of 55 $\pm$ 5 °C. The extraction was facilitated by a sonication step, followed by centrifugation and separation of the supernatant from the solids. The extraction procedure was repeated with the pellet from step 1 and the supernatants from both extraction steps were pooled. A vacuum centrifuge was used to evaporate ethanol and to concentrate the extracts before the final volume was adjusted to 10 mL with ultrapure water containing 50 mg/L NaN_3. The obtained fermentate extracts were filtered through 0.2 μm syringe-driven filters and stored frozen in sealed 1.5 mL screw-cap tubes until chromatographic analysis. The quantification of selected mono-, di-, and oligosaccharides and polyols (sucrose, glucose, fructose, mannitol, verbascose, stachyose/raffinose, galactose) was carried out as previously described by Ispiryan *et al.* [41] with a

Dionex ICS-5000+ system (Sunnyvale, CA) and pulsed amperometric detection. An external calibration was used for each of the compounds (0.1–20 ppm) and the separation was performed on two different columns: a Thermo Scientific Dionex CarboPac PA1 column (2×250 mm; Thermo Fisher Scientific, Waltham, MA, USA; used for quantification of sucrose, glucose, fructose, mannitol, galactose) and a Dionex CarboPac PA200 column (3 × 250 mm; Thermo Fisher Scientific, Waltham, MA, USA; used for quantification of verbascose, stachyose/raffinose). Both columns were equipped with the matching guard column. Flow rate, eluents and elution conditions (isocratic/gradient) were as specified by Ispiryan *et al.* [41]. Other mono-, di- and oligosaccharides such as arabinose, xylose, maltose and maltotriose were included in the screening but not detected in quantities higher than 0.02 %DM (throughout the fermentation process until 48 h of fermentation) and therefore not included in the reported results. The separation of acetic acid and lactic acid was performed using a Dionex UltiMate 3000 System (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Hi-Plex H column (7.7 × 300 mm; Agilent Technologies, Santa Clara, CA, USA) and the matching guard column, which was operated at a column temperature of 60 °C and with 5 mM sulphuric acid as eluent. The compounds were quantified using an external calibration (0.03–6 g/L) and an ultraviolet light/diode array detector (UV/DAD; Thermo Fisher Scientific, Waltham, MA, USA; UV spectra recorded between 190 and 400 nm; quantification at 210 nm). Additionally, the fermentation quotient, which represents the molar ratio of lactic and acetic acid (lactic acid [mmol/L]/acetic acid [mmol/L]), was calculated.

6.3.3.4 Level of Antifungal Compounds

The fermentates DFSD and PRSD were screened for the presence of 15 phenolic compounds with previously reported antifungal activity [42–45] (catechol, 4-hydroxyphenyllactic acid, 4-hydroxybenzoic acid, hydrocaffeic acid, vanillic acid, caffeic acid, phloretic acid, 3-phenyllactic acid, hydroferulic acid, *p*-coumaric acid, ferulic acid, benzoic acid, salicylic acid, hydrocinnamic acid, methylcinnamic acid). The extraction of these compounds was carried out based on the QuEChERS (quick, easy, cheap, effective, rugged and safe) procedure described by Brosnan *et al.* [46] with some modifications. Briefly, 1 g of the freeze-dried fermentates was dispersed in 10 mL of ultrapure water, followed by the addition of 10 mL of ethyl acetate containing 0.1% (v/v) formic acid and homogenisation using a vortex shaker. Subsequently, 1 g NaCl and 4 g $MgSO_4$ were added and the samples were thoroughly shaken by hand for one minute. The organic phase of the supernatants of the extracts was obtained and separated after centrifuging at 4800 g for 10 min and transferred into solid-phase extraction (SPE) tubes (Bond Elut QuEChERS

Dispersive kit; Agilent Technologies Inc., Santa Clara, CA, USA). The tube contents were first thoroughly homogenised and then centrifuged at 2300 g for 10 min. Five millilitres of the obtained supernatant was added to 100 μL dimethylsulfoxide (DMSO) and concentrated using a vacuum centrifuge (Scanvac, Scanspeed; 2 h, 500 rpm, 45 °C). The concentrated extracts were reconstituted by adding 400 μL 90/10 (v/v) ultrapure water/acetonitrile and filtered through 0.2 μm syringe-driven filters before chromatographic analysis. The separation and quantification of antifungal compounds was performed on a Dionex UltiMate 3000 RSLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a a Gemini C18 column (2 × 150 mm; Phenomenex Inc., Torrance, CA, USA), matching guard cartridge and a UV/DAD detector (Thermo Fisher Scientific, Waltham, MA, USA; UV spectra recorded between 190 and 400 nm; quantification at 210 nm). The column temperature was set to 30 °C and the following gradient elution based on two eluents (eluent A - ultrapure water containing 0.1% formic acid; eluent B - acetonitrile containing 0.1% formic acid) was applied at a constant flow rate of 0.2 mL/min: (1) 0–5 min, 95% A; (2) 5–15 min, 95–85% A; (3) 15–35 min, 85–60% A;(4) 35–45 min, 60–50% A; (5) 45–50 min, 50–5% A; (6) 50–70 min 95% A. An external calibration (5–50 ppm) was used to quantify antifungal compounds. Only five (4-hydroxybenzoic acid, caffeic acid, coumaric acid, ferulic acid, phenyllactic acid) of the 15 compounds were detected in levels above the limit of quantification (2.5 mg/100 g DM). Therefore, only the results of these five compounds are presented in this work.

6.3.4 Application of Sourdough-Like Fermentates and Product Analysis

For the application of DFSD and PRSD in bread (and for the determination of flour properties), the maximum fermentation time of 48 h was chosen. Five different bread formulations were prepared: a control wheat bread formulation based on wheat flour (CWB), two faba bean bread formulations with partial replacement of wheat flour by unfermented faba bean ingredients (DFB and PRB) and two faba bean bread formulations with the sourdough-like faba bean fermentates (DFSDB and PRSDB). The fermentates used for both flour analysis and baking trials were stored at 4 °C (for a maximum of 2 hours) after sample collection and conditioned in a water bath at 25 °C for 10 min immediately before use.

6.3.4.1 Flour Analysis

The properties of wheat flour (used for CWB) and FB flour mixtures (DF flour mix, DFSD flour mix, PR flour mix and PRSD flour mix used for DFB, PRB, DFSDB and

PRSDB, respectively) were analysed with regard to water absorption and gluten-aggregation behaviour. In FB flour mixtures, 15% of wheat flour were replaced by the respective faba bean ingredient (equivalent to the flour mixtures used for baking trials, see Table 6.1). For DFSD flour mix and PRSD flour mix, 15% of wheat flour were replaced by the fermentates based on their solid fraction (50% due to the chosen DY of 200). The liquid fraction that was brought into DFSD flour mix and PRSD flour mix by the fermentates was accounted for when recipes and results for the Farinograph and GlutoPeak measurements were calculated. Moreover, the moisture content of the FB flour mixtures was calculated based on the moisture content determined for the raw materials (wheat flour, DF and PR) and their ratios. The weigh-in of wheat flour and FB flour mixtures for each test was adjusted to match a theoretical moisture content of 14% according to AACC 82–23.01. The determination of Farinograph (Brabender GmbH and Co KG, Duisburg, Germany) water absorption (FWA) was performed according to AACC 54-21.02. Titration trials were performed in a triplicate, adjusting doughs from wheat flour and FB flour mixtures to a consistency of (500 ± 20) Farinograph units (FU). Subsequently, a full Farinograph measurement (over 20 min) with the initial water addition set to the mean FWA determined from titration trials was performed to confirm the previous results. The presented FWAs for DFSD and PRSD flour mixtures (Table 6.5) were corrected for the water amount originating from the fermentates. The gluten-aggregation properties of wheat flour and FB flour mixtures were evaluated according to the procedure previously reported by Hoehnel *et al.* [12] with a GlutoPeak device (Brabender GmbH and Co KG, Duisburg, Germany). Briefly, a flour/water slurry (50:50 ratio; adjusted based on AACC 82–23.01 and subtracting water from fermentates). The following test settings were applied: 36 °C; paddle speed 2750 rpm. The test variables torque maximum (TM, expressed in Brabender units BU) and peak maximum time (PMT,

Table 6.1: Recipes for control wheat bread (CWB) and faba bean formulations containing unfermented faba bean dehulled flour (DF), fermented faba bean dehulled flour (DFSD), unfermented faba bean high-protein flour (PR) or fermented faba bean high-protein flour (PRSD).

Ingredient	% based on flour	% based on recipe
Wheat flour	100.0 (85.0*)	60.46
Faba bean ingredient (DF/PR/DFSD/PRSD)	0.0 (15.00*, **)	0.00
Baker's yeast	2.0	1.21
NaCl	2.0	1.21
Oil	1.0	0.60
Water	60.4 (FWA*, ***)	36.52
Total	165.4	100.00

* For faba bean formulations.

** For fermentates: addition level was based on solids in fermentate (e.g. due to dough yield of 200, 30% of faba bean fermentates were used; water addition was adjusted accordingly.

*** FWA - Farinograph water absorption according to Table 6.5.

expressed in s) were retrieved from the test and are presented in addition to gluten-aggregation profiles.

6.3.4.2 Recipe Adaptation and Bread Production

Bread samples were prepared according to the procedure described by Hoehnel *et al.* [12]. For incorporation of FB ingredients (DF,PR, DFSD, PRSD), 15% of wheat flour were replaced (for DFSD and PRSD, based on solid fraction of fermentates). Water levels where applied as determined by Farinograph trials - FWAs and adjusted for the water originating from fermentates incorporated in DFSDB and PRSDB formulation (Table 6.1). The presented results represent the mean of three independently performed baking trials; in the case of DFSDB and PRSDB with fermentates prepared in three independent fermentation trials.

6.3.4.3 Determination of Dough pH

The pH of bread doughs was analysed with the same method as for FB fermentates (described above). After mixing, dividing and moulding, the dough was stored at 4 °C (for a maximum of 5 hours) until analysis. An Ultra Turrax (IKA Werke, Staufen, Germany) was used to homogenise the samples.

6.3.4.4 Bread Quality Analysis

Analysis of bread quality characteristics was performed according to the procedures and test settings reported by Hoehnel *et al.* [12]. The following bread quality characteristics have been determined for the breads produced in this study: bake loss (gravimetrically by weighing dough after shaping and bread loaf after cooling down), specific volume (SV; with a Volscan Profiler; Stable Micro Systems, Godalming, UK), crumb hardness (on days 0, 2 and 5 of storage; with a TA-XT2i Texture Analyser; Stable Micro Systems, Godalming, UK), crumb structure (with a C-Cell Imaging System; Calibre Control International Ltd, Warrington, UK) and lightness of crust and crumb (Colorimeter CR-400; Konica Minolta, Tokyo, Japan). Based on the crumb hardness determined on days 0 and 5 of storage, the staling rate of all five bread formulations was calculated with the following equation:

$$Staling\ rate = \frac{crumb\ hardness\ day\ 5 - crumb\ hardness\ day\ 0}{crumb\ hardness\ day\ 0} \quad (6.1)$$

The values obtained for lightness of crust (L* crust) and lightness of crumb (L* crumb) are reported using the CIELAB colour space.

6.3.4.5 Scanning Electron Microscopy

Scanning electron microscopy (SEM) of bread samples was performed according to the procedure described by Hoehnel *et al.* [13]. The samples were coated with a 5 nm layer of gold-palladium (80:20) and subsequently examined under the microscope; images were acquired at a constant accelerating voltage of 5 kV.

6.3.5 *In Vitro* Starch Digestion

In vitro starch digestion was performed to monitor the release of reducing sugars over time and to determine the hydrolysis index (HI) value. This value is calculated by dividing the area under the sugar release curve (30 to 240 min) of a certain sample (DFB, DFSDB, PRB and PRSDB in this study) by the area under the sugar release curve (30 to 240 min) of a reference/control sample (CWB in this study). The experiments were carried out using the bread crumb from defrosted bread samples and following the procedure reported by Brennan and Tudorica [47] with some modifications. The samples were subjected to a pepsin treatment (pH 1.5) followed by an incubation with pancreatic α-amylase (pH 6.9) for five hours in a dialysis tube. The release of reducing sugars into the buffer surrounding the dialysis tubing was monitored (sample collection every 30 min) and the content of reducing sugars in the samples was determined spectrophotometrically using 3,5-dinitrosalicylic acid (DNS; 10 g/L) as colouring reagent (100 μL of DNS solution was added to 100 μL of sample, the mixture was further diluted with 1 mL buffer and then incubated at 110 °C for 15 min before absorbance was read at 546 nm). Appropriate blanks (sample blank with deactivated enzymes; spiked sample blank with deactivated enzymes and added maltose; maltose blank) were run with the samples in order to evaluate whether differences in the release of reducing sugars are caused by an altered diffusion rate of reducing sugars or possibly a delayed/reduced starch degradation. The amount of released reducing sugars was expressed as percentage of the digestible starch present in the sample. Contents of resistant and digestible starch were determined using the enzyme kit K-RAPRS (Megazyme, Ireland) and freeze-dried samples of bread crumb.

6.3.6 Statistical Analysis

Both fermentation and baking trials were performed in triplicate. For the characterisation of fermentation profiles, measurements of cell count, pH, TTA, acids, sugars and antifungal compounds were performed in duplicate for each of the three independent fermentation trials. *In vitro* starch digestibility of bread samples was analysed in duplicate. Results are presented as the mean $\pm$ standard deviation.

Data analysis was carried out using RStudio, version 1.2.1335 with R version 3.6.1 (RStudio Inc, Boston, MA, USA; R Core Team, r-project). A correlation analysis (CA, significance level $p < 0.05$) was performed to determine correlation coefficients (r) for results of bread quality characteristics. One-way analysis of variance (ANOVA) with post-hoc pairwise Tukey's test was used to show significant differences ($p < 0.05$) between groups for data from both fermentation and baking trials. In addition, a two-way ANOVA was performed for fermentation characteristics in order to determine the influence of the substrate (DF/PR) and the fermentation time as well as the interactive effect of both of these factors.

6.4 Results and Discussion

6.4.1 Fermentation Profile

The growth and fermentation characteristics of *Ln. citreum* TR116 were monitored for a sourdough-like fermentation of two different FB ingredients over a fermentation time of 48 hours.

6.4.1.1 Microbial Growth and Acidification

The pH, TTA and cell count (Figure 6.1) of the fermentates were determined as key factors describing the fermentation characteristics of LABs in various matrices and media. While the results for pH and TTA reveal major differences in acidification, the cell counts are relatively similar and prove comparable growth of *Ln. citreum* TR116 in both DFSD and PRSD. The initial cell densities (0 h) in the fermentates reflect with 7.11 log CFU/g (DFSD) and 7.16 log CFU/g (PRSD) the inoculation level of 7 log CFU/g. Within the first six hours, cell densities substantially increased by approx. 2 log CFU/g to 8.95 log CFU/g in DFSD and 9.16 log CFU/g in PRSD. This point (6 h) marks the start of a stationary growth phase in both fermentates;

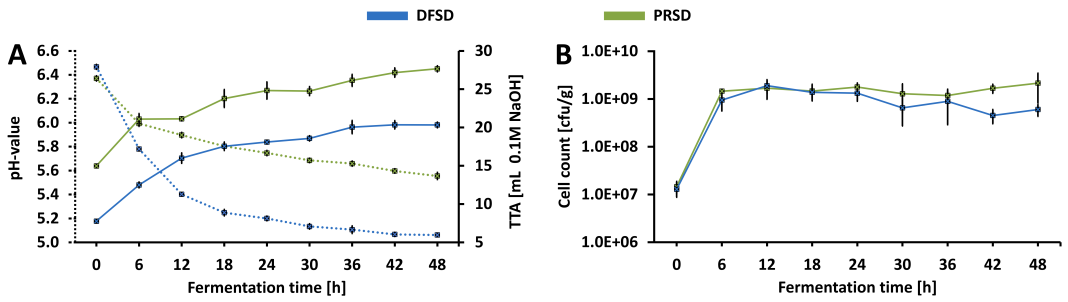


Figure 6.1: Acidification and microbial growth of *Leuconostoc citreum* TR116 in DFSD and PRSD during 48 h of fermentation: (**A**) Development of pH (represented by dotted lines) and TTA (represented by solid lines); (**B**) Determined cell densities.

no further significant increase in cell density was observed during the remaining fermentation time. While this stationary phase seems to last until the maximum fermentation time of 48 hours in PRSD, DFSD shows a tendency towards a decline of growth beginning at 30 hours. The determined cell densities in DFSD from 30 h forward are significantly lower than the corresponding cell counts in PRSD (except 36 h; no significant difference detected) as well as the maximum cell density in DFSD at 12 h (9.25 log CFU/g). This could be associated with increased stress and the beginning of a death phase of TR116 growth in DFSD. Sahin *et al.* [36] reported a similar growth pattern when *Ln. citreum* TR116 was used to ferment wheat flour (same DY and inoculation cell density) for 48 hours. However, the stationary phase of growth in wheat flour between 12 and 24 h seems to have been followed by a more pronounced death phase than in DFSD with a decline of cell density by 1.8 log CFU/g (compared to 0.5 log CFU/g in DFSD; calculated as difference to maximum cell density). The determined pH values in this study indicate a stronger acidification of DFSD than PRSD. While the initial pH at 0 h is similar for both fermentates (6.47 in DFSD and 6.37 in PRSD), this is followed by a much stronger pH drop in DFSD than in PRSD. Especially within the first 12 h, the acidification rate was with 0.09 h^{-1} more than twofold higher in DFSD than in PRSD with 0.04 h^{-1}. After this initial rapid decrease in pH, a more moderate decline was observed between 12 and 48 hours of fermentation with the same acidifcation rate of 0.0094 h^{-1} in both DFSD and PRSD. While this could have been associated with a higher production of organic acids in DFSD than in PRSD, the contents of acetic and lactic acid, which were additionally determined (Table 6.2), do not confirm such a correlation. On the contrary, slightly (but significantly; except 6 h) higher levels of acetic acid were produced in PRSD. The levels of lactic acid did not significantly differ (except 12 h) between DFSD and PRSD when the same fermentation time points are compared. This is also evident with regard to the generally slightly lower fermentation quotients obtained for PRSD. The fact that in PRSD both a smaller drop in pH and a slightly higher acid production was observed might be related to a higher buffering capacity. The significantly higher TTA values determined for PRSD support this theory. At all fermentation time points, more 0.1 M NaOH was needed to titrate PRSD fermentates to pH 8.5 than for DFSD fermentates. Considering the higher initial TTA of PRSD at 0 h and the relatively consistent difference of TTAs determined for DFSD and PRSD, it can be assumed that the buffering capacity does rather originate from compounds present in the raw material than from metabolites of TR116. Proteins are, besides peptides, amino acids and organic acids, the most important compounds affecting the buffering capacity in foods [48]. Due to the nearly twofold higher protein content in PR compared to DF, a higher buffering capacity can be expected in

PRSD. While in many sourdough-like fermentates the produced organic acids are likely to contribute to buffering capacity, this is expected to have only a minor impact in the fermentates in this study considering the pH ranges within which acetic acid (3.75–5.75) and lactic acid (2.86 – 4.86) have buffering capacity and the pH values reached in DFSD and PRSD [49]. Also minerals have been reported to cause increased buffering capacity in food matrices [48]. Hence, the higher abundance of minerals in PR than in DF (indicated by ash contents: 3.28 %DM in DF; 5.43 %DM in PR) could have further contributed to the elevated buffering capacity in PRSD. Since it is known that acidic environments can cause an inhibition of growth [31] for *Leuconostoc* species, the increased buffering capacity might also be the cause for the persisting stationary growth phase in PRSD by moderating both the pH drop and pH-associated stress for TR116. This is further in line with the previously mentioned findings of Sahin *et al.* [36] who reported lower pH values as well as a more pronounced decrease in cell density in wheat flour fermented with TR116. Two-way ANOVA identified a significant impact of both the chosen substrate (DF/PR) as well as the fermentation time on all variables discussed in this section (except FQ; only substrate impact was found significant). The impact of the interaction between substrate and fermentation was found to be significant only for the determined pH values. This is evident with regard to the curves displayed in Figure 6.1. The substrate influences the way the pH of the fermentates changes over time leading to strikingly different acidification rates within the first 12 hours of fermentation as mentioned above. In a study by Coda *et al.* [23] different air-classified faba bean fractions (faba bean dehulled

Table 6.2: Levels of lactic and acetic acid in faba bean fermentates, values expressed in %DM.

	Ferm. time [h]	Acetic acid^{S,T}	Lactic acid^{S,T}	Fermentation quotient^{S}
Faba bean	0	n.d.	n.d.	-
dehulled	6	0.45 ± 0.01^{l}	0.51 ± 0.01^{h}	0.76 ± 0.03^{cd}
flour	12	0.69 ± 0.00^{k}	0.95 ± 0.02^{g}	0.91 ± 0.02^{ab}
(DFSD)	18	0.75 ± 0.01^{jk}	1.13 ± 0.02^{ef}	1.00 ± 0.02^{a}
	24	0.84 ± 0.01^{ij}	1.23 ± 0.02^{def}	0.98 ± 0.02^{a}
	30	0.91 ± 0.01^{hi}	1.25 ± 0.01^{cdef}	0.92 ± 0.02^{ab}
	36	1.01 ± 0.02^{gh}	1.33 ± 0.02^{abcd}	0.88 ± 0.03^{abc}
	42	1.04 ± 0.03^{efg}	1.37 ± 0.04^{abc}	0.88 ± 0.06^{abc}
	48	1.16 ± 0.10^{cd}	1.32 ± 0.13^{bcd}	0.77 ± 0.15^{cd}
Faba bean	0	n.d.	n.d.	-
high-protein	6	0.56 ± 0.01^{l}	0.64 ± 0.02^{h}	0.75 ± 0.02^{cd}
flour	12	1.02 ± 0.00^{fg}	1.13 ± 0.02^{f}	0.74 ± 0.02^{d}
(PRSD)	18	1.07 ± 0.02^{defg}	1.25 ± 0.02^{cde}	0.78 ± 0.02^{cd}
	24	1.14 ± 0.03^{cde}	1.33 ± 0.03^{bcd}	0.78 ± 0.04^{cd}
	30	1.12 ± 0.05^{def}	1.35 ± 0.04^{abcd}	0.80 ± 0.06^{bcd}
	36	1.24 ± 0.06^{bc}	1.41 ± 0.06^{ab}	0.76 ± 0.07^{cd}
	42	1.29 ± 0.05^{ab}	1.45 ± 0.06^{a}	0.76 ± 0.07^{cd}
	48	1.37 ± 0.14^{a}	1.41 ± 0.13^{ab}	0.70 ± 0.14^{d}

Means $\pm$ standard deviation with different lower case letters in the same column are significantly different at $p < 0.05$.
n.d., Not detected or levels below 0.17 %DM
^{S} Two-way ANOVA confirmed significant effect ($p < 0.05$) of substrate (DF/PR) on this variable
^{T} Two-way ANOVA confirmed significant effect ($p < 0.05$) of fermentation time (0–6 h) on this variable

flour, air-classified protein-fraction; air-classified starch fraction) were fermented with a *Lactobacillus plantarum* strain (VTT E-133328) using the same dough yield, incubation temperature and inoculation cell density as applied in the present study. While the increase in cell density within the first 24 hours of fermentation was similar to the growth observed in this study, a significant difference was found between growth in the dehulled flour and the protein fraction (more growth in protein fraction). Moreover, they determined similar changes in pH for these two substrates and generally a more pronounced pH drop (ΔpH at 24 h: 1.85 (dehulled flour) or 1.86 (protein fraction) as opposed to 1.27 (DFSD) and 0.62 (PRSD) in this study). Although it is known that different LAB species show unique growth and acidification profiles in various substrates, a less pronounced pH drop due to increased buffering capacity of the protein fraction, as observed in the present study, could have been expected also for the fermentation with the respective *Lactobacillus plantarum* strain. While the ash content of the protein fraction investigated by Coda *et al.* [23] was with 5.98 %DM very similar to that of PR in the present study (5.43 %DM), a slightly lower protein content was reported (51.49 %DM in protein fraction in Coda *et al.* [23] as opposed to 61.25 %DM in PR). Potentially, this higher protein content in combination with the high ash level caused a substantially increased buffering capacity in PRSD.

6.4.1.2 Consumption and Degradation of Saccharides and Mannitol

The levels of relevant saccharides and mannitol were determined every 6 h over the total fermentation time of 48 hours and the obtained results are presented in Table 6.3. At 0 h of fermentation, only sucrose (and a small amount of glucose in PRSD) and the raffinose family oligosaccharides verbascose and stachyose/raffinose and galactose were detected in levels above 0.02 %DM for both DFSD and PRSD. The initially available amount of sucrose was with 1.93 %DM only slightly, but significantly, higher in DFSD than in PRSD with 1.42 %DM. The sucrose level rapidly decreased in both substrates and dropped below the LOQ of 0.02 %DM after 6 and 12 h in PRSD and DFSD, respectively. Glucose was only detected in very small quantities just above the LOQ at 6 h of fermentation (and 0 h in PRSD) in both substrates. Fructose was measured in both DFSD and PRSD at 6 and 12 h of fermentation with a decreasing trend. A considerable amount of mannitol, accounting for 0.60 %DM in DFSD and 0.51 %DM in PRSD, was produced within the first six hours of fermentation. The mannitol level then further increased significantly in both substrates reaching its maximum after 12 h in DFSD and after 18 h in PRSD. Thereafter, a relatively stable level of mannitol with a slight decreasing tendency was determined at all timepoints until the maximum fermentation time of 48 h in both substrates. *Leuconostoc* species have been reported to convert a

majority of sucrose into glucans (e.g. dextrans) and fructose via glucansucrases [31]. However, especially at early stages of fermentation, sucrose is primarily used as carbon source to support growth. High glucansucrase activity and high glucan yields are mainly observed subsequent to maximum growth rates (during stationary growth phase), for fermentations with sucrose supplementation and where cells were grown in sucrose containing media during inoculum preparation [31, 50–52]. For example, Xu *et al.* [27, 28, 29] and Wang *et al.* [26] reported substantial glucan production when fermenting sucrose supplemented faba bean flour with several *Leuconostoc* and *Weisella* strains (including the *Ln. citreum* strain DSM 5577) which were mostly grown in sucrose containing media prior to inoculation. The inoculums in the present study were prepared in MRS broth containing glucose as the main carbon source and little sucrose was available in both substrates. Therefore, it can be assumed that the majority of sucrose available in PRSD and DFSD was absorbed by the TR116 cells and subsequently converted into glucose-1-phosphate and fructose by sucrose phosphorylase utilising glucose as carbon source [34, 53]. This is further in line with the quick increase of lactic and acetic acid, (see Table 6.2) which are, besides ethanol, the main metabolites of glucose catabolism. While the proportions of the main metabolites generally depend on growth conditions, FQ values below 1, as observed in the present study, are mostly unusual in sourdough-like fermentations and lactic acid is commonly produced in molar excess of acetic acid. It has been reported, that under aerobic or semi-aerobic conditions and in the presence of alternative electron acceptors (other than O_2), the conversion of acetyl-P into acetate is preferred (additional gain of ATP) over its reduction to ethanol and simultaneous regeneration of NADH to NAD^+ [31, 34, 52]. This, potentially in combination with a partial incorporation of lactic acid in peptidoglycans, can lead to a substantial decrease in the molar ratio between lactic and acetic acid [31, 52, 54]. Several *Leuconostoc* species, and *Ln. citreum* TR116 in particular, have been reported to utilise fructose as an alternative electron acceptor to regenerate NAD^+ from NADH by reducing (mannitol-dehydrogenase) it to mannitol [31–34, 36]. This is in agreement with the determined fructose and mannitol concentrations in DFSD and PRSD in the present study, which simultaneously decrease (fructose) and increase (mannitol). The values indicate that the majority of fructose released from sucrose (considering the sucrose level in DFSD and PRSD at 0 h) was converted into mannitol. In agreement with the significantly higher sucrose level at 0 h in DFSD than in PRSD, DFSD exhibits higher concentrations of produced mannitol. Xu *et al.* [27, 28] also observed substantial mannitol production in combination with FQs as low as 0.77–0.9 for *Leuconostoc pseudomesenteroides* and *Leuconostoc citreum* strains when they performed single-strain fermentations of faba bean flour. However,

Table 6.3: Carbohydrate profile of faba bean fermentates, values expressed in %DM.

	Ferm. time [h]	Sucrose^{S,T}	Glucose^{S}	Fructose$^{T,S:T}$	Mannitol^{S}	Verbascose$^{S,T,S:T}$	Stachyose/Raffinose$^{S,T,S:T}$	Galactose$^{S,T,S:T}$
Faba bean	0	1.93 ± 0.05^{a}	n.d.	n.d.	n.d.	0.90 ± 0.02^{a}	0.96 ± 0.02^{cde}	0.04 ± 0.00^{l}
dehulled	6	0.03 ± 0.00^{c}	0.07 ± 0.01^{a}	0.39 ± 0.00^{a}	0.60 ± 0.01^{fgh}	0.84 ± 0.02^{b}	0.99 ± 0.02^{bcd}	0.28 ± 0.01^{j}
flour	12	n.d.	n.d.	0.03 ± 0.00^{d}	0.82 ± 0.02^{a}	0.72 ± 0.03^{c}	0.99 ± 0.03^{bcd}	0.48 ± 0.01^{i}
(DFSD)	18	n.d.	n.d.	n.d.	0.82 ± 0.01^{a}	0.56 ± 0.02^{e}	0.90 ± 0.03^{ef}	0.66 ± 0.02^{h}
	24	n.d.	n.d.	n.d.	0.77 ± 0.01^{ab}	0.49 ± 0.01^{f}	0.84 ± 0.01^{g}	0.75 ± 0.01^{g}
	30	n.d.	n.d.	n.d.	0.74 ± 0.01^{abc}	0.42 ± 0.03^{g}	0.77 ± 0.04^{hi}	0.86 ± 0.02^{f}
	36	n.d.	n.d.	n.d.	0.72 ± 0.03^{bcd}	0.37 ± 0.01^{h}	0.71 ± 0.02^{j}	0.95 ± 0.01^{e}
	42	n.d.	n.d.	n.d.	0.70 ± 0.02^{bcde}	0.32 ± 0.02^{ij}	0.63 ± 0.03^{k}	1.03 ± 0.03^{d}
	48	n.d.	n.d.	n.d.	0.65 ± 0.11^{defg}	0.28 ± 0.01^{j}	0.59 ± 0.03^{k}	1.12 ± 0.02^{c}
Faba bean	0	1.42 ± 0.04^{b}	0.03 ± 0.00^{b}	n.d.	n.d.	0.84 ± 0.04^{b}	0.95 ± 0.05^{cde}	0.13 ± 0.01^{k}
high-protein	6	n.d.	0.02 ± 0.01^{b}	0.25 ± 0.03^{b}	0.51 ± 0.03^{h}	0.81 ± 0.03^{b}	1.03 ± 0.04^{b}	0.46 ± 0.00^{j}
flour	12	n.d.	n.d.	0.17 ± 0.02^{c}	0.58 ± 0.02^{gh}	0.76 ± 0.02^{c}	1.09 ± 0.03^{a}	0.69 ± 0.01^{h}
(PRSD)	18	n.d.	n.d.	n.d.	0.67 ± 0.02^{cdef}	0.63 ± 0.02^{d}	1.01 ± 0.02^{bc}	0.87 ± 0.03^{f}
	24	n.d.	n.d.	n.d.	0.63 ± 0.02^{efg}	0.54 ± 0.01^{e}	0.93 ± 0.03^{def}	0.99 ± 0.03^{de}
	30	n.d.	n.d.	n.d.	0.63 ± 0.04^{efg}	0.47 ± 0.03^{f}	0.87 ± 0.04^{fg}	1.10 ± 0.06^{c}
	36	n.d.	n.d.	n.d.	0.61 ± 0.04^{efg}	0.42 ± 0.01^{g}	0.83 ± 0.01^{gh}	1.24 ± 0.02^{b}
	42	n.d.	n.d.	n.d.	0.60 ± 0.04^{fgh}	0.36 ± 0.01^{hi}	0.75 ± 0.01^{ij}	1.34 ± 0.03^{a}
	48	n.d.	n.d.	n.d.	0.52 ± 0.09^{h}	0.33 ± 0.02^{hi}	0.73 ± 0.04^{ij}	1.38 ± 0.05^{a}

Means ± standard deviation with different lower case letters in the same column are significantly different at $p < 0.05$.

n.d., Not detected or levels below 0.02 %DM.

^{S} Two-way ANOVA confirmed significant effect ($p < 0.05$) of substrate (DF/PR) on this variable.

^{T} Two-way ANOVA confirmed significant effect ($p < 0.05$) of fermentation time (0–6 h) on this variable.

$^{S:T}$ Two-way ANOVA confirmed significant interactive effect ($p < 0.05$) of substrate and fermentation time on this variable

low FQs primarily occurred when faba bean flour was supplemented with sucrose for fermentation. The impact of the conversion of fructose released from sucrose into mannitol on the organic acid profile (increase of acetate) has also been reported by Erten [55], Wisselink *et al.* [56] and Galle *et al.* [57] for several *Leuconostoc* and *Weissella* strains and by Rice *et al.* [34] for the *Ln. citreum* strain TR116 used in the present study. In addition to the carbohydrates discussed above, the levels of the α-galactosides verbascose and stachyose/raffinose as well as galactose were determined in this study (Table 6.3). α-Galactosides, also known as galactooligosaccharides or raffinose family oligosaccharides (RFO), are plant storage carbohydrates (particularly abundant in the seeds of legumes) consisting of α (1 →6) linked galactose molecules (one for raffinose, two for stachyose, three for verbascose) which are bound to sucrose. These compounds are not degraded during human gastro-intestinal digestion due to the absence of α-galactosidase and have therefore been associated with both antinutritional (causing flatulence) as well as prebiotic characteristics [58, 59]. In foods containing high levels of RFOs, a moderate reduction of these compounds seems generally desirable from a nutritional perspective. Similar concentrations of the saccharides were determined in DFSD and PRSD at 0 h. Verbascose and stachyose/raffinose were present in considerable amounts accounting for 0.90 %DM and 0.96 %DM (DFSD) and 0.84 %DM and 0.95 %DM (PRSD), respectively. Galactose on the other hand, was detected in much lower quantities with 0.04 %DM for DFSD and 0.13 %DM for PRSD. Throughout the fermentation process, a generally decreasing trend was observed for verbascose and stachyose/raffinose in both substrates, while galactose accumulated. The profile of stachyose/raffinose concentrations is characterised by an initial plateau (DFSD) or slight increase (PRSD) followed by a steady decrease starting at 18 h of fermentation in DFSD and between 24 and 30 h of fermentation in PRSD. This first accumulation of stachyose/raffinose is expected due to the equal abundance of verbascose and stachyose/raffinose and the fact that only a small proportion (approx. one sixth) of the initially detected stachyose/raffinose was identified as raffinose (additional separation on HPLC column, data not shown); which is in agreement with the findings of Xu *et al.* [27, 28, 29]. Therefore, the degradation of verbascose as well as stachyose to the respective breakdown products stachyose and raffinose causes an initial plateau/increase of the sum value for stachyose and raffinose. The observed degradation of RFOs is likely related to α-galactosidase activity during fermentation. Whether this α-galactosidase activity originates from endogenous α-galactosidase present in faba bean raw material [60] or from microbial α-galactosidase [61–63] expressed by *Ln. citreum* TR116 cannot be determined based on these results. However, the inability of *Leuconostoc citreum* to utilise RFOs due to the lack of α-galactosidase

has been mentioned in the literature as one of the distinguishing traits in comparison to other *Leuconostoc* species such as *Leuconostoc mesenteroides* [64, 65]. Therefore, a breakdown by endogenous faba bean α-galactosidase seems more likely. This is also in line with the findings of Xu *et al.* [27] who showed a substantial degradation (extant comparable to the present study) of RFOs as well as accumulation of galactose when they used the *Leuconostoc citreum* strain DSM 5577 to ferment native faba bean flour. Moreover, they observed no RFO degradation in a faba bean flour which was autoclaved prior to fermentation to inactivate plant-derived α-galactosidase. Considering the chemical composition of RFOs and the substantial accumulation of galactose in DFSD and PRSD in the present study, it can additionally be assumed that the RFO degradation led to the release of sucrose. However, since the decrease of RFO levels is quite slow, this sucrose could have been available only at later stages of fermentation when the growth of *Ln. citreum* had reached its stationary phase and in small quantities. Interestingly, no fructose accumulation and no further increase in mannitol was observed which indicates that fructose might have been utilised differently than at early stages of fermentation during exponential growth phase. Since the main metabolites of carbohydrate catabolism lactic and acetic acid still show a steady, but slower, increase after the first 18 to 24 h hours (see Table 6.2), both fructose and glucose released from sucrose were potentially used as carbon source. But also a utilisation of glucose for the production of small amounts of glucans via glucansucrases as observed by Xu *et al.* [27] for the fermentation of faba bean flour by *Ln. citreum* DSM 5577 cannot be excluded. Furthermore, the continuously low FQs (high acetate production relative to lactate) in combination with the lack of additional mannitol production indicates the presence of other alternative electron acceptors besides fructose. The observed accumulation of galactose is in agreement with the literature where the inability of *Leuconostoc citreum* strains to utilise galactose has repeatedly been reported [27, 64, 65]. However, considering the theoretical amount of galactose released from RFOs (mass balance), the quantity of accumulated galactose appears to be unexpectedly high and the origin of a proportion of galactose remains unexplained. It has been reported that faba beans contain considerable amounts of arabinogalactans and arabinogalactan-peptides, which belong to a class of compounds known as proteoglycans [66]. Due to proteolytic activity and acidification during the fermentation process, further galactose might have been released from these compounds.

6.4.1.3 Contents of Antifungal Phenolic Acids

Phenolic compounds, such as phenolic acids, are known to exert various biological activities. In plant-based food matrices, like faba bean flours, they often

occur esterified or bound to proteins or carbohydrates and only a smaller proportion is present in a free form as acids [67–69]. Phenolic compounds have also been shown to be metabolites of LAB and to be present in various fermented food products [70–72]. Besides their potential antioxidant activity *in vitro* and *in vivo* after ingestion and related health benefits [67, 73–75], phenolic acids have been reported to be advantageous with regard to the microbial shelf life of food products [76–80]. Specifically mould growth, which is one of the main causes of spoilage for bakery products [81], was found to be delayed in sourdough breads when high amounts of phenolic acids were produced/released during sourdough fermentation [45, 82, 83]. This positive impact on mould spoilage was attributed to a synergistic effect of the reduced pH, produced organic acids like lactic and acetic acid and additionally present phenolic acids [71, 72, 79, 82, 84]. In the present study, DFSD and PRSD were screened for the presence of 15 phenolic compounds/acids with antifungal activity. Only the five compounds presented in Table 6.4 were detected in quantities exceeding the LOQ of 2.50 mg/kg DM. 4-Hydroxybenzoic acid was found in both DFSD and PRSD at all fermentation time points. The levels are relatively stable throughout the fermentation. However, a slight increasing tendency was observed in DFSD; the corresponding difference between 0 h and 48 h levels thereby accounts for + 29%. Caffeic acid, coumaric acid and ferulic acid, which are hydroxycinnamic acid derivatives with similar chemical structures, were also found in the fermentates in this study. While ferulic acid could be quantified

Table 6.4: Contents of phenolic acids in faba bean fermentates, values expressed in mg/kg DM.

	Ferm. time [h]	4-Hydroxybenzoic acid$^{T,S:T}$	Caffeic acid$^{S,T,S:T}$	Coumaric acid$^{S,T,S:T}$	Ferulic acid$^{T,S:T}$	Phenyllactic acid^{T}
Faba bean dehulled flour (DFSD)	0	3.57 ± 0.05^{fg}	n.d.	n.d.	3.03 ± 0.16^{h}	n.d.
	6	3.80 ± 0.10^{cdef}	n.d.	n.d.	3.27 ± 0.21^{fgh}	n.d.
	12	3.60 ± 0.16^{efg}	4.80 ± 0.28^{gh}	n.d.	3.25 ± 0.18^{fgh}	7.57 ± 0.46^{gh}
	18	3.87 ± 0.04^{bcdef}	5.82 ± 0.21^{efg}	n.d.	3.69 ± 0.19^{defg}	10.45 ± 0.21^{fg}
	24	3.93 ± 0.07^{bcdef}	5.90 ± 0.13^{efg}	n.d.	4.02 ± 0.17^{de}	13.15 ± 0.14^{efg}
	30	4.10 ± 0.08^{abcdef}	5.99 ± 0.07^{ef}	2.52 ± 0.06^{e}	4.24 ± 0.153^{cd}	18.32 ± 1.20^{cde}
	36	4.40 ± 0.10^{abc}	6.41 ± 0.24^{def}	2.78 ± 0.07^{de}	4.64 ± 0.15^{bc}	21.27 ± 2.16^{c}
	42	4.54 ± 0.11^{a}	6.69 ± 0.20^{cde}	2.94 ± 0.03^{de}	5.05 ± 0.26^{ab}	24.02 ± 2.61^{bc}
	48	4.60 ± 0.08^{a}	6.45 ± 0.27^{def}	2.99 ± 0.07^{de}	5.22 ± 0.18^{a}	29.40 ± 5.30^{ab}
Faba bean high-protein flour (PRSD)	0	4.22 ± 0.53^{abcde}	n.d.	n.d.	3.15 ± 0.24^{gh}	n.d.
	6	4.15 ± 0.12^{abcdef}	3.60 ± 0.85^{h}	n.d.	3.86 ± 0.21^{def}	3.38 ± 0.08^{h}
	12	3.20 ± 0.43^{g}	5.46 ± 0.60^{fg}	n.d.	2.95 ± 0.29^{h}	7.94 ± 0.98^{gh}
	18	4.23 ± 0.13^{abcd}	7.77 ± 0.14^{bc}	3.95 ± 0.21^{cd}	3.69 ± 0.08^{defg}	14.66 ± 0.66^{def}
	24	3.75 ± 0.31^{defg}	7.42 ± 0.63^{bcd}	4.58 ± 0.43^{bc}	3.65 ± 0.25^{efg}	17.94 ± 2.42^{cde}
	30	4.27 ± 0.17^{abcd}	8.96 ± 0.64^{a}	5.26 ± 0.71^{ab}	4.11 ± 0.08^{cde}	20.30 ± 1.84^{cd}
	36	3.83 ± 0.50^{cdef}	8.36 ± 1.02^{ab}	5.26 ± 0.99^{ab}	3.98 ± 0.47^{de}	21.26 ± 3.21^{c}
	42	4.43 ± 0.43^{ab}	9.15 ± 0.88^{a}	5.89 ± 0.94^{a}	4.97 ± 0.38^{ab}	28.25 ± 3.22^{ab}
	48	4.23 ± 0.27^{abcd}	9.06 ± 0.45^{a}	6.27 ± 1.03^{a}	5.14 ± 0.48^{ab}	33.54 ± 7.12^{a}

Means ± standard deviation with different lower case letters in the same column are significantly different at $p < 0.05$.
n.d., Not detected or levels below 2.50 mg/kg DM.
^{S} Two-way ANOVA confirmed significant effect ($p < 0.05$) of substrate (DF/PR) on this variable.
^{T} Two-way ANOVA confirmed significant effect ($p < 0.05$) of fermentation time (0–6 h) on this variable.
$^{S:T}$ Two-way ANOVA confirmed significant interactive effect ($p < 0.05$) of substrate and fermentation time on this variable.

in DFSD and PRSD at 0 h, caffeic and coumaric acid were not present in levels above the LOQ at the start of the fermentation process. For all three hydroxycinnamic acid derivatives, a clear increase in concentrations was observed during fermentation reaching their highest levels at 42 h or 48 h of fermentation (except for coumaric acid in DFSD, where continuously low levels between 2.52 mg/kg DM (30 h) and 2.99 mg/kg DM (48 h) were measured). The determined increase in ferulic acid was similar for PRSD and DFSD with + 72% and + 63%, respectively. Caffeic acid and coumaric acid were present in levels exceeding the LOQ at shorter fermentation times in PRSD (6 h and 18 h, respectively) than in DFSD (12 h and 30 h, respectively). Additionally, significantly higher contents of caffeic and coumaric acid were reached after 48 hours of fermentation in PRSD (9.06 mg/kg DM and 6.27 mg/kg DM, respectively) than in DFSD (6.45 mg/kg DM and 2.99 mg/kg DM, respectively). The highest concentrations at the maximum fermentation time of 48 hours were observed for phenyllactic acid accounting for 29.40 mg/kg DM in DFSD and 33.43 mg/kg DM in PRSD. This represents a drastic increase since phenyllactic acid was below 2.50 mg/kg DM at 0 h of fermentation and started exceeding the LOQ only at 12 h of fermentation in DFSD and at 6 h in PRSD. While *Ln. citreum* TR116 has not previously been associated with the production of phenolic acids, the observed increase in phenolic acid contents during fermentation of DFSD and PRSD could be related to the metabolic pathways of this strain. Specifically phenyllactic acid, which is the phenolic acid with the highest increase in this study, has been described as a metabolite of LAB and was found to be produced by transamination and subsequent reduction of phenylalanine [85, 86]. The fact that FB protein exhibits a relatively high abundance of phenylalanine and that both DF and PR are relatively rich in protein, theoretically leads to a high availability of this precursor for phenyllactic acid in both substrates [19]. This is further supported by the tendency towards slightly higher phenyllactic acid levels reached in PRSD. However, also the possibility of a release of plant-derived phenolic acids due to a degradation of plant constituents the phenolic acids are usually bound to (proteins, carbohydrates) has been mentioned in the literature and seems to be a probable explanation for the trends observed in this study [87–90]. Especially the hydroxycinnamic acid derivatives might be released rather than produced during fermentation, since these compounds have been reported to occur largely as ester conjugates in faba bean plants [91–96]. The release can be mediated by proteolytic activity and acidification during fermentation or by activity of endogeneous or microbial enzymes. Feruloyl esterases, for example, have been found to be produced by various LAB species and to degrade the ester bonds of hydroxycinnamoyl esters [76, 87, 97]. The more pronounced increase of the three hydroxycinnamic acid derivatives in PRSD suggests that air-classification

might lead to a higher abundance of bound phenolic acids in the high-protein fraction (PR). The findings in the present study are in agreement with Coda *et al.* [98] who reported an increase in total phenolic content and antioxidant activity during sourdough-type propagation of a dehulled FB flour and an air-classified high-protein FB flour. They also associated this trend with improved extractability of phenolics due to lactic acidification and potential esterase activity. As mentioned above, the continuously low FQs (high acetate production relative to lactate) in combination with the lack of additional mannitol production at later stages of fermentation (from fructose liberated from RFOs) indicate the presence of other alternative electron acceptors besides fructose. It has been described that phenolic compounds such as hydroxycinnamic acids can be used as alternative electron acceptors by LAB [70, 87, 99]. With regard to a beneficial effect of the increased content of phenolic acids both their antioxidant and antifungal activity should be considered. Although a low pH was described to be part of the synergistic antifungal effect in fermented foods and the pH drop observed for DFSD and PRSD in this study was quite moderate, a positive influence on the microbial shelf life of breads containing DFSD and PRSD can be expected.

6.4.2 Baking Properties of Sourdough-Like Fermentates

To evaluate the baking performance of the produced FB fermentates, five different bread formulations were prepared. In addition to a wheat flour control, FB breads were produced by replacing 15% of wheat flour by either unfermented or fermented (48 h) FB ingredient (based on solids in ingredient). For the FB ingredients PR (unfermented) and PRSD (fermented) this replacement level ensures a protein content of > 20% of calories provided by protein in the final bread products (calculated based on compositional data of ingredients and recipe according to Table 6.1). This level is defined as the requirement for a 'high in protein' nutrition claim made on food products according to European legislation [100].

6.4.2.1 Water Absorption and Gluten-Aggregation

The influence of the partial replacement of wheat flour by unfermented and fermented FB ingredients on water absorption and gluten-aggregation characteristics was determined by Farinograph and GlutoPeak measurements. The water amount required to reach a dough consistency of 500 FU in the Farinograph measurements was determined for the five respective (theoretical) flour mixtures and used as optimal water addition level for the baking trials. The determined Farinograph water absorptions (FWAs) also reflect the impact of FB ingredient incorporation and FB ingredient fermentation on the water absorption characteristics in wheat

Table 6.5: Flour properties of wheat flour (used for control) and faba bean flour mixes (used for faba bean formulations) containing unfermented faba bean dehulled flour (DF), fermented faba bean dehulled flour (DFSD), unfermented faba bean high-protein flour (PR) or fermented faba bean high-protein flour (PRSD).

Variable	Wheat flour	DF flour mix	DFSD flour mix	PR flour mix	PRSD flour mix
Farinograph					
Farinograph water absorption (FWA)* [%]	60.6 ± 0.1^{c}	60.7 ± 0.1^{c}	61.3 ± 0.0^{b}	59.9 ± 0.1^{d}	62.4 ± 0.2^{a}
GlutoPeak					
Peak maximum time (PMT) [s]	46 ± 1^{b}	59 ± 1^{a}	37 ± 2^{c}	30 ± 1^{d}	32 ± 1^{d}
Torque maximum (TM) [BU]	77 ± 0^{a}	50 ± 1^{d}	57 ± 0^{bc}	55 ± 0^{c}	58 ± 1^{b}

* Measured water absorption corrected for water amount present in faba bean fermentates (DFSD and PRSD)
Means ± standard deviation with different lower case letters in the same row are significantly different at $p < 0.05$.

flour based bread dough systems. The obtained FWAs are displayed in Table 6.5. Although significant differences were detected, all of the FWAs determined in this study were relatively similar considering the big differences which can be observed just between different wheat flours [101]. The replacement of 15% wheat flour led to no change in FWA for DF flour mix and only a small decrease was observed for PR flour mix. The reduced FWA for PR flour mix might be associated with the higher protein content and the relatively high solubility of faba bean protein as described by Hoehnel *et al.* [12]. The incorporation of the fermented FB ingredients resulted in a significant increase in FWAs in comparison to the respective flour mixtures with unfermented FB ingredients as well as in comparison to wheat flour alone. Organic acids are known to increase FWAs [102, 103]. A higher number of charges (like dissociated acids) in the system increases intramolecular repulsive forces and therefore promotes the unfolding of gluten proteins which leads to more swelling and higher water absorption [104]. Furthermore, Vogelsang-O'Dwyer *et al.* [19] reported a drastically decreased solubility of faba bean protein between pH 3.6 and 6.0. Both FB fermentates in the present study exhibit pH values in this range. This can lead to an increased water absorption of the faba bean proteins in DFSD and PRSD compared to DF and PR, respectively. The fact that the FWA increase was more pronounced for PRSD flour mix, which contains more faba bean protein, than for DFSD flour mix further supports this hypothesis. The gluten-aggregation properties of wheat flour and flour mixtures containing FB ingredients were determined using the GlutoPeak device. The measurements indicated striking differences between the five samples and the variables obtained from the curves are presented in Table 6.5. A PMT of 45.7 s and a TM of 77.0 BU was measured for wheat flour. The partial replacement of wheat flour by DF and PR led to significantly lower TMs accounting for 49.7 BU and 54.7 BU, respectively. While the TM was reached significantly earlier for PR flour mix (32.0 s), a delay was observed for DF flour mix (58.5 s). The values obtained for DFSD flour mix and PRSD flour mix suggest that fermentation leads to a more similar impact of

the two ingredients on gluten-aggregation as opposed to the differing influences found for DF flour mix and PR flour. No significant difference was detected for the TMs of DFSD flour mix and PRSD flour mix with 56.7 BU and 58.3 BU, respectively. Furthermore, the TMs were reached significantly earlier than for wheat flour in both DFSD flour mix (36.7 s) and PRSD flour mix (32.0 s). A general trend with earlier and higher peaks for stronger wheat flours with higher contents and quality of gluten has been reported in the literature for measurements of pure wheat flours [105–107]. Due to the partial replacement of wheat flour, all FB flour mixtures contain considerably less gluten and generally lower as well as later gluten peaks could have been expected for these samples. Additionally, based on this general trend, the values indicate improved gluten-aggregation in DFSD flour mix (significantly lower PMT and higher TM) and PRSD flour mix (significantly higher TM) when compared to DF flour mix and PR flour mix, respectively. However, it has been shown that more factors than solely the gluten content affect GlutoPeak measurements [12, 13, 36, 108, 109] and, as previously suggested by Hoehnel *et al.* [12], partial wheat flour replacement leads to complex changes in gluten-aggregation profiles which do not necessarily follow this general trend. This is why, in addition to PMTs and TMs, a comparative evaluation of the gluten-aggregation profiles (Figure 6.2) is required. The GlutoPeak curve of wheat flour shows a well described profile of initial torque increase, equilibrium plateau, rapid torque increase and gluten peak followed by a decrease in torque caused by a beginning breakdown of the gluten-network. Goldstein *et al.* [110] associated a fast gluten-network development (fast torque increase), a sharp gluten peak and rapid breakdown with weak flours. The more pronounced equilibrium plateau in

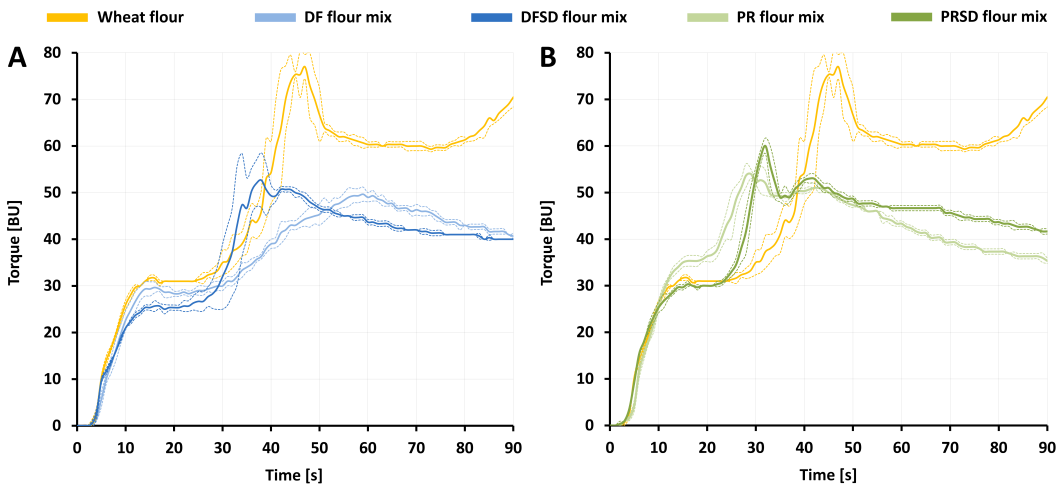


Figure 6.2: Flour properties of wheat flour and HP flour mix: (Gluten-aggregation profiles obtained by GlutoPeak test: (**A**) DF flour mix, DFSD flour mix compared to wheat flour; (**B**) PR flour mix and PRSD flour mix compared to wheat flour. Dashed curves represent standard deviation.

DF flour mix, DFSD flour mix and PRSD flour mix therefore suggests the formation of a stronger gluten-network when compared to PR flour mix. Bouachra *et al.* [108] described a drastically accelerated gluten-aggregation in the presence of lactic acid, which is in agreement with the difference in PMT between DF flour mix and DFSD flour mix. However, Bouachra *et al.* [108] also associated this trend with low pHs caused by the addition of lactic acid and a related increase in positive charges in the system, which increases intramolecular repulsive forces, unfolding of gluten proteins and their aggregation. The fact that there was no significant change in PMT between PR flour mix and PRSD flour mix could be related to the considerably less pronounced pH drop during fermentation of PRSD compared to DFSD. Another remarkable change in the gluten-aggregation profiles is the more distinct second peak in DFSD flour mix and PRSD flour mix when compared to the respective flour mixtures containing unfermented FB ingredients. This second peak has previously been attributed to potential co-networking of gluten and non-wheat proteins subsequent to a beginning breakdown of the gluten-network [12]. The presented results therefore suggest a stronger co-networking of faba bean proteins subjected to the fermentation process with gluten. The slightly more acidic pH faba bean proteins were subjected to during fermentation might have altered their structure and led to partial unfolding and an associated exposure of hydrophobic regions which are shielded within the native protein structure at a neutral pH. This could, in turn, facilitate co-networking with gluten proteins since hydrophobic interactions play an important role during gluten-network formation. Overall, the GlutoPeak measurements show inferior gluten-aggregation in flour mixtures containing FB ingredients in comparison to plain wheat flour. But also a substantial improvement of gluten-aggregation behaviour of flour mixtures containing FB ingredients induced by the fermentation process was indicated.

6.4.2.2 Bread Quality

Photographs of the five bread formulations produced in this study are displayed in Figure 6.3. A visual evaluation of the photographs immediately reveals striking differences in size, crumb structure, crust colour and crumb colour of these bread formulations. The determined technological bread quality characteristics confirm this observation and are presented in Table 6.6. Only small differences in dough pH were observed. The incorporation of unfermented FB ingredients DF and PR led to no significant changes in dough pH. Even the slightly lower pH of the PRSDB dough did not represent a significant difference compared to CWB. Only the incorporation of DFSD caused a significantly lower pH of the bread dough, which is in line with the more pronounced pH drop observed for DFSD than for PRSD. The bake loss of DFB (11.4%) and PRB (10.6%) was significantly lower than

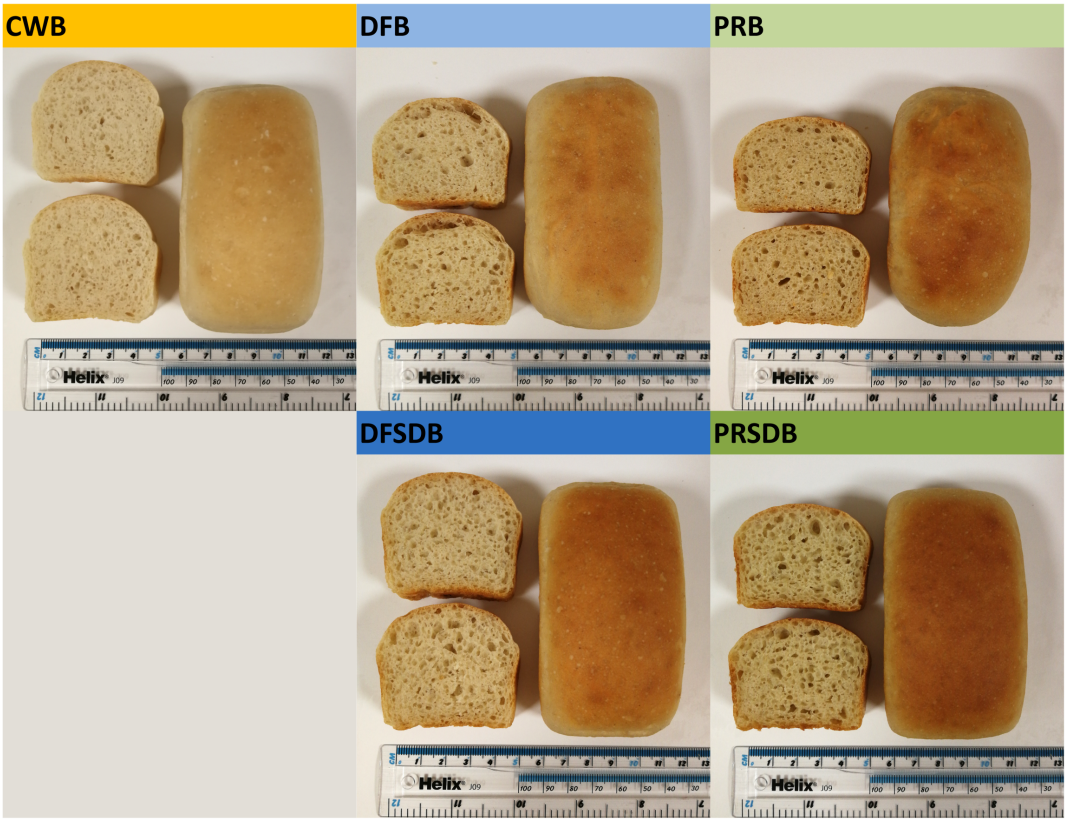


Figure 6.3: Photographs of control wheat bread and faba bean breads.

that of CWB (12.0%). This trend was not observed for the breads containing the fermented FB ingredients which showed bake losses higher than or similar to CWB with 12.8% (DFSDB) and 12.2% (PRSDB). With regard to one of the most indicative bread quality characteristics, the specific volume (SV), the incorporation of all FB ingredients (DF, DFSD, PR and PRSD) led to a significant decrease when compared to CWB with an SV of 3.09 mL/g. However, the extent of this general trend differed amongst the FB ingredient containing bread formulations. The incorporation of the

Table 6.6: Bread quality characteristics of control wheat bread (CWB) and faba bean breads containing unfermented faba bean dehulled flour (DFB), fermented faba bean dehulled flour (DFSDB), unfermented faba bean high-protein flour (PRB) or fermented faba bean high-protein flour (PRSDB).

Variable	CWB	DFB	DFSDB	PRB	PRSDB
pH of dough	5.76 ± 0.04^{a}	5.79 ± 0.07^{a}	5.47 ± 0.02^{b}	5.77 ± 0.02^{a}	5.69 ± 0.06^{a}
Bake loss [%]	12.0 ± 0.7^{b}	11.4 ± 0.5^{c}	12.8 ± 0.9^{a}	10.6 ± 0.9^{d}	12.2 ± 0.9^{b}
Specific volume (SV) [mL/g]	3.09 ± 0.12^{a}	2.42 ± 0.09^{d}	2.96 ± 0.10^{b}	2.16 ± 0.07^{e}	2.58 ± 0.09^{c}
Hardness day 0 [N]	6.31 ± 1.01^{e}	15.65 ± 1.51^{b}	8.02 ± 1.01^{d}	18.50 ± 1.4^{a}	11.44 ± 1.78^{c}
Hardness day 2 [N]	13.04 ± 1.46^{d}	30.41 ± 4.13^{b}	14.79 ± 1.48^{d}	33.51 ± 2.83^{a}	19.17 ± 2.26^{c}
Hardness day 5 [N]	20.65 ± 2.57^{e}	39.33 ± 3.26^{b}	22.66 ± 2.59^{d}	44.53 ± 4.99^{a}	28.04 ± 2.84^{c}
Staling rate [ΔN/d]	2.31 ± 0.36^{a}	1.53 ± 0.25^{c}	1.86 ± 0.43^{b}	1.42 ± 0.31^{c}	1.49 ± 0.34^{c}
Number of cells	1527 ± 95^{a}	1338 ± 94^{b}	1243 ± 80^{bc}	1454 ± 123^{a}	1144 ± 129^{c}
Cell area [%]	48.4 ± 1.1^{a}	46.9 ± 1.0^{b}	49.6 ± 1.0^{a}	46.3 ± 1.6^{b}	49.4 ± 1.0^{a}
Lightness of crumb (L*crumb)	76.9 ± 2.7^{a}	74.7 ± 2.0^{b}	71.5 ± 1.4^{cd}	73.2 ± 1.9^{bc}	70.6 ± 2.3^{d}
Lightness of crust (L*crust)	77.3 ± 2.6^{a}	70.5 ± 3.8^{b}	64.7 ± 2.5^{cd}	63.3 ± 5.0^{c}	61.7 ± 2.5^{d}

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

unfermented ingredient PR led to the lowest SV observed in this study (2.16 mL/g). For DF, only a moderate decrease in SV (to 2.42 mL/g) was found. This suggests a generally better baking performance of DF when compared to PR. Interestingly, fermentation seems to significantly improve the baking performance of both FB ingredients. The extent to which fermentation improved the FB ingredients' baking performance was similar. DFSDB showed with 2.96 mL/g an SV which was significantly higher than that of DFB and only slightly lower than that of CWB. Also PRSDB had with 2.58 mL/g a much higher SV than PRB and even reached a higher SV than DFB. This agrees well with the results obtained from GlutoPeak measurements which indicated a better gluten-aggregation behaviour with potentially improved gluten quality in the presence of both fermented FB ingredients as opposed to unfermented DF and PR. Crumb hardness is another important indicator of bread quality. A strong negative correlation of crumb hardness determined after 0 days of storage with SV was observed in this study (r:-0.99). Hardness values (0 d of storage) therefore follow the same trends (inverse) as described for SVs. The significantly lower crumb hardness of DFSDB and PRSDB (compared to DFB and PRB, respectively) represents further evidence that the performed sourdough-like fermentation of DF and PR leads to a substantial upgrade of the ingredients' baking performance. In addition, crumb hardness was determined after two and five days of storage. The calculated staling rates show that breads containing FB ingredients have a smaller increase in crumb hardness during storage, relative to their initial crumb hardness. However, also DFB, DFSDB, PRB and PRSDB still show substantial staling and, compared to CWB, significantly harder crumb after two and five days of storage. With regard to the bread formulations' crumb structure, the variables number of cells and cell area were evaluated. The number of cells significantly decreased when FB ingredients were included in the formulation (except for PRB, number of cells with 1454 not significantly different from that of CWB with 1527). This decrease was significantly more pronounced when the fermented instead of unfermented FB ingredients were applied. Also a reduced cell area was measured for DFB (46.9%) and PRB (46.3%) in comparison to CWB with 48.4%. The breads containing DFSD and PRSD on the other hand showed with 49.6% and 49.4% cell areas similar to CWB. These results indicate a denser crumb structure in DFB and PRB, while DFSDB and PRSDB exhibit crumb structures more similar to CWB with a slight tendency towards bigger cells, which can also be visually observed in the photographs presented in Figure 6.3. This, in addition to the higher SV, might have contributed to the lower crumb hardness values determined for DFSD and PRSD in comparison to DFB and PRB, respectively. Both crust and crumb lightness were reduced due to application of FB ingredients. Considering the fact that both raw materials PR and DF exhibit lower lightness values than

wheat flour (data not shown), such a trend was expected. Also Hoehnel *et al.* [12] reported a substantially reduced lightness of crust when 15% of wheat flour was replaced by PR. Additionally, the decrease in lightness of crust and crumb was stronger for breads containing DFSD and PRSD. This can be associated with a potential proteolytic activity during fermentation (either by activated plant-derived enzymes due to pH drop or mediated by microbial proteolytic enzymes produced by TR116). This is likely to increase the availability of components (mainly primary amines) involved in the Maillard reaction, which is besides caramelisation the main contributor to crust browning [111, 112]. Several previous studies investigating faba bean fermentation largely focused on EPS (dextrans) production as a potential tool to improve the techno-functional value of faba bean flours including its rheological properties and baking performance [26, 28, 113]. Wang *et al.* [26] prepared sourdough-like fermentates from faba bean flours with two different EPS producing LAB strains (*Weissella confusa* VTT E-143403 and *Leuconostoc pseudomesenteroides* DSM 20193) with and without sucrose supplementation to trigger EPS production. Breads were prepared by replacing 30% of wheat flour by faba bean fermentates (based on solids). While faba bean fermentates produced with the *Weissella* strain (with and without sucrose supplementation) significantly improved bread volume, a negative impact was observed for the *Leuconostoc* fermentate (+ sucrose). This difference was associated with a lower amount of dextrans produced by the *Leuconostoc* strain, a potentially different, and less beneficial, polymer structure of the dextrans as well as a higher acidity of the *Leuconostoc* fermentates (since intensive acidification was shown to negatively affect bread volume, crumb hardness and staling [114]). Also Coda *et al.* [115] observed a decreased bread volume and increased crumb hardness (in comparison to both a wheat flour control and composite faba bean/wheat bread) when 30% of wheat flour were replaced by a faba bean ingredient fermented with *P. pentosaceus* I02. In the present study, no major EPS production can be expected as explained in the sections concerning the fermentation profile of *Ln. citreum* TR116 in DFSD and PRSD above. However, a substantial improvement of the baking performance of both FB ingredients was achieved through fermentation. GlutoPeak results in combination with the fermentate characterisation and the obtained bread quality characteristics reveal that the improvement of the FB ingredients' baking performance is largely related to an improved gluten-aggregation in the presence of fermented FB ingredients as opposed to unfermented DF and PR.

6.4.3 *In Vitro* Starch Digestibility and Indication of Glycaemic Index

Besides technological characteristics, the nutritional quality of bread is crucial. The kinetics of starch digestibility *in vivo* are of major importance and indicative of the resulting blood sugar levels and, therefore, the glycaemic index (GI) of foods. An elevated risk for certain types of cancer, heart disease and diabetes has been associated with the consumption of high-GI and high-GL diets that are primarily based on food products containing high levels of rapidly digestible starch [116–120]. It has been reported that the food structure can have a major impact on the accessibility of starch by pancreatic α-amylase and, hence, on starch digestibility [121–123]. In pasta, for example, it is believed that the dense structure and coagulated protein network surrounding the starch granules, which is formed during the cooking process, delays starch accessibility by enzymes, leading to a low GI [124, 125]. Furthermore, Holt *et al.* [126] observed a lower glycaemic response for a high-protein bread when equal-energy portions of regular wheat bread and a high-protein bread were consumed. But also dietary fibre compounds, and specifically those that cause an increase in viscosity, can lead to a delay in starch digestibility [122]. The performance of a starch digestion *in vitro* and the evaluation of obtained sugar release curves can give an indication of sugar release and blood glucose response *in vivo*. The curves for the release of reducing sugars (expressed as a percentage of digestible starch) for all five bread samples investigated in the present study are displayed in Figure 6.4. The curves for all the five bread formulations appear quite similar at first sight, which indicates that their release of reducing sugars during amylolytic treatment is similar despite varying contents of digestible starch. A

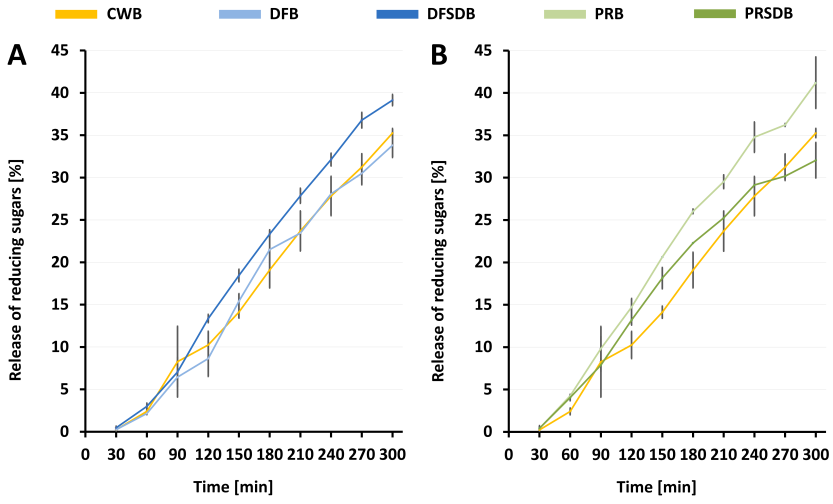


Figure 6.4: Sugar release curves expressed as reducing sugars as percentage of digestible starch: (**A**) DFB and DFSDB compared to CWB; (**B**) PRB and PRSDB compared to CWB.

closer look reveals several details which distinguish breads containing fermented FB ingredients from those that contain unfermented FB ingredients. Also a difference between DFB and DFSDB on one side and PRB and PRSDB on the other side can be observed. While DFB and DFSDB both exhibit similar sugar release as CWB within the first 90 min, further treatment with α-amylase thereafter leads to a gap between DFB and DFSDB. DFB closely follows the curve of CWB, whereas the starch in DFSDB seems to be more susceptible to a rapid breakdown. Interestingly, an assessment of the curves obtained for PRB and PRSDB reveals a trend opposing that of DFB and DFSDB. Here, the starch in the bread containing the unfermented ingredient (PRB) appears to be rapidly digested and PRSDB shows the flatter curve, more similar to that of CWB. However, the PRSDB curve still indicates a slightly faster starch digestion than for DFB. Furthermore, the initial release of reduced sugars within the first 150 min of *in vitro* digestion is generally higher for PRB and PRSDB than for DFB and DFSDB. These trends are also well reflected by the calculated hydrolysis indices (HIs) which are presented in Table 6.7. DFB exhibits a HI of 99.9%, which indicates a sugar release within 240 min of digestion that is very similar to CWB. Fermentation of DF led to an accelerated starch digestion represented by a HI of 119.0% determined for DFSDB. The highest HI in this study was obtained for PRB with 133.4%. For this FB ingredient, the HI of PRSDB (114.9%) indicates that fermentation caused a slower starch digestion. The fact that, for DF, fermentation seemed to accelerate or facilitate starch digestion in the present study does not follow the trends observed by Coda *et al.* [23] who performed *in vitro* starch digestion trials with both fermented faba bean flour as well as fermented air-classified faba bean high-protein flour. They found significantly decreased HIs in both fermented faba bean ingredients compared to the unfermented flours. However, these results were determined for the raw ingredients. Therefore, they do not account for the impact of FB ingredients on starch originating from wheat flour or for the influence of processing conditions during bread production on

Table 6.7: Values for resistant, digestible and total starch reported in %DM and hydrolysis indices (HIs) determined by *in vitro* starch digestion and monitoring of released reducing sugars over a time of 240 min for control wheat bread (CWB) and faba bean breads containing unfermented faba bean dehulled flour (DFB), fermented faba bean dehulled flour (DFSDB), unfermented faba bean high-protein flour (PRB) or fermented faba bean high-protein flour (PRSDB).

Variable	CWB	DFB	DFSDB	PRB	PRSDB
Resistant starch [%DM]	1.18 ± 0.02^{ab}	1.45 ± 0.16^{a}	1.26 ± 0.00^{a}	0.93 ± 0.04^{b}	0.95 ± 0.04^{b}
Digestible starch [%DM]	70.54 ± 0.65^{a}	66.75 ± 0.08^{b}	67.06 ± 0.35^{b}	61.96 ± 0.12^{c}	61.96 ± 1.17^{c}
Total starch [%DM]	71.72 ± 0.67^{a}	68.20 ± 0.08^{b}	68.32 ± 0.35^{b}	62.89 ± 0.15^{c}	62.91 ± 1.13^{c}
Hydrolysis index [%]	100*	99.9 ± 3.8^{c}	119.0 ± 3.7^{ab}	133.4 ± 4.4^{a}	114.9 ± 2.3^{b}

* HIs of DFB, DFSDB, PRB and PRSDB calculated as areas under their sugar release curves (30 to 240 min) relative to the area under CWB's sugar release curve (30 to 240 min).

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

starch digestibility. Still, in another study Coda *et al.* [115] reported a decreased GI when they replaced 30% of wheat flour by faba bean sourdough (based on solids). Altough Gobbetti *et al.* [77] mentioned a generally reducing effect of the use of sourdough technology on the glycaemic response of bread products, it was pointed out that varying results were reported in the literature depending on processing conditions, final bread quality and the application of dietary fibre or wholegrain ingredients. A decrease in GI in connection with sourdough fermentation was also associated with the presence of low pH values between 3.5 and 4.0 [77]. Furthermore, lactic acid was shown to be one of the main factors to lower the rate of starch digestion in *in vitro* trials [77, 122, 127]. Acetic acid and propionic acid on the other hand were found to play a role in reducing the rate of gastric emptying [77, 122, 127, 128] which is an effect that cannot be accounted for with the *in vitro* digestion method performed in the present study. Scazzina *et al.* [129] concluded that the mechanism through which sourdough fermentation reduces the GI of breads does not seem to be related to an alteration of the rate of starch hydrolysis. Another key factor for starch digestibility is its gelatinisation. A higher degree of gelatinisation generally correlates with higher rates of starch hydrolysis since the amorphus structure of gelatinised starch offers an increased availability of binding sites for α-amylase [130, 131]. The results of the present study do not seem to agree with these generally observed trends reported in the literature. However, the presented findings can potentially be explained considering the major differences between the applied FB ingredients. The primary constituent of DF is starch. PR on the other hand contains mainly protein and only a very small amount of starch. While the incorporation of DF did not seem to affect the rate of starch hydrolysis, the fermented version of DF, DFSD, led to an increased susceptibility of starch in DFSDB to hydrolysis. It can be hypothesised that the fermentation process made faba bean starch more accessible, more susceptible to gelatinisation during baking and, therefore, more rapidly digestible. In the case of PRB and PRSDB, however, this cannot be a valid explanation because of the very low starch content of PR. Water addition levels for the bread formulations in this study were determined in Farinograph trials. While faba bean protein is likely to contribute to this water absorption (late peak visible in farinograms, data not shown) it might not hold water as well as for example gluten. Gluten is known to have thermosetting properties, which leads to a moderate water redistribution during baking and facilitated starch gelatinisation. In the presence of faba bean protein, more water might be available for starch gelatinisation and a higher degree of gelatinisation could be the result. Higher water availability might be caused by a lower water holding capacity of faba bean proteins when heat treated or by the weakened gluten network in the presence of faba bean proteins as observed

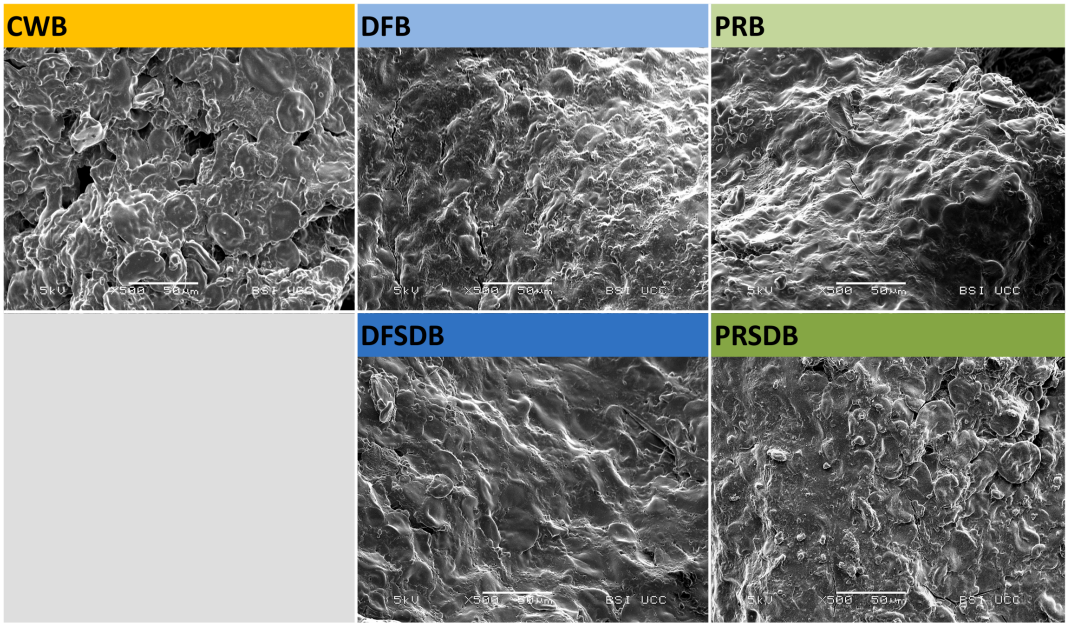


Figure 6.5: Micrographs (obtained by SEM) of crumb from control wheat bread and faba bean breads.

in GlutoPeak measurements. This would explain the substantially increased HI determined for PRB in the present study. It has been described above that fermentation and the associated moderate reduction of pH might induce changes in the faba bean protein structure (unfolding, agglomeration) in PRSD which is in agreement with the increased FWA and potentially improved co-networking with gluten proteins indicated by the GlutoPeak curve. These structural changes and the improved gluten-aggregation might lead to lower water availability for starch gelatinisation during baking when compared to PRB and could be an explanation for the lower HI of PRSDB in comparison to PRB. This also means that, specifically for PRB and PRSDB, an adjustment of the processing conditions during bread production, such as water addition level or mixing time, could be used as a tool to reduce rapid starch hydrolysis. The explanations given are also in agreement with the micrographs obtained from the crumbs of all five bread formulations in this study (Figure 6.5). The crumb of breads containing FB ingredients generally appears to have a smoother structure with starch granules being less visible than in CWB. This trend seems to be more pronounced in PRB and DFSDB, while for DFB and PRSDB individual exposed starch granules (like in CWB) can still be observed. This could indicate a higher degree of starch gelatinisation in PRB and DFSDB and explain the high HIs determined for these samples. The presented results show a similar or slightly more rapid/increased sugar release during *in vitro* starch digestion of breads containing FB ingredients in comparison to CWB. This could indicate slightly higher GIs, especially of DFSDB, PRB and PRSDB. However, as mentioned above, the GI reducing effect of sourdough fermentation is not necessarily related

to the rate of starch hydrolysis. Due to the relatively high levels of acetic acid in both DFSD and PRSD, a substantial delay of gastric emptying might be observed *in vivo* and therefore improved glycaemic responses in comparison to CWB.

6.5 Conclusion

This study compares the fermentation characteristics of the multifunctional *Ln. citreum* strain TR116 in two faba bean (FB) ingredients: faba bean dehulled flour (DF) and air-classified faba bean high-protein flour (PR). With regard to microbial growth, carbohydrate consumption as well as production of lactic and acetic acid, TR116 showed a similar metabolism in both substrates. The most important differences include a much more moderate pH drop, a slightly more pronounced galactose accumulation and a higher content of phenolic acids after 48 h of fermentation for PRSD in comparison to DFSD. Fermentation with *Ln. citreum* TR116 caused a striking improvement of the ingredients' baking performance when DFSD and PRSD were compared to their unfermented counterparts (including increased specific volume of bread loaves and decreased crumb hardness). GlutoPeak measurements allowed for an association of this observation with an improved gluten-aggregation in the presence of fermented FB ingredients. The high potential of the produced faba bean fermentates as functional ingredients for bakery applications is further underlined by the presence of phenolic acids with antifungal activity which might improve the microbiological shelf life of DFSD or PRSD containing breads. Also, the accumulation of small amounts of mannitol could positively influence sensory characteristics of the final bread products when compared to breads containing unfermented FB ingredients. In general, PRSD seems to represent a better choice than DFSD as a functional ingredient in bakery products. It offers a higher potential to improve protein quality in cereal products at lower levels of wheat flour replacement due to its higher protein content. This higher protein content also secures the opportunity to label breads in which 15% of wheat flour are replaced by PRSD with a 'high in protein' claim according to European legislation [100]. PRSD further offers better technological bread quality than both PR and DF as well as slightly higher levels of acetic acid and phenolic acids than DFSD, which is related to a higher potential to show improved microbial shelf life when incorporated in bakery products. In addition to this, HIs of DFSDB (119.0%) and PRSDB (114.9%) were similar, but a small tendency towards a lower HI of PRSDB was observed. While this might still leave DFSD in favour as a functional bakery ingredient, also the generally lower content of digestible starch should be considered (67.06 %DM (DFSDB) and 61.96 %DM (PRSDB)). This can be expected to result in a lower GL of breads containing PRSD (as opposed to breads

containing DFSD). Furthermore, the explanations for the increased HI of PRSDB (when compared to PRB and CWB) offered above, promise that a slight adjustment of processing conditions (water level, mixing time and speed) might further reduce starch digestibility. This could have an additional positive impact on quality characteristics of bread formulations containing PRSD.

6.6 Acknowledgements

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CHAPTER 7

Combining High-Protein Ingredients from Pseudocereals and Legumes for the Development of Fresh High-Protein Hybrid Pasta: Maintained Technological Quality and Adequate Sensory Attributes

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7.1 Abstract

The fortification of cereal foods, like pasta, with pseudocereal and legume ingredients promises a substantial improvement of their nutritional quality. However, partial replacement of wheat by pseudocereals and legumes in pasta formulations bears challenges regarding the products' technological and sensory quality. This study investigates the partial replacement of wheat semolina by a combination of high-protein ingredients (HPIs) from buckwheat, faba bean and lupin to reach a protein level of 20% of calories provided by protein. This high-protein hybrid pasta (HPHP) formulation was subjected to a thorough evaluation of technological quality characteristics and compared to regular wheat pasta and pasta formulations containing the single HPIs. Additionally, descriptive sensory profiling was performed to compare organoleptic properties of HPHP with regular wheat pasta. The quality of pasta formulations containing single HPIs was significantly reduced with regard to at least one of the determined quality characteristics. For the HPHP formulation containing all three HPIs, the technological quality was found to be equal to regular wheat pasta. No significant differences were detected for the most indicative quality characteristics cooking loss, firmness and stickiness. This was attributed primarily to compensating effects of the HPIs with respect to different quality characteristics. Sensory analysis revealed only slightly inferior overall quality of HPHP in comparison to regular wheat pasta, especially promoted by similar textural properties. The combination of selected HPIs offers the opportunity to produce high-protein hybrid pasta with technological and sensory quality similar to regular wheat pasta at a level of wheat semolina replacement of 25%.

7.2 Introduction

Pasta is a popular food product worldwide and the second most consumed wheat based staple food [1, 2]. It is a core component of many regular diets and widely accepted because of its palatability, convenience and ease of preparation at home. However, pasta quality can vary and depends on composition and physicochemical properties of the raw material as well as the chosen processing conditions [3]. Durum wheat (*Triticum durum*) semolina is considered the most suitable raw material for pasta production, resulting in pasta products with high quality [4]. High-quality pasta is generally characterised by high firmness and chewiness, low surface stickiness, and a bright and intense yellow colour. Desired cooking properties include a moderate water uptake and optimal cooking time. The cooking loss is one of the most important indicators of pasta cooking quality and lower cooking losses are generally associated with higher pasta quality [3, 5, 6]. Its popularity and unique structure make pasta an ideal matrix for a fortification with pseudocereal and legume ingredients, which promises a substantial improvement of its nutritional quality, specifically its protein quality. Many studies have investigated the impact of such ingredients on pasta quality, including technological, sensory and nutritional quality aspects. While Bustos *et al.* [6] state that chickpea flour and soy flour are the best studied ingredients in pasta fortification, also pseudocereals such as buckwheat and amaranth and legumes like lupin, lentil, pea, common bean and faba bean have been in the research focus in recent years [1, 7–17]. However, many of these studies report a substantial negative impact on technological quality characteristics as well as organoleptic properties of pasta. Increased cooking losses and stickiness as well as poor sensory quality seem to represent the most important challenges, especially at wheat semolina substitution levels above 10% [6, 16]. However, in order to reach a substantial improvement of nutritional quality, higher substitution levels are desirable. Furthermore, inferior pasta quality leads to poor consumer acceptance of such hybrid formulations containing cereals and legumes. Which is why the current study investigates the technological quality of a high-protein hybrid pasta (HPHP) formulation where a combination of pseudocereal (buckwheat) and legume (faba bean and lupin) high-protein ingredients (HPIs) is used to partially replace wheat semolina. Using HPIs, in contrast to pseudocereal or legume flours with lower protein contents, promises a better improvement of pasta protein quality at lower levels of wheat semolina replacement. This study provides a comparison of HPHP with regular wheat pasta as a reference as well as pasta formulations where the single HPIs (buckwheat, faba bean or lupin) are used to partially replace wheat semolina. The level of wheat semolina replacement was calculated for each pasta formulation to reach a protein level of at least 20%

of calories provided by protein. This level is specified in European legislation as requirement for a 'high in protein' nutritional claim [18]. Since technological quality characteristics are in close relation to sensory quality, also HPHP's organoleptic properties have been evaluated in a quantitative descriptive sensory analysis in comparison to regular wheat pasta. High technological and sensory quality promise high consumer acceptance, which is the key requirement for the success of non-traditional food products. The objective of this study is to identify whether the combination of HPIs from buckwheat, faba bean and lupin allows for the development of a high-protein hybrid pasta with a technological and sensory quality comparable to regular wheat pasta; and without the quality losses commonly observed when single pseudocereal or legume ingredients are applied.

7.3 Experimental

7.3.1 Materials

Three plant-based high-protein ingredients (HPIs) were applied in the high-protein hybrid pasta (HPHP) formulation. Buckwheat flour (protein content 22.52 %DM, fat 2.78 %DM, ash 3.22 %DM, fibre 1.6 %DM, carbohydrates by difference 69.9 %DM, total starch 54.72 %DM, obtained by dry fractionation) and faba bean flour (protein content 61.25 %DM, fat 3.81 %DM, ash 5.43 %DM, fibre 0.3 %DM, carbohydrates by difference 29.2 %DM, total starch 7.77 %DM [19]; obtained by dry fractionation) were experimentally produced and provided by Fraunhofer Institute IVV, Freising, Germany; lupin protein isolate (protein content 94.51 %DM, fat 2.94 %DM, ash 5.62 %DM, fibre < 0.1 %DM [19]) from Prolupin GmbH, Germany was also provided by Fraunhofer Institute IVV. Wheat semolina (protein content 17.4 %DM, fat 2.13 %DM, ash 1.4 %DM, fibre 4.2 %DM, carbohydrates by difference 74.9 %DM) was supplied by W G Buchanan & Son Ltd, Ireland; and salt by Glacia British Salt Ltd, UK.

7.3.2 Recipe Adaption and Pasta Production

Pasta samples were produced according to the recipes in Table 7.1. Formulations containing HPIs were established, based on the recipe of the reference wheat pasta (RWP), by partial replacement of wheat semolina by HPIs. The level of wheat semolina replacement was calculated, considering the composition of all ingredients, to reach a protein level of 20% of calories provided by protein (20 %E) in high-protein hybrid pasta (HPHP), buckwheat pasta (BP), faba bean pasta (FP) and lupin pasta (LP). The incorporation of HPIs can lead to a change in water

Table 7.1: Recipes for reference wheat pasta (RWP), reference wheat pasta (HPHP) and pasta formulations containing the single HPIs buckwheat flour (BP), faba bean flour (FP) or lupin protein isolate (LP); values given in % based on recipe unless stated otherwise.

Ingredient	RWP	HPHP	BP	FP	LP
Semolina	76.54	57.55	15.31	66.59	71.18
Buckwheat flour	–	13.02	61.23	–	–
Faba bean flour	–	3.97	–	9.95	–
Lupin protein isolate	–	2.01	–	–	5.36
NaCl	0.38	0.38	0.38	0.38	0.38
Water	23.08	23.08	23.08	23.08	23.08
Total	100.00	100.00	100.00	100.00	100.00
Level of wheat semolina replacement* [%]	0.00	24.81	80.00	13.00	7.00

* Values given in % based on HPI/wheat semolina mixture.

absorption of HPI/semolina mixtures in comparison to plain semolina. Therefore, water absorption indices for HPI/semolina mixtures and semolina were determined to characterise HPI/semolina mixtures and to evaluate whether an adjustment of the water level in the recipes was required. Water absorption indices (WAI) were obtained using a centrifugation method. In brief, the HPI/semolina mixtures or plain semolina (2.5 g) were dispersed in 10 mL distilled water (30 °C) for 15 s using an Ultra-Turrax equipped with a S10N-5G dispersing element (Ika-Labortechnik, Janke and Kunkel GmbH, Staufen, Germany) and subsequently shaken for 30 min at 1000 rpm on a platform shaker (UNI MAX 1010, Heidolph, Schwabach, Germany). After the mixture was thoroughly wetted, the sample was centrifuged at 3000 g for 10 min. Water absorption indices (WAIs) were calculated with the following equation:

$$WAI\,[\%] = \frac{weight\ of\ pellet\ after\ centrifugation\ and\ decantation\ [g]}{weight\ of\ semolina\ or\ HPI/semolina\ mix\ [g]} \times 100 \qquad (7.1)$$

Optimum water levels for recipes with HPIs can be adjusted by taking WAI of plain semolina and WAI of the HPI/semolina mixture as well as the optimum water level of the reference pasta into consideration. Pasta was produced using a benchtop cold extrusion single barrel system (Häussler, Germany) equipped with a spaghetti nozzle (diameter of holes: 2 mm). Dry ingredients were premixed with a whisk, water (30 °C) was added and the mass was mixed with a Kenwood chef mixer (Kenwood Ltd, UK; equipped with a K-beater attachment) for 10 min at minimum speed. The resulting crumbly dough was extruded, and the first 25 cm of spaghetti were discarded. The pasta product obtained after extrusion is referred to as 'raw pasta' throughout this work. The results for technological analyses were obtained from three independently produced pasta batches.

7.3.3 Pasta Quality Analysis

7.3.3.1 Cooking Quality

The cooking characteristics optimal cooking time, water uptake and cooking loss were determined in order to evaluate pasta quality. Optimal cooking time (OCT) is defined as the cooking time required to turn the starchy white core of raw pasta into a transparent one and was determined based on AACC 66-51.01. Twenty-five gram of spaghetti strands was weighed in and cooked in 300 mL distilled water in a 600 mL glass beaker on a stove. No salt was added in the cooking water and beakers were not covered. One strand of pasta was removed from the cooking water every 30 s, cooled down in cold distilled water and immediately squashed between two glass slides to evaluate transparency of the spaghetti core. The pasta product obtained with this cooking procedure is further referred to as 'cooked pasta' and was used to produce cooked pasta for each of the following technological measurements. The determined OCT was applied as cooking time and pasta was drained (not rinsed) and either stored in a covered glass beaker until analysis (for measurements of cooking loss, rheological properties and colour; resting time approx. 10 min) or left to cool down and cut for freeze-drying (microscopic analysis). Cooking loss was determined according to AACC 66-50.01. Twenty-five gram of spaghetti strands (5 cm length; exact weight recorded) was cooked according to the cooking procedure described above. Thereafter, the cooking water was transferred into a 500 mL volumetric flask and left to cool down. The volumetric flask was filled up with distilled water, shaken and 20 mL of its contents was transferred into a previously weighed aluminium dish. The aluminium dish containing the sample was weighed again to obtain the exact sample weight (by difference), placed into a drying-oven at 103 °C (until constant weight was recorded; approx. 15 h), left to cool down in a desiccator and weighed to obtain the dry weight of the sample. For determination of the water uptake during cooking, 42 g raw pasta was weighed in (exact weight noted), cooked, thoroughly drained and weighed again. Water uptake was calculated with the following equation:

$$Water\ uptake\,[\%] = \frac{weight\ of\ cooked\ pasta\ [g] - weight\ of\ raw\ pasta\ [g]}{weight\ of\ raw\ pasta\ [g]} \times 100 \quad (7.2)$$

7.3.3.2 Rheological Properties of Cooked Pasta

Rheological properties were measured with a texture analyser (TA.XTPlus, Stable Micro Systems, Surrey, UK) equipped with a 5 kg load cell. For determination of firmness, expressed as maximum cutting force (based on AACC 66-50.01 and

55-52.01), five pasta strands of 5 cm length were positioned parallel next to each other on the centre of the texture analyser platform (Heavy Duty Platform with a perspex plate). The test was performed with a perspex blade (Light Knife Blade) and five times per batch to obtain a total of 15 firmness values per formulation. The following test settings were applied: test speed 0.17 mm/s, post-test speed 10 mm/s, distance 4.5 mm, trigger force 0.05 N. Tensile strength of pasta is defined as resistance to uniaxial extension (expressed as maximum breaking strength) and was measured with a Spaghetti/Noodle Tensile Rig for ten strands of pasta (at least 10 cm long; one strand at a time) per batch with the following test settings: pre-test speed 1 mm/s, test speed 3 mm/s, post-test speed 10 mm/s, distance 100 mm, trigger force 0.05 N. Stickiness was measured with a Pasta Firmness/Stickiness Rig and is defined as the maximum peak force when the probe is retracted from the sample. For one measurement, five pasta strands of 5 cm length were positioned parallel next to each other under a rectangular aluminium probe and retained by a plate with rectangular opening. The following test settings were applied: pre-test speed 1 mm/s, test speed 0.5 mm/s, post-test speed 10 mm/s, distance 25 mm, trigger force 0.20 N.

7.3.3.3 Colour of Raw and Cooked Pasta

Colour measurements were carried out with a Colorimeter CR-400 (Konica Minolta, Japan) using the CIELAB colour space. Colour readings were taken from 30 separate points on the surface of the spaghetti strands. Results are expressed as L* (lightness between black (0) and white (100)), a* (colour space between magenta (positive) and green (negative)) and b* (colour space between yellow (positive) and blue (negative)). Additionally, differential colour indices (ΔE) were calculated with the following equation in order to assess colour changes in HPHP, BP, FP and LP compared to RWP.

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \tag{7.3}$$

ΔL^*.. $L^*_{control} - L^*_{sample}$
Δa^*.. $a^*_{control} - a^*_{sample}$
Δb^*.. $b^*_{control} - b^*_{sample}$

7.3.4 Scanning Electron Microscopy

In addition to pasta quality characteristics, the microstructure of RWP and HPHP was investigated using scanning electron microscopy (SEM). Short strands of raw pasta were frozen at -80 °C and freeze-dried. A thin slice was cut from a pasta strand using a scalpel (to prepare a cross section) and mounted onto a

plain aluminium stub with double-sided carbon adhesive tape. Scanning electron microscopy was performed as previously described by Hoehnel *et al.* [20]. In brief, the samples were coated with a 5 nm gold-palladium (80:20) layer and examined under high vacuum with a JOEL scanning electron microscope type 5510 (JOEL Technics Ltd., Tokyo, Japan).

7.3.5 Sensory Analysis

Descriptive sensory profiling (quantitative descriptive analysis – QDA) was carried out in order to characterise the pasta samples using an expert panel (n=8). The QDA procedure used in the study was in agreement with the standard ISO 13299:2016 and the ethical guidelines specified in EA-4/09 G 2017 ('Accreditation For Sensory Testing Laboratories'). The panelists' performance was evaluated prior to analysis using three parameters – repeatability, discrimination ability and homogeneity by applying analysis of variance. A 28-hour panel training was conducted on a range of pasta samples, including pasta from local supermarkets, to familiarise the panel with the characteristic sensory attributes of regular and novel pasta products. Panelists initially chose characteristics describing the samples individually; followed by the creation of a list of sensory attributes and their descriptions based on a joint agreement of the panelists. Continuous scales (10 cm long) with the extremes specified in Table 7.2 were used. Pasta was freshly cooked immediately before the sensory analysis. After cooking, pasta was tempered with cold water and each evaluator received 25 g of pasta.

Table 7.2: Sensory attributes and extremes of intensity scales used for QDA of pasta.

Attribute	Definition	Extremes
Odour		
Flour	Odour characteristic of wheat wholemeal flour	None – very intense
Legume	Odour characteristic of legume flour in hot water	None – very intense
Appearance		
Beige colour	Pasta strand colour intensity	Light – dark
Texture (manual)		
Elasticity	The extent to which pasta strands return to their original length when stretched	Low - high
Flavour		
Flour	Flavour characteristic of wheat wholemeal flour	None – very intense
Salty	Taste characteristic of NaCl (1% in water)	None - very intense
Legume	Flavour characteristic of legume flour in hot water	None – very intense
Sweet	Taste characteristic of sucrose (3% in water)	None – very intense
Bitter	Taste characteristic of caffeine (diluted in water)	None – very intense
Aftertaste	Lingering sensation after swallowing the sample	None – very intense
Texture (oral)		
Hardness	Force required to bite through the sample when placed between the front teeth	Low – high
Chewiness	Extent of chewing necessary to prepare food for swallowing	Low – high
Adhesiveness	Degree of adhesiveness when chewing the food 10 times	Low – high
Overall quality	Conclusive evaluation of all attributes and their harmonic balance	Bad – very good

7.3.6 Statistical Analysis

All measurements were performed in triplicate unless stated otherwise. Data analysis was performed using RStudio, version 1.2.1335 with R version 3.6.1 (RStudio Inc, USA; R Core Team, r-project). One-way analysis of variance (ANOVA) with post-hoc pairwise Tukey's test was used to identify significant differences ($p < 0.05$ unless stated otherwise). A correlation analysis (CA, significance level $p < 0.05$) determining correlation coefficients (r) was carried out for technological pasta characteristics. When available, values are given as the mean ± standard deviation.

7.4 Results and Discussion

In a series of preliminary trials, a total of 15 plant-based HPIs (both commercial and experimental ingredients) from pseudocereals (quinoa, amaranth, buckwheat), legumes (soy, pea, carob, lupin, chickpea, lentil, faba bean), cereals (wheat gluten, zein) and potato were screened for their impact on quality of high-protein pasta. In all recipes, the level of wheat semolina replacement by HPI was calculated, considering the composition of all ingredients, to reach a protein level of 20% of calories provided by protein (20 %E). Based on technological quality characteristics and a preliminary descriptive sensory evaluation (data not shown), three HPIs (buckwheat flour, faba bean flour and lupin protein isolate) were identified as most suitable. Due to a different performance of these ingredients regarding pasta quality criteria like firmness, tensile strength, stickiness and cooking loss (see Table 7.3), response surface methodology (RSM) was applied to 17 formulations containing all three HPIs buckwheat flour, faba bean flour and lupin protein isolate in different ratios (data not shown). Hence, a combination of these three HPIs for partial wheat semolina replacement with optimised technological characteristics was identified, which led to the establishment of the HPHP formulation. The amount of lupin protein isolate was kept to a minimum due to its allergenicity and its limited capacity to compensate for the lack of lysine [21] in wheat semolina protein with respect to a potential improvement of protein quality. Water absorption indices and pasta quality characteristics were determined and evaluated for HPHP and RWP as well as for the formulations containing the single ingredients (BP, FP, LP) for comparison reasons. Investigations of the microstructure and sensory attributes, however, were focused on the optimised formulation HPHP and RWP.

7.4.1 Water Absorption Indices of HPI/Semolina Mixtures

Water absorption indices were determined in order to characterise the HPI/semolina mixtures and to evaluate whether an adjustment of the water level in HPHP,

BP, FP and LP is required. No significant differences were found between WAIs of wheat semolina and the HPI/semolina mixtures used for BP, FP and LP. Only small tendencies towards lower (faba bean) or higher (buckwheat, lupin) WAIs were observed. The HPI/semolina mixture used for HPHP reached with 168.2% the highest WAI in this study. Even though it is significantly higher than the WAI of plain wheat semolina (161.9%), this only represents a relative increase by 4%. Changes in WAI can be related to a variety of reasons. These include the water absorption capacity of HPIs, which depends on their chemical composition and protein and starch properties. Also the level of wheat semolina replacement, which determines the extent to which wheat starch and wheat gluten are diluted, can affect WAIs. The fact that the HPI/semolina mixture for HPHP had the highest WAI could be related to the combination of HPIs in this formulation. While faba bean and lupin mainly contribute to a replacement of wheat semolina by non-wheat protein, buckwheat also contains a considerable amount of non-wheat starch. In the HPI/semolina mixture for HPHP, both components potentially contribute to an increase in WAI. However, the differences in WAI observed for the formulations in this study remain relatively small. Therefore, it was decided to apply a 5% threshold to differences in WAIs with regard to adjustments of water levels in the formulations. Hence, the same water level was used for the RWP, HPHP, BP, FP and LP.

7.4.2 Pasta Quality Characteristics

Photographs of raw and cooked pasta produced from RWP and HPHP formulation are displayed in Figure 7.1. A visual comparison reveals primarily a striking difference in colour for the raw pasta, while cooked RWP and HPHP are more similar in appearance. This is also evident with regard to the determined pasta quality characteristics for RWP and HPHP, including colour values for raw and cooked pasta, which are presented in Table 7.3. For comparison reasons, quality characteristics are additionally given for the pasta formulations BP, FP and LP, which contain only one of the HPIs buckwheat flour, faba bean flour or lupin protein isolate, respectively for partial wheat semolina replacement (see Table 7.1).

7.4.2.1 Cooking Quality

During the cooking of pasta, protein coagulation and starch gelatinisation are the two main processes that characterise structural changes. The coagulating protein matrix entraps starch granules which slowly gelatinise when water penetrates the protein network [3, 22–24]. While in the outer layer, gelatinisation of starch is primarily mediated by hydration, it can be attributed to a heat-induced crystallite

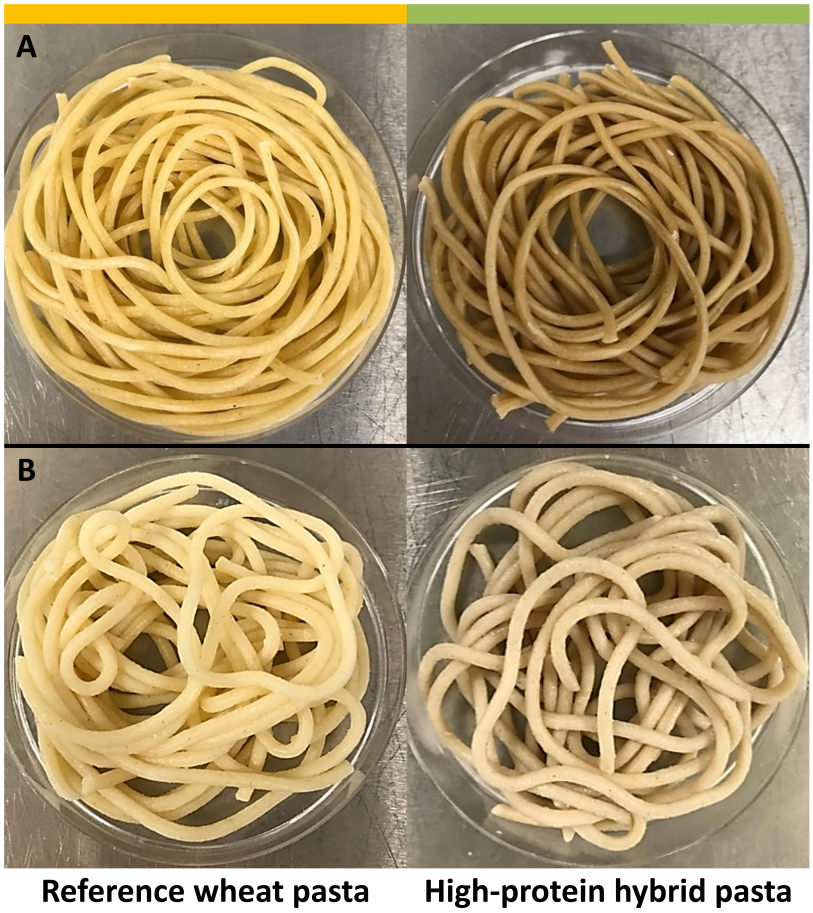


Figure 7.1: Photographs of RWP and HPHP: (**A**) Raw pasta; (**B**) Cooked pasta.

melting in the pasta core[25]. Its unique protein (gluten) and starch properties make wheat particularly suitable for pasta production. A dilution of wheat gluten and starch, due to the incorporation of non-wheat ingredients, has often been reported to negatively affect pasta quality [1, 3, 26–28]. Cooking quality of pasta

Table 7.3: Pasta quality characteristics of reference wheat pasta (RWP), reference wheat pasta (HPHP) and pasta formulations containing the single HPIs buckwheat flour (BP), faba bean flour (FP) or lupin protein isolate (LP).

Variable	RWP	HPHP	BP	FP	BP
WAI [%]	161.9 ± 0.6^{bc}	168.2 ± 1.5^{a}	165.4 ± 2.1^{ab}	159.3 ± 2.3^{c}	163.4 ± 1.4^{abc}
Optimal cooking time [min]	6.5	8.0	7.0	7.0	6.5
Cooking loss [%]	5.21 ± 0.29^{ab}	5.54 ± 0.40^{a}	4.39 ± 0.23^{b}	5.15 ± 0.19^{ab}	5.39 ± 0.09^{a}
Water uptake [%]	78.5 ± 2.1^{b}	82.5 ± 0.9^{ab}	88.9 ± 3.1^{a}	83.9 ± 3.3^{ab}	79.6 ± 1.6^{b}
Firmness* [N]	3.24 ± 0.10^{b}	3.54 ± 0.20^{b}	2.30 ± 0.11^{c}	4.04 ± 0.15^{a}	3.36 ± 0.19^{b}
Tensile strength* [N]	0.295 ± 0.047^{b}	0.334 ± 0.051^{b}	0.162 ± 0.071^{c}	0.407 ± 0.088^{a}	0.317 ± 0.053^{b}
Stickiness* [N]	3.18 ± 0.89^{b}	3.41 ± 0.56^{b}	3.55 ± 0.67^{b}	5.86 ± 0.72^{a}	4.23 ± 0.58^{b}
L*	76.54 ± 1.32^{a}	69.82 ± 2.13^{bc}	65.10 ± 2.10^{c}	72.78 ± 2.38^{ab}	73.82 ± 1.76^{ab}
a*	-2.26 ± 0.20^{d}	0.79 ± 0.11^{b}	3.69 ± 0.22^{a}	-1.34 ± 0.24^{c}	-2.30 ± 0.20^{d}
b*	15.34 ± 0.68^{b}	13.15 ± 0.45^{b}	9.60 ± 0.52^{c}	18.06 ± 1.32^{ab}	20.74 ± 1.67^{a}
$\Delta E^{*/**}$	-	7.70 ± 0.84^{b}	14.12 ± 0.80^{a}	4.74 ± 1.24^{c}	6.05 ± 1.08^{bc}
ΔE^{**} raw pasta	-	20.62 ± 0.74^{bc}	28.55 ± 0.29^{a}	20.04 ± 1.46^{c}	22.44 ± 0.73^{b}

Means ± standard deviation with different lower case letters in the same row are significantly different at $p < 0.05$.
* Results refer to cooked pasta unless stated otherwise.
** Colour differences expressed as differential colour indices ΔE.

is described by optimal cooking time (OCT), cooking loss and water uptake. The cooking loss is largely related to the continuity and strength of the protein matrix and its ability to prevent starch, and specifically amylose from the swelling starch granules, from leaching out of the pasta strands into the cooking water [6, 22, 23, 26]. In the present study, LP performed similar to RWP regarding the three aforementioned pasta quality characteristics and no significant differences were found. This can be associated with the low level of wheat semolina replacement in LP and, therefore, little dilution of the technologically most important constituents wheat gluten and wheat starch. Also the FP formulation shows a cooking loss and water uptake similar to RWP; but its OCT is with 7.0 min slightly longer than for RWP with 6.5 min. Laleg *et al.* [29] and Petitot *et al.* [16] both reported a tendency towards decreased OCT when wheat semolina was partially replaced by faba bean flour. Laleg *et al.* [29] additionally observed an increase in cooking loss for composite faba bean/wheat semolina pastas. However, the levels of wheat semolina replacement in these studies were much higher (Laleg *et al.* [29] 50–100% and Petitot *et al.* [16] 35%) than in the present study. Furthermore, the faba bean ingredients used in these studies had much lower protein contents (24–29 %DM) than the faba bean HPI applied in FP and HPHP. In regular wheat pasta, longer OCTs are associated with slower water penetration rates due to higher protein contents [22, 30]. While this trend mainly refers to the unique networking properties of wheat gluten, it can be hypothesised that faba bean protein can interact with wheat gluten as indicated by GlutoPeak measurements by Hoehnel *et al.* [19]. This could strengthen the protein matrix surrounding the starch granules and delay water penetration. Also, the even higher OCT of 8.0 min determined for HPHP suggests a strong protein matrix, potentially built by interactions between wheat, buckwheat, faba bean and lupin proteins. No significant differences were found between HPHP's and RWP's cooking loss and water uptake indicating comparable cooking quality of both formulations. With regard to cooking characteristics, the BP formulation represents an exception amongst the pasta formulations in this study. While its OCT is with 7.0 min very similar to FP, the cooking loss is with 4.39% the lowest determined in this study. The water uptake on the other hand is with 88.9% approximately 13% higher than that of RWP with 78.5%. Buckwheat also represents the formulation with the highest level of wheat semolina replacement of 80%. The associated high dilution of gluten could have been expected to lead to an increased cooking loss due to a weak protein matrix and insufficient entrapping of starch granules. The fact that BP shows a tendency towards lowered cooking loss instead, might be related to the presence of a considerable amount of non-wheat starch originating from buckwheat flour (30% in BP formulation, calculated based on buckwheat flour composition). Buckwheat

starch possesses unique physicochemical properties distinguishing it from wheat starch as well as from other pseudocereal starches like amaranth and quinoa. The average size of buckwheat starch granules is with 5.8 μm considerably smaller than for wheat starch with 13.8 μm [31]. Soh *et al.* [32] showed that an increase of the ratio between smaller and bigger wheat starch granules improves overall pasta quality and decreases cooking loss. This was attributed to a more uniform distribution of starch granules within the protein matrix [3]. Also the amylose content has been associated with cooking loss. While some publications report higher cooking losses in relation to elevated amylose contents, other articles mention the relevance of amylose and its interactions with the protein matrix to improve pasta quality and minimise cooking loss [22, 32, 33]. Buckwheat starch contains a higher amount of amylose compared to wheat starch, which could lead to more interactions of amylose with buckwheat or wheat proteins preventing it from leaching into the cooking water. Biney and Beta [7] also observed a decrease in cooking loss when buckwheat flour was used to replace 20% of wheat semolina in a pasta formulation. However, they reported an increased cooking loss compared to the control when the level of replacement was raised to 30% or higher. Another reason for the low cooking loss observed for BP in the present study could be the low solubility of buckwheat starch in comparison to wheat starch, especially at temperatures higher than 75 °C [32, 34]. Hence, amylose from buckwheat starch might be less prone to leach into cooking water. Also the high water uptake of BP can be linked to buckwheat starch properties. The swelling power of buckwheat starch has been reported to considerably exceed that of wheat starch at temperatures above 75 °C [34, 35]. Qian *et al.* [31] also reported a higher water binding capacity in comparison to wheat starch. Therefore, a higher water uptake during cooking can be expected for BP in comparison to RWP.

7.4.2.2 Rheological Characteristics of Cooked Pasta

Rheological properties of cooked pasta are another major aspect of pasta quality and are largely related to the sensory acceptance of pasta products. Firmness and tensile strength showed a high positive correlation for the pasta formulations investigated in this study (cc: 0.999). For both variables, no significant differences were found between RWP and LP, which can be associated with the small level of wheat semolina replacement in LP. The BP formulation shows a significant decrease in firmness and tensile strength by approximately 29% and 45%, respectively when compared to RWP. For FP on the other hand, firmness increased by 25% and tensile strength by 38% relative to RWP. Firmness and tensile strength values of the HPHP formulation, however, are not significantly different from those of RWP. The negative impact of buckwheat flour and the positive effect of faba bean

flour on rheological pasta quality seem to compensate each other when combined in HPHP. High firmness and elasticity of pasta after cooking have been mainly attributed to a strong coagulated protein/gluten matrix and high gluten content [3, 22, 36]. The drastically decreased firmness of BP could, therefore, be related to the high level of gluten dilution. While the unique properties of buckwheat starch seemed to cause a slight improvement of cooking quality of BP despite a high dilution of wheat gluten and wheat starch, a similar trend could not be observed for rheological pasta properties. Even though buckwheat starch has been reported to form stronger and more stable gels after heat treatment than wheat starch [37], this seems to not sufficiently compensate for the lack of gluten in the formulation with regard to rheological pasta properties. Additionally, higher water uptake during cooking (which was observed for BP) has been associated with increased softening of pasta [6, 38]. The increase in pasta firmness observed for FP, which occurs despite a lower gluten content than in RWP, is in agreement with the findings of Petitot *et al.* [16] who replaced 35% of wheat semolina by faba bean flour. Also Teterycz *et al.* [39], Zhao *et al.* [14] and Bahnassey *et al.* [40] found an increase in pasta firmness when pasta was fortified with legume flours or legume protein concentrates. An interaction of faba bean protein with gluten and a resulting strengthening effect, as explained above and suggested by GlutoPeak measurements reported by Hoehnel *et al.* [19], could be a possible explanation for this trend. Petitot *et al.* [17] postulated a weak protein network when legume ingredients are incorporated in pasta formulations based on microscopy images and investigations of protein interactions. The introduction of legume proteins lowered disulphide bonds and covalent interactions and increased non-covalent hydrophobic interactions of pasta protein molecules. However, also hydrophobic intermolecular interactions have been reported to have a major stabilising effect on the pasta protein matrix [6]. Stickiness or adhesiveness of pasta is often discussed in relation to cooking loss in literature. It has been reported that higher cooking losses, due to a higher extent of amylose leaching out into the cooking water, lead to higher amounts of amylopectin left in the outer layer of pasta strands, which causes increased stickiness [6, 41]. However, no correlation between cooking loss and stickiness was found for the formulations in this study (cc: 0.063). It can be hypothesised, that also the properties of the composite protein networks, containing wheat gluten and non-wheat proteins, contribute to pasta stickiness. Stickiness values of the formulations in this study range from 3.18 N for RWP up to 4.23 N for LP. Only FP represents an exception with a significantly increased stickiness of 5.86 N. Laleg *et al.* [29] investigated the microstructure of 100% faba bean pasta and observed coalesced swollen starch granules in the external region of pasta strands. These starch granules formed a thin continuous starch phase at

the pasta surface (excluding proteins) surrounding an otherwise continuous protein network with embedded starch granules. Also the nature of the wheat/faba bean protein network in FP in the present study might favour the formation of a starch layer at the pasta surface similar to that observed by Laleg *et al.* [29] (however, FP contains mainly wheat starch as opposed to faba bean starch in Laleg *et al.* [29]). This could explain the high stickiness of the pasta surface in FP, while cooking loss and other pasta quality characteristics remain unchanged or improved.

7.4.2.3 Colour of Raw and Cooked Pasta

Results obtained from colour measurements are presented in Table 7.3. Colour differences between RWP and pasta formulations containing HPIs were more pronounced in raw pasta than in cooked pasta. While ΔE ranges from 20.04 (FP) to 28.55 (BP) for raw pasta, considerably lower values between 4.74 (FP) and 14.12 (BP) were obtained for cooked pasta. Jukić *et al.* [42] reported that fresh pasta shows higher colour losses during cooking than pasta that has been subjected to a drying process after extrusion. Furthermore, the colour differences (ΔE values) for the cooked pasta formulations observed in this study are in the same range as values reported by Tazrart *et al.* [8], Teterycz *et al.* [39] at similar levels of wheat semolina replacement by legume ingredients. Colour values (L* and a*) measured for RWP, HPHP, BP, FP and LP correlate well with their levels of wheat semolina replacement (cc(L*): -0.955, cc(a*): 0.967). The incorporation of HPIs generally leads to a darker (L* values) and redder (a* values) appearance of cooked pasta, which is most pronounced in BP. While the addition of faba bean flour in FP and lupin protein isolate in LP leads to a yellower pasta colour, buckwheat flour caused a slightly bluer appearance of BP. The combination of these HPIs in HPHP results in a b* value similar to that of RWP and no significant difference was detected. Pasta appearance, besides cooking properties and texture, is considered as one of the most important indicators of quality and consumer acceptance. Yellower and lighter pasta colour are commonly regarded as desirable and preferred amongst consumers [3, 5, 6]. The darker and redder appearance of HPHP can, therefore, be considered as a slight decrease in pasta quality.

7.4.3 Pasta Microstructure

The SEM images of raw RWP and HPHP after extrusion (cross sections through spaghetti strand) are presented in Figure 7.2. The pictures obtained from both magnifications reveal a more porous structure of HPHP than of RWP. However, the areas with higher porosity seem to be located rather in the pasta core than in outer layers of the pasta strand. In images with higher magnification (× 500), it can

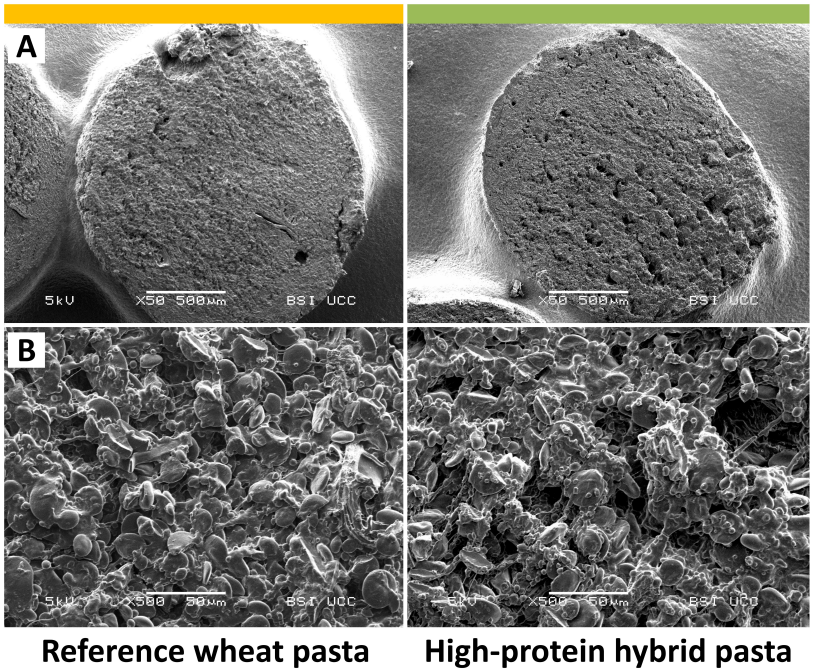


Figure 7.2: Scanning electron micrographs of RWP and HPHP: (**A**) Cross section of pasta strands (raw pasta; after extrusion) with × 50 magnification; (**B**) Cross section of pasta strands (raw pasta; after extrusion) with × 500 magnification.

be observed that the size and shape of starch granules is more inhomogeneous in HPHP than in RWP. This is caused by the incorporation of buckwheat flour which contains a substantial amount of buckwheat starch. As described above, buckwheat starch is characterised by, on average, smaller starch granules (in comparison to wheat starch) with polygonal shape [31, 43]. The protein layer surrounding the starch granules appears slightly thicker in HPHP, which can be explained by its higher protein content. Laleg *et al.* [29] also observed a thicker protein network with entrapped starch granules in faba bean pastas with increased protein contents (80 or 100% of wheat semolina replaced by faba bean flour).

7.4.4 Sensory Characteristics

Sensory characteristics are highly related to a products' technological quality and determine, to a large extent, the consumer acceptance of the food product. Formulations with increased protein content and enhanced nutritional profile, like HPHP, can be categorised as functional foods. However, consumer surveys have shown that functional foods are evaluated by consumers the same way as conventional foods. Functional features cannot outweigh inferior sensory quality and are merely perceived as added value [44]. Sensory analysis for RWP and HPHP was carried out with a trained panel and jointly selected descriptors for pasta quality (Figure 7.3). The most notable differences in the sensory profile between RWP and

HPHP were observed for odour and colour. The RWP formulation had a significantly stronger flour odour than HPHP, while the legume odour was much more pronounced for HPHP. Furthermore, the perception of a beige colour was much stronger for HPHP than for RWP. This is in agreement with a visual assessment of the photographs of both pasta formulations presented in Figure 7.1. Also the results of instrumental pasta colour measurements indicated a darker and redder colour for HPHP than for RWP, which might be perceived as a more beige appearance. Similar to flour and legume specific odour, also flour flavour is more pronounced in RWP while legume flavour is stronger in HPHP. Amongst consumers, legume flavour is often associated with a negative sensory experience [45–48]. However, this negative association is largely related to bitterness and unpleasant aftertaste, which are often present in legume or pseudocereal containing foods [48, 49]. Bitterness and aftertaste have been evaluated as separate attributes in this study and both are only slightly more pronounced for HPHP than for RWP, as opposed to the much bigger difference observed in legume odour and flavour. Furthermore, a nutty flavour, which is typically part of the legume flavour profile [48], has been previously reported as a desirable sensory attribute for pasta. Minor differences between RWP and HPHP were detected for texture attributes. The HPHP formulation was perceived slightly (but significantly) harder, chewier, more adhesive, and

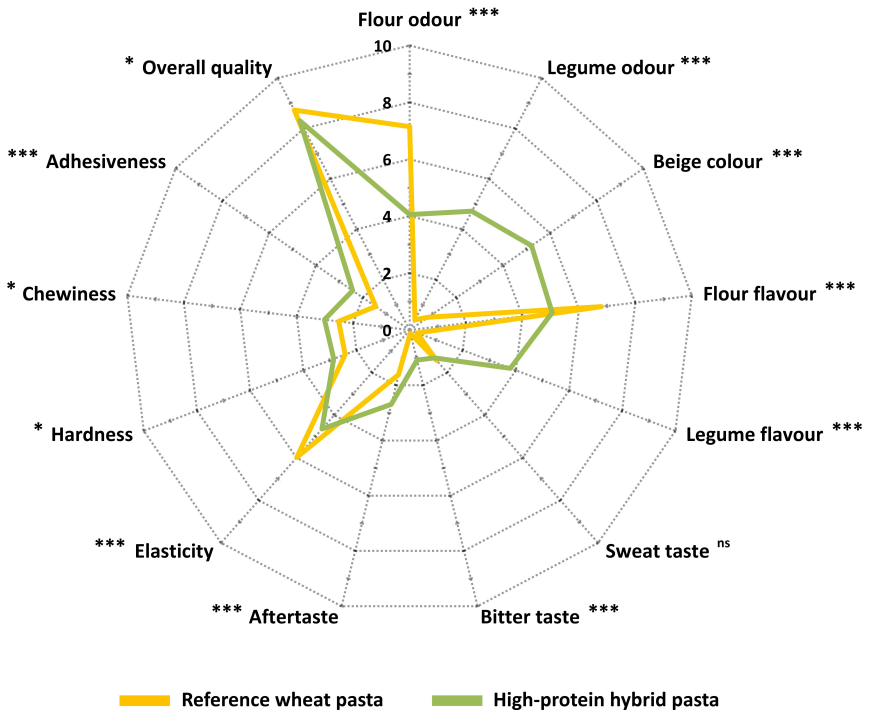


Figure 7.3: Sensory characteristics of reference wheat pasta and high-protein hybrid pasta; asterisks indicate attributes which showed significant differences between RWP and HPHP (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns no significant difference detected).

less elastic than RWP. This is in line with the results of instrumentally measured rheological pasta properties, where no significant differences between HPHP and RWP were detected, but a small tendency towards higher firmness for HPHP. Instrumental measurements additionally suggested a tendency towards increased stickiness in HPHP. However, this value is an indicator of surface stickiness, while the sensory attribute adhesiveness is evaluated orally during chewing. It should be considered that these two variables describe different rheological properties and do not necessarily correlate. The fact that HPHP scored only slightly lower than RWP in overall quality, indicates that, despite a more pronounced legume odour and flavour, HPHP's overall harmonic balance of organoleptic properties is similar to RWP. This also suggests that, for pasta, texture attributes influence the perception of overall sensory quality to a larger extent than odour and flavour, which has been previously reported in literature [5]. A similar trend was observed by Laleg *et al.* [29] who performed sensory analysis for a range of different pasta formulations; including regular wheat pasta, whole wheat pasta, gluten-free pasta (containing maize, millet and rice) and faba bean pastas with wheat semolina replacement levels of 80 and 100%. The results suggested a bigger impact of texture attributes than flavour attributes on the obtained liking scores for the pasta formulations. They further suggested a preference of pastas which were perceived as stickier (determined orally during chewing), less elastic and less firm. These trends (except for firmness) were observed in the present study for HPHP in comparison to RWP. This identifies HPHP as a pasta formulation with adequate organoleptic properties and similar sensory quality to RWP, which suggests high consumer acceptance.

7.5 Conclusion

The present study compares the technological quality of regular wheat pasta as a reference (RWP) with high-protein hybrid pasta formulations containing HPIs from buckwheat, faba bean and lupin. The HPIs were incorporated at levels to reach protein contents of at least 20% of calories provided by protein. The results show that the incorporation of the single HPIs lead to inferior product quality in at least one of the determined quality characteristics. However, when a combination of all three HPIs (ratio of HPIs determined in preliminary trials, data not shown) was applied in the proposed HPHP formulation, technological quality was found to be equal to RWP. No significant differences were detected with regard to the most indicative quality characteristics cooking loss, firmness, tensile strength and stickiness. Only the colour change observed in HPHP (darker and redder) can be considered a slight decrease in pasta quality. The HPHP formulation also scored

high in overall sensory quality. Especially sensory attributes describing pasta texture were evaluated similarly for HPHP and RWP suggesting high consumer acceptance. The study provides evidence that a combination of selected HPIs offers the opportunity to produce high-protein hybrid pasta with a technological and sensory quality similar to regular wheat pasta (at a level of wheat semolina replacement of approx. 25%); especially due to compensating effects of different HPIs. Furthermore, the incorporation of buckwheat, faba bean and lupin HPIs in a wheat based food promises a substantial improvement of the product's nutritional profile.

7.6 Acknowledgements

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CHAPTER 8

Combining High-protein Ingredients from Pseudocereals and Legumes for the Development of Fresh High-Protein Hybrid Pasta: Enhanced Nutritional Profile

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8.1 Abstract

The fortification of wheat based staple foods, such as pasta, with pseudocereal and legume flours has received growing research interest in recent years. While it is associated with many challenges regarding technological and sensory quality of the products, it promises a substantial improvement of the nutritional value of pasta. However, investigations of the nutritional quality of fortified pasta often focus on the carbohydrate/starch fraction and information on changes in protein quality is relatively scarce. This study evaluates the nutritional profile of a high-protein hybrid pasta (HPHP) formulation where a combination of three high-protein ingredients (HPIs) from buckwheat, faba bean and lupin is used to partially replace wheat semolina. The formulation's macronutrient composition, protein quality and the contents of antinutritional compounds are assessed in comparison to regular wheat pasta. The HPHP formulation represents a more favourable macronutrient profile compared to regular wheat pasta, particularly in relation to the isocaloric replacement of wheat starch by non-wheat protein. Furthermore, a more balanced amino acid profile, improved N utilisation and increased protein efficiency ratio (*in vivo*) were determined for HPHP which conclusively suggests a substantially enhanced protein quality. The cooking process was shown to significantly reduce levels of vicine/convicine and trypsin inhibitor activity originating from HPIs. The small remaining levels seem to not adversely affect HPHP's nutritional quality. This significant upgrade of the pasta's nutritional value identifies HPHP, and similar hybrid formulations, as a healthy food choice and valuable alternative to regular wheat pasta, specifically for a protein supply of adequate quality in mostly plant-based diets.

8.2 Introduction

The focus of research concerning durum wheat (*Triticum durum*) pasta has shifted in recent years. While technological quality, texture properties and sensory still represent the main topics of interest, nutrition and health aspects of pasta have gained increasing attention. This is in close relation to the evolving market potential of functional foods that have the ability to improve wellness and health of consumers [1]. According to Jenkins *et al.* [2] there is a necessity to transition from diets with high nutrient density to diets with high nutritional density, particularly in the context of decreasing physical activity. This refers to an increased intake of essential macro- and micronutrients at lower total caloric intakes. Pasta has been described as a promising vehicle for the addition of both macro- and micronutrients through the incorporation of functional ingredients [3]. The fortification with pseudocereal and legume ingredients, which are particularly rich in protein and micronutrients, offers an upgrade of nutritional quality and nutritional density of pasta; for example due to increased protein content and enhanced protein quality [4, 5]. While protein intakes in many parts of the world exceed the average daily requirement, this is often related to an overconsumption of animal protein or food in general [6]. Many dietary guidelines recommend a substantial reduction of protein from animal sources and increased intake of plant-based protein [6]. Furthermore, the urgently required transition to a food system with improved environmental sustainability largely relies on a shift to predominantly plant-based human diets [6]. Several studies have investigated the impact of pasta fortification with pseudocereal and legume ingredients on technological, sensory and nutritional product quality [7–13]. However, many of these articles report major challenges with regard to textural properties, cooking quality and sensory characteristics. From a nutritional point of view, the research was often focused on the carbohydrate/starch fraction (e.g. starch digestibility) and information on changes in protein quality of fortified pasta is rather scarce [14]. One of the concerns regarding nutritional quality when pseudocereal and legume ingredients are incorporated in foods refers to antinutritional compounds (ANCs). These compounds are molecules which are naturally present in plants and can, for example, decrease a product's sensory quality (primarily tannins and saponins), protein digestibility (trypsin inhibitors) and the bioavailability of minerals (phytate) [5, 15, 16]. The pyrimidine glycosides vicine and convicine, which can trigger favism, are also considered ANCs and are mainly found in faba beans [17, 18]. The analysis of important ANCs should be considered when the nutritional quality of pseudocereal and legume containing foods is evaluated. In this study, a combination of three high-protein ingredients (HPIs; from the pseudocereal buckwheat and the legumes faba bean and lupin) was used

to partially replace wheat semolina and to produce a high-protein hybrid pasta (HPHP). This HPHP formulation was subjected to a thorough assessment of its nutritional quality with a focus on macronutrient composition, starch digestibility (*in vitro*), protein quality (amino acid profile, *in vitro* and *in vivo* protein digestion) and ANCs. Technological and sensory quality of this formulation were previously validated by Hoehnel *et al.* [19] and found to be similar to regular wheat pasta. While favourable texture attributes and organoleptic properties remain the most important determinators for consumer acceptance of foods, also the products' nutritional value and potential health benefits have received growing interest from consumers. The objective of this study is to compare the nutritional quality of the HPHP formulation to regular wheat pasta and to identify whether the combination of HPIs from buckwheat, faba bean and lupin leads to an enhanced nutritional value in addition to the previously confirmed adequate technological and sensory quality [19].

8.3 Experimental

8.3.1 Materials

Reference wheat pasta (RWP) and high-protein hybrid pasta (HPHP) were produced from the same ingredients as specified in Hoehnel *et al.* [19]. Buckwheat flour (protein content 22.52 %DM, lipids 2.78 %DM, ash 3.22 %DM, fibre 1.6 %DM, carbohydrates by difference 69.9 %DM, total starch 54.72 %DM; obtained by dry fractionation), faba bean flour (protein content 61.25 %DM, lipids 3.81 %DM, ash 5.43 %DM, fibre 0.3 %DM, carbohydrates by difference 29.2 %DM, total starch 7.77 %DM [20]; obtained by dry fractionation) and lupin protein isolate (protein content 94.51 %DM, lipids 2.94 %DM, ash 5.62 %DM [20]) were provided by Fraunhofer Institute IVV, Freising, Germany. Wheat semolina (protein content 17.4 %DM, lipids 2.13 %DM, ash 1.37 %DM, fibre 4.2 %DM, carbohydrates by difference 74.86 %DM) was purchased from W G Buchanan & Son Ltd, Ireland; and salt by Glacia British Salt Ltd, UK. The following ingredients were used for the preparation of diets for *in vivo* nitrogen balance trials: casein (C) from Lacpol Co., Poland; soy protein isolate (SPI) ISOPRO 900 HI (non-GMO) from EDMIR-POL Co., Poland; soy flour (SF) SOPRO TB 200 from EDMIR-POL Co., Poland; α-cellulose (C8002) from Sigma Aldrich, Missouri, USA; soy oil from ZPT Co., Poland; choline chloride from SIGMA, Poland; cholesterol from PPH Standard Co., Poland; sucrose from POCH SA Co., Poland; and corn starch from Avebe, The Netherlands. For *in vitro* digestion trials, enzymes were purchased from Sigma-Aldrich, Missouri, USA: pepsin from porcine gastric mucosa; EC 3.4.23.1; P7000; 727 U/mg and pancreatin

from porcine pancreas; 4 × USP; P1750. All other chemicals were also purchased from Sigma-Aldrich, Missouri, USA unless stated otherwise.

8.3.2 Recipe Adaption and Pasta Production

Pasta (spaghetti) samples were produced according to Table 8.1 and the procedure described by Hoehnel *et al.* [19]. In brief, the HPHP formulation was established based on the recipe of the reference wheat pasta (RWP), by partial replacement of wheat semolina by HPIs. The impact of 15 plant-based HPIs from cereals, potato, pseudocereals and legumes on the quality of high-protein pasta was screened in a series of preliminary trials. The levels of wheat semolina replacement were calculated to reach a protein level of 20% of calories provided by protein (20 %E). The results identified three HPIs (buckwheat flour, faba bean flour and lupin protein isolate) as most suitable with regard to technological pasta quality. Response surface methodology was used to determine a combination of these three HPIs to produce pasta with optimised technological quality. Due to its allergenicity and its limited capacity to compensate for the lack of lysine in wheat semolina protein, the amount of lupin protein isolate in the formulation was kept to a minimum (see Section 8.4.2). The fresh pasta product obtained after extrusion is referred to as 'raw pasta' throughout this work. In order to prepare the product which is further referred to as 'cooked pasta', the optimal cooking time (OCT) of RWP and HPHP determined by Hoehnel *et al.* [19] was applied as cooking time. After cooking (tap water, no salt added), pasta was drained (not rinsed) and left to cool down. The strands were cut into small pieces of approx. 1 cm length, frozen at -80 °C and freeze-dried. Freeze-dried pasta was milled prior to nutritional analysis. Results are expressed as contents per dry matter considering the moisture of the freeze-dried pasta powders unless stated otherwise.

Table 8.1: Recipes for reference wheat pasta (RWP) and high-protein hybrid pasta (HPHP) (as reported by Hoehnel *et al.* [19]), values given in % based on recipe unless stated otherwise.

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

* Values given in % based on HPI/wheat semolina mixture.

8.3.3 Compositional Analysis

Compositional analysis was performed as previously described by Hoehnel *et al.* [21]. In brief, analysis of the following compositional data was performed by Concept Life Science Ltd., UK, based on the indicated validated methods: energy (calculated considering protein, lipids, available carbohydrates and fibre), protein (Dumas method, modified after AOAC 1977.992.15; nitrogen-to-protein conversion factor 6.25), ash (removal of organic matter by oxidation at 550 °C, based on ISO 936:1998), lipids (low resolution proton nuclear magnetic resonance (NMR), based on MQC-23-35 Oxford Instruments application note), fatty acid profile (GC-FID of fatty acid methyl esters; triglyceride conversion factor 0.956), total dietary fibre (gravimetric method, based on AOAC 991.43), sodium (flame photometry after removal of organic matter). Moisture was measured based on the air-oven method (AACC 44-15.02) using a moisture analyser (Mettler Toledo, Ohio, US). The contents of total, digestible and resistant starch were analysed with the enzyme kit K-RAPRS (Megazyme, Ireland).

8.3.4 *In Vitro* Starch Digestion

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

8.3.5 Amino Acid Analysis

Determination of protein amino acid composition was performed by Mérieux NutriSciences CHELAB S.r.l., Italy, based on ionic chromatography with post-column ninhydrin derivatisation (fluorescence detection; UV detection for tryptophan) after adequate extraction and protein hydrolysis (separate procedures for tryptophan, sulphur containing amino acids and remaining amino acids).

8.3.6 Antinutritional Compounds

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

8.3.7 *In Vitro* Protein Digestion

In vitro simulation of gastro-pancreatic protein digestion was performed as described by Hoehnel *et al.* [21] based on a previously reported static multi-step method to determine *in vitro* protein digestibility (IVPD) [23, 26]. In short, sample amounts containing 50 ± 1 mg protein were subjected to enzymatic hydrolysis: pepsin digestion (37 °C, pH 1-2, 1 h) followed by a sequential pancreatin digestion (37 °C, pH 7-8, short-term: +1 h, medium-term: +3 h, long-term: +24 h). The enzyme/substrate ratio (w/w) was kept constant at 1:50 (pepsin stage) and 1:10 (pancreatin stages). IVPD in % was determined using a trinitrobenzenesulfonic acid (TNBS) assay. Results are expressed as the concentration of free α-amino groups in the digested samples in relation to an alanine standard solution representing 100% protein digestibility.

8.3.8 *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. *In vivo* nitrogen balance was investigated according to the procedure described by Hoehnel *et al.* [21]. The experiment was performed with growing male Wistar rats (average bodyweight of 173.2 g), which were randomly divided into groups of seven animals. The following diets were used for experimental feeding: a standard control diet based on casein as main protein source (supplemented with 0.2% *DL*-methionine), a second control diet based on soy protein isolate (without any supplementation), a third control diet based on soy flour (without any supplementation); and the experimental diets containing RWP and HPHP (Table 8.2). All experimental diets

Table 8.2: Composition of diets for *in vivo* nitrogen balance trials containing either casein (C), soy protein isolate (SPI), soy flour (SF), reference wheat pasta (RWP) or high-protein hybrid pasta (HPHP) as protein source, values given in % of diet.

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

[1] AIN-93G-MX: Mineral mixture as specified by Reeves [27]
[2] AIN-93G-VX: Vitamin mixture as specified by Reeves [27]

represent modifications of the AIN-93G diet for laboratory rodents recommended by the American Institute of Nutrition [27] (dietary protein level was lowered to approx. 11% to measure protein digestibility and utilisation rate). In order to determine nitrogen (N) digestibility and utilisation, faeces and urine of all rats (7 per diet group) were thoroughly collected for 5 days (after a 9-day preliminary period). The total N content of each diet, faecal sample and urinal sample was analysed in duplicate (AOAC 979.09). Additionally, bodyweight (BW) gain (BWs recorded at beginning and end of the study) and diet intake (daily record) were monitored for all rats to enable calculation of the protein efficiency ratio (PER).

8.3.9 Statistical Analysis

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

8.4 Results and Discussion

The nutritional profile of the cooked pasta formulations RWP and HPHP (photographs of raw and cooked pasta displayed in Figure 8.1) was evaluated with a focus on macronutrient composition, ANCs and protein quality. The analysis of ANCs and *in vitro* digestibility was additionally performed with raw pasta samples

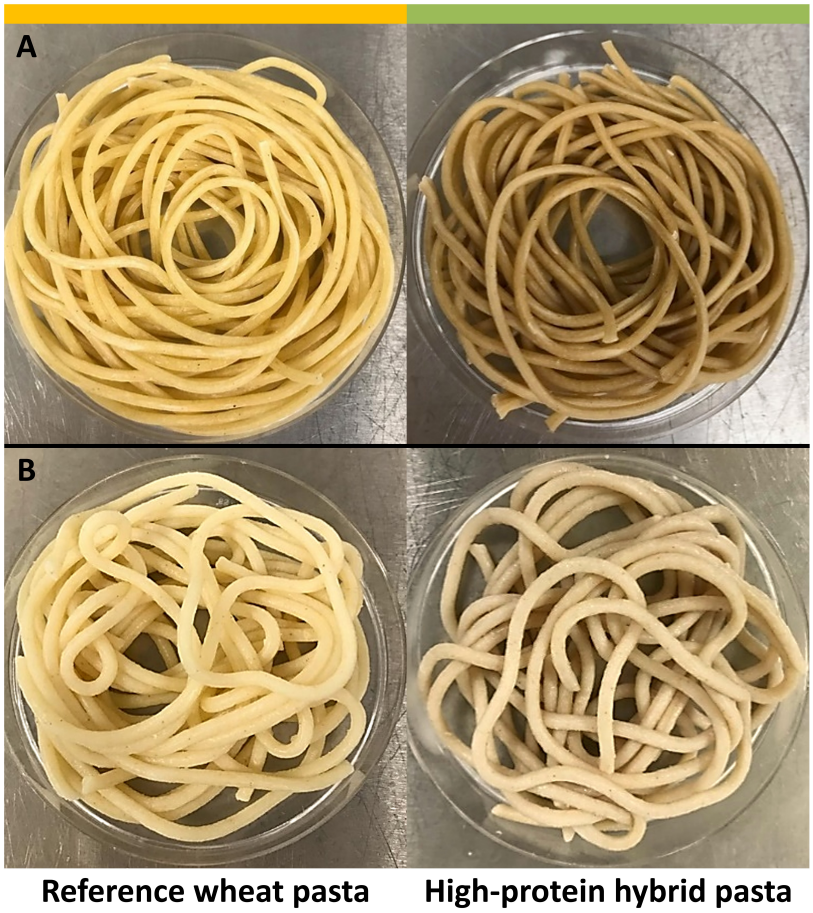


Figure 8.1: Photographs of RWP and HPHP (as reported by Hoehnel *et al.* [19]): (**A**) Raw pasta; (**B**) Cooked pasta.

in order to monitor the effect of heat treatment and aqueous extraction during cooking on nutritional pasta quality.

8.4.1 Macronutrient Composition and *In Vitro* Starch Digestibility

The macronutrient composition of RWP and HPHP was analysed to assess compositional changes caused by the partial replacement of wheat semolina by plant-based HPIs (Table 8.3). An increased protein level and decreased carbohydrate content, amongst other minor differences to RWP, characterise the macronutrient profile of HPHP. The HPHP formulation contains with 23.2 %DM approximately 6 %DM more protein than RWP with 17.3 %DM. The content of total starch accounts for 64.70 %DM in HPHP and 72.10 %DM in RWP. Hence, the partial replacement of wheat semolina by HPIs primarily causes a substitution of wheat starch by non-wheat protein. The determined energy contents are with 393.0 kcal/100 g DM (RWP) and 396.8 kcal/100 g DM (HPHP) very similar in both formulations. Therefore, the compositional changes in HPHP can additionally be considered an isocaloric replacement of wheat starch by non-wheat protein. It has been reported that

Table 8.3: Composition of reference wheat pasta (RWP, cooked) and high-protein hybrid pasta (HPHP, cooked)); contents expressed in %DM unless stated otherwise.

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

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

* Calculated based on energy content, protein content and 4 kcal/g protein.

** Calculated by difference.

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

the isocaloric replacement of dietary carbohydrate by protein in diets reduces blood lipid concentrations and blood pressure [28]. This effect was attributed to the reduced intake of carbohydrate rather than the increased intake of protein [28]. However, the metabolic effects of dietary carbohydrate are influenced by its glycaemic index (GI) [29]. Consequently, the isocaloric replacement of dietary carbohydrate by protein might be more or less effective (with regard to blood lipid concentrations, blood glucose or other metabolic conditions) depending on the GI of the replaced carbohydrate. Appel *et al.* [28] observed a positive influence on blood lipid concentrations and blood pressure when medium GI (between 68 and 75) carbohydrate was replaced by protein. Even though pasta contains refined wheat starch, which is a rapidly digestible carbohydrate with high GI, pasta is considered a low GI food [14]. This is related to its unique and dense structure, which seems to prevent or delay enzymatic degradation of starch [13, 14]. While in other carbohydrate foods, like bread, the incorporation of plant-based protein ingredients has been reported to significantly lower GI [30], only small effects or no significant changes have been reported for pasta [7, 13, 31]. The HI values determined in this study (Table 8.3) suggest a decreased starch digestibility of HPHP (86.0%) compared to RWP (100%). This trend is also evident with regard to the sugar release curves obtained from *in vitro* starch digestion trials which are displayed in Figure 8.2. This indicates a lower GI of HPHP in comparison to RWP, but additionally a lower GL due to the significantly lower level of digestible starch in

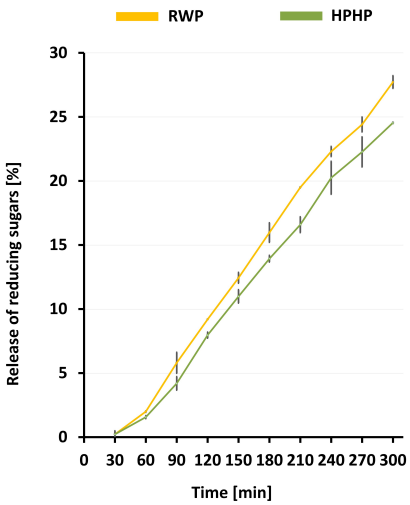


Figure 8.2: Sugar release curves of RWP and HPHP obtained from *in vitro* starch digestion; values expressed as amount of reducing sugars relative to digestible starch.

HPHP than in RWP (Table 8.3). This suggests HPHP as favourable pasta formulation compared to RWP, since diets with high GI and/or GL have been associated with elevated risk for heart disease, diabetes and certain types of cancer [32–34]. The isocaloric replacement of wheat starch by non-wheat proteins in HPHP also brings the ratio of calories provided by protein up to 23.4% compared to the lower ratio of 17.6% in RWP. This is particularly important with regard to European legislation (regulation (EC) No 1924/2006 [35]), where a protein level of at least 20% of calories provided by protein is specified as requirement for a 'high in protein' nutritional claim made on foods. Besides non-wheat proteins, also a considerable amount of non-wheat starch is brought into the HPHP formulation by buckwheat flour; accounting for approx. 9.4 %DM (calculated based on composition of raw materials and HPHP recipe). Buckwheat is known to contain substantial amounts of resistant starch (RS), which has been linked to several health benefits and could further improve HPHP's nutritional value [36, 37]. However, the contents of RS determined for RWP and HPHP are both relatively low and HPHP contains with 0.56 %DM even slightly less RS than RWP with 0.91 %DM (Table 8.3). This is in line with the literature where cooking has been reported to cause a substantial decrease of RS in buckwheat groats [36]. Other minor compositional changes include the contents of lipids, ash and fibre. The HPHP formulation contains with 1.97 % slightly more lipids than RWP with 1.33 %DM. However, this can only be considered a small difference and both formulations are low in fat (threshold for 'low fat' nutritional claim in European legislation [35]: 3 g of fat per 100 g of solids). Furthermore, studies have shown that the fatty acid balance of a diet is more critical than the total fat intake [38]. Although dietary recommendations refer to the whole diet, RWP's and HPHP's contribution to a balanced fatty acid

profile can be compared. A desired ratio of SFA:MUFA:PUFA in a diet seems to approximate 1:1.3:1 [38]. Both formulations contribute with 1:1.3:2.6 (RWP) and 1:1.4:2.3 (HPHP) similarly elevated amounts of PUFA to complement other low-PUFA diet components (e.g. milk fat). The increased ash content of 1.4 %DM in HPHP (1.0 %DM in RWP) is likely related to a higher concentration of minerals caused by the incorporation of HPIs. Vogelsang-O'Dwyer *et al.* [39] determined the mineral composition of the lupin protein isolate used in the present study and reported high levels of the nutritionally valuable minerals Ca, Fe and Zn. Tazrart *et al.* [8] and Petitot *et al.* [12] found substantially elevated levels of Ca, Fe and Zn in pasta fortified with faba bean flour. The determined fibre content for HPHP is with 3.8 %DM slightly lower than for RWP with 4.8 %DM. However, the analytical standard method applied in this study (AOAC 991.43) has been reported to not sufficiently quantitate food components like resistant starch, fructans and galactooligosaccharides (GOS), which are considered as dietary fibre according to more recent definitions [40]. Ispiryan *et al.* [41] characterised the FODMAP profile of selected cereal-product ingredients and reported a high GOS content (raffinose, stachyose and verbascose) of 4.87 %DM for the faba bean flour used in the present study. Considering this, it could be expected that the amount of total dietary fibre in HPHP was underestimated and might be equal to or higher than in RWP.

8.4.2 Amino Acid Profile

Wheat based staple foods, like regular wheat pasta, represent an important source of plant-based dietary protein. However, wheat protein has low quality which is related to its unbalanced amino acid (AA) profile and a lack of indispensable AA; specifically lysine [42]. Besides changes in the macronutrient profile, the incorporation of pseudocereal and legume HPIs in HPHP also introduces a shift in the AA pattern. The AA contents of RWP and HPHP are presented in Table 8.4 and expressed in percent relative to the formulations' protein content in order to allow for a direct comparison of the AA profiles. Amongst the dispensable AAs, the contents of asparagine/aspartic acid, glutamine/glutamic acid, proline and arginine differ substantially between RWP and HPHP. Wheat is rich in glutamine/glutamic acid and proline but contains relatively small amounts of asparagine/aspartic acid and arginine [42]. Buckwheat, faba bean and lupin represent a complementary pattern with respect to these AAs [37, 39, 43]. Therefore, reduced levels of glutamine/glutamic acid and proline and raised levels of asparagine/aspartic acid and arginine are present in HPHP. The profiles of indispensable AAs in RWP and HPHP also exhibit several differences, specifically with regard to threonine, tryptophan, lysine and sulphur containing AAs (SAAs). The incorporation of HPIs leads to a

Table 8.4: Amino acid (AA) composition of protein of reference wheat pasta (RWP) and high-protein hybrid pasta (HPHP).

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

Amino acid contents ± uncertainty values.

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

** Sum of sulphur containing amino acids (SAAs).

*** Sum of aromatic amino acids (AAAs).

small increase in the level of threonine in HPHP compared to RWP, which is related to high contents of this AA in faba bean, lupin and particularly in buckwheat [39, 43, 44]. The tryptophan level was below the limit of quantification (LOQ; equals 0.29 %Protein for RWP) and above the limit of determination (LOD; equals 0.58 %Protein for RWP) for RWP. For HPHP, a tryptophan content of 0.84 %Protein was determined. While wheat semolina, faba bean and lupin all contain similar amounts of this AA, buckwheat is relatively rich in tryptophan [37, 39, 42, 43]. Therefore, an increased tryptophan level is achieved in HPHP due to the use of buckwheat HPI in addition to legume HPIs. Lysine represents the indispensable AA with the biggest discrepancy between RWP and HPHP. The HPHP formulation contains 3.90 %Protein which represents a by 34% increased lysine level in comparison to RWP with 2.58 %Protein. In contrast to this increase observed for lysine, the results show that the substitution of wheat semolina by HPIs leads to a decrease in SAAs, which account for 3.39 %Protein in RWP and 2.99 %Protein in HPHP. These differences in indispensable AAs might seem small when levels expressed in %Protein are compared. But some of these differences represent a significant improvement of the pasta's amino acid balance. This becomes apparent when AA levels are expressed relative to a reference pattern of AA intake

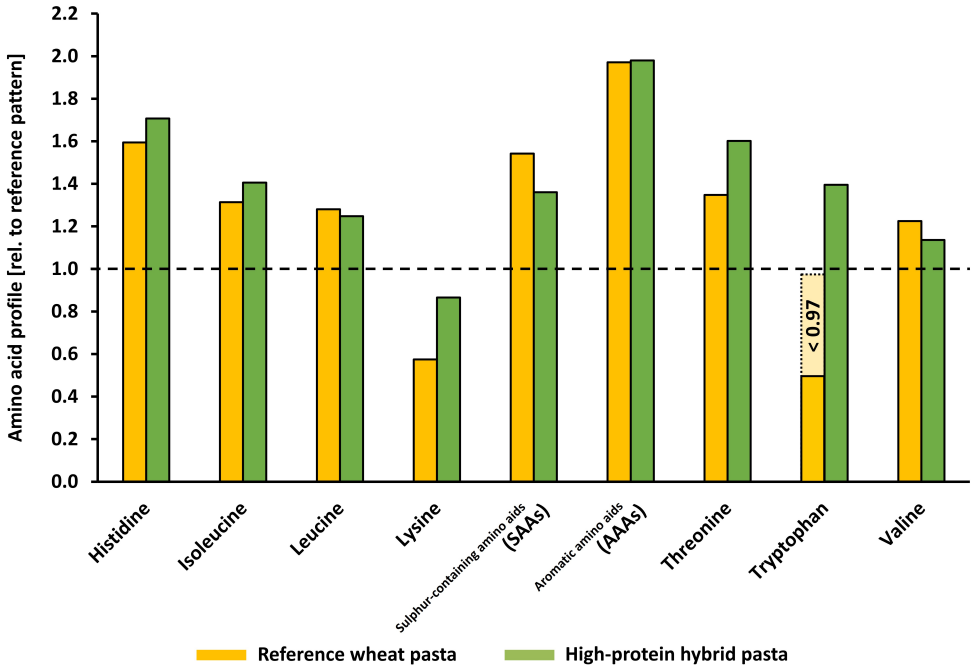


Figure 8.3: Profile of indispensable amino acids of reference wheat pasta and high-protein hybrid pasta expressed relative to the requirement pattern (WHO 2007 [45]) and based on an average intake of 0.66 g protein/kg; the level of tryptophan in RWP was below the limit of quantification (LOQ) and above the limit of detection (LOD) which equals a range between 0.48 and 0.97 calculated relative to tryptophan in the reference pattern.

for adults recommended by WHO [45] (Figure 8.3). The comparison of the AAs in RWP and HPHP with the reference pattern shows that lysine does not reach the recommended level (= 1). Thus, lysine represents the limiting AA in the protein from both formulations. However, the increased lysine content in HPHP reaches 87% of the recommended level as opposed to 57% in RWP. Furthermore, the incorporation of buckwheat flour secures an adequate level of tryptophan in HPHP by raising its content to 140% of the required amount. This makes the HPHP AA profile, which nearly covers the recommended intake of all indispensable AAs, much more balanced. Amino acid scores (AASs) represent the content of the limiting AA of a protein calculated relative to the reference pattern and are commonly used to interpret protein quality. Table 8.5 summarises the AASs of RWP, HPHP and the raw materials used for their production. The AASs indicate that the combination of buckwheat flour, faba bean flour and lupin protein isolate for partial wheat semolina substitution leads to an upgrade of the protein quality of all protein sources. Only buckwheat flour represents an exception and possesses a desirable AA profile on its own. The partial substitution of wheat semolina by legumes has previously been reported to improve the AA profile of pasta protein; specifically through increased lysine levels [9]. However, an optimal substitution ratio (legume:wheat) has been discussed with regard to SAAs [9]. While lysine

Table 8.5: Amino acid scores (AASs) for pasta formulations and their raw materials.

Protein source	AAS	Limiting Amino acids
Reference wheat pasta (RWP)	0.57	Lysine; Tryptophan (<0.97)
High-protein hybrid pasta (HPHP)	0.87	Lysine
Semolina*	0.57	Lysine
Buckwheat flour*	- (1.13)***	- (Leucine)***
Faba bean flour**	0.66	SAAs
Lupin protein isolate**	0.70	SAAs; Valine (0.93); Lysine (0.98)

* Calculated from amino acid composition; determined as for RWP and HPHP (data not shown).
** Calculated from amino acid composition; determined as for RWP and HPHP and reported by Vogelsang-O'Dwyer *et al.* [39, 43].
*** Not strictly limiting (≥ 1), but represents AA with lowest level relative to reference pattern.

levels rise with increased legumes:wheat ratios, SAA contents drop; due to low levels of SAAs in legumes. The fact that only a moderate decrease in SAA content was observed for HPHP in this study is related to the high level of SAAs in buckwheat which even exceeds that of wheat semolina [42, 44]. The use of AASs to evaluate protein quality is based on the assumption that the considered daily intake of 0.66 g protein per kg bodyweight is entirely covered by the concerning protein source. In a real diet, AA deficiencies of one protein source can potentially be covered by another. Nevertheless, the assessment of a protein's ability to cover AA requirements is considered an adequate approach to compare proteins from different sources and their nutritional quality.

8.4.3 Antinutritional Compounds

The activity/contents of trypsin inhibitors and the pyrimidine glycosides vicine and convicine have been determined for both raw and cooked RWP and HPHP (Table 8.6). Trypsin inhibitors are some of the most relevant ANCs with regard to a product's protein quality. These molecules can form complexes with the intestinal enzyme trypsin (or chymotrypsin) thereby inhibiting their proteolytic activity and potentially causing a decrease in protein digestibility [15]. They have also been reported to cause other adverse physical conditions like pancreatic hypertrophy and increased pancreatic secretory activity [46]. The TIAs determined for raw and cooked HPHP were higher than those for RWP (both raw and cooked).

Table 8.6: Contents of antinutritional compounds of reference wheat pasta (RWP) and high-protein hybrid pasta (HPHP), contents refer to dry matter as indicated.

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

Means $\pm$ standard deviation.
n.d., Not detected.

While the lupin protein isolate applied in HPHP reportedly only has a very low remaining TIA [39], the HPIs from faba bean and buckwheat are likely to cause the higher TIA in HPHP. Vogelsang-O'Dwyer *et al.* [43] found a TIA of 2.34 TIU/mg DM in the faba bean flour applied in HPHP. Also the low digestibility of buckwheat protein has been associated with a high trypsin inhibitor activity [5, 47]. The results in the present study additionally show that the cooking process leads to a substantial decrease in TIA in both RWP and HPHP formulation. A very low TIA of 0.38 TIU/mg DM was measured for raw RWP and no TIA was detected in cooked RWP. For HPHP, the cooking process caused a more than threefold reduction of TIA from 3.3 TIU/mg DM in raw HPHP to 0.91 TIU/mg DM in cooked HPHP. Several articles have addressed the impact of various processing techniques on TIA in foods [16, 46, 48]. Thermal treatments such as boiling, cooking and roasting have been mentioned as the most efficient procedures to inactivate legume trypsin inhibitors. The mechanism of this inactivation primarily relies on conformational changes of their active site which prevents complex formation with and inhibition of trypsin. Soybean trypsin inhibitors, for example, seem to be sufficiently inactivated by boiling at 100 °C for approx. 9 min [48]. Similar conditions were applied in the cooking procedure of HPHP. The achieved reduction of TIA is in agreement with results reported in literature, where usually a decrease to 15–51% residual TIA in cooked pasta is reached [10, 11]. Zhao *et al.* [11] even reported no residual TIA in cooked spaghetti fortified with 15–20% pea and lentil flour. The small remaining TIA in this study could be related to the lack of a heat drying process (which is performed in most studies investigating pasta quality) after extrusion of RWP and HPHP which could contribute to trypsin inhibitor inactivation. Furthermore, trypsin inhibitors originating from buckwheat seeds were found to be relatively thermostable (specifically at acidic pH) when subjected to heat treatment at 100 °C for 30 min [49]. Faba beans are known to contain the thermostable ANCs vicine and convicine [50]. These pyrimidine glycosides can trigger an adverse physical condition called favism in human individuals that are deficient in glucose-6-phosphate dehydrogenase (G6PD) [18]. Since triggered favism leads to acute haemolytic anaemia in G6PD deficient consumers [18],it is important to monitor vicine and convicine levels in faba bean containing foods. Recent efforts in plant breeding with the objective to reduce pyrimidine glycoside contents led to cultivars with levels as low as 0.01–0.02 %DM. However, in faba bean seeds from most varieties, a VC (sum of vicine and convicine) level of approx. 1 %DM is present [17, 50]. While for RWP, expectedly, no vicine and convicine were detected, a VC level of 0.300 %DM (vicine 0.161 %DM; convicine 0.139 %DM) was determined in raw HPHP. This is in agreement with the findings of Vogelsang-O'Dwyer *et al.* [43] who reported a VC content of 1.25 %DM in the faba bean flour applied in HPHP (expected

amount of VC in HPHP approximates 0.50 %DM based on calculation considering recipe and ingredient composition). Cooked HPHP contains only 0.050 %DM of VC (vicine 0.028 %DM; convicine 0.022 %DM) which represents an 83 % reduction of VC content in HPHP induced by the cooking procedure. Besides enzyme treatment, also aqueous extraction at elevated temperatures, has been mentioned as efficient technique to eliminate or reduce vicine and convicine contents in faba beans [17, 50, 51]. A recent study by Gallo *et al.* [52] investigated the consumption of large quantities of faba beans (500 g) with a low VC content of 0.016% (in wet weight as ingested) by G6PD deficient men. Favism was not triggered and the results suggested that this level of VC intake is safe for G6PD deficient individuals. In the context of these findings, also the consumption of approx. 300 g cooked HPHP (exceeds the daily intake of cooked pasta recommended by dietary guidelines of European countries [53]) can be considered safe. While the results in the present study confirm a substantial reduction of TIA and VC content due to cooking of pasta, also the dilution of both ANCs due to incorporation of the HPIs in a complex pasta formulation should be addressed. The separate consumption of protein from sources with complementary AA patterns (cereals, pseudocereals, legumes) can in theory provide a balanced AA intake. However, higher ANC levels in raw materials, as opposed to hybrid formulations like HPHP, can impair protein digestibility and, therefore, bioavailability of AA from some of these sources.

8.4.4 Protein Digestibility and Utilisation

In vitro digestion was performed with raw and cooked pasta in order to evaluate the effect of the cooking procedure on the samples' susceptibility to degradation by pepsin and pancreatin. The results are presented in Table 8.7. The pepsin treatment and subsequent short term treatment with pancreatin mimics the protein digestion of RWP and HPHP in the human gastrointestinal tract. Medium and long term treatment with pancreatin were carried out in order to obtain an indication of the maximum achievable protein degradation under the same conditions. Two major trends were observed. For pepsin treatment as well as short and medium term pancreatin treatment of both formulations, raw pasta reached significantly (except RWP after medium term pancreatin treatment) higher IVPDs than cooked pasta. This trend was clearly detected even for the HPHP formulation, where a substantial reduction of TIA (by 73%) was observed due to the cooking process, which could have been expected to cause an increase in protein digestibility. Petitot *et al.* [14] who reviewed the impact of pasta processing on starch and protein digestibility concluded that thermally induced protein aggregation could increase resistance to pepsin/pancreatin degradation of protein in cooked pasta. It is also possible

Table 8.7: *In vitro* protein digestibility and *in vivo* nitrogen balance of reference wheat pasta (RWP) and high-protein hybrid pasta (HPHP).

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

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

that, similar to observations reported for *in vitro* starch digestion [13, 14], the pasta structure created during the cooking process (coagulated protein network entrapping starch granules) slows down enzymatic degradation of proteins. The fact that IVPDs of cooked pasta after long term pancreatin treatment are not smaller than for raw pasta further supports this theory. According to these results, changes in structure and substrate accessibility induced by the cooking procedure seem to have a larger impact on protein digestibility in HPHP than TIA. Furthermore, the difference in IVPD between raw and cooked pasta seems to be more pronounced for HPHP than for RWP. This is in line with the previous findings of Hoehnel *et al.* [19]. Their results of technological quality analysis of RWP and HPHP suggested a thicker protein network in HPHP than RWP; and a strengthened protein network in HPHP through interactions between wheat and non-wheat proteins. The second clear trend with regard to IVPDs concerns the difference between RWP and HPHP. Both raw and cooked HPHP reach higher IVPDs for all digestive treatments (except long term pancreatin treatment) than their RWP equivalent. Again, higher IVPDs were observed despite higher detected TIAs for these samples. This indicates that TIA levels might have been too low to affect enzymatic protein degradation. Also Laleg *et al.* [54] and Tazrart *et al.* [8] found slightly increased protein digestibility of pasta formulations fortified with legume ingredients when compared to wheat semolina controls. A higher protein digestibility in formulations containing pseudocereal and legume ingredients could be related to a higher abundance of target AA for the cleavage by trypsin and chymotrypsin (lysine, arginine, AAAs, tryptophan) as already suggested by Hoehnel *et al.* [21]. The results of *in vivo* nitrogen balance trials performed with rats are presented in Table 8.7. The most important variables are N intake (which is used to calculate relative N losses in urine and faeces) as well

as N digestibility, N utilisation and PER, which are indicative of the pastas' nutritional value. No significant difference was observed for the N intake of rats which were fed diets containing either RWP or HPHP as protein source. Since the content of RWP and HPHP in the experimental diets was adjusted so they contained the same amount of protein, this indicates that similar amounts of total diet were consumed by RWP and HPHP rats. The values determined for N digestibility were not significantly different between RWP (88.0%) and HPHP (88.4%). *In vitro* digestion, however, indicated higher digestibility of HPHP. According to literature, *in vitro* and *in vivo* digestibility data usually show good correlations; but it has also been reported that legumes tend to reach higher digestibility *in vitro* than *in vivo* [55]. The protein digestibility corrected amino acid score (PDCAAS) represents another commonly used indicator of protein quality and is calculated considering faecal N digestibility and AASs (discussed in amino acid section above). Due to the similar N digestibility of RWP and HPHP, PDCAAS values follow the same trend as AAS values. While RWP has a PDCAAS of only 0.50, HPHP reaches a value of 0.79 owing to its higher lysine level. The significantly lower urinary N loss measured for HPHP rats results in a by 55.6% increased N utilisation rate for HPHP (43.1%) compared to RWP (27.7%). This is likely related to the improved AA balance of HPHP. The lack of one or more indispensable AAs, amongst absorbed AAs, has been reported to cause a plateau in AA retention while AAs which are present in excess of the limiting AA (relative to the required AA pattern) are excreted with the urine after oxidisation [45, 56]. Furthermore, a positive correlation between balance of dietary AAs and AA utilisation was found in both animal and human studies and a higher degree of AA imbalance led to limited protein synthesis [57]. In agreement with the higher N utilisation rate, also the PER determined for rats with HPHP diet (2.13 g/g) was significantly higher than that determined for RWP rats (1.37 g/g). The PER reflects a protein's value with respect to AA requirements for growth. Both *in vitro* and *in vivo* models to determine protein quality for human nutrition have their limitations and true protein quality depends on the AA profile which is absorbed and utilised to achieve specific metabolic actions in the human body [56, 58]. However, AA profile and values like IVPD, N digestibility, N utilisation rate and PER provide a valid comparison of protein from RWP and HPHP and conclusively indicate improved protein quality in HPHP.

8.5 Conclusion

The present study evaluates the nutritional profile of a high-protein hybrid pasta (HPHP) formulation. In this formulation, a combination of three HPIs from buckwheat, faba bean and lupin was used to partially substitute wheat semolina. The

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

8.6 Acknowledgements

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CHAPTER 9

General Discussion and Conclusions

9.1 General Discussion and Conclusions

It has been conclusively demonstrated, in the literature and in this thesis, that our current food system needs to undergo a fundamental transformation to preserve environmental and human health [1–10]. Multiple measures and strategies will need to be employed at the same time to achieve this transformation [1, 11–13]. In addition to reducing food waste and improving agricultural processes and technologies, a global shift to predominantly plant-based diets promises substantially improved environmental sustainability of our food system as well as improved human health [1]. Since animal foods provide large amounts of high-quality protein in currently consumed diets, the question whether and how adequate protein supply from predominantly plant-based diets can be achieved, deserves extensive research attention. Therefore, current protein intakes were reviewed in **Chapter 2** of this thesis and discussed in the context of recommended protein intakes and anticipated diet shifts. Current protein intakes seem to suggest that protein is overconsumed in many high/medium-income countries and that the pressure on future food systems to provide plant-based protein could be reduced by avoiding protein overconsumption. However, considerable uncertainties remain with regard to recommended protein intakes for optimal health (as opposed to maintenance of nitrogen balance) and, therefore, with regard to the extent of current protein overconsumption. High-protein diets (protein intakes above currently recommended intake level of 0.83 g/kg/day [14]) have been associated with several health benefits (reduced prevalence of obesity and diabetes type 2, improved blood pressure and blood lipid levels), especially when protein intake was covered by plant-based protein sources [15–21]. Moreover, such high-protein diets have been reported to promote increased satiety [20]. This means that a moderate 'overconsumption' of protein from predominantly plant-based diets could help to reduce calorie overconsumption, which is an issue (with environmental and human health implications) of currently consumed diets in many high/medium-income countries [13]. The often inferior protein quality of individual plant-based protein sources can be overcome by consuming slightly increased quantities (to compensate for limited protein digestibility) and by consuming sufficiently diverse plant-based diets (to compensate for the lack of certain IAAs in individual plant-based protein sources) [22, 22–26]. Protein supply data presented in **Chapter 2** show that plant-based protein supply in currently consumed diets lacks diversity and is primarily covered by cereal foods. Hence, an increased inclusion of non-cereal protein in predominantly plant-based diets is desirable. Nonetheless, where diet shifts are concerned, it is important to also consider consumer perspectives. Several studies have found that limited consumer acceptance of healthy (and environmentally sustainable)

diets, based on less meat and more whole-grain foods, legumes, nuts, fruit and vegetables, is often related to a lack of convenience and affordability [13, 27, 28]. This highlights the substantial demand for tasty, affordable and convenient plant-based protein-foods. A targeted approach, considering scientific knowledge on environmental sustainability, human health and consumer preferences, should be chosen for the development of these plant-based protein-foods in order to support food system transformation. This approach was used to establish five principles of targeted plant-based protein-food development, which were introduced in **Chapter 2** of this thesis. However, the formulation of plant-based protein-foods according to these principles depends on the availability of suitable plant-based protein ingredients (PIs). While the plant-based food sector is growing fast and the number of commercially available plant-based PIs is rising, the majority of these ingredients are derived from the following four plant sources: soy, pea, wheat and rice. To increase diversity in plant-based protein intake by consumers, product developers rely on an improved availability of PIs from a larger range of promising plant sources.

In this thesis, a large range of plant-based high-protein ingredients (HPIs) from cereals, pseudocereals, potato and legumes were characterised and investigated regarding their applicability in cereal-based staple foods. Both commercially available as well as experimental plant-based HPIs were included to support progress in ingredient development. A thorough physicochemical characterisation of HPIs builds the foundation for identifying appropriate processing techniques and food applications for such experimental. In **Chapter 3**, various extraction procedures were compared with regard to their suitability to extract short-chain carbohydrates from plant-based HPIs for subsequent separation and quantification with HPLC-RID. Commonly used aqueous extraction of short-chain carbohydrates can lead to practical complications when applied to plant-based HPIs due to their high water absorption and gelation capacity. This is particularly problematic when low liquid/solid are required during extraction to obtain concentrated extracts for quantification with low-sensitivity detectors (e.g. RID). Further complications can be related to enzymatic degradation of di-, oligo- and polysaccharides during extraction, co-extraction of polysaccharides or microbial growth [29–31]. A sonication-assisted ethanolic extraction in combination with vacuum centrifugation to concentrate the extract and the use of NaN_3 as antimicrobial agent in the final aqueous extract was identified as most suitable to accommodate the aforementioned challenges. A high liquid/solid ratio and the high ethanol content during extraction avoid practical problems linked to water absorption and gelation. Besides, the high proportion of ethanol in the extraction solvent (80/20 (v/v) in

UPW) also prevents enzymatic conversion and degradation of carbohydrates [29]. While ethanol levels above 50% in the extraction solvent have been found to reduce extractability of short-chain carbohydrates [29, 32], this seems to have been compensated by the use of sonication-assisted extraction, which was shown to result in more efficient sugar extraction than stirring or shaking [33, 34]. The evaporation of ethanol by vacuum centrifugation assures sufficiently high extract concentration for subsequent quantification with HPLC-RID and the use NaN_3 prevents microbial growth and associated consumption or conversion of short-chain carbohydrates in the final aqueous extracts.

The applicability of plant-based HPIs in bread formulations (**Chapter 4**) was investigated by replacing 15% of wheat flour by plant-based HPIs to obtain breads that are 'high in protein' in accordance with European legislation (at least 20% of calories provided by protein [35]). The outcome was HPI specific and a large range of bread qualities, from substantially impaired (lupin protein isolate) to substantially improved (wheat gluten) in comparison to a control wheat bread, was observed. A bread formulation's baking performance is largely dependent on rheological dough properties and the dough's response to heat treatment (and subsequent cooling). Both were found to be affected by the incorporation of plant-based HPIs. The analysis of gluten-aggregation behaviour in the presence of plant-based HPIs was identified as a useful tool to explain the impact of plant-based HPIs on bread quality. Gluten-aggregation profiles indicated a weakened gluten network in formulations containing pea and potato HPIs. For formulations containing carob and zein HPIs, which showed improved bread quality (compared to control wheat bread), a secondary network formation between non-wheat proteins and gluten was postulated based on the appearance of a second peak maximum in gluten-aggregation profiles resulting in delayed network breakdown. Despite the weakened gluten-aggregation of the formulation containing potato HPI, its overall bread quality was similar to the control wheat bread. This was attributed to the potato protein's unique gelling behaviour in mixture with wheat flour upon heat treatment. From a nutritional point of view, the application of legume HPIs in bread formulations seems most promising due to the complementing amino acid profiles of cereal and legume proteins [36]. Amongst the tested legume HPIs, the experimental faba bean HPIs (in addition to the commercial carob HPI) resulted in breads with adequate technological quality only slightly inferior compared to the control wheat bread. This is particularly remarkable since this experimental faba bean ingredient was produced by dry fractionation, which has been described as sustainable processing option for protein sources [37, 38].

In **Chapter 5**, faba bean, carob and gluten HPIs were chosen to create a high-protein hybrid bread (HPHB) formulation with optimised technological quality. The analysis of dough and bread characteristics confirmed that technological bread quality was equal to that of regular wheat bread. Similar to observations in **Chapter 4**, a delayed network breakdown in gluten-aggregation profiles (here, in the form of a plateau rather than a second peak maximum) was associated with good baking performance. The nutritional characterisation of HPHB revealed a substantial improvement of the bread's nutritional value in comparison to regular wheat bread. While it is difficult to judge true protein quality for human nutrition without performing human intervention studies, the conducted experiments conclusively indicated enhanced protein quality with an amino acid composition more suitable for human nutrition, increased *in vitro* protein digestibility, substantially elevated *in vivo* nitrogen utilisation and higher protein efficiency ratio. Also, other nutritional aspects were found to be improved. The macronutrient profile was characterised by an increased fibre content and an isocaloric replacement of starch by protein, which promises a decreased glycaemic load of HPHB. Furthermore, a significantly higher antioxidant potential was measured while only low levels of antinutritional compounds (ANCs) were detected. Considering the fact that, additionally, sensory analysis proved adequate sensory quality of HPHP, this formulation represents a valuable plant-based protein-food developed in accordance with the principles of targeted plant-based protein-food development introduced in **Chapter 2**.

Fermentation technology as a tool to improve the baking performance of two faba bean PIs (dehulled flour (DF) and dry fractionated high-protein flour (PR)) was explored in **Chapter 6**. While the faba bean high-protein flour was also utilised for HPHB in **Chapter 5**, it required the combination with other, better performing HPIs (carob and gluten) and the addition of functional ingredients like xylanase to achieve technological bread quality equal to regular wheat bread. Ingredient functionalisation by fermentation might reduce the need for such functional ingredients, which could further improve the value of faba bean PIs. The single strain fermentation with the multifunctional LAB strain *Leuconostoc citreum* TR116 resulted in much more moderate acidification (lower pH) but much higher total titratable acidity (TTA) in PR than in DF. This was possibly related to a higher buffering capacity of PR mediated by the higher protein and/or mineral content. Most importantly, fermentation caused a striking improvement of the baking performance of both faba bean PIs primarily represented by higher loaf volume and lower crumb hardness (compared to breads containing unfermented faba bean PIs). Again, this improved baking performance was indicated by stronger gluten network formation and

delayed network breakdown observed in the gluten-aggregation profiles. During ingredient fermentation, levels of phenolic acids with potentially antioxidant activity (and associated health benefits [39–42]) and antifungal activity [43–45] were monitored. More phenolic acids were released during fermentation of the high-protein flour. Therefore, fermented PR might be more desirable for bakery applications than fermented DF, since the presence of antifungal phenolic acids due to sourdough fermentation has been associated with potentially improved microbiological shelf life of breads [46–50]. Additionally, due to its higher protein content, fermented PR represents a higher capacity to diversify plant-based protein supply when incorporated in breads and the breads containing fermented PR reach the protein level required for a 'high in protein' label according to European legislation [35].

A combination of pseudocereal (buckwheat) and legume (faba bean and lupin) HPIs were applied in pasta in **Chapter 7** to create a high-protein hybrid pasta (HPHP) formulation. The level of wheat semolina replacement (25% in HPHP) was chosen to reach the previously mentioned threshold of 20% of calories provided by protein in the final products (requirement for 'high in protein' nutrition claim [35]). The main challenges associated with the incorporation of plant-based HPIs in pasta seem to be increased cooking losses, increased stickiness and poor sensory attributes [51, 52]. In the experiments performed for **Chapter 7**, faba bean HPI was identified to increase stickiness (faba bean) but, at the same time, to improve pasta texture (increased firmness and tensile strength). Buckwheat HPI, on the other hand, led to an impaired pasta texture and did not significantly impact pasta stickiness. The use of a combination of HPIs in HPHP was found to be beneficial to allow for a compensation of each others' effects on specific pasta quality characteristics. Therefore, technological quality characteristics of HPHP were equal those of regular wheat pasta (except darker pasta colour). Additionally, sensory attributes of HPHP were similar to regular wheat pasta with only moderately increased legume odour and flavour. The nutritional value of HPHP was thoroughly investigated in **Chapter 8** and found to be similarly improved as for HPHB in **Chapter 5**. A substantially improved protein quality was suggested by the results of amino acid analysis, *in vitro* protein digestibility and *in vivo* nitrogen balance trials. HPHP, like HPHB, was also characterised by an isocaloric replacement of starch by plant-based protein in comparison to regular wheat pasta. While this already suggests a lowered glycaemic load as long as the glycaemic index of HPHP an regular wheat pasta is similar, starch digestion of both pasta formulations was studied *in vitro* to further define HPHP's nutritional value. Starch digestion was shown to be similar for HPHP and regular wheat pasta, however, with a small

tendency towards slower release of reducing sugars from starch, which supports the above hypothesis about HPHP's glycaemic load. Levels of ANCs (trypsin inhibitor activity, vicine, convicine) were determined in both raw and cooked HPHP and it was found that the cooking process reduced ANC contents significantly (trypsin inhibitor activity by 73% and vicine and convicine by 83% and 84%, respectively). With the two primary HPIs applied in HPHP being dry fractionated pseudocereal and legume HPIs and considering HPHP's adequate technological and sensory quality and the improved nutritional value, it can be concluded that also HPHP represents a valuable plant-based protein-food developed in accordance with the principles of targeted plant-based protein-food development introduced in **Chapter 2**.

This thesis demonstrates that both bread and pasta can be produced with a technological and sensory quality similar to regular wheat bread and pasta but with a substantially improved nutritional profile by incorporating a combination of carefully selected and potentially functionalised non-cereal plant-based HPIs. These cereal/non-cereal staple food hybrids provide valuable protein, specifically for predominantly plant-based diets, and represent an excellent opportunity to diversify plant-based protein supply for consumers. For the functionalisation of plant-based high-protein ingredients, lactic acid fermentation was proven to be a valuable tool and should be further exploited to advance the protein transition.

9.2 References

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APPENDIX A: Supplementary Information to Chapter 2

Table A.A1: Countries included in world regions based on classification from FAO, 2011 (Global Food Losses and Food Waste: Extent, Causes and Prevention. Rome. Annex 1, p. 23) with some modifications due to missing data in FAOSTAT or GDD for 2015.

Region 1 Europe	**Region 2** Northern America, Australia, New Zealand	**Region 3** Industrialised Asia	**Region 4** Sub-Saharan Africa	**Region 5** North Africa, West and Central Asia	**Region 6** South and Southeast Asia	**Region 7** Latin America
Albania	Australia	China	Angola	Algeria	Afghanistan	Argentina
Armenia	Canada	Japan	Benin	Egypt	Bangladesh	Belize
Austria	New Zealand	South Korea	Botswana	Iraq	Cambodia	Bolivia
Azerbaijan	United States of America		Burkina Faso	Israel	India	Brazil
Belarus			Cameroon	Jordan	Indonesia	Chile
Belgium			Central African Rep	Kazakhstan	Iran	Colombia
Bosnia and Herzegovina			Chad	Kuwait	Malaysia	Costa Rica
Bulgaria			Congo	Kyrgyzstan	Myanmar	Cuba
Croatia			Côte d'Ivoire	Lebanon	Nepal	Dominican Rep
Cyprus			Ethiopia	Mongolia	Pakistan	Ecuador
Czech Rep			Gabon	Morocco	Philippines	El Salvador
Denmark			Ghana	Oman	Sri Lanka	Guatemala
Estonia			Guinea	Saudi Arabia	Thailand	Guyana
Finland			Guinea-Bissau	Tajikistan	Vietnam	Haiti
France			Kenya	Tunisia		Honduras
Georgia			Lesotho	Turkey		Jamaica
Germany			Liberia	United Arab Emirates		Mexico
Greece			Malawi	Uzbekistan		Nicaragua
Hungary			Mali	Yemen		Panama
Iceland			Mauritania			Peru
Ireland			Mozambique			Paraguay
Italy			Namibia			Suriname
Latvia			Niger			Uruguay
Lithuania			Nigeria			Venezuela
Luxembourg			Rwanda			
North Macedonia			Senegal			
Rep of Moldova			Sierra Leone			
Montenegro			South Africa			
Netherlands			Sudan			
Norway			Togo			
Poland			Uganda			
Portugal			Zambia			
Romania			Zimbabwe			
Russian Federation						
Serbia						
Slovakia						
Slovenia						
Spain						
Sweden						
Switzerland						
Ukraine						
United Kingdom						
∑ 42	∑ 4	∑ 3	∑ 33	∑ 19	∑ 14	∑ 24

Table A.A2: List of protein ingredients (PIs) included in market overview in Chapter 2; last updated 25/02/2021.

Company	Headquarters	Product name	Source	Type	Protein [%]	Link
Ingredients Inc.	USA	Adzuki Protein	Adzuki bean	Protein		https://www.ingredientsinc.com/ingredients
Bioriginal Food & Science Corp	Canada	Alfapro®Alfalfa Juice Protein	Alfalfa	Protein	50	https://www.bioriginal.com/products/alfapro/
Osage Food Products	USA	Alfalfa Protein	Alfalfa	Protein		http://osagefood.com/protein-products/plant-proteins/
Glanbia plc	Ireland	AlgaVia™ Whole Algal Protein	Algae (unspecified)	Protein		https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
TerraVia	USA	AlgaVia®protein-rich whole algae	Algae (unspecified)	Protein	63	https://algavia.com/ingredients/proteins/
All Organic Treasures GmbH	Germany	Almond Protein, organic	Almond	Protein	53	https://www.all-organic-treasures.com/assortment-food/mandelprotein-bio-lebensmittel.html
Harris Woolf California Almond	USA	Almond Protein Powder	Almond	Protein		https://harriswoolfalmonds.com/products/almond-protein-powder/
Naturz Organics	The Netherlands	Almond Protein 45% - Powder - Premium	Almond	Protein	45	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Almond Protein 50% - Powder - Premium	Almond	Protein	50	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Almond Protein 50% - Powder - Regular	Almond	Protein		https://naturzorganics.com/our-products/proteins-fibers-starches/
Nut Butter Concepts LLC	USA	Almond Protein Powder	Almond	Protein	58	https://nutbutterconcepts.com/almond-protein-powder/
Osage Food Products	USA	Almond Protein	Almond	Protein	70	http://osagefood.com/protein-products/plant-proteins/
Evergrain, LLC by AB-InBev	USA	EverPro™	Barley	Protein	85	https://evergrainingredients.com/our-products/
Evergrain, LLC by AB-InBev	USA	EverVita™	Barley	Protein	35	https://evergrainingredients.com/our-products/
BioExx Specialty Proteins Ltd	Canada	Isolexx®	Rapeseed/Canola	Protein isolate	90	http://teutexx.com/
BioExx Specialty Proteins Ltd	Canada	Vitalexx®	Rapeseed/Canola	Protein	80	http://teutexx.com/
Burcon NutraScience Corporation	Canada	Supertein®	Rapeseed/Canola	Protein isolate		https://www.ulprospector.com/en/na/Food/Detail/12268/354065/Supertein
Burcon NutraScience Corporation	Canada	Puratein®	Rapeseed/Canola	Protein isolate		https://www.ulprospector.com/en/na/Food/Detail/12268/354066/Puratein
Burcon NutraScience Corporation	Canada	Nutratein®	Rapeseed/Canola	Protein isolate		https://burcon.ca/products/canola-proteins/
DSM	The Netherlands	CanolaPRO	Rapeseed/Canola	Protein	90	https://www.dsm.com/corporate/solutions/nutrition-health/canolapro-plant-protein.html
Dupont Nutrition & Biosciences	USA	GRINDSTED®VEG PRO Carob Protein	Carob	Protein		https://www.dupontnutritionandbiosciences.com/product-range/carob-protein.html
Osage Food Products	USA	Cashew Protein	Cashew	Protein	90	http://osagefood.com/protein-products/plant-proteins/
Distribution Nexxus Inc	Canada	Chia Protein	Chia	Protein	35	http://nexxusfoods.com/en/specialty-ingredients/#proteines
Glanbia plc	Ireland	HarvestPro™	Chia	Protein	30	https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
Glanbia plc	Ireland	CuisineChia™ Protein	Chia	Protein concentrate	30	https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
Glanbia plc	Ireland	BevChia™ Protein	Chia	Protein concentrate	30	https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
NP Nutra	USA	Chia Protein powder (organic)	Chia	Protein		https://npnutra.com/wholesale-chia.html
Taijo GmbH	Germany	XiaPure®Chia Protein	Chia	Protein	42	https://taiyogmbh.com/en/start-en/products/chia-protein/
InnovoPro	Israel	CP-Pro 70	Chickpea	Protein concentrate	70	https://innovopro.com/products/
Lehmann & Voss & Co.	Germany	Chickpea Protein 68%	Chickpea	Protein concentrate	68	https://www.lehvoss-nutrition.com/ingredients/plant-derived-ingredients.html
Nutriati Inc.	USA	Artesa Chickpea Protein	Chickpea	Protein	60	http://nutriati.com/ingredients.html
Prinova Group LLC	USA	Chickpea Protein	Chickpea	Protein		https://www.prinovaeurope.com/ingredients/protein-ingredient-suppliers/
Top Health Ingredients	Canada	AdvantaChick-p™ Resolve 65	Chickpea	Protein	65	https://www.tophealthingredients.com/advantachick-p
Top Health Ingredients	Canada	AdvantaChick-p™ Nourish 65	Chickpea	Protein	65	https://www.tophealthingredients.com/advantachick-p
Green Source Organics	USA	Chlorella Powder	Algae (Chlorella)	Protein		https://www.gsoextracts.com/products/chlorella
Green Source Organics	USA	Chlorella Powder (cracked cell)	Algae (Chlorella)	Protein		https://www.gsoextracts.com/products/chlorella
Naturz Organics	The Netherlands	Chlorella Powder - Spray-Dried	Algae (Chlorella)	Protein		https://naturzorganics.com/our-products/microalgae/
Naturz Organics	The Netherlands	Chlorella Powder - Broken Cell Wall - Spray-Dried	Algae (Chlorella)	Protein		https://naturzorganics.com/our-products/microalgae/
NP Nutra	USA	Chlorealla (Cracked Cell) Powder Organic	Algae (Chlorella)	Protein		https://npnutra.com/chlorella_details.html
Osage Food Products	USA	Coconut Protein	Coconut	Protein		http://osagefood.com/protein-products/plant-proteins/

Cargill Food Ingredients	USA	Corn protein	Corn	Protein	70-90	https://www.cargill.com/food-bev/na/proteins/corn-protein
Industrias Mexstarch SAPI De CV	Mexico	Fortius®Corn Protein Concentrate	Corn	Protein concentrate	50	https://www.ulprospector.com/en/na/Food/Detail/33353/368259/Fortius-Corn-Protein-Concentrate
Penta Manufacturing Company	USA	Zein	Corn	Protein		https://www.ulprospector.com/en/na/Food/Detail/16153/406202/Zein
Qingdao Free Trade Zone United International	China	Corn Gluten Meal	Corn	Protein	60	https://www.ulprospector.com/en/na/Food/Detail/15382/428731/Corn-Gluten-Meal
Vitacyclix	USA	Cranberry Protein Powder	Cranberry	Protein		https://www.morretec.com/divisions/vitacyclix/proteins-fibers/
Osage Food Products	USA	Cucumber Seed Protein	Cucumber seed	Protein		http://osagefood.com/protein-products/plant-proteins/
Hinoman Ltd.	Israel	Mankai™	Duckweed	Protein		https://www.eatmankai.com/
Parabel USA Inc	USA	LENTEIN®Complete	Duckweed	Protein	40-55	https://lentein.com/products/lentein-complete/
Parabel USA Inc	USA	LENTEIN®Protein Concentrate	Duckweed	Protein	70-75	https://lentein.com/products/lentein-concentrate/
Parabel USA Inc	USA	LENTEIN®Isolate	Duckweed	Protein	85	https://lentein.com/products/lentein-isolate/
All Organic Treasures GmbH	Germany	Faba Bean Protein, organic	Faba bean	Protein	55	https://www.all-organic-treasures.com/assortment-food/favabohnenprotein-55-bio-lebensmittel.html
Blattmann Schweiz AG	Schweiz	BecoPulse Fava 60 Bio	Faba bean	Protein concentrate	60	https://www.blattmannschweiz.com/products/fava-bean-protein-and-flour/
Distribution Nexxus Inc	Canada	Fava Bean Protein	Faba bean	Protein		http://nexxusfoods.com/en/specialty-ingredients/#proteines
Dutch Organic International Trade	The Netherlands	Fava bean protein powder 60%	Faba bean	Protein	60	https://www.organic.nl/ingredients/plant-protein/plant-protein/20334-fava-bean-protein-powder-60-org-15-kg.html
Herba Ingredients BV	The Netherlands	HerbaPRO FB65	Faba bean	Protein	65	http://www.herbaingredients.com/pulses/faba-bean-ingredients/faba-bean-protein
Ingredients Inc.	USA	Fava Bean Protein	Faba bean	Protein		https://www.ingredientsinc.com/ingredients
Ingredion	USA	VITESSENCE™ Pulse 3600	Faba bean	Protein concentrate	60	https://www.ulprospector.com/en/na/Food/Detail/12852/705464/VITESSENCE-Pulse-CT-3602
Lehmann & Voss & Co.	Germany	Fava Bean Protein 88%	Faba bean	Protein concentrate	88	https://www.lehvoss-nutrition.com/ingredients/plant-derived-ingredients.html
Meelunie B.V.	The Netherlands	Fava bean protein concentrate	Faba bean	Protein concentrate	60	https://meelunie.com/products/product-catalogue/
Naturz Organics	The Netherlands	Fava Bean Protein 65% - Powder	Faba bean	Protein	65	https://naturzorganics.com/our-products/proteins-fibers-starches/
Osage Food Products	USA	Fava Bean Protein	Faba bean	Protein		http://osagefood.com/protein-products/plant-proteins/
Prinova Group LLC	USA	Fava Bean Protein	Faba bean	Protein		https://www.prinovaeurope.com/ingredients/protein-ingredient-suppliers/
Roquette Frères	France	Nutralys®TF-C	Faba bean	Textured protein		https://www.roquette.com/food-nutrition-protein-textured-plant
The Scoular Company	USA	Fava Bean Protein	Faba bean	Protein		https://www.scoular.com/food-ingredient-products/proteins/fava-bean-protein/
Top Health Ingredients	Canada	AdvantaFava™ Resolve 90	Faba bean	Protein	90	https://www.tophealthingredients.com/advantafava
Top Health Ingredients	Canada	AdvantaFava™ Nourish 90	Faba bean	Protein	90	https://www.tophealthingredients.com/advantafava
Bioriginal Food & Science Corp	Canada	Organic Flax Protein Powder	Flax seed	Protein	35	https://www.bioriginal.com/products/flax-protein-powder/
Glanbia plc	Ireland	HarvestPro™ Flax Protein	Flax seed	Protein	26-36	https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
Glanbia plc	Ireland	SnackFlax™ Protein	Flax seed	Protein concentrate	35	https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
Glanbia plc	Ireland	BevFlax™ Protein Organic	Flax seed	Protein concentrate	26-36	https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
Ingredients Inc.	USA	LeinPRO	Flax seed	Protein		https://www.ingredientsinc.com/ingredients
Naturz Organics	The Netherlands	Flaxseed Protein 30% Brown - Powder	Flax seed	Protein	30	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Flaxseed Protein 30% Gold - Powder	Flax seed	Protein	30	https://naturzorganics.com/our-products/proteins-fibers-starches/
All Organic Treasures GmbH	Germany	Hemp Protein 50, organic	Hempseed	Protein	50	https://www.all-organic-treasures.com/assortment-food/hanfprotein-50-bio-lebensmittel.html
All Organic Treasures GmbH	Germany	Hemp Protein 70, organic	Hempseed	Protein	66	https://www.all-organic-treasures.com/assortment-food/hanfprotein-50-bio-lebensmittel.html
Axiom Foods Inc.	USA	Cannatein®	Hempseed	Protein		http://axiomfoods.com/cannatein-hemp-protein/
Bioriginal Food & Science Corp	Canada	Hemp Protein Powder	Hempseed	Protein	35	https://www.bioriginal.com/products/hemp-protein-powder/
Bioriginal Food & Science Corp	Canada	Hemp Protein Powder	Hempseed	Protein	50	https://www.bioriginal.com/products/hemp-protein-powder/
Distribution Nexxus Inc	Canada	Hemp Protein	Hempseed	Protein	43	http://nexxusfoods.com/en/specialty-ingredients/#proteines
Euroduna Food Ingredients	Germany	Hemp Protein	Hempseed	Protein		http://www.euroduna-food.com/en/ingredients/hempproducts.php

Fresh Hemp Foods Ltd.	Canada	HempBev 65 Hemp Protein	Hempseed	Protein	65	https://www.fieldcraft.com/co/hempoilcanada/products
Fresh Hemp Foods Ltd.	Canada	Hemp Protein Powder	Hempseed	Protein	50	https://www.fieldcraft.com/co/hempoilcanada/products
Green Source Organics	USA	Hemp Protein Powder	Hempseed	Protein		https://www.gsoextracts.com/products/hemp
Hemp Oil Canada	Canada	Hemp Protein Powder 50%	Hempseed	Protein	50	https://www.ulprospector.com/en/na/Food/Detail/11059/334486/Hemptein-Hemp-Protein-Powder-43
Hemp Oil Canada	Canada	Hemp Protein Powder 65%	Hempseed	Protein	65	https://www.ulprospector.com/en/na/Food/Detail/11059/334487/Hemptein-Hemp-Protein-Powder-50
Hemp Oil Canada	Canada	HempBev 65	Hempseed	Protein	65	https://hempoilcanada.com/
Minnesota Hemp Farms, Inc.	USA	Hemp Protein Powder	Hempseed	Protein	50	https://mnhempfarms.com/products/wholesale-hemp-protein
Minnesota Hemp Farms, Inc.	USA	Hemp Protein Powder	Hempseed	Protein	33	https://mnhempfarms.com/products/wholesale-hemp-protein
Minnesota Hemp Farms, Inc.	USA	Hemp Protein Powder	Hempseed	Protein	23	https://mnhempfarms.com/products/wholesale-hemp-protein
Naturz Organics	The Netherlands	Hemp Protein 30% - Powder	Hempseed	Protein	30	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Hemp Protein 50% - Powder	Hempseed	Protein	50	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Hemp protein 65% - Powder	Hempseed	Protein	65	https://naturzorganics.com/our-products/proteins-fibers-starches/
NP Nutra	USA	Hemp Protein Flour	Hempseed	Protein	30	https://npnutra.com/wholesale-bulk-organic-hemp.html
NP Nutra	USA	Hemp Protein Powder	Hempseed	Protein	45	https://npnutra.com/wholesale-bulk-organic-hemp.html
Osage Food Products	USA	Hemp Protein	Hempseed	Protein		http://osagefood.com/protein-products/plant-proteins/
Prinova Group LLC	USA	Hemp Protein	Hempseed	Protein		https://www.prinovaeurope.com/ingredients/protein-ingredient-suppliers/
Top Health Ingredients	Canada	AdvantaHemp™	Hempseed	Protein	55	https://www.tophealthingredients.com/advantahemp
Ingredients Inc.	USA	Lentil Protein	Lentil	Protein		https://www.ingredientsinc.com/ingredients
Ingredion	USA	VITESSENCE®Pulse 2550 Lentil protein	Lentil	Protein concentrate	55	https://emea.ingredion.com/findingredients/proteins.html
Prinova Group LLC	USA	Red Lentil Protein	Lentil	Protein		https://www.prinovaeurope.com/ingredients/protein-ingredient-suppliers/
FRANK Food Products	The Netherlands	FRALU-CON	Lupin	Protein concentrate		https://www.frankfoodproducts.com/index.asp?objectid=2&titel=#id53
Ingredients Inc.	USA	Lupin Protein	Lupin	Protein		https://www.ingredientsinc.com/ingredients
Koralta Agri-Business Inc.	Canada	Lupin Bean Protein	Lupin	Protein	60	https://www.fieldcraft.com/co/koraltaagri/products
Prolupin GmbH	Germany	Lupine Protein Isolate	Lupin	Protein isolate	90	https://www.prolupin.com/lupine-protein-isolate-lpi.html
hining Seas Imports	USA	Organic Moringa Seed Protein Powder	Algae (Moringa)	Protein	60	http://shiningseasimports.com/ingredients/organic-moringa-seed-protein-powder/
Ingredients Inc.	USA	Mung Bean Protein	Mung bean	Protein		https://www.ingredientsinc.com/ingredients
Naturz Organics	The Netherlands	Mung Bean Protein 80% - Powder	Mung bean	Protein	80	https://naturzorganics.com/our-products/proteins-fibers-starches/
Prinova Group LLC	USA	Mung Bean Protein	Mung bean	Protein		https://www.prinovaeurope.com/ingredients/protein-ingredient-suppliers/
The Scoular Company	USA	Mung Bean Protein	Mung bean	Protein		https://www.scoular.com/food-ingredient-products/proteins/mung-bean-protein/
Top Health Ingredients	Canada	AdvantaMung™ Resolve 60	Mung bean	Protein	80	https://www.tophealthingredients.com/advantamung
Top Health Ingredients	Canada	AdvantaMung™ Nourish 80	Mung bean	Protein	80	https://www.tophealthingredients.com/advantamung
MycoTechnology Inc.	USA	FermentIQ™ Protein	Fungal mycelium	Protein	77	https://www.mycoiq.com/ingredients/fermentiq
Fazer	Finland	Fazer Aurora Oat Protein 20 (OP 20)	Oat	Protein concentrate	20	https://www.fazermills.com/products/
Ingredients Inc.	USA	Oat Protein	Oat	Protein	20	https://www.ingredientsinc.com/ingredients
Lantmännen Oats	Sweden	PrOatein™	Oat	Protein concentrate	55	https://www.tateandlyle.com/sites/default/files/2017-06/PrOatein%20Oat%20Protein_product%20leaflet_1703_A4_EN-GB.pdf
Naturz Organics	The Netherlands	Oat Protein 30% - Powder	Oat	Protein	30	https://naturzorganics.com/our-products/proteins-fibers-starches/
Oat Tech Inc.	USA	Oat Protein 55	Oat	Protein	55	https://www.ulprospector.com/en/na/Food/Detail/6116/222995/Oat-Protein-55
ADM (Archer Daniels Midland)	USA	ProFam®Pea 580	Pea	Protein	80	https://www.adm.com/products-services/food/proteins/ingredients/pea-proteins
ADM (Archer Daniels Midland)	USA	Arcon T 513 Textured Pea Protein	Pea	Textured protein		https://www.adm.com/products-services/food/proteins/ingredients/pea-proteins
ADM (Archer Daniels Midland)	USA	Arcon T514 Textured Pea Protein	Pea	Textured protein		https://www.adm.com/products-services/food/proteins/ingredients/pea-proteins
All Organic Treasures GmbH	Germany	Pea Protein 80, organic	Pea	Protein	80	https://www.all-organic-treasures.com/assortment-food/erbsenprotein-80-bio.html
AMCO Proteins	USA	Pea Protein 80%	Pea	Protein concentrate	80	https://www.amcoproteins.com/plant-proteins-peaproteins#Pea_Protein_80_
AMCO Proteins	USA	Pea protein 85%	Pea	Protein concentrate	85	https://www.amcoproteins.com/plant-proteins-peaproteins#Pea_Protein_80_
Axiom Foods Inc.	USA	VegOtein P™ 80	Pea	Protein concentrate	80	http://axiomfoods.com/vegotein-pea-protein-powder/

Axiom Foods Inc.	USA	VegOtein P™ 85	Pea	Protein concentrate	85	http://axiomfoods.com/vegotein-pea-protein-powder/
Axiom Foods Inc.	USA	VegOtein MA	Pea	Protein concentrate	80	http://axiomfoods.com/vegotein-ma-pea-protein-powder/
Axiom Foods Inc.	USA	VegOtein N™	Pea	Protein concentrate	80	http://axiomfoods.com/veg-o-tein-pea-protein-powder-neutral/
Axiom Foods Inc.	USA	VegOtein TX80™	Pea	Textured protein	80	http://axiomfoods.com/vegotein-tx-pea-protein-powder/
Axiom Foods Inc.	USA	VegOtein TX65™	Pea	Textured protein	65	http://axiomfoods.com/vegotein-tx-pea-protein-powder/
Axiom Foods Inc.	USA	VegOtein HP80™	Pea	Protein	80	http://axiomfoods.com/veg-o-tein-pea-protein-powder-hydrolyzed/
Batory Foods	USA	Pea Protein	Pea	Protein		http://www.batoryfoods.com/product-categories/protein-ingredients/#plant-based
Blattmann Schweiz AG	Schweiz	BecoPulse Pea 50 Bio	Pea	Protein concentrate	50	https://www.blattmannschweiz.com/products/brown-pea-protein-and-flour/
Burcon NutraScience Corporation	Canada	Peazac®	Pea	Protein	80	https://burcon.ca/products/pea-proteins/
Burcon NutraScience Corporation	Canada	Peazac®850	Pea	Protein		https://burcon.live-website.com/merit-functional-foods/
Burcon NutraScience Corporation	Canada	Peazazz®	Pea	Protein isolate		https://www.ulprospector.com/en/na/Food/Detail/12268/354067/Peazazz
Cargill Food Ingredients	USA	PURIS™ Pea Protein	Pea	Protein	80	https://www.cargill.com/food-bev/na/pea-protein
CBH Qingdao Co., Ltd.	China	Pea Protein Isolate	Pea	Protein isolate	80	http://www.cbhcn.com/pro_info.asp?id=606&classID=443&parentID=430
CBH Qingdao Co., Ltd.	China	Pea Protein Isolate	Pea	Protein isolate	85	http://www.cbhcn.com/pro_info.asp?id=606&classID=443&parentID=430
Ciranda	USA	Pea Protein Flour	Pea	Protein	50	https://www.ciranda.com/ingredients/flours-starches/pea-protein-flour/pea-protein-flour
Cosucra Groupe Warcoing SA	Belgium	Pisane®Pea Protein	Pea	Protein isolate	88-90	https://www.stauberusa.com/our-partners/pea-protein-isolate/
Dealers Ingredients Inc.	Canada	Nutralein Pea Protein Isolate	Pea	Protein isolate	80	https://dealersingredients.com/products/proteins/
Distribution Nexxus Inc	Canada	ProNex™ Pea Protein	Pea	Protein	80	http://nexxusfoods.com/en/specialty-ingredients/#proteines
DONGYU USI	USA	Pea Protein Isolate	Pea	Protein isolate	80	https://www.ulprospector.com/en/na/Food/Detail/11625/722265/Pea-Protein-Isolate
DONGYU USI	USA	Soy Protein Isolate	Pea	Protein isolate	90	https://dongyu.us/product/soy-protein/
Dupont Nutrition & Biosciences	USA	TRUPRO®Pea Protein	Pea	Protein	83	https://www.dupontnutritionandbiosciences.com/products/trupro-2000-pea-protein.html
Dutch Organic International Trade	The Netherlands	Pea protein powder 80%	Pea	Protein	80	https://www.organic.nl/ingredients/plant-protein/plant-protein/20330-pea-protein-powder-80-org.-20-kg*.html
Dutch Organic International Trade	The Netherlands	Pea protein powder 50%	Pea	Protein	50	https://www.organic.nl/ingredients/plant-protein/plant-protein/20331-pea-protein-powder-org.-50-15-kg.html
Emsland-Stärke GmbH	Germany	Empro®E 86	Pea	Protein	87	https://www.ulprospector.com/en/na/Food/Detail/5383/584580/Empro-E-86
Emsland-Stärke GmbH	Germany	Empro®E 86 HV	Pea	Protein isolate	87	https://www.ulprospector.com/en/na/Food/Detail/5383/642190/Empro-E-86-HV
Euroduna Food Ingredients	Germany	Pea Protein 80%	Pea	Protein isolate	80	https://www.ulprospector.com/en/na/Food/Detail/11798/461080/Pea-Protein
Euroduna Food Ingredients	Germany	TPP55	Pea	Textured protein		http://www.euroduna-food.com/en/ingredients/pea_products.php
Euroduna Food Ingredients	Germany	VegTein	Pea	Textured protein		http://www.euroduna-food.com/en/ingredients/pea_products.php
Farbest Brands	USA	Jianyuan Pea Protein JY-PP85	Pea	Protein isolate	85	https://www.ulprospector.com/en/na/Food/Detail/11855/603505/Jianyuan-Pea-Protein-JY-PP85
Farbest Brands	USA	Jianyuan Pea Protein JY-PP80	Pea	Protein isolate	80	https://farbest.com/ingredients/proteins/plant-proteins/pea-protein/
Farbest Brands	USA	Jianyuan Pea Protein JY-PP-BLS	Pea	Protein isolate	80	https://farbest.com/ingredients/proteins/plant-proteins/pea-protein/
Farbest Brands	USA	Farbest Pea Protein 80%, Fine Mesh FB600	Pea	Protein isolate	80	https://farbest.com/ingredients/proteins/plant-proteins/pea-protein/
Farbest Brands	USA	Jianyuan Pea Protein JYPP-OP	Pea	Protein isolate	80	https://farbest.com/ingredients/organic-ingredients/organic-plant-proteins/
Fenchem, Inc.	USA	VegePro™ Plant Protein	Pea	Protein isolate		http://www.fenchem.com/index.php/industry/industrycenter/1/
Foodchem International Corporation	China	Pea Protein	Pea	Protein	75	https://www.ulprospector.com/en/na/Food/Detail/12674/351541/Pea-Protein
Foodchem International Corporation	China	Concentrated Pea Protein	Pea	Protein concentrate	55	http://www.foodchem.cn/product/Proteins/2
Foodchem International Corporation	China	Isolated Pea Protein	Pea	Protein isolate	80	http://www.foodchem.cn/product/Proteins/2
Fooding Group Limited	China	Hydrolyzed Vegetable Protein	Pea	Protein		http://www.chinafooding.com/Hydrolyzed_Vegetable_Protein
Fooding Group Limited	China	Pea Protein	Pea	Protein		http://www.chinafooding.com/Pea_Protein
Glanbia plc	Ireland	HarvestPro™ Pea Proteins	Pea	Protein	75-85	https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
Glanbia plc	Ireland	BarHarvest™ Pea Protein	Pea	Protein concentrate		https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
Glanbia plc	Ireland	BevEdge™ Pea Protein	Pea	Protein concentrate		https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0

Green Source Organics	USA	Pea Protein Powder	Pea	Protein	80	https://www.gsoextracts.com/products/pea-powder
Gremount International Company Limited	China	Pea Protein	Pea	Protein	55	http://www.gremount.com.cn/index.php?m=content&c=index&a=lists&catid=37#product_right
H & A Canada Inc.	Canada	Pea Protein Concentrate/Isolate	Pea	Protein		https://hacanada.com/pea-protein-concentrate-isolate/
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Hydrolyzed Pea Protein	Pea	Protein	80	http://www.zxchem.com.cn/index/product/detail/id/Hydrolyzed_Pea_Protein_Peptide.html
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Hydrolyzed Pea Protein	Pea	Protein	85	http://www.zxchem.com.cn/index/product/detail/id/Hydrolyzed_Pea_Protein_Peptide.html
Herba Ingredients BV	The Netherlands	HerbaPro YP55	Pea	Protein	55	http://www.herbaingredients.com/pulses/pea-ingredients/pea-protein
Herba Ingredients BV	The Netherlands	Texturized Pea Protein	Pea	Textured protein	55	http://www.herbaingredients.com/pulses/pea-ingredients/texturized-pea-protein
Hi-Food	Italy	HI-Protein PEA VIII	Pea	Protein		https://www.hifood.it/product-range/plant-based-proteins/3
Ingredients Inc.	USA	Pea Protein	Pea	Protein	50-80	https://2720b1d5-6c89-4528-8139-053781c82d9e.filesusr.com/ugd/4eb45d_b0bbd27caf754fd09c2c19c83ff89d7e.pdf
Ingredients Inc.	USA	Textured Pea Protein	Pea	Textured protein	75-80	https://2720b1d5-6c89-4528-8139-053781c82d9e.filesusr.com/ugd/4eb45d_c79682b3a2fc4fce9a676ea00949cf92.pdf
Ingredion	USA	VITESSENCE™ Pulse 1550	Pea	Protein concentrate	55	https://www.ulprospector.com/en/na/Food/Detail/12852/705463/VITESSENCE-Pulse-CT-1552
Ingredion	USA	VITESSENCE®Pulse 1803 Pea Protein	Pea	Protein isolate	80	https://emea.ingredion.com/findingredients/proteins.html
Kerry Group plc	Ireland	Hyprol™ Pea Protein	Pea	Protein		https://www.kerry.com/products/nutrition/proteins/hyprol
Kerry Group plc	Ireland	ProDiem™ Pea Protein	Pea	Protein		https://www.kerry.com/products/nutrition/proteins/prodiem
Kerry Group plc	Ireland	ProDiem™ Refresh Pea Protein	Pea	Protein		https://www.kerry.com/products/nutrition/proteins/prodiem-refresh
Lehmann & Voss & Co.	Germany	Pea Protein Concentrate 80% ODB	Pea	Protein concentrate	80	https://www.lehvoss-nutrition.com/ingredients/plant-derived-ingredients.html
Lehmann & Voss & Co.	Germany	Pea Protein Concentrate 80% ODB (Organic)	Pea	Protein concentrate	80	https://www.lehvoss-nutrition.com/ingredients/plant-derived-ingredients.html
Meelunie B.V.	The Netherlands	Pea Protein	Pea	Protein	80	https://meelunie.com/products/product-catalogue/
MGP	USA	Proterra™ 2200	Pea	Textured protein		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Proterra™ 2350	Pea	Textured protein		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MTC Industries, Inc.	USA	Pea Protein 80%	Pea	Protein	80	https://www.ulprospector.com/en/na/Food/Detail/4006/646115/Pea-Protein-80
MTC Industries, Inc.	USA	Organic Pea Protein	Pea	Protein		https://www.mtcindustries.com/protein
Naturz Organics	The Netherlands	Pea Protein 50% - Textured	Pea	Textured protein	50	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Pea Protein 60% - Textured	Pea	Textured protein	60	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Pea Protein 70% - 80% - Textured	Pea	Textured protein	70	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Pea Protein 80% - Powder	Pea	Protein	80	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Pea Protein 80% - Powder - Premium	Pea	Protein	80	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Pea Protein 80% - Powder - Regular	Pea	Protein	80	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Pea Protein 80% - Textured	Pea	Textured protein	80	https://naturzorganics.com/our-products/proteins-fibers-starches/
NP Nutra	USA	Pea Protein Powder 80% Organic	Pea	Protein		https://www.ulprospector.com/en/na/Food/Detail/4145/633488/Pea-Protein-Powder-Organic
Nutra Food Ingredients, LLC	USA	Pea Protein Isolate, organic	Pea	Protein isolate	>80	https://www.ulprospector.com/en/na/Food/Detail/14051/353649/Pea-Protein
Nutra Food Ingredients, LLC	USA	Pea Protein Isolate	Pea	Protein isolate	>80	https://www.ulprospector.com/en/na/Food/Detail/14051/353649/Pea-Protein
Nutra Food Ingredients, LLC	USA	Pea Protein Concentrate	Pea	Protein isolate	>80	https://www.ulprospector.com/en/na/Food/Detail/14051/353649/Pea-Protein
Nutra Food Ingredients, LLC	USA	Pea Protein Concentrate, organic	Pea	Protein isolate	>80	https://www.ulprospector.com/en/na/Food/Detail/14051/353649/Pea-Protein
Nutri-Pea Ltd.	Canada	Propulse™	Pea	Protein isolate	80	https://nutripea.com/products/pulsepro-pea-protein/
Osage Food Products	USA	Pea Protein	Pea	Protein		http://osagefood.com/protein-products/plant-proteins/
Prinova Group LLC	USA	Pea Protein	Pea	Protein		https://www.prinovaeurope.com/ingredients/protein-ingredient-suppliers/

Puris Proteins, LLC	USA	PURIS Textured Pea Protein 80% Low Sodium (TPP80 LS)	Pea	Textured protein		https://puris.com/products/puris-textured-pea-protein-80-low-sodium-tpp80-ls
Puris Proteins, LLC	USA	PURIS Textured Pea Protein 80% Firm Bite (TPP80 FB)	Pea	Textured protein		https://puris.com/products/puris-textured-pea-protein-80-firm-bite-tpp80-fb
Puris Proteins, LLC	USA	PURIS Pea Protein 870 LS (P870 LS)	Pea	Protein	80	https://puris.com/products/puris-pea-protein-870-ls-op870-ls
Puris Proteins, LLC	USA	PURIS Textured Pea Protein 70% (TPP70)	Pea	Textured protein	70	https://puris.com/products/puris-textured-pea-protein-70-tpp70
Puris Proteins, LLC	USA	PURIS Textured Pea Protein 80% (TPP80)	Pea	Textured protein		https://puris.com/products/puris-textured-pea-protein-80-tpp80
Puris Proteins, LLC	USA	PURIS Textured Pea Protein 65% (TPP65)	Pea	Textured protein	65	https://puris.com/products/puris-textured-pea-protein-65-tpp65
Puris Proteins, LLC	USA	PURIS Pea Protein 875 (P875)	Pea	Protein	80	https://puris.com/products/puris-pea-protein-875-p875
Puris Proteins, LLC	USA	PURIS Pea Protein 870 MV (P870 MV)	Pea	Protein		https://puris.com/products/puris-pea-protein-870-mv-p870-mv
Puris Proteins, LLC	USA	PURIS Pea Protein 870H (P870H)	Pea	Protein	80	https://puris.com/products/puris-pea-protein-870H-p870H
Puris Proteins, LLC	USA	PURIS Pea Protein 860 (P860)	Pea	Protein	80	https://puris.com/products/puris-pea-protein-860-p860
Puris Proteins, LLC	USA	PURIS Pea 870 (P870)	Pea	Protein	80	https://puris.com/products/puris-pea-870-p870
Puris Proteins, LLC	USA	Organic PURIS Textured Pea Protein 70% (OTPP70)	Pea	Textured protein	70	https://puris.com/products/organic-puris-textured-pea-protein-70-otpp70
Puris Proteins, LLC	USA	Organic PURIS Textured Pea Protein 80% (OTPP80)	Pea	Textured protein		https://puris.com/products/organic-puris-textured-pea-protein-80-otpp80
Puris Proteins, LLC	USA	Organic PURIS Textured Pea Protein 65% (OTPP65)	Pea	Textured protein	65	https://puris.com/products/organic-puris-textured-pea-protein-65-ottp65
Puris Proteins, LLC	USA	Organic PURIS Pea Protein 875 (OP875)	Pea	Protein	80	https://puris.com/products/organic-puris-pea-protein-875-op875
Puris Proteins, LLC	USA	Organic PURIS Pea Protein 870 MV (OP870 MV)	Pea	Protein		https://puris.com/products/organic-puris-pea-protein-870-mv-op870-mv
Puris Proteins, LLC	USA	Organic PURIS Pea Protein 870H (OP870H)	Pea	Protein	80	https://puris.com/products/organic-puris-pea-protein-870h-op870h
Puris Proteins, LLC	USA	Organic PURIS®Pea Protein 870 LS (OP870 LS)	Pea	Protein	80	https://puris.com/products/organic-puris-pea-protein-870-ls-op870-ls
Puris Proteins, LLC	USA	Organic PURIS Pea Protein 870 (OP870)	Pea	Protein	80	https://puris.com/products/organic-puris-pea-protein-870-op870
Puris Proteins, LLC	USA	Organic PURIS Pea Protein 860 (OP860)	Pea	Protein	80	https://puris.com/products/organic-puris-pea-protein-860-op860
Qingdao Free Trade Zone United International	China	Pea Protein Isolated	Pea	Protein isolate	70	https://www.ulprospector.com/en/na/Food/Detail/15382/428736/Pea-Protein-Isolated
Roquette Frères	France	NUTRALYS®B85F	Pea	Protein isolate	84	https://www.roquette.com/food-nutrition-protein-pea-non-functional
Roquette Frères	France	NUTRALYS®F85F	Pea	Protein isolate	83	https://www.roquette.com/food-nutrition-protein-pea-functional
Roquette Frères	France	NUTRALYS®F85M	Pea	Protein isolate	83	https://www.roquette.com/food-nutrition-protein-pea-functional
Roquette Frères	France	NUTRALYS®S85 PLUS D	Pea	Protein isolate	83	https://www.roquette.com/food-nutrition-protein-pea-soluble
Roquette Frères	France	NUTRALYS®S85 PLUS N	Pea	Protein isolate	84	https://www.roquette.com/food-nutrition-protein-pea-soluble
Roquette Frères	France	NUTRALYS®S85F	Pea	Protein isolate	84	https://www.roquette.com/food-nutrition-protein-pea-soluble
Roquette Frères	France	NUTRALYS®S85M	Pea	Protein isolate	84	https://www.roquette.com/food-nutrition-protein-pea-soluble
Roquette Frères	France	NUTRALYS®S85XF	Pea	Protein isolate	84	https://www.roquette.com/food-nutrition-protein-pea-soluble
Roquette Frères	France	NUTRALYS®T70S	Pea	Textured protein	70	https://www.roquette.com/food-nutrition-protein-textured-plant
Roquette Frères	France	NUTRALYS®T70S-02	Pea	Textured protein		https://www.roquette.com/food-nutrition-protein-textured-plant
Roquette Frères	France	NUTRALYS®TP65M	Pea	Textured protein	63	https://www.roquette.com/food-nutrition-protein-textured-plant
Roquette Frères	France	NUTRALYS®TP70G	Pea	Textured protein		https://www.roquette.com/food-nutrition-protein-textured-plant
The Scoular Company	USA	Textured Pea Protein	Pea	Textured protein	60	https://www.ulprospector.com/en/na/Food/Detail/3988/1000388/Textured-Pea-Protein
The Scoular Company	USA	Organic Pea Protein JYPP-B 80%	Pea	Protein	80	https://www.ulprospector.com/en/na/Food/Detail/3988/424365/Organic-Pea-Protein-JYPP-B-80
The Scoular Company	USA	Pea Protein Flour - Prestige	Pea	Protein	50	https://www.ulprospector.com/en/na/Food/Detail/3988/200671/Pea-Protein-Flour—Prestige

The Scoular Company	USA	Pea Protein JYPP-B 80%	Pea	Protein	80	https://www.ulprospector.com/en/na/Food/Detail/3988/342060/Pea-Protein-JYPP-B-80
The Scoular Company	USA	Pea Protein YS 85%	Pea	Protein	85	https://www.ulprospector.com/en/na/Food/Detail/3988/342065/Pea-Protein-YS-85
Top Health Ingredients	Canada	AdvantaPea™ Essential 80	Pea	Protein	80-85	https://www.tophealthingredients.com/advantapea
Top Health Ingredients	Canada	AdvantaPea™ Resolve 85	Pea	Protein	85	https://www.tophealthingredients.com/advantapea
Top Health Ingredients	Canada	AdvantaPea™ Nourish 85	Pea	Protein	85	https://www.tophealthingredients.com/advantapea
Vitacyclix	USA	Propulse™ Pea Protein	Pea	Protein isolate	82	https://www.morretec.com/divisions/vitacyclix/proteins-fibers/
Vitacyclix	USA	Pea Protein Isolate C9	Pea	Protein isolate		https://www.morretec.com/divisions/vitacyclix/proteins-fibers/
All Organic Treasures GmbH	Germany	Pistachio Flour, organic	Pistachio	Protein	40-80	https://www.all-organic-treasures.com/assortment-food/pistazienmehl-bio.html
All Organic Treasures GmbH	Germany	Pomegranate Flour, organic	Pomegranate seed	Protein	15-20	https://www.all-organic-treasures.com/assortment-food/granatapfelmehl-bio-lebensmittel.html
Osage Food Products	USA	Pomegranate Protein	Pomegranate seed	Protein		http://osagefood.com/protein-products/plant-proteins/
Avebe	The Netherlands	Solanic®potato protein	Potato	Protein isolate		https://www.avebe.com/producten/solanic/
KMC	Denmark	KMC PotaPro 1500	Potato	Protein	78	http://www.kmc.dk/kmc-ingredients/kmc-ingredients
KMC	Denmark	Pratofay	Potato	Protein		http://www.kmc.dk/kmc-ingredients/kmc-ingredients/protafy
Osage Food Products	USA	Potato Protein	Potato	Protein		http://osagefood.com/protein-products/plant-proteins/
All Organic Treasures GmbH	Germany	Pumpkin protein, unroasted	Pumpkin seed	Protein	60	https://www.all-organic-treasures.com/assortment-food/kuerbisprotein-60-ungeroestet-bio-lebensmittel.html
All Organic Treasures GmbH	Germany	Pumpkin protein, roasted	Pumpkin seed	Protein	60	https://www.all-organic-treasures.com/assortment-food/kuerbisprotein-60-geroestet-bio-lebensmittel.html
Axiom Foods Inc.	USA	Cucurbotein™ OR55	Pumpkin seed	Protein	55	http://axiomfoods.com/cucurbotein-pumpkin-protein-powder/
Axiom Foods Inc.	USA	Cucurbotein™ OR65	Pumpkin seed	Protein	65	http://axiomfoods.com/cucurbotein-pumpkin-protein-powder/
Axiom Foods Inc.	USA	Toasted Cucurbotein™ OT59	Pumpkin seed	Protein		http://axiomfoods.com/cucurbotein-pumpkin-protein-powder/
Bioriginal Food & Science Corp	Canada	Pumpkin Protein	Pumpkin seed	Protein	60	https://www.bioriginal.com/products/pumpkin-protein-powder/
Fenchem, Inc.	USA	Pumpkin Seed Protein	Pumpkin seed	Protein	60	http://www.fenchem.com/index.php/industry/industrycenter/1/
Ingredients Inc.	USA	PumpkinPRO	Pumpkin seed	Protein	60	https://www.ingredientsinc.com/ingredients
Lehmann & Voss & Co.	Germany	Pumpkin Seed Protein 60% ODB (Organic)	Pumpkin seed	Protein concentrate	60	https://www.lehvoss-nutrition.com/ingredients/plant-derived-ingredients.html
MTC Industries, Inc.	USA	Pumpkin Seed Protein	Pumpkin seed	Protein		https://www.mtcindustries.com/protein
Naturz Organics	The Netherlands	Pumpkin Protein 60% - Powder	Pumpkin seed	Protein	60	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Pumpkin Protein 70% - Powder	Pumpkin seed	Protein	70	https://naturzorganics.com/our-products/proteins-fibers-starches/
Nutra Food Ingredients, LLC	USA	Pumpkin Protein 60%	Pumpkin seed	Protein	60	https://www.nutrafoodingredients.com/products/pumpkin-protein-powder/
Osage Food Products	USA	Pumpkin Protein	Pumpkin seed	Protein		http://osagefood.com/protein-products/plant-proteins/
Prinova Group LLC	USA	Pumpkin Protein	Pumpkin seed	Protein		https://www.prinovaeurope.com/ingredients/protein-ingredient-suppliers/
Andean Naturals Inc.	USA	Quinoa Protein Concentrate™	Quinoa	Protein concentrate	70	https://www.ulprospector.com/en/na/Food/Detail/10788/329951/Quinoa-Protein-Concentrate
Ingredients Inc.	USA	Quinoa Protein	Quinoa	Protein		https://www.ingredientsinc.com/ingredients
Avena Nordic Grain Oy	Finland	BlackGrain from yellow fields™	Rapeseed/Canola	Protein		https://blackgrain.com/#whatis
AG Commodities, Inc.	USA	Organic Rice Protein 80%	Rice	Protein	80	https://www.ulprospector.com/en/na/Food/Detail/23999/577696/Organic-Rice-Protein-80
All Organic Treasures GmbH	Germany	Rice Protein 80 White, organic	Rice	Protein	80	https://www.all-organic-treasures.com/assortment-food/reisprotein-bio-lebesmittel.html
Axiom Foods Inc.	USA	Oryzatein®SG-BN	Rice	Protein concentrate	80	https://www.ulprospector.com/en/na/Food/Detail/10857/384707/Organic-Oryzatein-SG-BN
Axiom Foods Inc.	USA	Oryzatein®Ultra 90	Rice	Protein isolate	90	http://axiomfoods.com/axiom-foods-ingredients-granted-non-gmo-project-verification/
Axiom Foods Inc.	USA	Oryzatein® Silk 90	Rice	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/10857/385177/Organic-Oryzatein-Silk-90

Axiom Foods Inc.	USA	Oryzatein®90	Rice	Protein isolate	90	http://axiomfoods.com/oryzatein-brown-rice-protein-powder/
Axiom Foods Inc.	USA	Oryzatein®SG-MA	Rice	Protein		https://www.ulprospector.com/en/na/Food/Detail/10857/384905/Oryzatein-SG-MA
Axiom Foods Inc.	USA	Oryzatein®Ultra 80	Rice	Protein concentrate	80	https://www.ulprospector.com/en/na/Food/Detail/10857/385189/Organic-Oryzatein-Ultra-80
Axiom Foods Inc.	USA	Oryzatein®80	Rice	Protein concentrate	80	https://www.ulprospector.com/en/na/Food/Detail/10857/384636/Organic-Oryzatein-80
Axiom Foods Inc.	USA	Oryzatein®SG-FE	Rice	Protein concentrate		https://www.ulprospector.com/en/na/Food/Detail/10857/384918/Oryzatein-SG-FE
Axiom Foods Inc.	USA	Oryzatein®Silk 80	Rice	Protein concentrate	80	https://www.ulprospector.com/en/na/Food/Detail/10857/385150/Oryzatein-Silk-80
Axiom Foods Inc.	USA	Oryzatein®SG-X	Rice	Protein concentrate		https://www.ulprospector.com/en/na/Food/Detail/10857/384939/Oryzatein-SG-X
Beneo GmbH	Germany	Remypro®N80+(G)	Rice	Protein	79	https://www.beneo.com/ingredients/human-nutrition/specialty-rice-ingredients/rice-protein
Distribution Nexxus Inc	Canada	ProNex™ Rice Protein	Rice	Protein	90	http://nexxusfoods.com/en/specialty-ingredients/#proteines
Dutch Organic International Trade	The Netherlands	Rice protein powder 80%	Rice	Protein	80	https://www.organic.nl/ingredients/plant-protein/plant-protein/20336-rice-protein-powder-80-org.-20-kg*.html
Euroduna Food Ingredients	Germany	Rice Protein	Rice	Protein concentrate	75-87	http://www.euroduna-food.com/en/ingredients/proteins.php
Farbest Brands	USA	Brown Rice Protein	Rice	Protein isolate	80	https://www.ulprospector.com/en/na/Food/Detail/11855/603404/Organic-Rice-Protein
Fenchem, Inc.	USA	Rice Protein	Rice	Protein		http://www.fenchem.com/index.php/industry/industrycenter/1/
Foodchem International Corporation	China	Rice Protein	Rice	Protein		https://www.ulprospector.com/en/na/Food/Detail/12674/351533/Rice-Protein
Fooding Group Limited	China	Rice protein powder	Rice	Protein isolate		http://www.chinafooding.com/Rice_Protein_Powder
Green Source Organics	USA	Brown Rice Protein Powder	Rice	Protein		https://www.gsoextracts.com/products/rice
Green Source Organics	USA	Organic Brown Rice Protein Powder 80%	Rice	Protein	80	https://www.gsoextracts.com/products/rice
Gremount International Company Limited	China	Rice Protein	Rice	Protein		http://www.gremount.com.cn/index.php?m=content&c=index&a=lists&catid=37#product_right
H & A Canada Inc.	Canada	Rice Protein Powder	Rice	Protein		https://hacanada.com/rice-protein-powder/
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Hydrolyzed Rice Protein	Rice	Protein	80	http://www.zxchem.com.cn/index/product/detail/id/Hydrolyzed_Rice_Protein_.html
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Hydrolyzed Rice Protein	Rice	Protein	85	http://www.zxchem.com.cn/index/product/detail/id/Hydrolyzed_Rice_Protein_.html
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Hydrolyzed Rice Protein	Rice	Protein	90	http://www.zxchem.com.cn/index/product/detail/id/Hydrolyzed_Rice_Protein_.html
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Rice Protein ZX R80	Rice	Protein	80	https://www.ulprospector.com/en/na/Food/Detail/13009/353659/Rice-Protein-ZX-R80
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Rice Protein ZX C80	Rice	Protein	80	https://www.ulprospector.com/en/na/Food/Detail/13009/353660/Rice-Protein-ZX-C80
Hefei TNJ Chemical Industry Co., Ltd.	China	Rice Protein	Rice	Protein	80	https://www.tnjchem.com/rice-protein_p8180.html
Hefei TNJ Chemical Industry Co., Ltd.	China	Rice Protein	Rice	Protein	90	https://www.tnjchem.com/rice-protein_p8180.html
Hi-Food	Italy	HI-PROTEIN RICE 75+	Rice	Protein		https://www.hifood.it/product-range/plant-based-proteins/3
Ingredients Inc.	USA	Rice Protein	Rice	Protein		https://www.ingredientsinc.com/ingredients
Kerry Group plc	Ireland	Hyprol™ Rice Protein	Rice	Protein		https://www.kerry.com/products/nutrition/proteins/hyprol
Kerry Group plc	Ireland	ProDiem™ Rice Protein	Rice	Protein		https://www.kerry.com/products/nutrition/proteins/prodiem
MTC Industries, Inc.	USA	Rice Protein	Rice	Protein		https://www.mtcindustries.com/protein
Naturz Organics	The Netherlands	Rice Bran Protein 75% - Powder	Rice	Protein	75	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Rice Protein 60% Purple - Powder	Rice	Protein	60	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Rice Protein 80% Brown - Powder	Rice	Protein	89	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Rice Protein 80% White - Powder	Rice	Protein	89	https://naturzorganics.com/our-products/proteins-fibers-starches/
NP Nutra	USA	Brown Rice Protein Powder 80% (organic)	Rice	Protein	80	https://npnutra.com/wholesale-bulk-organic-rice-protein.html

Nutra Food Ingredients, LLC	USA	Rice Protein	Rice	Protein	80	https://www.nutrafoodingredients.com/products/rice-protein/
Nutra Food Ingredients, LLC	USA	Organic Rice Protein	Rice	Protein	80	https://www.nutrafoodingredients.com/products/organic-rice-protein/
Osage Food Products	USA	Rice Protein	Rice	Protein		http://osagefood.com/protein-products/plant-proteins/
Prinova Group LLC	USA	Rice Protein	Rice	Protein		https://www.prinovaeurope.com/ingredients/protein-ingredient-suppliers/
RiceBran Technologies	USA	Proryza™ P-35	Rice	Protein	28-35	https://www.ulprospector.com/en/na/Food/Detail/13513/364030/Proryza-P-35
RiceBran Technologies	USA	Proryza™ PF-20/50	Rice	Protein	15-25	https://www.ulprospector.com/en/na/Food/Detail/13513/363992/Proryza-PF-20-50
Shafi Gluco-Chem (Pvt.) Ltd.	Pakistan	Organic Rice Protein 65%	Rice	Protein	65	https://www.ulprospector.com/en/na/Food/Detail/13660/373752/Organic-Rice-Protein-65
Shafi Gluco-Chem (Pvt.) Ltd.	Pakistan	Organic Rice Protein 50%	Rice	Protein	50	https://www.ulprospector.com/en/na/Food/Detail/13660/373751/Organic-Rice-Protein-50
Suzanne's Specialties, Inc.	USA	Rice Proteins	Rice	Protein		https://www.ulprospector.com/en/na/Food/Detail/13891/411457/Rice-Proteins
The Scoular Company	USA	Rie Protein Isolate	Rice	Protein isolate	80	https://www.scoular.com/food-ingredient-products/proteins/rice-protein/
Top Health Ingredients	Canada	AdvantaRice™ Premier 300	Rice	Protein concentrate	80	https://www.ulprospector.com/en/na/Food/Detail/6673/531944/AdvantAmino-80—300-Mesh
Top Health Ingredients	Canada	AdvantaRice™ 800 Smooth	Rice	Protein concentrate	80	https://www.ulprospector.com/en/na/Food/Detail/6673/531945/AdvantAmino-800-Smooth
Top Health Ingredients	Canada	AdvantaRice™ Flex	Rice	Protein concentrate	80	https://www.tophealthingredients.com/advantarice
Top Health Ingredients	Canada	AdvantaRice™ Peak	Rice	Protein concentrate	80	https://www.tophealthingredients.com/advantarice
Vitacyclix	USA	Rice Protein Powder	Rice	Protein		https://www.morretec.com/divisions/vitacyclix/proteins-fibers/
Axiom Foods Inc.	USA	Incatein™	Sacha inchi	Protein		https://www.ulprospector.com/en/na/Food/Detail/10857/384534/Incatein
Axiom Foods Inc.	USA	Organic Incatein™	Sacha inchi	Protein	60	https://www.ulprospector.com/en/na/Food/Detail/10857/384540/Organic-Incatein
NP Nutra	USA	Sacha Inchi Protein Powder	Sacha inchi	Protein		https://npnutra.com/sacha_inchi_details.html
Osage Food Products	USA	Sacha Inchi Protein	Sacha inchi	Protein		http://osagefood.com/protein-products/plant-proteins/
Dipasa USA	USA	Sesam-Ina	Sesame seed	Protein	60	https://www.ulprospector.com/en/na/Food/Detail/1768/64265/Sesam-Ina
Green Source Organics	USA	Sesame Seed Protein Powder	Sesame seed	Protein	50	https://www.gsoextracts.com/products/sesame
Naturz Organics	The Netherlands	Sesame Protein 50% - Powder	Sesame seed	Protein	50	https://naturzorganics.com/our-products/proteins-fibers-starches/
A.H.A International Co., Ltd	China	Sunsoy 6901J	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/6107/209954/Sunsoy-6901J
ADM (Archer Daniels Midland)	USA	Arcon®F Soy Protein Concentrate	Soy	Protein concentrate	65-90	https://www.adm.com/products-services/food/proteins/ingredients/soy-protein-concentrates
ADM (Archer Daniels Midland)	USA	Arcon®G Soy Protein Concentrate	Soy	Protein concentrate	65-90	https://www.adm.com/products-services/food/proteins/ingredients/soy-protein-concentrates
ADM (Archer Daniels Midland)	USA	Arcon®S Soy Protein Concentrate	Soy	Protein concentrate	65-90	https://www.adm.com/products-services/food/proteins/ingredients/soy-protein-concentrates
ADM (Archer Daniels Midland)	USA	Arcon®SJ Soy Protein Concentrate	Soy	Protein concentrate	65-90	https://www.adm.com/products-services/food/proteins/ingredients/soy-protein-concentrates
ADM (Archer Daniels Midland)	USA	Arcon®SM Soy Protein Concentrate	Soy	Protein concentrate	65-90	https://www.adm.com/products-services/food/proteins/ingredients/soy-protein-concentrates
ADM (Archer Daniels Midland)	USA	Arcon®T Soy Protein Concentrate	Soy	Textured protein	50-85	https://www.adm.com/products-services/food/proteins/ingredients/textured-soy-proteins-crisps
ADM (Archer Daniels Midland)	USA	Arcon®VF Soy Protein Concentrate	Soy	Protein concentrate	65-90	https://www.adm.com/products-services/food/proteins/ingredients/soy-protein-concentrates
ADM (Archer Daniels Midland)	USA	Pro-Fam®646 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®648 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®781 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins

ADM (Archer Daniels Midland)	USA	Pro-Fam®825 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®873 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®875 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®891 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®892 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®922 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®930 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®931 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®932 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®955 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
ADM (Archer Daniels Midland)	USA	Pro-Fam®974 Isolated Soy Protein	Soy	Protein isolate	90	https://www.adm.com/products-services/food/proteins/ingredients/isolated-soy-proteins
América Pampa Agroindustrial SA	Argentina	Micronized Soy Protein	Soy	Protein		https://www.ulprospector.com/en/na/Food/Detail/16598/404484/Micronized-Soy-Protein
América Pampa Agroindustrial SA	Argentina	Micronized Soy Protein Full Fat Active	Soy	Protein		https://www.ulprospector.com/en/na/Food/Detail/16598/404458/Micronized-Soy-Protein-Full-Fat-Active
América Pampa Agroindustrial SA	Argentina	Texturized Soy Protein	Soy	Textured protein		https://www.ulprospector.com/en/na/Food/Detail/16598/404507/Texturized-Soy-Protein
Batory Foods	USA	Soy Protein Concentrate	Soy	Protein concentrate		http://www.batoryfoods.com/product-categories/protein-ingredients/#plant-based
Batory Foods	USA	Soy Protein Isolate	Soy	Protein isolate		http://www.batoryfoods.com/product-categories/protein-ingredients/#plant-based
Burcon NutraScience Corporation	Canada	CLARISOY®100	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/12268/354063/CLARISOY-100
Burcon NutraScience Corporation	Canada	CLARISOY®150	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/12268/354064/CLARISOY-150
Cargill Food Ingredients	USA	Prosanté®Textured Soy Flour	Soy	Textured protein	50	https://www.ulprospector.com/en/na/Food/Detail/6220/387173/Prosant-Textured-Soy-Flour
Daqing Sun Moon Star Protein Co., Ltd.	China	Concentrated Soybean Protein	Soy	Protein concentrate	70	https://www.ulprospector.com/en/na/Food/Detail/16865/410014/Concentrated-Soybean-Protein
Daqing Sun Moon Star Protein Co., Ltd.	China	Defatted Soybean Protein	Soy	Protein	50	https://www.ulprospector.com/en/na/Food/Detail/16865/410034/Defatted-Soybean-Protein
Daqing Sun Moon Star Protein Co., Ltd.	China	Isolated Soy Protein	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/16865/409992/Isolated-Soy-Protein
Distribution Nexxus Inc	Canada	Soy Protein Isolate	Soy	Protein		http://nexxusfoods.com/en/specialty-ingredients/#proteines
DKSH	Schweiz	Wilpro G100	Soy	Protein isolate	90	https://www.ulprospector.com/en/eu/Food/Detail/101881/1018437/SOYBEAN-PROTEIN
DKSH	Schweiz	TexVeg Soy Granules	Soy	Textured protein	52	https://www.ulprospector.com/en/na/Food/Detail/11592/359275/Soya-Texturized-Vegetable-Protein
Dupont Nutrition & Biosciences	USA	ALPHA®	Soy	Protein concentrate		https://www.dupontnutritionandbiosciences.com/product-range/protein/soy-protein-applications.html
Dupont Nutrition & Biosciences	USA	CENTEX®	Soy	Textured protein	50-55	https://www.dupontnutritionandbiosciences.com/products/centex-soy-flour.html

Dupont Nutrition & Biosciences	USA	PROCON®	Soy	Protein concentrate		https://www.dupontnutritionandbiosciences.com/product-range/protein/soy-protein-applications.html
Dupont Nutrition & Biosciences	USA	RESPONSE®	Soy	Textured protein		https://www.dupontnutritionandbiosciences.com/product-range/protein/soy-protein-applications.html
Dupont Nutrition & Biosciences	USA	SUPRO®	Soy	Protein isolate		https://www.dupontnutritionandbiosciences.com/product-range/protein/soy-protein-applications/supro-soy-protein-beverages.html
Dupont Nutrition & Biosciences	USA	SUPRO®EX	Soy	Protein isolate		https://www.dupontnutritionandbiosciences.com/product-range/protein/soy-protein-applications.html
Dupont Nutrition & Biosciences	USA	SUPRO®XT 221D	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/3882/646345/SUPRO-XT-221D
Dupont Nutrition & Biosciences	USA	SUPRO®XT 55	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/3882/713627/SUPRO-XT-55
Dutch Organic International Trade	The Netherlands	TVP soya chunks coarse	Soy	Textured protein	50	https://www.organic.nl/ingredients/plant-protein/plant-protein/11223-tvp-soya-chunks-coarse-org.-10-kg.html
Dutch Organic International Trade	The Netherlands	TVP soya chunks extra fine	Soy	Textured protein	50	https://www.organic.nl/ingredients/plant-protein/plant-protein/11221-tvp-soya-chunks-extra-fine-org.-10-kg.html
Dutch Organic International Trade	The Netherlands	TVP soya chunks fine	Soy	Textured protein	50	https://www.organic.nl/ingredients/plant-protein/plant-protein/11233-tvp-soya-chunks-fine-org.-10-kg.html
Euroduna Food Ingredients	Germany	Dunapro 95M	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/11798/361767/Dunapro-95M
Euroduna Food Ingredients	Germany	Dunasoy 60F	Soy	Protein concentrate	68	https://www.ulprospector.com/en/na/Food/Detail/11798/458119/Dunasoy-60F
Euroduna Food Ingredients	Germany	Dunasoy 70 C	Soy	Protein concentrate	68	https://www.ulprospector.com/en/na/Food/Detail/11798/361764/Dunasoy-70-C
Euroduna Food Ingredients	Germany	Dunasoy 90	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/11798/361765/Dunasoy-90
Euroduna Food Ingredients	Germany	Dunasoy 95	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/11798/416334/Dunasoy-95
Euroduna Food Ingredients	Germany	Soy Protein Isolate YP 901	Soy	Protein isolate	90	http://www.euroduna-food.com/en/ingredients/soy_products.php
Euroduna Food Ingredients	Germany	Soy Protein Isolate YP 928	Soy	Protein isolate	90	http://www.euroduna-food.com/en/ingredients/soy_products.php
Farbest Brands	USA	Gushen Soy Protein GS5100	Soy	Protein		https://www.ulprospector.com/en/na/Food/Detail/11855/603513/Gushen-Soy-Protein-GS5100
Farbest Brands	USA	Gushen Soy Protein GS5100D	Soy	Protein		https://farbest.com/ingredients/proteins/plant-proteins/soy-protein/
Farbest Brands	USA	Gushen Soy Protein GS5200A	Soy	Protein		https://farbest.com/ingredients/proteins/plant-proteins/soy-protein/
Farbest Brands	USA	Gushen Soy Protein GS5300A	Soy	Protein		https://www.ulprospector.com/en/na/Food/Detail/11855/603511/Gushen-Soy-Protein-GS5300A
Farbest Brands	USA	Gushen Soy Protein GS5300D	Soy	Protein isolate		https://www.ulprospector.com/en/na/Food/Detail/11855/603508/Gushen-Soy-Protein-GS5300D
Farbest Brands	USA	Gushen Soy Protein GS5300DC-Calcium Fortified	Soy	Protein isolate		https://farbest.com/ingredients/proteins/plant-proteins/soy-protein/
Farbest Brands	USA	Gushen Soy Protein GS5300S	Soy	Protein		https://farbest.com/ingredients/proteins/plant-proteins/soy-protein/
Farbest Brands	USA	Organic Soy Protein	Soy	Protein		https://farbest.com/ingredients/organic-ingredients/organic-plant-proteins/
Fenchem, Inc.	USA	Soy Protein	Soy	Protein		http://www.fenchem.com/index.php/industry/industrycenter/1/
Food Ingredient Technology Co., Ltd.	Thailand	Isolated Soy Protein (ISP)	Soy	Protein isolate		https://www.fit-biz.com/proteins.html
Food Ingredient Technology Co., Ltd.	Thailand	Isolated Soy Protein (ISP) for Beverage	Soy	Protein isolate		https://www.fit-biz.com/proteins.html
Food Ingredient Technology Co., Ltd.	Thailand	Textured Soy Protein (TSP)	Soy	Textured protein		https://www.fit-biz.com/proteins.html
Foodchem International Corporation	China	Soy Protein Concentrated	Soy	Protein concentrate	68	https://www.ulprospector.com/en/na/Food/Detail/12674/351495/Soy-Protein-ConcentratedFood-Grade
Foodchem International Corporation	China	Soy Protein Isolate	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/12674/351493/Soy-Protein-Isolated
Foodchem International Corporation	China	Textured Soya Protein	Soy	Textured protein		https://www.ulprospector.com/en/na/Food/Detail/12674/351535/Textured-Soya-Protein
Fooding Group Limited	China	Soy Protein Concentrated	Soy	Protein concentrate		http://www.chinafooding.com/Soy_Protein_Concentrate
Fooding Group Limited	China	Soy Protein Isolated	Soy	Protein isolate		http://www.chinafooding.com/Soy_Protein_Isolated
Fooding Group Limited	China	Textured vegetable protein	Soy	Textured protein		http://www.chinafooding.com/Textured_vegetable_protein

Fooding Group Limited	China	Teextured soy protein	Soy	Textured protein		http://www.chinafooding.com/Textured_soy_protein
Gremount International Company Limited	China	Soy Protein	Soy	Protein		http://www.gremount.com.cn/index.php?m=content&c=index&a=lists&catid=37#product_right
Gujarat Ambuja Exports Ltd.	India	Textured Vegetable Protein TVP	Soy	Textured protein		https://www.ambujagroup.com/product/soya-granules-soya-nuggets-tvp/
Gushen Biological Technology Group Co., Ltd	China	GS 5000 - Soy Protein Isolate	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/13365/402665/GS-5000—Soy-Protein-Isolate
Gushen Biological Technology Group Co., Ltd	China	GS 5100-W - Soy Protein Isolate	Soy	Protein isolate	92	https://www.ulprospector.com/en/na/Food/Detail/13365/402592/GS-5100-W—Soy-Protein-Isolate
Gushen Biological Technology Group Co., Ltd	China	GS 5200 - Isolated Soy Protein	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/13365/402543/GS-5200—Isolated-Soy-Protein
Gushen Biological Technology Group Co., Ltd	China	GS 5200I - Isolated Soy Protein	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/13365/402532/GS-5200I—Isolated-Soy-Protein
Gushen Biological Technology Group Co., Ltd	China	GS 5300B - Isolated Soy Protein	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/13365/402743/GS-5300B—Isolated-Soy-Protein
Gushen Biological Technology Group Co., Ltd	China	GS 5300C - Isolated Soy Protein	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/13365/402718/GS-5300C—Isolated-Soy-Protein
Gushen Biological Technology Group Co., Ltd	China	GS 5300H - Isolated Soy Protein	Soy	Protein isolate	80	https://www.ulprospector.com/en/na/Food/Detail/13365/402450/GS-5300H—Isolated-Soy-Protein
Gushen Biological Technology Group Co., Ltd	China	GS 6000 - Soy Protein Concentrate	Soy	Protein concentrate	68	https://www.ulprospector.com/en/na/Food/Detail/13365/402890/GS-6000—Soy-Protein-Concentrate
Gushen Biological Technology Group Co., Ltd	China	GS 6100 - Concentrated Soy Protein	Soy	Protein concentrate	68	http://www.gsjt.com/en/product-5840-5583-14803.html
Gushen Biological Technology Group Co., Ltd	China	GS 8100 - Concentrated Soy Protein	Soy	Protein concentrate	70	http://www.gsjt.com/en/product-5840-5583-14803.html
Gushen Biological Technology Group Co., Ltd	China	Textured Soy Protein GS51	Soy	Textured protein	50	http://www.gsjt.com/en/product-5840-5583-14804.html
Gushen Biological Technology Group Co., Ltd	China	Textured Soy Protein GS52	Soy	Textured protein	70	http://www.gsjt.com/en/product-5840-5583-14804.html
Gushen Biological Technology Group Co., Ltd	China	S Fibertex 1000	Soy	Textured protein	55-75	http://www.gsjt.com/en/product-5840-5583-14805.html
Gushen Biological Technology Group Co., Ltd	China	S Fibertex 2000	Soy	Textured protein	55-75	http://www.gsjt.com/en/product-5840-5583-14805.html
H & A Canada Inc.	Canada	Soya Protein Isolated Emulsion Isopro 900EM	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/4946/188449/Soya-Protein-Isolated-Emulsion
H & A Canada Inc.	Canada	Soy Protein Concentrate/Isolate	Soy	Protein		https://hacanada.com/soy-protein-concentrate-isolate/
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Isolated Soy Protein	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/13009/353451/Isolated-Soy-Protein
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Textured Soy Protein	Soy	Textured protein	50	https://www.ulprospector.com/en/na/Food/Detail/13009/353866/Textured-Soy-Protein
Hefei TNJ Chemical Industry Co., Ltd.	China	Soy Protein Isolate	Soy	Protein isolate	90	https://www.tnjchem.com/isolated-soy-protein-cas-9010-10-0_p8925.html
Improved Nature, LLC	USA	Nature's PRIME™	Soy	Textured protein	64-68	https://improvednature.com/products/
Ingredients Inc.	USA	Soy Protein	Soy	Protein		https://www.ingredientsinc.com/ingredients
Kansas Protein Foods LLC	USA	Imagic®	Soy	Textured protein		https://www.ksprotein.net/products
Kansas Protein Foods LLC	USA	Ultra-Soy®	Soy	Textured protein	50	https://www.ksprotein.net/products
Linyi Shansong Biological Products Co., Ltd	China	Concentrated Soy Protein	Soy	Protein concentrate	65-86	http://www.shansong.com/product/16.html

Linyi Shansong Biological Products Co., Ltd	China	Isolated Soy Protein-Dispersion Type	Soy	Protein isolate	90	http://www.shansong.com/product/21.html
Linyi Shansong Biological Products Co., Ltd	China	Isolated Soy Protein-Gel & Emulsion Type	Soy	Protein isolate	90	http://www.shansong.com/product/19.html
Linyi Shansong Biological Products Co., Ltd	China	Isolated Soy Protein-Injection Type	Soy	Protein isolate	90	http://www.shansong.com/product/20.html
Linyi Shansong Biological Products Co., Ltd	China	Textured Soy Protein	Soy	Textured protein	50-70	http://www.shansong.com/product/15.html
Meelunie B.V.	The Netherlands	Soy Protein Concentrate	Soy	Protein isolate	65-89	https://meelunie.com/products/product-catalogue/
Meelunie B.V.	The Netherlands	Soy Protein Isolate	Soy	Protein concentrate	90	https://meelunie.com/products/product-catalogue/
Naturz Organics	The Netherlands	Soy Protein 50% - Textured	Soy	Textured protein	50	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Soy Protein 65% - Powder	Soy	Protein	65	https://naturzorganics.com/our-products/proteins-fibers-starches/
Naturz Organics	The Netherlands	Soy Protein 90% - Powder	Soy	Protein	90	https://naturzorganics.com/our-products/proteins-fibers-starches/
Nutra Food Ingredients, LLC	USA	Soy Protein (Concentrate, 70%)	Soy	Protein concentrate	70	https://www.ulprospector.com/en/na/Food/Detail/14051/359188/Soy-Protein-Concentrate-70
Nutra Food Ingredients, LLC	USA	Soy Protein (Isolate 90%)	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/14051/359187/Soy-Protein-Isolate-90
Nutra Food Ingredients, LLC	USA	Soy Protein (Textured, 70%)	Soy	Textured protein	70	https://www.ulprospector.com/en/na/Food/Detail/14051/359189/Soy-Protein-Textured-70
Osage Food Products	USA	Soy Protein Concentrate	Soy	Protein concentrate		http://osagefood.com/protein-products/plant-proteins/
Osage Food Products	USA	Soy Protein Isolate	Soy	Protein isolate		http://osagefood.com/protein-products/plant-proteins/
Pacific Soybean & Grain	USA	High Protein Soy Powder - Bakery & Snacks	Soy	Protein		https://www.ulprospector.com/en/na/Food/Detail/13193/367270/High-Protein-Soy-Powder—Bakery-and-Snacks
Pacific Soybean & Grain	USA	High Protein Soy Powder - Dairy & Meat	Soy	Protein		https://www.ulprospector.com/en/na/Food/Detail/13193/367272/High-Protein-Soy-Powder—Dairy-and-Meat
Pacific Soybean & Grain	USA	Soy Protein Concentrate Powder	Soy	Protein concentrate		https://www.ulprospector.com/en/na/Food/Detail/13193/367276/Soy-Protein-Concentrate-Powder
Pacific Soybean & Grain	USA	Textured Soy Protein	Soy	Textured protein		https://www.ulprospector.com/en/na/Food/Detail/13193/367264/Textured-Soy-Protein
Pacific Soybean & Grain	USA	Textured Soy Protein Concentrate	Soy	Textured protein		https://www.ulprospector.com/en/na/Food/Detail/13193/367266/Textured-Soy-Protein-Concentrate
Prinova Group LLC	USA	Soy Protein	Soy	Protein		https://www.prinovaeurope.com/ingredients/protein-ingredient-suppliers/
Qingdao Free Trade Zone United International	China	Soya Protein Isolate	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/15382/363999/Soya-Protein-Isolate
Qingdao Tianwei Biotechnology Co., Ltd.	China	Concentrated Soy Protein - TW700	Soy	Protein concentrate		https://www.ulprospector.com/en/na/Food/Detail/17606/453420/Concentrated-Soy-Protein—TW700
Qingdao Tianwei Biotechnology Co., Ltd.	China	Emulsified Isolated Soy Protein - TW910	Soy	Protein isolate		https://www.ulprospector.com/en/na/Food/Detail/17606/452919/Emulsified-Isolated-Soy-Protein—TW910
Qingdao Tianwei Biotechnology Co., Ltd.	China	Functional Concentrated Soy Protein - TW710	Soy	Protein concentrate		https://www.ulprospector.com/en/na/Food/Detail/17606/453083/Functional-Concentrated-Soy-Protein—TW710
Qingdao Tianwei Biotechnology Co., Ltd.	China	Gel Isolated Soy Protein - TW900	Soy	Protein isolate		https://www.ulprospector.com/en/na/Food/Detail/17606/452810/Gel-Isolated-Soy-Protein—TW900
Qingdao Tianwei Biotechnology Co., Ltd.	China	Injectable Isolated Soy Protein - TW920	Soy	Protein isolate		https://www.ulprospector.com/en/na/Food/Detail/17606/452953/Injectable-Isolated-Soy-Protein—TW920
Qingdao Tianwei Biotechnology Co., Ltd.	China	Scattered Isolated Soy Protein - TW950	Soy	Protein isolate		https://www.ulprospector.com/en/na/Food/Detail/17606/453031/Scattered-Isolated-Soy-Protein—TW950
Qingdao Tianwei Biotechnology Co., Ltd.	China	Textured Soy Protein	Soy	Textured protein		http://www.soybeanproduct.com/Textured-Soy-Protein_187.html

Ruikang Food Co.,Ltd.	China	Doreckonsoy 100	Soy	Protein isolate	89	https://www.ulprospector.com/en/na/Food/Detail/16408/398172/Doreckonsoy-100
Ruikang Food Co.,Ltd.	China	Doreckonsoy 200	Soy	Protein isolate	89	https://www.ulprospector.com/en/na/Food/Detail/16408/398177/Doreckonsoy-200
Ruikang Food Co.,Ltd.	China	Doreckonsoy 70	Soy	Protein concentrate	68	https://www.ulprospector.com/en/na/Food/Detail/16408/398139/Doreckonsoy-70
Ruikang Food Co.,Ltd.	China	Doreckonsoy 80	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/16408/398149/Doreckonsoy-80
Ruikang Food Co.,Ltd.	China	Doreckonsoy 90	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/16408/398156/Doreckonsoy-90
Shandong Sinoglory Health Food Co., Ltd.	China	Soy Protein Isolate ISOPRO 510A	Soy	Protein isolate		https://sinogloryhealth.en.ecplaza.net/products/soy-protein-isolated-non-gmo-isopro_3775335
Shandong Sinoglory Health Food Co., Ltd.	China	Soy Protein Isolate ISOPRO 510B	Soy	Protein isolate		https://sinogloryhealth.en.ecplaza.net/products/soy-protein-isolated-non-gmo-isopro_3775429
Shandong Sinoglory Health Food Co., Ltd.	China	Soy Protein Isolate ISOPRO 515A	Soy	Protein isolate		https://sinogloryhealth.en.ecplaza.net/products/soy-protein-isolated-non-gmo-isopro_3775387
Shandong Sinoglory Health Food Co., Ltd.	China	Soy Protein Isolate ISOPRO 516B	Soy	Protein isolate		https://sinogloryhealth.en.ecplaza.net/products/soy-protein-isolated-non-gmo-isopro_3775518
Shandong Sinoglory Health Food Co., Ltd.	China	Soy Protein Isolate ISOPRO 600	Soy	Protein isolate		https://www.ingredientsnetwork.com/conpro-600-prod138316.html
Shandong Sinoglory Health Food Co., Ltd.	China	Soy Protein Isolated Non-GMO ISOPRO 618B	Soy	Protein isolate		https://sinogloryhealth.en.ecplaza.net/products/soy-protein-isolated-non-gmo-isopro_3775526
Shandong Sinoglory Health Food Co., Ltd.	China	Soy Protein Isolate ISOPRO 815A	Soy	Protein isolate		https://sinogloryhealth.en.ecplaza.net/products/soy-protein-isolated-non-gmo-isopro_3775476
Shandong Sinoglory Health Food Co., Ltd.	China	Textured Soy Protein	Soy	Protein isolate		https://sinogloryhealth.en.ecplaza.net/products/textured-soy-protein_3775460
Shandong Wonderful Industrial Group Co., Ltd.	China	Textured Soy Protein	Soy	Textured protein	50	http://www.wandefugroup.com/html/TEXTURED_SOY_PROTEIN1/20181120_65.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 880	Soy	Protein isolate	88	wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_71.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 930M	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_59.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 930E	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_59.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 930F	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_59.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 961T	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_59.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 950E	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_61.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 951E	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_61.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 952E	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181123_72.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 955E	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_61.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 960T	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_60.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 962T	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_60.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 963T	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_62.html

Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 970W	Soy	Protein isolate	90	http://www.wandefugroup.com/html/SOY_PROTEIN_ISOLATES/20181120_62.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFSPC 680	Soy	Protein concentrate	68	http://www.wandefugroup.com/html/SOY_PROTEIN_CONCENTRATE_NON_FUNCTIONAL/20181120_63.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFSPC 690	Soy	Protein concentrate	69	http://www.wandefugroup.com/html/SOY_PROTEIN_CONCENTRATE_NON_FUNCTIONAL/20181120_63.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 690	Soy	Protein concentrate	67	http://www.wandefugroup.com/html/SOY_PROTEIN_CONCENTRATE_NON_FUNCTIONAL/20181120_64.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 692	Soy	Protein concentrate	67	http://www.wandefugroup.com/html/SOY_PROTEIN_CONCENTRATE_NON_FUNCTIONAL/20181120_64.html
Shandong Wonderful Industrial Group Co., Ltd.	China	WDFPRO 695	Soy	Protein concentrate	69	http://www.wandefugroup.com/html/SOY_PROTEIN_CONCENTRATE_NON_FUNCTIONAL/20181120_64.html
Shandong Yuwang Industrial Co., Ltd.	China	Isolated Soy Protein YP901E Vegetarian Type	Soy	Protein isolate	90	http://en.yuwangcn.com/index.php?c=show&id=23
Shandong Yuwang Industrial Co., Ltd.	China	Isolated Soy Protein YP932A Fermentation type	Soy	Protein isolate	90	http://en.yuwangcn.com/index.php?c=show&id=21
Shandong Yuwang Industrial Co., Ltd.	China	Isolated Soy Protein YP928A Beverage Type	Soy	Protein isolate	90	http://en.yuwangcn.com/index.php?c=show&id=20
Shandong Yuwang Industrial Co., Ltd.	China	Isolated Soy Protein YP901A Meat Type	Soy	Protein isolate	90	http://en.yuwangcn.com/index.php?c=show&id=22
Shandong Yuwang Industrial Co., Ltd.	China	Concentrated Soy Protein YP701B Functional Concentrated	Soy	Protein concentrate	67	http://en.yuwangcn.com/index.php?c=show&id=25
Shandong Yuwang Industrial Co., Ltd.	China	Concentrated Soy Protein YP701A Functional Concentrated	Soy	Protein concentrate	65	http://en.yuwangcn.com/index.php?c=show&id=24
Shanghai Yancui Import and Export Corporation	China	Soy Protein Isolate	Soy	Protein isolate	90	http://www.yancui.com/cgi/search-en.cgi?f=product_en_1_+company_en_1_&id=5025&t=product_en_1_
Shanghai Yancui Import and Export Corporation	China	Soya Protein Concentrate 70%	Soy	Protein concentrate	70	http://www.yancui.com/cgi/search-en.cgi?f=product_en_1_+company_en_1_&id=4935&t=product_en_1_
Shijiazhuang Yuanchem Imp. & Exp. Corp. Ltd.	China	Soy Protein Concentrate	Soy	Protein concentrate	67	http://www.yuanchem.com/template/product_06_32.html
Shijiazhuang Yuanchem Imp. & Exp. Corp. Ltd.	China	Soy Protein Isolate	Soy	Protein isolate	90	http://www.yuanchem.com/template/product_06_31.html
Sonic Biochem	India	JEM®NUTRIMAX Soya Protein Concentrate	Soy	Protein concentrate		https://www.sonicbiochem.co.in/products-and-applications/
Sonic Biochem	India	JEM®NUTRIMAX Functional Soya Protein Concentrate	Soy	Protein concentrate		https://www.sonicbiochem.co.in/products-and-applications/
Sonic Biochem	India	JEM®NUTRIMAX Textured Soya Protein Concentrate	Soy	Textured protein		https://www.sonicbiochem.co.in/products-and-applications/
Sonic Biochem	India	JEM®Soya TVP Natural	Soy	Textured protein	56	https://www.sonicbiochem.co.in/products-and-applications/
Sonic Biochem	India	JEM®Soya TVP Natural Light	Soy	Textured protein	56	https://www.sonicbiochem.co.in/products-and-applications/
Sonic Biochem	India	JEM®Soya TVP Caramel Light	Soy	Textured protein	56	https://www.sonicbiochem.co.in/products-and-applications/
Sonic Biochem	India	JEM®Soya TVP Red	Soy	Textured protein	56	https://www.sonicbiochem.co.in/products-and-applications/
The Scoular Company	USA	ISOPRO 900EM - Soy Protein Isolate	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/3988/112529/ISOPRO-900EM—Soy-Protein-Isolate

The Scoular Company	USA	ISOPRO 900HI - Soy Protein Isolate	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/3988/112528/ISOPRO-900HI—Soy-Protein-Isolate
The Scoular Company	USA	ISOPRO 956 Hi Soy Protein Isolate	Soy	Protein isolate	90	https://www.ulprospector.com/en/na/Food/Detail/3988/204029/ISOPRO-956-Hi-Soy-Protein-Isolate
The Scoular Company	USA	Soy Protein Concentrate	Soy	Protein concentrate	70	https://www.scoular.com/food-ingredient-products/proteins/soy-protein/
Top Health Ingredients	Canada	AdvantaSoy™ Resolve 90	Soy	Protein	90	https://www.tophealthingredients.com/advantasoy
Top Health Ingredients	Canada	AdvantaSoy™ Nourish 90	Soy	Protein	90	https://www.tophealthingredients.com/advantasoy
Vippy Industries Limited	India	Non-GMO Textured Soya Protein / TVP	Soy	Textured protein		https://vippysoya.com/products/
W. Ruitenberg Czn N.V.	The Netherlands	Rudin®ProVega	Soy	Textured protein		https://ruitenberg.com/rudin/rudin-provega/
Zhejiang Wecan Biotechnology Co., Ltd.	China	Soy Protein Isolate	Soy	Protein isolate		http://www.wecanbio.com/pro02_en/typeid/5.html
Blattmann Schweiz AG	Schweiz	Becodur Dinkel®	Spelt	Protein		https://www.blattmannschweiz.com/products/spelt-wheat-proteins/vital-spelt-gluten/
Kröner-Stärke GmbH	Germany	Vital Spelt Gluten	Spelt	Protein		https://www.kroener-staerke.de/Products.7.0.html?&L=1
Fenchem, Inc.	USA	Spirulina Protein	Algae (Spirulina)	Protein		http://www.fenchem.com/index.php/industry/industrycenter/1/
Green Source Organics	USA	Spirulina Powder	Algae (Spirulina)	Protein		https://www.gsoextracts.com/products/spirulina
MTC Industries, Inc.	USA	Spirulina (Protein 58-62)	Algae (Spirulina)	Protein	58-62	https://www.mtcindustries.com/protein
Naturz Organics	The Netherlands	Spirulina Powder - Spray-Dried	Algae (Spirulina)	Protein		https://naturzorganics.com/our-products/microalgae/
NP Nutra	USA	Spirulina Powder (organic)	Algae (Spirulina)	Protein	60	https://npnutra.com/spirulina_details.html
Osage Food Products	USA	Spirulina Protein	Algae (Spirulina)	Protein		http://osagefood.com/protein-products/plant-proteins/
All Organic Treasures GmbH	Germany	Heliaflor®45, Organic	Sunflower	Protein	45	https://www.all-organic-treasures.com/assortment-food/sonnenblumenprotein-bio-heliaflor-45-lebensmittel.html
All Organic Treasures GmbH	Germany	Heliaflor®55, Organic	Sunflower seed	Protein	55	https://www.all-organic-treasures.com/assortment-food/sonnenblumenprotein-bio-heliaflor-55-lebensmittel.html
Ingredients Inc.	USA	Sunflower Protein	Sunflower seed	Protein		https://www.ingredientsinc.com/ingredients
Kerry Group plc	Ireland	ProDiem™ Sunflower Protein	Sunflower seed	Protein		https://www.kerry.com/products/nutrition/proteins/prodiem
Naturz Organics	The Netherlands	Sunflower Protein 50% - Powder	Sunflower seed	Protein	50	https://naturzorganics.com/our-products/proteins-fibers-starches/
Osage Food Products	USA	Sunflower Protein	Sunflower seed	Protein		http://osagefood.com/protein-products/plant-proteins/
Sunbloom Proteins GmbH	Germany	Sunbloom Protein	Sunflower seed	Protein	60	https://www.sunbloom.de/produkte/
NP Nutra	USA	Tara Protein Powder	Tara seed	Protein	45	https://npnutra.com/wholesale-bulk-organic-tara.html
ADM (Archer Daniels Midland)	USA	Provim ESP	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
ADM (Archer Daniels Midland)	USA	Whetpro 75	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
ADM (Archer Daniels Midland)	USA	Whetpro 80	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
ADM (Archer Daniels Midland)	USA	SuperGluten80	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
ADM (Archer Daniels Midland)	USA	Prolite®LF	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
ADM (Archer Daniels Midland)	USA	Prolite®MeatTEX	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
ADM (Archer Daniels Midland)	USA	Prolite®MeatXT	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
ADM (Archer Daniels Midland)	USA	Prolite®100	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
ADM (Archer Daniels Midland)	USA	Prolite®200	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
ADM (Archer Daniels Midland)	USA	SmartBind	Wheat	Protein		https://www.adm.com/products-services/food/proteins/ingredients/wheat-proteins
AG Commodities, Inc.	USA	Vital Wheat Gluten	Wheat	Protein	77	https://www.ulprospector.com/en/na/Food/Detail/23999/568747/Vital-Wheat-Gluten
AMCO Proteins	USA	Hydrolyzed Vital Wheat Gluten HG 80	Wheat	Protein concentrate	71	https://www.amcoproteins.com/plant-proteins-peaproteins#Pea_Protein_80_
Batory Foods	USA	Vital Wheat Gluten	Wheat	Protein		http://www.batoryfoods.com/product-categories/protein-ingredients/#plant-based
Batory Foods	USA	Wheat Protein Isolate	Wheat	Protein isolate		http://www.batoryfoods.com/product-categories/protein-ingredients/#plant-based
Beneo GmbH	Germany	BeneoPro W-Tex Flakes	Wheat	Textured protein	67	https://www.beneo.com/ingredients/human-nutrition/functional-proteins/textured-wheat-protein
Beneo GmbH	Germany	BeneoPro W-Tex Chunks	Wheat	Textured protein	67	https://www.beneo.com/ingredients/human-nutrition/functional-proteins/textured-wheat-protein

Beneo GmbH	Germany	BeneoPro VWG 75	Wheat	Protein		https://www.beneo.com/ingredients/human-nutrition/functional-proteins/vital-wheat-gluten
Blattmann Schweiz AG	Schweiz	Becodur GS	Wheat	Protein	75	https://www.blattmannschweiz.com/products/spelt-wheat-proteins/vital-wheat-gluten/
Blattmann Schweiz AG	Schweiz	Gluvital™	Wheat	Protein		https://www.blattmannschweiz.com/products/spelt-wheat-proteins/vital-wheat-gluten/
Blattmann Schweiz AG	Schweiz	Becodur-Tex Wheat	Wheat	Textured protein	63-67	https://www.blattmannschweiz.com/products/
CBH Qingdao Co., Ltd.	China	Vital Wheat Gluten	Wheat	Protein	75	https://www.ulprospector.com/en/na/Food/Detail/16835/416599/Vital-Wheat-Gluten
Dutch Organic International Trade	The Netherlands	Wheat Gluten (11035)	Wheat	Protein	78	https://www.ulprospector.com/en/na/Food/Detail/15231/356727/Wheat-Gluten-11035
Dutch Organic International Trade	The Netherlands	Wheat Gluten (11032)	Wheat	Protein	80	https://www.ulprospector.com/en/na/Food/Detail/15231/356731/Wheat-Gluten-11032
Euroduna Food Ingredients	Germany	Wheat Protein	Wheat	Protein concentrate	75	https://www.ulprospector.com/en/na/Food/Detail/11798/461183/Wheat-Protein
Food Ingredient Technology Co., Ltd.	Thailand	Wheat Gluten	Wheat	Protein		https://www.fit-biz.com/proteins.html
Foodchem International Corporation	China	Vital Wheat Gluten	Wheat	Protein	75	https://www.ulprospector.com/en/na/Food/Detail/12674/351503/Vital-Wheat-Gluten
Foodchem International Corporation	China	Wheat Gluten	Wheat	Protein	75	https://www.ulprospector.com/en/na/Food/Detail/12674/351522/Wheat-Gluten
Fooding Group Limited	China	Vital Wheat Gluten	Wheat	Protein	75-85	http://www.chinafooding.com/vital_wheat_gluten
Glanbia plc	Ireland	HarvestPro™	Wheat	Protein	85	https://www.glanbianutritionals.com/en-gb/what-we-do/solutions/proteins-0
Glico Nutrition Co., Ltd.	Japan	Wheat Protein V-75	Wheat	Protein concentrate		https://www.glico.com/nutrition/en/product/ingredient/wprotein/
Glico Nutrition Co., Ltd.	Japan	Wheat Protein MG-400	Wheat	Protein concentrate		https://www.glico.com/nutrition/en/product/ingredient/wprotein/
Glico Nutrition Co., Ltd.	Japan	Wheat Protein SS Gluten	Wheat	Protein concentrate		https://www.glico.com/nutrition/en/product/ingredient/wprotein/
Glico Nutrition Co., Ltd.	Japan	Wheat Protein NP Gluten	Wheat	Protein concentrate		https://www.glico.com/nutrition/en/product/ingredient/wprotein/
Green Source Organics	USA	Wheat Gluten	Wheat	Protein		https://www.gsoextracts.com/products/wheat
Gremount International Company Limited	China	Vital Wheat Gluten	Wheat	Protein		http://www.gremount.com.cn/index.php?m=content&c=index&a=lists&catid=37#product_right
H & A Canada Inc.	Canada	Vital Wheat Gluten	Wheat	Protein		https://hacanada.com/vital-wheat-gluten/
Hainan Zhongxin Wanguo Chemical Co., Ltd.	China	Vital Wheat Gluten	Wheat	Protein	75	https://www.ulprospector.com/en/na/Food/Detail/13009/353841/Vital-Wheat-Gluten
Ingredients Inc.	USA	Wheat Gluten	Wheat	Protein		https://www.ingredientsinc.com/ingredients
Kerry Group plc	Ireland	Hyprol™ Wheat Protein	Wheat	Protein		https://www.kerry.com/products/nutrition/proteins/hyprol
Kröner-Stärke GmbH	Germany	Vital Wheat Fluten	Wheat	Protein		https://www.kroener-staerke.de/Products.7.0.html?&L=1
Kröner-Stärke GmbH	Germany	GLUSTAR	Wheat	Protein		https://www.kroener-staerke.de/Products.7.0.html?&L=1
Kröner-Stärke GmbH	Germany	GLUSOL	Wheat	Protein		https://www.kroener-staerke.de/Products.7.0.html?&L=1
Manildra Group	USA	Gem of the West™ Vital Wheat Gluten	Wheat	Protein	75	https://www.ulprospector.com/en/na/Food/Detail/11035/334014/Gem-of-the-West-Vital-Wheat-Gluten
Manildra Group	USA	GemPro®HPG	Wheat	Protein	90	https://www.ulprospector.com/en/na/Food/Detail/11035/334006/GemPro-HPG
Manildra Group	USA	GemPro®DVG	Wheat	Protein	75	https://www.manildrausa.com/products
Manildra Group	USA	GemPro®4400	Wheat	Protein	85	https://www.manildrausa.com/products
Manildra Group	USA	GemPro®Plus	Wheat	Protein	85	https://www.manildrausa.com/products
Manildra Group	USA	GemPro®3300	Wheat	Protein	90	https://www.manildrausa.com/products
Manildra Group	USA	GemPro®Prime-E	Wheat	Protein	90	https://www.manildrausa.com/products
Manildra Group	USA	GemPro®Prime-W	Wheat	Protein	90	https://www.manildrausa.com/products
Manildra Group	USA	GemPro®7700	Wheat	Protein	75	https://www.manildrausa.com/products
Manildra Group	USA	GemPro®Ultra	Wheat	Protein	90	https://www.manildrausa.com/products
Manildra Group	USA	GemPro®Track	Wheat	Protein		https://www.manildrausa.com/products
Manildra Group	USA	GemPro®Extend	Wheat	Protein		https://www.manildrausa.com/products
Manildra Group	USA	Organic Gem of the West™ Vital Wheat Gluten	Wheat	Protein		https://www.manildrausa.com/products

Manildra Group	USA	Organic GemPro®HPG	Wheat	Protein	90	https://www.manildrausa.com/products
Manildra Group	USA	Organic GemPro®DVG	Wheat	Protein	75	https://www.manildrausa.com/products
Manildra Group	USA	Organic GemPro®Plus	Wheat	Protein	85	https://www.manildrausa.com/products
Manildra Group	USA	Organic GemPro®Prime-E	Wheat	Protein	90	https://www.manildrausa.com/products
Manildra Group	USA	Organic GemPro®7700	Wheat	Protein	90	https://www.manildrausa.com/products
Manildra Group	USA	Organic GemPro®Ultra	Wheat	Protein	90	https://www.manildrausa.com/products
Manildra Group	USA	Organic GemPro®Track	Wheat	Protein		https://www.manildrausa.com/products
Meelunie B.V.	The Netherlands	Vital Wheat Gluten	Wheat	Protein	75	https://meelunie.com/products/product-catalogue/
MGP	USA	FP™ 600	Wheat	Protein concentrate	75	https://www.mgpingredients.com/food-ingredients/products/line/fp/fp-600.html
MGP	USA	FP™ 300	Wheat	Protein concentrate	75	https://www.mgpingredients.com/food-ingredients/products/line/fp/fp-300.html
MGP	USA	Optein®	Wheat	Protein		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Proterra™ 1100 Series	Wheat	Textured protein		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Proterra™ 1150M Series	Wheat	Textured protein		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Proterra™ 1200 Series	Wheat	Textured protein		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Proterra™ 1300 Series	Wheat	Textured protein		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Proterra™ 1350 Series	Wheat	Textured protein		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	HWG 2009™	Wheat	Protein		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Arise®5000	Wheat	Protein isolate		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Arise®6000	Wheat	Protein isolate		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Arise®8000	Wheat	Protein isolate		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Arise®8100	Wheat	Protein isolate		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
MGP	USA	Arise®8200	Wheat	Protein isolate		https://www.mgpingredients.com/food-ingredients/products/ingredient/protein
Naturz Organics	The Netherlands	Wheat Protein (Gluten) 80% - Powder	Wheat	Protein	80	https://naturzorganics.com/our-products/proteins-fibers-starches/
Osage Food Products	USA	Vital Wheat Gluten	Wheat	Protein		http://osagefood.com/protein-products/plant-proteins/
Osage Food Products	USA	Wheat Protein	Wheat	Protein		http://osagefood.com/protein-products/plant-proteins/
Purefield Ingredients	USA	Gro-Tein 80 Wheat Protein - Vital Wheat Gluten	Wheat	Protein	80	https://www.fieldcraft.com/co/purefield/products
Purefield Ingredients	USA	Heartland 75 Wheat Protein - Vital Wheat Gluten	Wheat	Protein	75	https://www.fieldcraft.com/co/purefield/products
Qingdao Free Trade Zone United International	China	Vital Wheat Gluten	Wheat	Protein	75	https://www.ulprospector.com/en/na/Food/Detail/15382/363958/Vital-Wheat-Gluten
Roquette Frères	France	NUTRALYS®W	Wheat	Protein	83	https://www.ulprospector.com/en/na/Food/Detail/1129/341748/NUTRALYS-W-Hydrolyzed-Wheat-Gluten
Roquette Frères	France	Roquette®Vital Wheat Gluten	Wheat	Protein		https://www.ulprospector.com/en/na/Food/Detail/1129/204202/Roquette-Vital-Wheat-Gluten
Shanghai Yancui Import and Export Corporation	China	Vital Wheat Gluten	Wheat	Protein	75	http://www.yancui.com/cgi/search-en.cgi?f=product_en_1_+company_en_1_&id=96801&t=product_en_1_
The Scoular Company	USA	Hydrolyzed Wheat Protein	Wheat	Protein	78.5	https://www.ulprospector.com/en/na/Food/Detail/3988/194398/Hydrolyzed-Wheat-Protein
Angel Yeast Co., Ltd.	China	AnPro®Yeast Protein	Yeast	Protein	>70	https://en.angelyeast.com/products/human-health/yeast-protein-anpro.html
Angel Yeast Co., Ltd.	China	Angel®Yeast Extract FH02	Yeast	Protein	>55	https://en.angelyeast.com/products/human-health/yeast-extract.html
Biospirnger by Lesaffre	France	Springer Proteissimo 101	Yeast	Protein	75	https://biospringer.com/en/natural-products/springer-proteissimo-101/
H & A Canada Inc.	Canada	Autolyzed Yeast Cells	Yeast	Protein		https://hacanada.com/autolyzed-yeast-cells/

APPENDIX B: Supplementary Information to Chapter 3

Table A.B1: Sugar levels determined in wheat flour, wholemeal wheat flour and soy protein isolate using different extraction procedures.

	Wheat flour			Wholemeal wheat flour			Soy protein isolate		
Extraction	Sucrose/maltose	Glucose	Fructose	Sucrose/maltose	Glucose	Fructose	Sucrose/maltose	Glucose	Fructose
A1	1.87 ± 0.16^{a}	0.26 ± 0.03^{a}	0.21 ± 0.03^{a}	0.53 ± 0.04^{bc}	0.66 ± 0.05^{a}	0.81 ± 0.07^{a}	-*	-*	-*
A2	1.71 ± 0.02^{a}	0.34 ± 0.01^{a}	0.26 ± 0.00^{a}	0.45 ± 0.04^{c}	0.69 ± 0.02^{a}	0.91 ± 0.02^{a}	-*	-*	-*
A3	1.11 ± 0.17^{b}	0.13 ± 0.02^{b}	0.07 ± 0.01^{b}	0.79 ± 0.03^{a}	0.27 ± 0.01^{b}	0.18 ± 0.01^{c}	-*	-*	-*
A4	0.47 ± 0.23^{c}	0.02 ± 0.01^{c}	0.03 ± 0.01^{c}	0.61 ± 0.06^{abc}	0.05 ± 0.00^{c}	0.05 ± 0.00^{c}	-*	-*	-*
A5	0.60 ± 0.03^{bc}	0.03 ± 0.00^{c}	0.04 ± 0.00^{c}	0.63 ± 0.00^{ab}	0.06 ± 0.00^{c}	0.06 ± 0.00^{c}	-*	-*	-*
A6	2.09 ± 0.26^{a}	0.15 ± 0.06^{b}	0.08 ± 0.02^{b}	0.63 ± 0.08^{ab}	0.35 ± 0.05^{b}	0.39 ± 0.09^{b}	-*	-*	-*
E1	0.43 ± 0.02^{c}	0.03 ± 0.00^{c}	0.04 ± 0.00^{c}	0.58 ± 0.04^{bc}	0.03 ± 0.00^{c}	0.05 ± 0.00^{c}	0.33 ± 0.03^{a}	0.04 ± 0.01^{a}	0.04 ± 0.00^{a}
E2	0.45 ± 0.01^{c}	0.03 ± 0.00^{c}	0.04 ± 0.00^{c}	0.60 ± 0.00^{bc}	0.04 ± 0.00^{c}	0.05 ± 0.00^{c}	0.30 ± 0.01^{a}	0.04 ± 0.01^{a}	0.04 ± 0.00^{a}
E3	0.48 ± 0.01^{c}	0.03 ± 0.00^{c}	0.04 ± 0.00^{c}	0.62 ± 0.06^{abc}	0.04 ± 0.00^{c}	0.05 ± 0.00^{c}	0.36 ± 0.01^{a}	0.05 ± 0.00^{a}	0.04 ± 0.00^{a}

Means $\pm$ standard deviation with different letters in the same column were significantly different ($p < 0.05$).

* Method not applicable for this ingredient.

Table A.B2: Sugar levels determined in HPIs using extraction procedure E2.

Ingredient	Sugar-Pak I column			High-Performance Carbohydrate column			
	Sucrose/maltose	Glucose	Fructose	Sucrose	Maltose	Glucose	Fructose
Soy	0.31 ± 0.02^{i}	0.04 ± 0.00^{g}	0.04 ± 0.00^{e}	0.28 ± 0.02^{f}	n.d.	n.d.	n.d.
Pea	0.77 ± 0.11^{h}	0.01 ± 0.00^{i}	0.04 ± 0.00^{ef}	0.71 ± 0.11^{e}	n.d.	n.d.	n.d.
Lupin	0.15 ± 0.00^{j}	n.d.	0.03 ± 0.00^{f}	n.d.	n.d.	n.d.	n.d.
Gluten	0.95 ± 0.00^{g}	0.06 ± 0.00^{f}	0.07 ± 0.00^{b}	0.16 ± 0.01^{f}	0.78 ± 0.05^{b}	n.d.	n.d.
Lentil IEP*	0.81 ± 0.01^{h}	0.12 ± 0.00^{e}	0.06 ± 0.00^{c}	0.61 ± 0.01^{e}	0.19 ± 0.04^{d}	0.13 ± 0.02^{b}	n.d.
Lentil UF*	0.05 ± 0.01^{j}	0.02 ± 0.00^{h}	0.02 ± 0.00^{g}	n.d.	n.d.	n.d.	n.d.
Carob	4.94 ± 0.08^{c}	0.25 ± 0.01^{b}	0.07 ± 0.00^{b}	4.60 ± 0.08^{b}	0.22 ± 0.04^{d}	n.d.	0.26 ± 0.01^{a}
Chickpea	1.56 ± 0.00^{e}	0.15 ± 0.00^{c}	0.03 ± 0.00^{ef}	1.18 ± 0.02^{d}	0.38 ± 0.04^{c}	n.d.	0.15 ± 0.01^{b}
Quinoa	7.04 ± 0.03^{a}	0.45 ± 0.00^{a}	0.14 ± 0.00^{ef}	5.86 ± 0.13^{a}	1.17 ± 0.05^{a}	0.43 ± 0.00^{a}	n.d.
Amaranth	5.18 ± 0.03^{b}	n.d.	0.05 ± 0.00^{d}	4.61 ± 0.20^{b}	0.44 ± 0.03^{c}	n.d.	n.d.
Buckwheat	2.28 ± 0.05^{d}	0.14 ± 0.00^{d}	0.07 ± 0.00^{b}	2.32 ± 0.11^{c}	n.d.	n.d.	n.d.
Faba bean	1.19 ± 0.02^{f}	n.d.	0.04 ± 0.00^{ef}	1.16 ± 0.05^{d}	n.d.	n.d.	n.d.
Lentil	0.95 ± 0.02^{g}	0.05 ± 0.00^{g}	0.04 ± 0.00^{ef}	0.82 ± 0.09^{e}	0.20 ± 0.03^{d}	n.d.	n.d.

Means $\pm$ standard deviation with different letters in the same column were significantly different ($p < 0.05$).

n.d., Not detected or levels below 0.01 %DM (SP column) or 0.15 %DM (HPC column).

* Lentil protein isolates produced by isoelectric precipitation (IEP) and ultrafiltration (UF).

APPENDIX C: List of Publications and Presentations

First Author Publications

Hoehnel, A., Axel, C., Bez, J., Arendt, E.K., Zannini, E., 2019. Comparative analysis of plant-based high-protein ingredients and their impact on quality of high-protein bread. Journal of Cereal Science 89, 102816. doi:10.1016/J.JCS.2019.102816

Hoehnel, A., Bez, J., Petersen, I.L., Amarowicz, R., Juśkiewicz, J., Arendt, E.K., Zannini, E., 2020. Enhancing the nutritional profile of regular wheat bread while maintaining technological quality and adequate sensory attributes. Food and Function 11, 4732–4751. doi:10.1039/D0FO00671H

Hoehnel, A., Bez, J., Sahin, A.W., Coffey, A., Arendt, E.K., Zannini, E., 2020. *Leuconostoc citreum* TR116 as a Microbial Cell Factory to Functionalise High-Protein Faba Bean Ingredients for Bakery Applications. Foods 9, 1706. doi:10.3390/foods9111706

Hoehnel, A., Bez, J., Amarowicz, R., Arendt, E.K., Zannini, E., 2020. Combining high-protein ingredients from pseudocereals and legumes for the development of fresh high-protein hybrid pasta: maintained technological quality and adequate sensory attributes. Journal of the Science of Food Science and Agriculture. doi:10.1002/jsfa.10994

Hoehnel, A., Bez, J., Petersen, I.L., Amarowicz, R., Juśkiewicz, J., Zannini, E., Arendt, E.K., 2020. Combining high-protein ingredients from pseudocereals and legumes for the development of fresh high-protein hybrid pasta: enhanced nutritional profile. Journal of the Science of Food Science and Agriculture. doi:10.1002/jsfa.11015

Hoehnel, A., Salas García, J., Coffey, C., Zannini, E., Arendt, E.K., 2021. Comparative study of sugar extraction procedures for HPLC analysis and proposal of an ethanolic extraction method for plant-based high-protein ingredients. Journal of the Science of Food Science and Agriculture. doi:10.1002/jsfa.11204

Publications in Progress

Hoehnel, A., Zannini, E., Arendt, E.K., 2021. Targeted formulation of plant-based protein-foods: supporting the food system's transformation in the context of human health, environmental sustainability and food trends. Submitted to Trends in Food Science & Technology.

Other Publications

Ispiryan, L., Heitmann, M., Hoehnel, A., Zannini, E., Arendt, E.K., 2019. Optimization and Validation of an HPAEC-PAD Method for the Quantification of FODMAPs in Cereals and Cereal-Based Products. Journal of Agricultural and Food Chemistry 67, 15, 4384-4392. doi:10.1021/acs.jafc.9b00382

Bellut, K., Michel, M., Zarnkow, M., Hutzler, M., Jacob, F., Atzler J.J., Hoehnel, A., Lynch, K., Arendt, E.K., 2019. Screening and Application of *Cyberlindnera* Yeasts to Produce a Fruity, Non-Alcoholic Beer. Fermentation 5(4), 103. doi:10.3390/fermentation5040103

Presentations

Hoehnel, A., Axel, C., Zannini, E., Arendt, E.K. Comparative analysis of high protein ingredients and their impact on quality of high-protein bread. AACC International Annual Meeting, London, 2018. *(Oral Presentation)*

Hoehnel, A., Bez, J., Petersen, I.L., Amarowicz, R., Juśkiewicz, J., Arendt, E.K., Zannini, E.. High-protein breads. PROTEIN2FOOD Final Meeting/Conference - Food for the Future: Accelerating the Protein Transition, Brussels, 2020. *(Oral Presentation)*

Hoehnel, A., Axel, C., Zannini, E., Arendt, E.K. Plant Protein-Rich Bread - Comparative Analysis of Plant-Based High-Protein Ingredients and their Impact on Quality of High-Protein Bread. School of Food and Nutritional, University College Cork, Sciences Internal Conference, Cork, 2019. *(Oral Presentation)*

Hoehnel, A., Bez, J., Sahin, A.W., Coffey, A., Arendt, E.K., Zannini, E. *Leuconostoc citreum* TR116 as a Microbial Cell Factory to Functionalise High-Protein Faba Bean Ingredients for Bakery Applications. Smart Protein WP 3 Meeting, 2020. *(Oral Presentation)*

Hoehnel, A., Bez, J., Sahin, A.W., Coffey, A., Arendt, E.K., Zannini, E. *Leuconostoc citreum* TR116 as a Microbial Cell Factory to Functionalise High-Protein Faba Bean Ingredients for Bakery Applications. Smart Protein 2nd Annual Meeting, 2021. *(Poster Presentation)*

Awards

Best poster presentation of the Smart Protein 2nd Annual Meeting, 2021. Hoehnel, A., Bez, J., Sahin, A.W., Coffey, A., Arendt, E.K., Zannini, E., 2021. *Leuconostoc citreum* TR116 as a Microbial Cell Factory to Functionalise High-Protein Faba Bean Ingredients for Bakery Applications. Smart Protein 2nd Annual Meeting.