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Ollscoil na hÉireann
National University of Ireland

Coláiste na hOllscoile, Corcaigh
University College Cork

School of Food and Nutritional Sciences



UCC

Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

**Evaluation and improvement of technological
and nutritional properties of plant-based milk
substitutes**

Thesis presented by
Stephanie Jeske
BEng in Food Technology

For the degree of
Doctor of Philosophy - Ph.D. in Food Science and Technology

Under the supervision of
Prof. D.Sc. Dr. Elke K. Arendt

Head of School
Prof. Dr. Kevin Cashman

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Declaration

I hereby declare that this thesis is my own work and effort, and that it has not been submitted for another degree, neither at the National University Ireland, Cork nor elsewhere. Where other sources of information have been used, they have been acknowledged.

Signature

A handwritten signature in black ink, appearing to read 'S. J. de'.

Date:

09/05/2019

Abbreviations

ACE	Angiotensin-converting enzyme
ANS	1-Anilino-8-naphthalenesulfonate
BMS	based milk substitutes
CD	Circular dichroism
DH	Degree of hydrolysis
DTT	Dithiothreitol
EAI	Emulsifying activity
ESI	Emulsifying stability
EPS	Exopolysaccharide
FAC	Fat absorption capacity
FODMAPs	Fermentable oligo-, di-, mono-saccharides and polyols
GI	Glycaemic index
GL	Glycaemic load
HCT	Heat coagulation time
HPLC	High performance liquid chromatography
IEP	Isoelectric precipitation
LAB	Lactic acid bacteria
LCA	Life cycle assessment
LGC	Least gelling concentration
LPI	Lentil protein isolates

MRS	de Man Rogosa and Sharp
MW	Molecular weight
n.d.	Not determined
ND	Not detectable
pI	Isoelectric point
PBDS	Plant-based dairy substitutes
PSD	Particle size distribution
QBMS	Quinoa-based milk substitute
SAT	Sensory acceptance testing
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S ₀	Surface hydrophobicity
TPA	Texture profile analysis
UF	Ultrafiltration
UHT	Ultra-high temperature
WHC	Water holding capacity
WI	Whiteness index

Abstract

Plant-based milk substitutes are suspensions of extracted and disintegrated plant material in water. However, nutritional values and physico-chemical properties of these products vary greatly and depend largely on the raw material. Plant-based milk substitutes are perceived to be healthy by consumers, but this study unveiled that most products lack in quality. Only soya-based products showed good nutritional and functional properties. Accordingly, this thesis addresses the investigation of several techniques to improve the nutritional and physico-chemical properties of plant-based milk substitutes. Fermentation in conjunction with enzymatic treatments was used as a tool for sugar reduction in a quinoa-based product. *Leuconostoc citreum* TR116 was identified as a potent mannitol producer, whereby some of the sweetness was preserved during fermentation. The glucose content was reduced by 40% and the glycaemic load by 35% compared to the untreated control. Moreover, a quinoa-based milk substitute was treated with proteolytic enzymes to improve protein stability and functionality. It was found that endoproteases were the most effective to increase the protein solubility. At the same time, product properties, like foaming or colloidal stability were not affected considerably. The protein content of most commercial plant-based milk substitutes was found to be less than 0.5%. Therefore, lentil protein isolates were investigated as a protein enriching ingredient. Isoelectric precipitation and ultrafiltration were investigated as potential extraction methods for the production of protein isolates from brown lentils. Overall, both lentil protein isolates exhibited promising performances, while scoring better environmental standards when compared to cow milk proteins. Ultrafiltration resulted in a more pure protein isolate (93.7%) that exhibited better functional properties, such as solubility, emulsifying stability and gelling properties. Based on these results the lentil protein isolate obtained by ultrafiltration was chosen to create emulsions with a protein and fat content similar to cow's milk. With high-pressure homogenisation at 900 bar and a heat treatment at 85 °C for 2 minutes emulsions with good colloidal stability (2%/h), appearance, textural and organoleptic profiles were created. These studies assessed the broad range of products available and provided a variety of methods for the formulation of nutritional and functional improved plant-based milk substitutes.

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Chapter 1: Introduction

1.1 Introduction

Agriculture is a major contributor to total greenhouse gas output and is responsible for up to 30% of global emissions. Mainly accountable for these emissions is livestock farming (Aleksandrowicz *et al.*, 2016). Farming of animals also expends a majority of available natural resources such as fresh water and cultivatable land (FAO, 2018). Seventy six percent less farmland would be required for food production by changing to a diet that excludes animal products (Poore and Nemecek, 2018) and 25 million additional people could be fed by replacing dairy with nutritionally comparable plant-based products (Shepon *et al.*, 2018). The conversion efficiency of animal feed to actual food products is generally low; only 14% of feed protein is converted to consumed cow's milk protein, the remaining 86% is "lost" in terms of human nutrition (Shepon *et al.*, 2016). Therefore, one of the most impactful actions to feed the growing world population, save our environment and avert a climate catastrophe may be to reduce our consumption of animal-derived products. One product category which could help achieving this goal are plant-based milk substitutes (PBMSs). While they have recently gained popularity, PBMSs have long been consumed traditionally all over the world (Blandino *et al.*, 2003). Soya-based milk substitutes (BMSs) are the most common products worldwide (MarketsandMarkets, 2017) and have been consumed for centuries in Asia (Golbitz, 1995). Nowadays, the market is expanding rapidly with a predicted growth rate of 11.7% between 2017 and 2022 and new products based on different nuts, seeds, legumes and cereal grains being explored (MarketsandMarkets, 2017).

Generally, PBMSs are colloidal systems of dissolved and disintegrated plant material extracted in water. While soya-BMSs are studied fairly well, the amount of research on the development of other products, like almond, rice, and other nut and seed-BMSs is quite limited, but has been increasing in recent years (Bernat, Cháfer, *et al.*, 2015; Pineli *et al.*, 2015; Briviba *et al.*, 2016; Mårtensson *et al.*, 2000; Gul *et al.*, 2017; Hickisch *et al.*, 2016). Additionally, nutritional properties of such products gained interest and importance. Many studies pointed out the inferior properties of some of these products (Sousa *et al.*, 2017; Katz *et al.*, 2005; Le Louer *et al.*, 2014). Vanga and Raghavan (2018) recently compared the four most popular PBMSs available on the market; they pointed out that soya-BMSs are the best alternative to cow's milk based on nutrients. While almond-BMSs lack nutrient density, rice- and

coconut-BMSs are rich in sugar, and fat, while containing little protein. This imbalance of nutrients poses a potential health issue. Therefore, new raw materials and ingredients merit greater attention. Two crops with great promise are quinoa and lentils. Although very different in their composition and properties, both offer unique possibilities.

Quinoa (*Chenopodium quinoa*) has gained much attention over the past years. The FAO identified quinoa as "one of humanity's most promising crops" that can help to achieve food security in the 21st century (FAO, 2011b). In addition NASA considered quinoa as a crop for the Controlled Ecological Life Support System (Schlick and Bubenheim, 1993). Quinoa is a pseudo-cereal, and the seeds have comparable chemical composition to cereal grains. However, significant differences to cereals are found in its nutritional value. The protein content of quinoa seeds is considerably higher (12 – 23%), with a highly balanced amino acid composition and high levels of lysine and methionine (Abugoch James, 2009). Additionally, quinoa seeds contain considerable amounts of fibre and minerals, such as calcium and iron (Ando *et al.*, 2002) and are also rich in antioxidants like polyphenols (Repo-Carrasco-Valencia *et al.*, 2010). Along with its high nutrient content, quinoa's ability to grow in adverse and highly stressful environmental conditions (e.g. salinity, acidity, drought, frost) makes it a promising crop for the future of agriculture (Jacobsen *et al.*, 2003). As quinoa is rich in starch, a similar approach as for rice-BMSs can be applied. Due to hydrolysis of the starch using glucosidases such as α -amylase, mono- and disaccharides are released, and a low-viscosity liquid is obtained (Mitchell *et al.*, 1981). From a nutritional point of view, the high amounts of free sugar are, however, not favourable. Fermentation is often used to reduce carbohydrate contents, and furthermore, can improve the nutritional and organoleptic profile and enrich the substrate with functional metabolites (Lorusso *et al.*, 2018). With respect to sugars and their sweetness, one such metabolite is mannitol. It has a sweetness of 0.6 relatively to sucrose (Nutrients Review, 2016) but has no effect on blood sugar level and has a low caloric value, since it is not metabolised by the human intestine (European Food Safety Authority, 2011). Some lactic acid bacteria (LAB) strains are able to reduce fructose to mannitol, which decreases the carbohydrate content and at the same time allows some of the sweetness of the product to be maintained. Furthermore, another nutritional disadvantage is the low

solubility of quinoa seed proteins, which can lead to low extraction levels in the final product (Pineli *et al.*, 2015). In this regard, specific proteases can be applied for controlled hydrolysis to increase the solubility and functionality of the protein. It has been proven to be a low-cost, energy-efficient process, which allows to amend proteins gently (Panyam, 1996).

Lentils (*Lens culinaris*), on the other hand, are a staple food in many parts of the world, especially in South Asia (Joshi *et al.*, 2017). They are one of the most economic sources of plant proteins and an important source of dietary protein in developing countries (Kumar *et al.*, 2013). Like most legumes, lentils contain high amounts of protein, ranging from 20.6% to 31.4% (Jarpa-Parra, 2018). Lentils also have other desirable nutritional properties, as they are a good source of carbohydrates, dietary fibre, vitamins and minerals (Jarpa-Parra *et al.*, 2015), and contain phytochemicals including phenolic acids, flavanols and condensed tannins, with antioxidant properties (Durazzo *et al.*, 2013). However, lentils comprise also some antinutritional compounds, such as lectins (carbohydrate-binding proteins), enzyme inhibitors, and contain considerable amounts of FODMAPs (fermentable oligo-, di-, mono-saccharides and polyols), leading to low protein digestibility and flatulencies and compromising some of the aforementioned health benefits (Tuck *et al.*, 2018; Roy *et al.*, 2010; Nosworthy *et al.*, 2018). Preparation and processing methods, such as soaking, cooking, dehulling, extrusion, or isolation of major fractions provides an opportunity to improve the digestibility of lentils (Joshi *et al.*, 2017). In particular, the isolation of protein fractions is of great interest since it opens new possibilities for product formulations, and proteins can be applied specifically for their functional use. Studies on lentil protein isolates (LPIs) have shown promising properties related to gelation, water and oil absorption capacity, and foam and emulsion formation and may be used as substitutes for soya or animal proteins (Toews and Wang, 2013; Boye, Zare, *et al.*, 2010; Ma *et al.*, 2016; Primožic *et al.*, 2018; Joshi *et al.*, 2011; Alsohaimy *et al.*, 2007; Primožic *et al.*, 2017). Lentil protein isolates were previously used to stabilize emulsions with good colloidal stabilities (Primožic *et al.*, 2018; Joshi *et al.*, 2012), however not yet with the aim and formulation to imitate cow's milk.

With the increasing popularity of PBMSs and awareness of the environmental impacts associated with animal-derived products, there is a growing need to identify plant sources and technologies that can nourish and preserve the health of both humans and the Earth. The objective of this thesis is to increase the level of fundamental knowledge and understanding about PBMSs as complex systems and to identify processing techniques to improve nutritional and functional properties of such products, based on the development of quinoa- and lentil-BMSs.

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***Chapter 2: Literature review - Past, present and future;
the strength of plant-based dairy substitutes based on
gluten-free raw materials***

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2.1 Abstract

Plant-based foods are gaining popularity and the market is developing fast. This trend is based on several factors, like the change of lifestyle, interest in alternative diets, and the increasing awareness about sustainable production of food and especially proteins. Plant-based dairy substitutes can serve as an option to traditional food products, meeting many of these interests. However, the market is in its infancy and needs to progress. Trends show, that the market will change from being focused on mainly soya, almond and rice-based products, due to their unsustainable farming, and nutritional concerns, like genetic modification and low protein content. The market is likely to shift towards alternative plants to meet consumers' needs and desire for healthy, flavourful and intriguing products. In this regard, the aspect of allergy-free, like gluten-free products gain in importance. Research studies are approaching the nutritional quality of plant-based dairy substitutes, such as improving the protein quality and glycaemic properties. Furthermore, the application of these products or plant proteins as functional ingredients or substitutes for cow's milk in dairy products like cheese and yoghurt are disseminated. However, there is still a need for much more diversified studies in order to overcome stability, textural, nutritional and sensory problems.

2.2 Introduction

The interest in alternative foods is increasing. Driven by the need to feed the growing world population, sustainable, and nutritious food sources are becoming an omnipresent concern, for companies and consumers likewise. Although plant-based dairy substitutes (PBDSs) have been consumed for centuries as a traditional part of various cultures, a new interest is developing, and the market for such products is expanding rapidly. Traditional products include Spanish Horchata and also Asian soya milk, whereas today the most popular cow's milk alternative is still soya milk besides almond- and rice-based milk substitutes (BMSs) (Mäkinen, Wanhala, Zannini, & Arendt, 2016). The market for PBDSs is driven by many interests and influenced by different opinions. Nowadays, the majority of consumers are not choosing PBDSs out of necessity, but out of preference (Mintel Group Ltd, 2016). Especially, protein sources are on everyone's minds, since livestock farming is expending a lot of energy and producing greenhouse gases (Day, 2013). However, such concerns are not the only aspects driving the market; an awakening awareness and longing for tradition and culture can be observed. Ray *et al.* (2016) described it as "Folk to function" and pointed out the treasure of fermentation and heritage that we can find in this tradition, which has been preserved from generation to generation. Therefore, traditional products like Horchata or fermented plant-based foods like tofu, sufu, or other new developed probiotic products are gaining in popularity. There has been an increase in consumer awareness of the rapid environmental deterioration over the past few decades (Min and Galle, 1997). The shift toward plant based diets, as people become aware of their beneficial health implications also improves food sustainability and environmental impact. Furthermore, consumer aloofness regarding allergens is growing. Not only dairy-free but nut-free, and gluten-free products are gaining in market share (Theodore, 2015). The avoidance of allergens is increasingly seen as part of a healthy lifestyle.

For all these reasons, the market and therefore as well the interest of research is changing and developing quickly. This review summarises the recent achievements and technological, nutritional, and environmental aspects of PBDSs. This review aims to emphasise the importance of these products in terms of nutrition and sustainability and to give an overview on the market situation and consumer interest.

2.3 *Past; traditional plant-based beverages*

Plant-based beverages and derived products have been consumed in early civilisations all over the world. Most of them are available just at the local market or, are prepared traditionally at home on a very small scale in order to provide for the family or the small local community (Blandino *et al.*, 2003). There is a wide range of indigenous plant-based beverages from around the world. For example, many different rice-based beverages originate from Asia; Sikhye, based on cooked rice, malt extract and sugar from Korea; Amazake, a sweet, low or non-alcoholic fermented rice drink from Japan. But similar and other beverages derive from different regions around the world: Atole is a Mexican drink, traditionally prepared with maize. Chicha is a term used for any fermented or unfermented beverage consumed in the Andes based on many different grains and fruits. Bushera is a fermented drink made of sorghum or millet from Uganda. Boza is a fermented drink made of wheat, rye, millet or maize originated from Bulgaria, Albania, Turkey and Romania (Blandino *et al.*, 2003). The “tiger-nut milk” Horchata is an unfermented beverage of milky appearance from Spain (Cortés *et al.*, 2005). Soya-based food products date back to the cornerstone of the traditional Asian diet. Today it is the most widely consumed plant-based milk substitute (PBMS). The first commercially available soya-BMS was produced in Asia in 1940 and spread to the Western World rapidly (Mäkinen *et al.*, 2016). This success is due to much research on the production of milk flavoured soya milk to meet the consumer expectations and the development of technologies for large scale production.

2.4 *Present of plant-based milk substitutes; current market situation*

Consumer demand for cow’s milk alternatives has increased as a result of people being intolerant to cow’s milk, including lactose intolerance and cow’s milk allergy. Soya-BMS is still the most common milk-substitute. However, 14% of the individuals who suffer from cow’s milk allergy also have reactions against soya (Zeiger *et al.*, 1999). Additionally, throughout the Western World concepts of nutritional needs are expanding from survival and hunger satisfaction to using food to promote health and overall state of well-being (Granato *et al.*, 2010). Consumer interest in health and wellbeing is growing rapidly across the world (Ali *et al.*, 2015) and has been led by the increase in knowledge in chronic diseases and the many health claims that are now associated with different foods available. Effectively,

most people consume PBMSs out of preference nowadays, rather than necessity due to an allergy (Mintel Group Ltd, 2016). PBMSs are often perceived as healthy, possibly due to the health claims associated with them e.g. regarding vitamins, fibre, or cholesterol. This is pushing the market, alongside with the negative perceptions people often associated with cow's milk. Negative perceptions of cow's milk include the possibility to contribute to some human diseases along with the high fat content (Dewhurst *et al.*, 2006). In 2015, over 130 variants of different PBMSs were available on the European market and worth 1.5 billion U.S.\$. (Mintel Group Ltd, 2016). Additionally, fermented food and beverages are in the spotlight of consumer attention, which is broadening the non-dairy market even wider. New products, like fermented yoghurt and cheese alternatives are occurring on the market. However, the scientific attention is still little for these products and they have not received the attention they deserve in the last decades.

2.5 *Plant-based milk substitutes*

PBMSs are water extracts of dissolved and disintegrated plant material. Several processing steps can be applied in their production. However, the general outline of a modern industrial scale process is essentially the same: the plant material is either soaked and wet-milled or the raw material is dry-milled and the flour is extracted in water afterwards. Often this slurry is filtered or decanted, to remove the grinding waste and insoluble plant material. Standardisations and addition of other ingredients like oil, flavourings, sugar, and stabilizer may be applied afterwards, depending on the desired product. To improve the suspension and microbial stability, homogenisation and pasteurisation or ultra-high temperature (UHT) treatment take place at the end of the process (Diarra, Nong & Jie 2005). Depending on the plant source and or production steps, the solutions are either colloidal suspensions or emulsions. The finalised product resembles cow's milk in appearance. The general manufacturing steps are displayed in Figure 2-1. Mäkinen *et al.* (2016) described the production of PBMSs in detail recently therefore it will not be reviewed in this article. This review will focus on related products such as substitutes for yoghurt, cheese and other derived products and their product technologies, with special emphasis on the fermentation process.

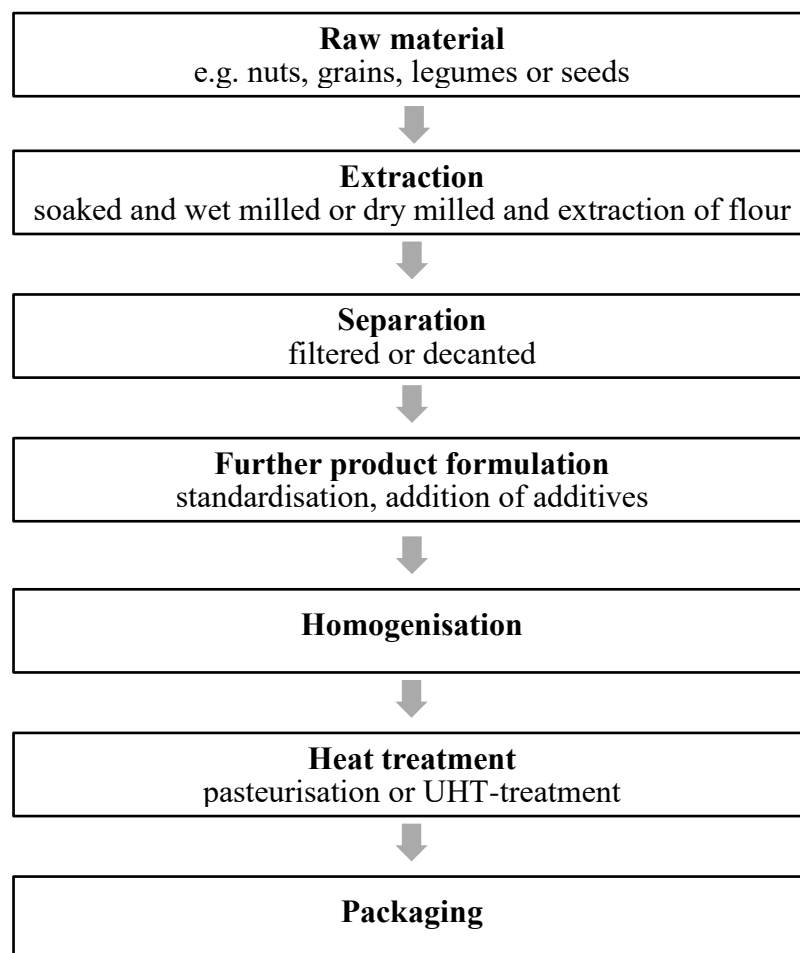


Figure 2-1 General manufacturing steps for the production of PBMSs.

2.6 Fermentation in plant-based products

Fermentation is one of the oldest forms of food preservation; additionally it improves nutritional quality and enhances the sensory attributes of the resulting products while being natural and economical (Ross *et al.*, 2002). Currently, over 5000 different fermented foodstuffs are consumed by humans throughout the world. In 2014 the fermented milk market was worth 46 billion € worldwide, accounting for almost 77% of the fermented food market (Marsh *et al.*, 2014). Many communities around the world produce fermented cereal products, similar to PBMSs, both small and large scale, using different cereals such as rice, wheat, corn or sorghum (Blandino *et al.*, 2003). The microbes present in these products are often poorly characterised due to the small-scale home preparation of the products using skills and recipes that have been passed down through generations. Yeasts, bacteria and fungi are involved in the fermentation, solely or in mixed cultures, working in parallel or sequentially with a changing dominant flora. The commonly used yeasts are species

of *Saccharomyces*, which results in alcoholic fermentation (Piškur *et al.*, 2006). These products are not discussed in this review. Likewise, fungi are not frequently used to ferment PBMSs. However, the majority of non-alcoholic fermented products are soured and mainly based on lactic acid fermentations. Bacteria genus of interest for cereal fermentation are *Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Bifidobacterium* and *Bacillus* (Steinkraus, 1997). During cereal fermentation many different volatile chemicals are emitted out and perceived by the consumer as aromas; these volatile compounds contribute to the overall flavour of the fermented cereal product (Blandino *et al.*, 2003; Peyer *et al.*, 2016). Fermentation has been shown to significantly increase the nutritional quality of cereals. It can also help to enrich the pool of available amino acids, vitamins and minerals, and consequently fermentation increases the overall digestibility and sensory attributes of the food (Ray *et al.*, 2016). Another enzymatic process of huge value is the reduction of oligosaccharides, responsible for digestion problems like flatulence and occurring in beans and vegetables. Due to fermentation with a *Bifidobacterium*, α -galactosidase were released, which minimized the content of oligosaccharides in soya-BMSs (Donkor *et al.*, 2007). The same study showed an increase of angiotensin-converting enzyme (ACE) inhibitory activity, which results in an antihypertensive effect. ACE-inhibitory peptides occur in several food proteins, including the storage proteins of cereals (Hu *et al.*, 2011; Donkor *et al.*, 2007).

Different parameters can be observed which influence the growth of microorganism; the composition and processing of the cereal grains, the substrate formulation, the growth capability and productivity of the starter culture. In general, cereals support microbial growth well and even have been proposed as prebiotics, since they are rich in indigestible matter, like dietary fibre, or certain peptides, proteins and lipids to promote the growth of beneficial bacteria (FAO/WHO, 2001). Inulin and a range of other oligosaccharides, present in some fruits, vegetables and cereals, are getting most of the scientific attention and have demonstrated in several studies the ability to support microbial growth (Capriles and Arêas, 2013; Bernat, Chafer, *et al.*, 2015; Vijaya Kumar *et al.*, 2015).

2.6.1 Fermented drinks

Besides aroma compounds and other minor products, acids are produced most notably during the fermentation of food with bacteria. This results in a sour product with a certain aroma profile. Depending on the base, cereal products can be diluted or blended with juice, aromas and flavours are added or carbonized in order to get a beverage (Kreisz *et al.*, 2008). Arora *et al.* (2010) and Sharma *et al.* (2014) worked on germinated barley and wheat beverages fermented with *Lactobacillus acidophilus*. Both came to the conclusion that a combination of germination and fermentation is a successful way to improve nutritional quality: it ensured significant improvement by reducing the content of sugars while enhancing the levels of thiamine, niacin, lysine, and soluble dietary fibre in the case of the wheat-based study (Arora *et al.*, 2010) and in the barley-based study it was shown that germination increases the cell count effectively (Sharma *et al.*, 2014). It can be observed that a lot of research is based on probiotics and functional food in non-dairy as in dairy products. It is not surprising that a lot of products contain oats; Oats are renowned for their β -glucan content, which has been officially acknowledged by the FDA and allows products to claim a reduced risk of heart diseases (U.S. Food and Drug Administration 2013). Oats were shown in several studies to be a suitable substrate for several bacteria. Several studies from Mårtensson are based on oat-based yoghurt like product (Mårtensson, Maite-Duenas-Chascob, *et al.*, 2002; Mårtensson *et al.*, 2001), which is described in the section pertaining to yoghurt and further information about oats can be found in the section on nutrition. Probiotics are living microorganisms which contribute to the health of the host, when consumed in adequate amounts (FAO/WHO, 2001). Traditionally, dairy products have been the main vehicle of probiotics in the human diet but nowadays, consumer demand is rising for plant-based alternatives. Therefore, synbiotics, the combination of probiotics and prebiotics (FAO/WHO, 2001) are investigated recently. Casarotti *et al.* (2014) supplemented milk with quinoa flour to study the probiotic activity. No changes regarding the growth of *Bifidobacterium animalis* ssp. *lactis* or *Lactobacillus acidophilus* were observed when low levels (up to 3%) of quinoa were added to cow's milk. Even this neutral effect is a positive outcome, since it shows that highly valuable quinoa can be added to dairy products to improve nutrition, while not affecting the probiotic quality. Furthermore, it is not only new developed products that are investigated: the interest in traditional products and their complex

microbial flora is growing. For example, the traditional Bulgarian beverage Boza was assessed regarding its microbial flora. The isolated *Lactobacillus plantarum*, *Candida rugosa* and *Candida lambica* demonstrated probiotic properties, been resistant to bile up to a concentration of 2% (Gotcheva *et al.*, 2002). In 2008, Todorov *et al.* showed that LAB isolated from Boza produced bacteriocins, which were active against a number of pathogens, e.g. *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Mridula & Sharma (2015) investigated the utility of sprouted wheat, barley, pearl millet and green gram in combination with oats, and with or without soya-BMSs in a probiotic drink. Each of the sprouted cereals improved the growth of *Lactobacillus acidophilus* with increasing levels of cereals.

2.6.2 Yoghurt substitutes

Traditionally, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are commonly used as starter cultures in yoghurt production based on cow's milk. They live symbiotically and grow rapidly during fermentation. Typically, cow's milk is fermented at 42 -43 °C until pH 4.5 to 4.2 is reached, which corresponds to 1.2 to 1.4% lactic acid. The bacteria reach finally a population of 20×10^8 cells/mL (Robinson, 2002; Chandan, 2007). Cow's milk yoghurt is a gel based matrix. Gelation is a critical first step in both cheese making and yoghurt manufacture (Lucey, 2002). In the case of yoghurt, cow's milk is normally heated at a pH (6.6) distant to the isoelectric point (pI) of cow's milk proteins, which causes denaturation and formation of soluble aggregates. During the fermentation with LAB, the pH is decreasing towards the pI (4.6), resulting in gelation as caseins form a continuous network (Lee and Lucey, 2004). Dairy alternative yoghurts can have structural disadvantages compared to dairy yoghurts made with animal milks, because of the nature of the proteins. Mäkinen *et al.* (2014) studied the structure of acidified bovine milk, soya- and quinoa-BMSs. Even though all proteins showed gel-like characters with $G' > G''$, the quinoa sample showed a weaker gel structure compared to soya and bovine sample. This could be due to either stronger intermolecular association or simply less: the used quinoa-BMS contained little protein with 1.26 compared to 3.32 and 2.95 g/100g for bovine milk and soya-BMS respectively. Another study based on oat-BMS showed similar results: chemically acidified samples with glucono-delta-lactone did not increase the viscosity. The authors hypothesised that

the proteins are not likely to coagulate and that the protein content was too low to show changes in viscosity (Mårtensson *et al.*, 2000). Further investigation of Mäkinen *et al.* (2015) exhibited the sensitive nature of proteins to their environment, such as the pH: heat-denaturation of a quinoa protein isolate at high pH (10.5) prior to an acid induced gel formation led to a strong gel, whereas a heat treatment at lower, neutral pH resulted in a weak gel. At pH 10.5 the proteins were highly soluble and heat-aggregation was retarded, due to increased surface charge. During acidification, the soluble aggregates cross-linked and formed a regular and very fine network. In contrast to that, at a lower pH the proteins formed random disulphide bonds and aggregated already during heating, which resulted in a coarse coagulum. Accordingly, recent studies on oat-protein-isolates investigated the characteristics of gelling in detail. Different levels of glucono-delta-lactone were added to heated oat-protein-isolates at pH 8, which enabled them to produce different gels, with changing gel network structure and strength. A high pH reduction rate and final pH near the pI resulted in gels with small pores, dense wall microstructure, and superior mechanical strength, comparable to egg white gel (Yang *et al.*, 2017). In contrast to the study of Mårtensson *et al.* (2000), Yang *et al.* (2017) used oat protein isolates, controlled pH, applied heating and adjusted acidification. By taking all these factors into account, protein gels for many applications can be achieved. However, applying this to a food system can be difficult, since the system might be much more complex and altering the conditions might influence other ingredients or is simply too cost and work intensive. Most commercial yoghurt-like products contain thickeners and emulsifiers to alter the firmness but several attempts have been made to improve the quality of plant-based yoghurt substitutes, e.g. by the addition of different ingredients like inulin, raffinose and glucose (Donkor *et al.*, 2007), fructose (Buono *et al.*, 1990), or even whey proteins (Lee *et al.*, 1990), lactose (Cheng *et al.*, 1990) and evaporated milk (Buono *et al.*, 1990), or pre-treatments such as ultra-high pressure homogenisation (Fernandez-Avila and Trujillo, 2016) or microwave processing (Bhattacharya and Jena, 2007). Many consumers reject these additives in products. The clean label trend is growing and the use of animal ingredients excludes the vegetarian/vegan consumer segment. One potential method to improve the texture of yoghurt substitutes and to avoid additives is the use of bacteria that produce exopolysaccharides (EPS). EPS such as dextran, with predominant α -(1 \rightarrow 6) linkages produced by the enzyme dextransucrase could find applications as food

hydrocolloids. Certain strains of LAB can produce EPS, which can cause a consistency similar to that of dairy yoghurt. Effects of EPS include improved mouth feel, limited syneresis, ropiness, and increased gel firmness based on its ability to bind water and interact with proteins (Hickisch *et al.*, 2016). The amount and composition of the EPS produced by the LAB depends on many different factors such as growth of LAB strain, temperature, initial pH, carbon source and the availability of different minerals, vitamins and other medium components (Grobben *et al.*, 2000; Amari *et al.*, 2013; Shukla and Goyal, 2011). While the interaction between EPS and milk proteins is extensively studied the interactions of EPS with plant-proteins have rarely been investigated. Soya proteins and EPS were embedded in a network, formed a stable network, and maintained a high apparent viscosity (966.43 mPa s) during 21 days of storage at 4 °C (Li *et al.*, 2014). Another study on lupin-based yoghurt alternatives found that EPS yields were comparable to EPS yields reported for cow's milk yoghurt. *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Lactobacillus brevis* were used as starter cultures with glucose as a carbon source, and yielded in apparent viscosities significantly lower than cow's milk yoghurt. At 50 s⁻¹ viscosities of ~350–1180 mPas were measured compared to ~2000–2400 mPas respectively. They stated that EPS was very likely to be incorporated in a network with proteins and other constituents, but no structural characterizations were performed to investigate the nature of the network (Hickisch *et al.*, 2016). Different heat treatments were applied as well, and like mentioned earlier, this is affecting the protein-network itself, but could change the interaction with EPS also. A similar product based on *Lupinus campestris* seeds fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* yielded a viscosity comparable to dairy yoghurts after 72 h fermentation at 25 °C (Jiménez-Martínez *et al.*, 2003a). In addition, fermentation not only provides texture by altering the pH or forming EPS, it is essential for flavour development. This point is discussed in the section about consumer acceptance and sensory in detail.

2.6.3 Cheese substitutes

Milk-based cheese is naturally made with the use of enzymes from rennet and LAB for acidification. The kind and degree of fermentation determines characteristic properties, like the rheology, texture, and taste. Depending on this, many different cheese styles can be achieved (Lucey, 2002). Cheese analogues, just like traditional

cheese, are emulsions of oil-in-water, wherein proteins function as emulsifiers and provide structure throughout a gel matrix similar to natural cheese (Bachmann, 2001). However, the production of cheese substitutes follows a different regime, due to the different nature of plant-proteins; in cow's milk, casein is changed to para-casein by the rennet, which interacts with calcium-salts and forms a strong, reticulated curd (Chavan and Jana, 2007). Literature dealing with enzymatic coagulation of peanut- and soya-BMSs showed generally weak structures and especially hard style cheeses are difficult to obtain (Santos *et al.*, 1989). The so-called cheese of Asia, sufu dates back to ancient Chinese history. It is produced by coagulating soya-BMS, pressing the resulting curds and following fungal and/or bacterial fermentation (Ahmad *et al.*, 2008). As coagulate salts like calcium sulphate are traditionally used. The general production steps are displayed in Figure 2-2. The structure is described as tender but brittle. By using other ingredients, or methods, like acidulants, or the application of enzymes, smoother textures can be achieved since the structure relies on the pore sizes and other microscopic properties of the matrix (Berk, 1992), similar to the system of yoghurt. On the Western market, most of the cheese analogues contain dairy ingredients like casein, or milk fat, and are supplying the low value market with cheap products, the most abundant of which being a mozzarella analogue for pizza (Bachmann, 2001). On the other hand, the exclusion of dairy ingredients and inclusion of high value plant-based ingredients serves the market with functional, healthy and diversified food products. Most of the products available on the market consists of partly hydrogenated vegetable oils, plant proteins, stabilizers, emulsifiers, emulsifying salts, acidulants, salt, colouring, flavouring, preservatives, and water, without using starter cultures and enzymes. The ingredients are blended together and most of the time the products are non-matured (Bachmann, 2001). Plant-based cheese-like products have attained little scientific attention and most of the studies only partially replace dairy ingredients with plant ingredients. To the author's knowledge, no research has dealt with cereal-based cheese analogues, and research is focused rather on soft cheese-like spreads based on soya and peanut.

Chavan and Jana (2007) reported inferior product properties of cheese analogues containing high levels of vegetable proteins instead of casein: adhesive/sticky consistency, lower hardness, stretchability and elasticity, and often poor flavour.

Therefore, some studies use pastes, rather than milk-like beverages to overcome the texture problems; An imitation cheese spread obtained from a peanut paste resulted in a texture similar to commercial dairy-based products (Santos *et al.*, 1989). Further, peanut was described as favourable due to its bland flavour and light colour (Chavan and Jana, 2007). Soybeans on the other hand are known to have an undesirable beany taste and to result in a grainy texture (Li *et al.*, 2013). In contrast to dairy products, plant-based products like sufu attained little scientific interest but the adaption of sufu processing technologies, shown in Figure 2-2, could be an interesting tool for other PBMSs.

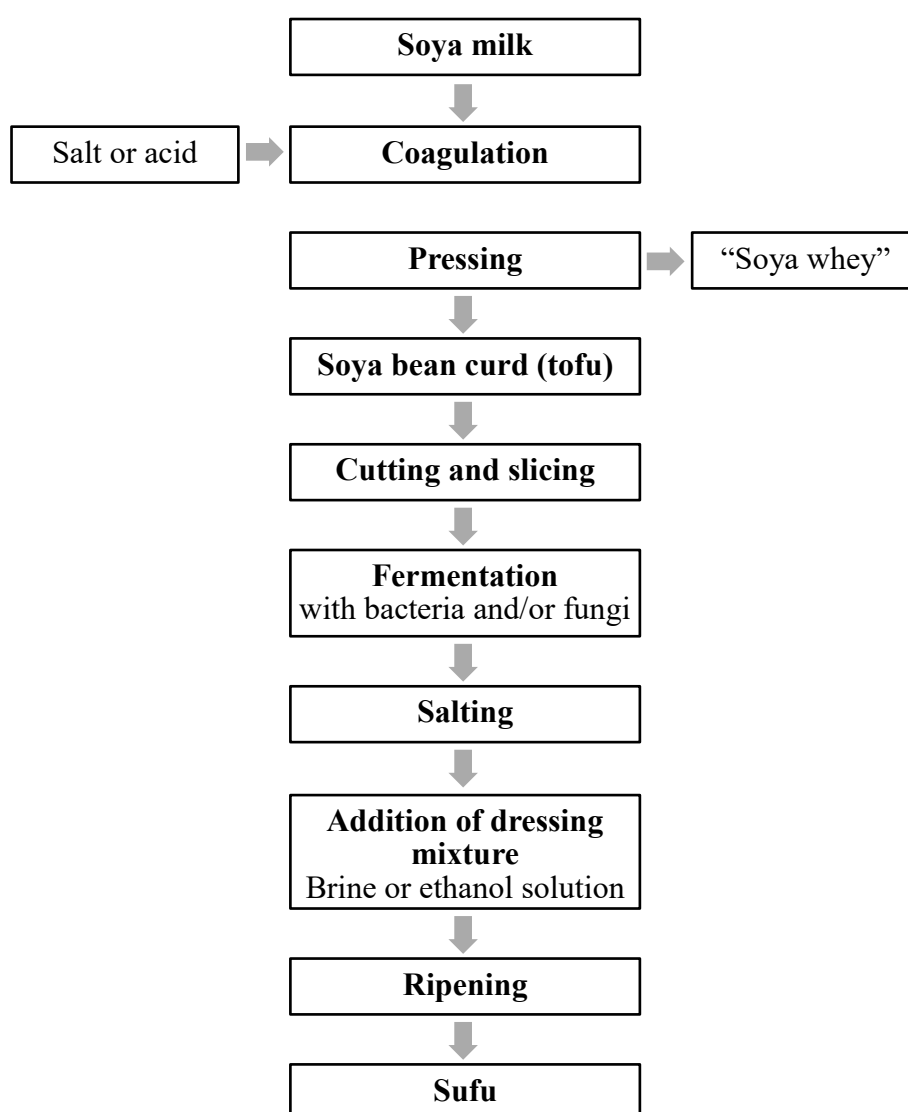


Figure 2-2 Traditional process of “sufu” (soya cheese).

Some research tried to incorporate other plant ingredients into traditional dairy products: Lupin-BMS was incorporated in tofu, which showed a neutral effect on texture, flavour and overall acceptability when replacing 40% of soya-BMS by lupin-BMS (Jayasena *et al.*, 2010). Lupin-BMS was also used in dairy based cheese and was shown to enhance taste, texture, flavour, and overall acceptability of both fresh and matured cheese at concentrations of 25% (Elsamani *et al.*, 2014). Sesame protein isolate was shown to increase hardness, cohesiveness, adhesiveness, and gumminess in cheddar cheese since the sesame proteins contributed to the micro-structure via protein micelle cluster interaction in the curd (Lu *et al.*, 2010). Such research shows promising opportunities for products since they are nutritionally more valuable than the traditional products with a higher protein content, however, the use of dairy ingredients excludes dairy-free diets followers. Nevertheless, these products followed the traditional cheese production steps, unlike most of the commercial dairy-free products, which are produced by blending ingredients simply together and incorporating different additives to achieve the desirable texture and taste (Chavan and Jana, 2007). Zulkurnain *et al.* (2008), for example, used tofu, and blended it with oil, salt, and three different polysaccharides, in order to achieve a similar texture to cream cheese: Carrageenan was used to impart firmness, maltodextrin provided body to the product, while pectin prevented syneresis but introduced viscous behaviour to the final product. Valuable insights about different production strategies for a soya-based cheese spread are given by Li *et al.* (2013); the results showed that a combination of glucono-delta-lactone and LAB fermentation together with enzymatic hydrolysis resulted in the best spreadability, more acceptable sensory features, and a stable homogeneous structure.

Generally, plant proteins possess very different molecular and functional properties compared to casein due to their larger molecular size and complex quaternary structure (Bachmann, 2001). As described in the section about yoghurt-like products, various technologies, like the application of proteases, acids, or heat treatments, need to be considered to improve the solubility, emulsification, and coagulability of those proteins. For example, the melting ability of imitation mozzarella cheese was improved by partial enzymatic hydrolysis of soya protein isolate using a mixture of amylases and rennet (Nishiya *et al.*, 1989). Not only proteins play a crucial role in the cheese matrix: fat determines the properties

equally. It contributes to a creamy mouth feel, flavour, and texture. Fat globules occupy space within the protein matrix and prevent the formation of a dense network, which would result in a hard, corky product (Bachmann, 2001). Generally, the phase state of fat, i.e. solid or liquid, determines the texture of the product. The study of Lobato-Calleros *et al.* (1997) exhibited this clearly: Soybean fat imparted hardness and adhesiveness to cheese analogues, but decreased cohesiveness and springiness, while the opposite effect was observed for soybean oil and butterfat. The melting points of plant fats are generally low, around room temperature. To achieve the desired hardness in a cheese matrix, vegetable oils are often hydrogenated to increase the melting point. Since not only the physical, but also the chemical properties, play a crucial role, partial hydrogenation can help further to improve the texture: Saturated fatty acids promote the adsorption of lipophilic groups of the proteins onto the surface of fat globules, thus improving the emulsion and forming a reticulated matrix (Lobato-Calleros *et al.*, 1997). However, this procedure reduces the nutritional value of plant fats drastically and increases the risk of cardiovascular and coronary heart diseases (Mozaffarian and Clarke, 2009). Moreover, the application of EPS producing strains could offer opportunities to improve the hardness, and structure of plant-based cheese analogues, since this showed good results for fat reduced dairy cheeses (Awad *et al.*, 2005; Zhang *et al.*, 2015).

2.7 Other related products

Cow's milk can be further formulated to other products and so can PBMSs; cream alternatives, ice cream, chocolate, and infant formulas to name just a few. Although not much literature is available on these kinds of products, the ones available show promising results with new approaches. Certain processing steps are applied to achieve different products. Bastioğlu *et al.* (2016) spray-dried a melon seed-BMS to obtain an instant powder. The product was obtained by a simple extraction, meaning crushing and blending of the melon seeds with water prior to a filtration. The spray-dried powder prolonged the shelf life of the product and the prepared beverage maintained the quality characteristics of fresh melon seed-BMS. Another usage of dehydrated PBMSs was studied by Aidoo *et al.* (2010): peanut-and cowpea-BMSs were drum dried and formulated into a fine powder with a hammer mill and used to prepare dark chocolate. Unfortunately, the recipe of the chocolate is not published, but the obtained chocolates showed good acceptability in a sensory

test, with no significant differences to the control chocolate, prepared with skimmed milk powder. A tiger-nut-BMS was used to replace cow's milk in an ice-cream formulation, the results showed that even a complete replacement led to no significant differences in physical properties, like whipping, viscosity, overrun and meltdown, and sensory properties when compared to a dairy product. Additionally, the tiger-nut-BMS enhanced the growth of the incorporated probiotic bacteria strains (El-Shenawy *et al.*, 2016).

2.8 Law, governmental and global interest about dairy substitutes

Product names like "milk" and "cheese" are traditional dairy terms and consumers relate such products essentially to be dairy-derived. However, PBMSs are labelled with the same descriptors to achieve familiar expectations quite often. The discussion from both sides (dairy, and non-dairy) whether this is misbranding and consumer deception, is not uniformly answered yet. The European commission regulates the term "milk" clearly, to be exclusively used for "the normal mammary secretion obtained from one or more milkings without either addition thereto or extraction therefrom" (Council of the European Union, 2007). Further designations related to dairy products are reserved also exclusively for milk products, i.e. whey, cream, butter, buttermilk, butteroil, caseins, anhydrous milkfat, cheese, yogurt, kephir, koumiss, viili/fil, smetana, fil (Council of the European Union, 2007). The European commission allows exceptions for traditional products (European Commission 2010). Such exceptions include products like "Leberkäse", a meat product from Germany, "Crème d'anchois", a fish spread from France, coconut milk, and "Leche de almendras", almond milk from Spain. Therefore, coconut milk and almond milk are the only PBMSs allowed to be labelled as milk in the European Union. To the author's knowledge, in no other country regulations like this are applied.

The idea of sustainable food production has become very important to local governments along with many food industries around the world. PROTEIN2FOOD is a project funded from the European Union to improve human health, environmental sustainability, and biodiversity. The aim of this project is to increase the production and consumption of high value plant proteins. The project takes the whole product chain into account, from field to fork, to enhance the sustainability of

their production and processing and to create innovative, high quality, protein-rich food products, including dairy-substitutes (PROTEIN2FOOD, 2015). The government of the United Kingdom recommends, in their recent publication “The Eatwell Guide”, to replace dairy products with plant-based alternatives for a healthy diet (NHS England, 2016). Furthermore, “Protein Challenge 2040”, which is a global coalition of stakeholders coming from various backgrounds, was founded to create a worldwide network of international businesses and non-governmental organisation to face the growing demand of proteins (Forum for the Future, 2016). These projects are just a few selected examples, but represent well the interest of different stakeholders. A key interest for all of them is to improve the public awareness, and knowledge regarding food and proteins. People’s purchasing decision is highly related to cultural and social norms. Promoting plant-based food, and educating about health and sustainability can change this cultural and social environment.

2.9 Impact on climate and land use; Sustainability

Cereals are one of the most important energy sources in the world; they contribute to about 50% of the average daily energy intake in most populations, and in some developing countries they can contribute up to 70% of the energy intake (De Anton Migliorati *et al.*, 2015). The world’s population is increasing rapidly and is putting enormous pressure on food systems to provide enough food to feed the growing population. Food systems depend heavily on our natural resources like land, water, and energy resources like fossil fuels and natural gas, and increased pressure on these resources contributes massively to many of the environmental problems of today, such as greenhouse gas emissions and eutrophication (Jeswani *et al.*, 2015). The growth in food consumption per capita worldwide is also putting an increased pressure on the cereal production in the agricultural-food industry (Henry *et al.*, 2016). Even though land plants are an important source of energy in the human diet, relatively cheap and very abundant source of protein, the direct consumption of plant proteins is still relatively low (Day, 2013). There has been a move towards increased meat and dairy consumption in the last decade, which is especially evident in developed countries and consequently increasing the demand for cereals further as a main source of feed for the animals with the U.S. animal feed industry being the largest feed producer in the world producing over 120 million tonnes of animal feed in 2004 since the consumption of meats per capita in the U.S. in 2003 was 90.5

kg/year (Sapkota *et al.*, 2007). However, the conversion of plant proteins to animal proteins is very inefficient, since 75- 90% of the grain and consequently 65-90% of the plant protein fed to livestock is lost in conversion (Grigg, 1995). Meat and dairy production are responsible for a massive share in the food related environmental pressure, with the production of animal proteins requiring about 100 fold more water compared to the production of the same amount of plant-based proteins (Day, 2013). Due to the constant rise of the global population, food security is becoming an increasing problem for the agricultural-food industry. Better use of plant-based proteins is becoming more critical for sustainable food consumption, as the supply of animal-based proteins reaches its maximum production capacity (Day, 2013). In this regard, PBDSs have the opportunity to expand their market and serve as a plant-based food, but especially, as a plant-based protein source in the diet.

However, the production of soya and almond, base for the most popular PBMSs, are facing already environmental problems; the rapidly expanding market for soya is causing a threat to the environment with a huge impact especially in South America. The expansion of soybean plantations into fragile ecosystems, like the rainforest, or savannahs, is contributing to climate change, and jeopardizing biodiversity and endangered species (Fearnside, 2001). A similar problem is faced by the almond agriculture: California is currently producing 80% of the world's almonds (Theodore, 2015). Almonds have large water requirements, however California is suffering from its fifth year of severe drought (California Water Science Center, 2016). Therefore, alternatives need to be found. Plants, which are more resistant to climate changes, like peanuts, quinoa or peas for example, are already gaining in popularity (Theodore, 2015).

2.10 Nutritional properties

Dairy products negatively affect a great number of the world's population, as they cause adverse reactions within the body of the affected individual, including lactose intolerance and cow's milk allergy (Crittenden, Bennett 2005). At the same time, it needs to be noted that milk and dairy products are a fundamental source of nutrition for many people all around the world (FAO, 2013) and furthermore, beneficial effects associated with the consumption of these products have been shown in many studies (Mckinley, 2005; Michalski and Januel, 2006). However,

consumers are avoiding dairy products based on health concerns, like cholesterol and antibiotic residues in cow's milk (Organic Monitor, 2005). PBDSs are purchased for their health and wellness benefits (Mintel Group Ltd, 2016; Organic Monitor, 2005). One major downside of PBMSs is the low protein content of most of the products available on the market. Half of the samples analysed by Jeske *et al.* (2017) (Chapter 3) contained less than 0.5% proteins and only samples based on soya reached values comparable to cow's milk with 3.7%. Even if for most of the population an overconsumption of protein is the case (Ranganathan *et al.*, 2016), if cow's milk is replaced by PBMSs, consumer awareness is still very important. Inappropriate use of PBMSs in the diet of young infants can lead to severe malnutrition. Le Louer *et al.* (2014) reported for several cases protein-calorie malnutrition and deficiencies of minerals and vitamins with severe consequences for the infants. In addition to the low protein quantity in some PBMSs, the quality of plant-based proteins can be inferior compared to cow's milk proteins, which has a protein digestibility-corrected amino acid score of 121. Plant proteins show lower values ranging from 91, 90 for soya and cashew, and down to 23 for almond (Schaafsma, 2000; Freitas, 2012; Ahrens *et al.*, 2005). However, the market is developing and products are changing: So Delicious Dairy Free released an almond-based beverage, which claims to contain 5 times more protein than other almond milks available on the market. This product is enriched with pea and rice protein to reach 2 g of protein per 100 mL of product (So Delicious Dairy Free 2016). Furthermore, fermentation has been shown to improve the protein digestibility (Taylor and Taylor, 2002; Holzapfel, 1997), in addition to the bioavailability of minerals and other micronutrients (Watzke, 1998; Greffeuille *et al.*, 2011). Plants are a superior source for nutrients like vitamins, dietary fibres, antioxidants, and flavonoids, which have shown nutritious and health-promoting properties. For example, the level of plasma cholesterol and low-density lipoprotein cholesterol was decreased when cow's milk was replaced by an oat-based beverage due to the β -glucans in oats (Önning *et al.*, 1998). Also, fermented oat-BMS showed a lower postprandial glucose response than cow's milk yoghurt (Lindström *et al.*, 2015). Again, in this study the β -glucans were proposed to be responsible for this difference. The impact of EPS was studied as well but appeared to have no impact on the glycaemic response (Lindström *et al.*, 2015). In general, the glycaemic index for several PBMSs is moderate with values from 47 to 64. Rice- and coconut-BMSs showed however high values, reaching values of 100 for the

glycaemic index. Due to the low carbohydrate content of the coconut-BMS, this sample had a low glycaemic load (4.81), whereas the rice-BMSs showed values comparable to Coca-Cola (>16) (Jeske *et al.*, 2017; Chapter 3). Cow's milk is an important source for calcium, iodine, vitamin B12 and riboflavin. To overcome the risk of malnutrition most of the commercial PBMSs are fortified, but consumer awareness of this is very important. In this regard an interesting point is the potential renal acid load, which indicates the amount of acid produced during metabolism. The consumption of animal proteins increases the acid load in the body, whereas fruits and vegetables generally decrease it. The created acid needs to be neutralized. For this purpose, the body uses mainly calcium, which acts as a very effective base (Barzel and Massey, 1998; Thorpe and Evans, 2011). To draw a line back to the calcium contents in cow's milk and PBMSs, it is not only important how much calcium is provided by the diet, but the way the entire food system is metabolised is essential. Some plants can contain anti-nutritional compounds, like phytates and trypsin inhibitors, which impart the bioavailability of nutrients. These can be combated by fermentation, germination, chelating agents, or exogenous phytase (Savelkoul *et al.*, 1992; Buddrick *et al.*, 2014; Kumar *et al.*, 2010), or heat treatments (Friedman, 1996). Processing steps like heat treatments can destroy heat-sensitive vitamins like B₁, B₆, B₁₂, and D₃ and therefore, should be applied carefully. Further health benefits can be attained from probiotics used during fermentation. As aforementioned, probiotic strains grow successfully in PBDSs enhancing functional properties of the product. Probiotics are believed to suppress potentially harmful organism in the intestine, stimulate immune system, and prevent cancer (Kreis *et al.*, 2008). PBDSs and related products meet the increasing demand for healthy food and beverages and satisfy diets such as vegetarianism and veganism (Corbo *et al.*, 2014). They are free of lactose and cow's milk proteins, but a lot of products are based on soya and peanut which are allergens also. Since 14% of the individuals who suffer from cow's milk allergy also have reactions against soya (Zeiger *et al.* 1999), other PBMSs should be considered to exclude more allergens and include more consumers. The avoidance of allergens is increasing and part of a healthy lifestyle for many people. An increasing number of consumers seek consciously for products which are gluten-free, nut-free or even completely allergen-free (Theodore, 2015).

2.11 Consumer acceptance and sensory characteristics

Consumers expect plant products that replace animal product to be similar according to the law of similarity (Adise *et al.*, 2015). However, a good approach from industry and consumers would be to appreciate the taste of the plant ingredients. Would a costumer not expect to taste quinoa in a quinoa-BMS, especially considering the high value and price of quinoa? Conflicting with this, simply the visual similarity of a plant-based animal product substitute and labelling it as such is increasing primarily the willingness of purchase, but if the sensory properties do not match the expectations, then the consumer might be disappointed and dislike the product even more (Zellner *et al.*, 2004). Also, an important point in this regard is a phenomenon called neophobia; Novelty of a product can evoke prejudices, simply because consumers are not familiar with the product. They expect the taste to be unpleasant and sometimes even assume the food to be dangerous (Adise *et al.*, 2015). Dealing with these problems, the advertisement of such plant food alternatives is a key point. In an internet survey, 48% stated that they would purchase PBMSs because of its good taste (Mintel Group Ltd, 2016). However, sensory evaluations by Mäkinen *et al.* (2014), including lactose free bovine milk, soya-, oat-, quinoa- and rice-BMSs, showed hedonic ratings were the highest for bovine milk, and slightly lower likings were obtained for the PBMSs, besides the quinoa-based one, which was described as “dislike moderately”. Furthermore, the panellists were asked about their future intention of consuming more PBMSs; 86% of the panellists stated the need to improve the taste of PBMSs, and 73% would consume more if the products had proven health benefits. Proof of environmentally friendliness was considered to be an argument of purchase by 43% (Mäkinen *et al.*, 2014). Since PBMSs are more or less suspensions of disintegrated plant material in water, the products can have a chalky mouth feel due to large insoluble particles (Durand *et al.*, 2003), which can be removed by homogenisation or filtration. Many legume-BMSs are perceived as “beany” and “painty”, which occurs as a result of lipoxygenase activity, or possess of compounds with off-flavours. Quinoa, for example contains saponins, which cause bitterness and cause other disadvantages like decreased protein bioavailability (Pineli *et al.*, 2015). By applying certain processing steps, like blanching, or soaking and maceration in acidified saline solution, enzymes can be easily inactivated (Yuan *et al.*, 2008; El-Shenawy *et al.*, 2016), or undesired compounds can be removed (Pineli *et al.*, 2015).

In several studies fermentation proved to be a useful tool to control texture, and especially, a desired flavour, as indicated earlier. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are commonly used as starter cultures in yoghurt production, which provide a huge range of flavour compounds; Lactic acid itself is one of the most important components contributing to yoghurt flavour. Together with formic, butanoic, and propanoic acids and especially the group of carbonyl compounds like acetaldehyde, acetone, acetoin, and diacetyl, it belongs to the most important aromatic components (Routray and Mishra, 2011). A screening of different strains applied to an oat-based beverage produced by Mårtensson *et al.* (2000) resulted in low consumer acceptance if fermented with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, while samples fermented with *Leuconostoc mesenteroides* achieved pleasant flavours and a good taste in the oat-BMS. Several aroma compounds were identified in cereal- and soya-based yoghurt-like beverages which were fermented with different strains including *Lactobacillus plantarum*, *Lactobacillus rossiae*, *Weissella cibaria* and *Pediococcus pentosaceus*. Alcohols and aldehydes were the most abundant ones amongst other kind of aroma compounds. Products were not rated hedonically, but a characterization of sensory attributes was performed. Most of them were described as fruity, sour, and with cereal attributes but with little to no dairy notes (Coda *et al.*, 2012). Mårtensson *et al.* (2002) did some further work on oat-based yoghurt-like product fermented with *Pediococcus damnosus*. They succeeded to produce a product that could not be differentiated from a dairy equivalent by a sensory preference test.

Considering the cheese-like products, Ahmad *et al.* (2008) reviewed the flavour improvement of a soya-based cheese substitute and found that fermentation leads to better acceptability since cheese flavours are mainly produced by LAB with cultures like *Lactococcus lactis* ssp. *lactis*, *Lactobacillus helveticus*, *Lactobacillus casei*, *Streptococcus lactis* ssp. *maltigenes* and *Lactococcus lactis* ssp. *cremoris*. However, the flavour in dairy cheese is a complex system of several compounds, and especially ripened cheese has an intense flavour resulting from the breakdown of casein proteins and milk fat into amino acids, amines, and fatty acids (Ahmad *et al.*, 2008). Most studies deal with cream cheese analogues and to the author's knowledge no study exist exploring the sensory attributes of a ripened cheese analogue in detail. Soya-based products fermented by *Lactobacillus casei* ssp. *ramnosus* showed

cream cheese compatible flavour and texture, containing diacetyl as a flavour component (Hofmann and Marshall, 1985).

2.12 Future of plant-based dairy substitutes and thriving trends

The market of PBDSs is growing rapidly and is becoming more and more popular all over the world. Figure 2-3 summarizes the different influences and mechanisms that determine the variety of products available on the market. The European market is still in its infancy; however it is believed to grow during the next 5 years by nearly 50%. While soya is still the leading crop, other plants are trending and the variety of PBDSs is growing (Mintel Group Ltd, 2016). In the period of 2014-2015, more products based on other ingredients than soya were launched. Consumers are opting for a variety of flavours and are getting more willing to experience new products. Soya has some disadvantages: first and foremost, it is among one of the most common food allergens. By using allergens, a big part of consumers is excluded. In total, a fifth of the population is affected by allergic diseases (including asthma), and more than a fifth of U.S. consumers is avoiding nut-/peanut-containing foods (Theodore, 2015). The avoidance of allergens is increasing, even for consumers who are not directly suffering from it. Therefore, the use of allergens will discourage more and more consumers from purchasing such products. Furthermore, soya beans are more than likely to be genetically modified: In the US, 94% of the soya varieties are genetically engineered (United States Department of Agriculture, 2016). Consumers are not well educated about this topic but 16% of PBDS-consumers are avoiding ingredients that are genetically modified (Mintel Group Ltd, 2016). Moreover, the environmental impact of soya and almonds is alarming as discussed in the chapter pertaining to climate impact. For all those reasons, the market is expected to change. This can be observed already in the “new” European market: rice/grain/nut/seed-based drinks grew by 380% from 2012 to 2015, whereas soy-based drinks “just” doubled their market in Germany. New plants already arose in or from these markets recently, like lupine or tiger nut. Tiger nuts are not real nuts but tubers and traditionally used in Spanish Horchata. It gained popularity in the U.S., possibly since it is fitting into the meal plan of the trending paleo-diet (Theodore, 2015). Lupine is a new plant to be explored as an ingredient in PBDSs. It shows promising qualities due to its high protein, dietary fibre, and antioxidants contents (Sujak *et al.*, 2006). It is part of the European project

PROTEIN2FOOD and is incorporated already in many products, including milk-, ice cream- and yoghurt-like foods by a German start-up company (Theodore, 2015).

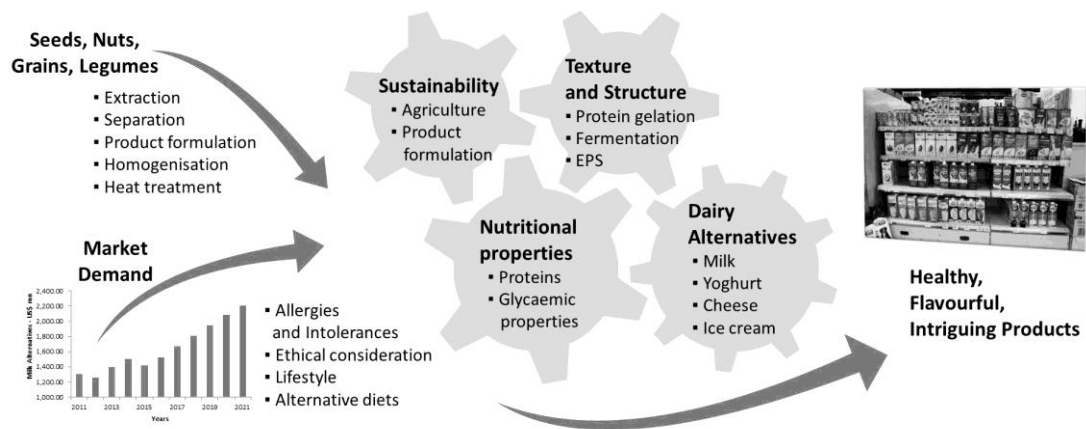


Figure 2-3 Influences and mechanisms that determine the variety of PBDSs on the market.

These new products are just examples, which are starting the new driving, innovative change in the market. Consumers are going to look out for more than just milk substitutes. They opt for healthy functional foods, which benefit their overall wellbeing. Indeed, the dairy-market is going to be more of a competing market for the non-dairy products, since most non-dairy milk drinkers also drink dairy milk (Mintel Group Ltd, 2016). Another trend, observed in the dairy industry may spill over to the non-dairy market also: Dairy products, like yoghurt are getting popular as a healthy option to conventional snacks (van den Bos, 2016). The demand for food is expected to grow by 70% until 2050 (from 2006 on). Due to urbanization, people change their diets to increased calorie and especially protein intake. While the expected protein consumption is believed to grow by 80% until 2050, the source of protein needs to change from being primarily consumed from resource intensive foods, like meat and dairy products to plant-based proteins (Ranganathan *et al.*, 2016). Forty-two % of consumers are already consuming PBMSs as a source of protein and 30% would be encouraged to drink more PBMS if the products contain more protein (Mintel Group Ltd, 2016). Therefore, the protein content is supposed to be one of the driving factors for the development of new products being thriving on the market. On the other hand, the lack of protein in some of the non-dairy milks, like rice- and almond-BMSs could be a limiting factor for their future success.

2.13 Conclusion

The market for PBDSs is growing fast and the demand is increasing. The presented work showed clearly that the product quality needs to improve to meet the consumers' aspiration in future times. Sustainability and nutritional quality will need to be further improved if PBDSs are substituting dairy products commercially successful. Keeping up with the competitive request of the market research is facing the challenge: further studies on functional properties, stability, and sensory acceptance are needed while keeping the cost and environmental impact in mind.

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***Chapter 3: Evaluation of physicochemical and glycaemic
properties of commercial plant-based milk substitutes***

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3.1 Abstract

The market for plant-based dairy-type products is growing as consumers replace bovine milk in their diet, for medical reasons or as a lifestyle choice. A screening of 17 different commercial plant-based milk substitutes based on different cereals, nuts and legumes was performed, including the evaluation of physicochemical and glycaemic properties. Half of the analysed samples had low or no protein contents ($<0.5\%$). Only samples based on soya showed considerable high protein contents, matching the value of cow's milk (3.7%). An *in vitro* method was used to predict the glycaemic index. In general, the glycaemic index values ranged from 47 for bovine milk to 64 (almond-based) and up to 100 for rice-based samples. Most of the plant-based milk substitutes were highly unstable with separation rates up to $54.39\%/h$. This study demonstrated that nutritional and physicochemical properties of plant-based milk substitutes are strongly dependent on the plant source, processing and fortification. Most products showed low nutritional qualities. Therefore, consumer awareness is important when plant-based milk substitutes are used as an alternative to cow's milk in the diet.

3.2 Introduction

Consumer demand for cow's milk alternatives arose as a result of people being intolerant to cow's milk, including lactose intolerance and cow's milk allergy. Nowadays, the avoidance of dairy products is additionally based on health concerns, like cholesterol and antibiotic residues in cow's milk. Further, it is part of different lifestyles including vegetarian and vegan diets or based on ethical considerations against the consumption of cow's milk. The market for non-dairy milks was growing by 9% in 2015 to reach \$1.9 billion (Mintel Group Ltd, 2016) with 138 different variants of plant-based milk substitutes (PBMSs) just in Europe (Mintel Group Ltd., 2015). Generally, PBMSs are extracts of plant material in water, which resemble cow's milk in appearance. Technologically, water is used to extract the plant material, followed by the liquid separation for the beverage production. Processing steps beforehand and afterwards like the addition of other ingredients, heat treatments or homogenisation can be applied to formulate the final product (Mäkinen *et al.*, 2016). Soya-BMS (based milk substitute) is the most common milk substitute. However, 14% of the individuals who suffer from cow's milk allergy also have reactions against soya (Zeiger *et al.*, 1999). Beside soya, other plant sources are used for developing non-dairy milk products, like oat, almond, coconut, rice and quinoa and their market share is increasing. Consumers are purchasing PBMSs for their health and wellness benefits (Mintel Group Ltd, 2016; Organic Monitor, 2005). Hence, health claims regarding vitamins, fibre, or cholesterol values are very common in this category. Sixty-nine % of Americans trust that non-dairy milks are nutritious for kids (Mintel Group Ltd, 2016). Nevertheless, some products have extremely low protein contents. Recently, Mäkinen *et al.* (2016) pointed out the risk of replacing cow's milk with PBMSs, especially for young children, and emphasised the importance of consumer awareness. However, the choice of raw material has a great impact on the product quality as well as the process technology adopted. Due to the fact that soya-BMSs are already successful on the market for more than 70 years, a lot of research and literature is available. A huge selection of different soya beans with specially designed processing characteristics or physical properties are available (Riaz, 2006). On the other hand there are more and more varieties of PBMSs available on the market but the quality of those products has not been yet fully investigated. Therefore, a screening of 17 different commercial milk substitutes, based on different cereals, nuts and legumes was performed. The aim of this study is

to give an overview on physicochemical and nutritional properties of different PBMSs.

3.3 *Materials and Methods*

3.3.1 *Samples*

Seventeen different commercial PBMSs based on different cereals, nuts and legumes were purchased on the market. Table 3-1 and Table 3-2 show the list of samples selected for this research with the labelled ingredients, and nutrients, respectively. Two different batches of each commercial sample were analysed. Bovine milk was used as a control. The samples were stored at 4 °C and used within 2-3 days of opening the packaging.

3.3.2 *Compositional analysis*

Total nitrogen content of samples was analysed using the Kjeldahl method (MEBAK 1.5.2.1). Nitrogen-to-protein conversion factors were selected according to WHO/FAO (2003) guidelines for plant ingredient; 5.18 for nut- and seed-BMSs, 5.83 for oat 5.95 for rice-, 5.71 for soya-BMSs and 6.38 for bovine milk. Moisture was determined by drying in an oven at 103 °C until constant mass was reached. Ash was analysed by incineration in a muffle furnace: Samples were pre-heated in crucibles for 1 h at 100 °C and ashed for 4 h at 600 °C. Fat content was determined by extracting total fat using Soxhlet technique with hot solvent and gravimetry. For sugar analysis, samples were filtered (0.25 µm) and diluted with water. Sugar profiles were analysed using an Infinity 1260 HPLC system equipped with a refractive index detector (Agilent Technologies, Palo Alto, CA) with a Hi-Plex H, 300 x 7.7 mm, 8 µm HPLC column (Agilent Technologies, Palo Alto, CA) at a flow rate of 1 mL/min of water. Sugar concentrations were determined using sucrose, maltose, glucose, fructose, lactose and galactose as external standards. For maltose and sucrose qualification samples were diluted with water/acetonitrile (75:25 v/v) and analysed with a Supelcosil LC-NH₂, 250 x 4.6 mm, 5µm HPLC column (Sigma-Aldrich) at a flow rate of 1 mL/min of water/acetonitrile (75:25 v/v) using the same HPLC system. Sum of sugars was determined as the sum of all sugars detected in one sample. Total starch was determined using the enzyme kit K-TSTA supplied by Megazyme, Ireland.

Table 3-1 List of selected commercial plant-based milk substitutes and their ingredients.

Brand	Name	Ingredients
The good little Cook alpro	Almond MLK	Water, 20% almonds
	Almond original	Water, sugar, almond (2%), tri-calcium phosphate, sea salt, stabilisers (locust bean gum, gellan gum), emulsifier (sunflower lecithin), vitamins B2, B12, E, D2)
Provamel	Organic almond drink	Water, almond (7%), sea salt
The good little Cook	Carob almond MLK	Water, almonds, carob, dates, maple syrup
Provamel	Organic cashew drink	Water, roasted cashew (6%), agave syrup (3.5%), sea salt
alpro	Coconut original	Water, coconut milk (5.3%) (coconut cream, water), rice (3.3%), tri-calcium phosphate, stabilisers (carrageenan, guar gum, Xanthan gum), sea salt, vitamins (B12, D2), flavourings
	Hazelnut original	Water, sugar, hazelnuts (2.5%), tri-calcium phosphate, sea salt, stabilisers (locust bean gum, gellan gum), emulsifier (sunflower lecithin), vitamins B2, B12, E, D2)
Braham & Murray	Hemp Milk Unsweetened	Water, hemp cream (3%), tri-calcium phosphate, emulsifier (sucrose ester), natural flavouring, stabiliser (xanthan gum), sea salt, stabiliser (gellan gum), vitamin D2
Provamel	Organic macadamia drink	Water, macadamia nuts (4%), agave syrup (3.5%), sea salt
Oatly	Organic oat drink	Oat base (water, oats 10%), sea salt

continued

Brand	Name	Ingredients
EcoMil	Quinoa drink	Water, quinoa (7%), agave syrup, corn maltodextrin, almond oil
Vitariz	Organic rice drink natural	Organic rice, water, organic sunflower oil, sea salt
Rude Health	Organic brown rice drink	Water, organic brown rice (14%), sunflower oil, sea salt.
Provamel	Organic soya drink, calcium	Water, hulled soya beans (7,2%), apple concentrate (3,3%), algue lithothamnium calcereum (0,4%), sea salt
Sojade	Plain UHT organic soya drink	Water, hulled organic soya beans (6.5%)
alpro	Soya organic, wholebean	Water, hulled soya beans (8%).
alpro	Soya original	Water, hulled soya beans (6%), sugar, acidity regulators (mono-potassium phosphate, di-potassium phosphate), calcium carbonate, flavouring, sea salt, stabiliser (gellan gum), vitamins (B2, B12, D2)
Clona Product Ltd.	Dairy Fresh milk, pasteurised & homogenised	Milk

Table 3-2 Nutritional values in 100 g of product as stated on the packaging of the selected products.

Brand	Name	Energy [kcal]	Protein [g]	Carbohy- drates [g]	Sugars [g]	Fat [g]	Saturated fat [g]
The good little Cook	Almond MLK	-	-	-	-	-	-
alpro	Almond original	24	0.5	3	3	1.1	0.1
Provamel	Organic almond drink	33	0.9	0.2	0.2	3	0.3
The good little Cook	Carob almond MLK	-	-	-	-	-	-
Provamel	Organic cashew drink	47	0.9	4.4	2.9	2.8	0.5
alpro	Coconut original	20	0.1	2.7	1.9	0.9	0.9
alpro	Hazelnut original	29	0.4	3.1	3.1	1.6	0.2
Braham & Murray	Hemp Milk Unsweetened	23	<0.1	<0.1	<0.1	2.7	0.3
Provamel	Organic macadamia drink	34	0.5	2.4	2.1	2.4	0.4
Oatly	Organic oat drink	35	1	6.5	4	0.5	0.1
EcoMil	Quinoa drink	46	1.5	3.7	2.5	2.8	0.7
Vitariz	Organic rice drink natural	64	0.7	10.5	9.5	1.9	0.2

continued

Brand	Name	Energy [kcal]	Protein [g]	Carbohy- drates [g]	Sugars [g]	Fat [g]	Saturated fat [g]
Rude Health	Organic brown rice drink	59	0.3	11	5	1.3	0.4
Provamel	Organic soya drink, calcium	188	3.7	2.4	2.4	2.1	0.4
Sojade	Plain UHT organic soya drink	154	3.8	0.45	0.45	2.1	0.3
alpro	Soya organic, wholebean	132	3.3	2.4	2.4	1.9	0.3
alpro	Soya original	167	3	2.8	2.7	1.8	0.3
Clona Dairy Product Ltd.	Fresh milk, pasteurised & homogenised	64	3.3	4.8	4.8	3.5	2.3

3.3.3 Glycaemic index and Glycaemic load

In vitro determination of the glycaemic index (GI) was evaluated according to Magaletta & DiCataldo (Magaletta and DiCataldo, 2009) using a calculation designed by an artificial neural network. An equivalent to 0.5 g of available carbohydrates (based on the results of sugar and starch analysis) was digested by a multi-enzyme preparation. The digestate was analysed for glucose, fructose, lactose and galactose with the HPLC described as above. These results, together with the results from the protein and fat content of the samples, were used to feed the calculation:

$$GI = 26.264529 - 1.048186 \cdot \text{Protein [\%]} - 0.248138 \cdot \text{Fat [\%]} \\ + 621.7824 \cdot \text{Glucose [\%]} - 52.7993 \cdot \text{Fructose [\%]} \\ - 233.67679 \cdot \text{Lactose [\%]} - 61.21071 \cdot \text{Galactose [\%]}$$

Glycaemic load (GL) was calculated according to Atkinson *et al.* (2008):

$$GL = (GI \cdot \text{available carbohydrate (g) per portion}) / 100$$

The portion size was set to 250 g.

3.3.4 Colour measurement

The colour values were measured using the CIE L*a*b* colour system and obtained using illuminant D65. The instrument used was a colorimeter (CR-400, Konica Minolta, Osaka, Japan). Colour of samples was characterised according to whiteness index (WI), defined as:

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

3.3.5 Rheology

The rheological behaviour of the products was characterised using a controlled stress rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a sensor system of coaxial cylinders (C-CC27-T200/SS, Anton Paar GmbH, Austria). The shear stress (σ) was measured as a function of shear rate ($\dot{\gamma}$) ranging from 0.5 to 100 s⁻¹ within 500 seconds. The power law model was fitted to the experimental points to determine the flow behaviour index (n):

$$\sigma = K(\dot{\gamma})^{n-1}$$

The measurements were carried out at 10 °C. The apparent viscosity measured at 10 s⁻¹ is referred to as “viscosity”.

3.3.6 Suspension stability

Stability was determined through phase separation analysis using an analytical centrifuge (LUMiSizer; LUM GmbH, Berlin, Germany). The instrumental parameters used were as follow: 1000 rpm for 30 min followed by 3000 rpm for 60 min at 24 °C. Height of sediment and creaming layer in mm, and separation rate in %/h were determined.

3.3.7 Particle size distribution

Analyses of particle size distribution were carried out using a static laser light diffraction unit (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK). Samples were applied to the instrument with ultrapure water as dispersion medium at 2,800 rpm until an obscuration rate of 5% was obtained. The refractive index was determined using a hand-held refractometer (Atago R5000, Atago, Tokyo, Japan).

3.3.8 Statistical analysis

All analyses were carried out at least in triplicate. Means were compared using one-way analysis of variance (ANOVA) and Tukey’s post hoc-test using Minitab release 16 (Minitab Inc. State College, Pa., USA). The level of significance was determined at $p < 0.05$. Linear correlation measurements of results were performed using Pearson’s correlation.

3.4 Results and discussion

3.4.1 Composition and glycaemic response

The compositional data of the samples is given in Table 3-3 and 3-4. Protein contents ranged from 0.07% for brown rice-BMS to 3.70% for bovine milk and the soya-BMS from Sojade. Half of the analysed samples had low or no protein contents (<0.5%). Only samples based on soya showed considerable high protein contents. Compared to the labelled values most of the measured values coincide but two, which differed greatly. The measured values of the quinoa- and Provamel soya-based samples were 85 and 26% lower than the labelled values. However, this is still within the discrepancy range, set by the European Commission (European Commission, 2012). In addition to the low protein content in most of the PBMSs, plant proteins have a lower protein quality in terms of digestibility compared to animal derived proteins; Cow's milk protein has a protein digestibility-corrected amino acid score of 121, whereas literature values for the plant proteins are all lower: soya, cashew, quinoa, rice, hemp, oat and almond have values of 91, 90, 67, 56, 49-53, 41-51, and 23, respectively (Schaafsma, 2000; Freitas, 2012; Ruales *et al.*, 2002; Eggum *et al.*, 1993; House *et al.*, 2010; Pedó *et al.*, 1999; Ahrens *et al.*, 2005). Hence, if these products are consumed to replace cow's milk in the diet and used as a protein source, this can cause a protein deficit and severe illnesses. Especially for young infants, an appropriate healthy diet tailored to their requirements is important since about 40% of the protein is needed for growth. Adults do need protein for maintenance, therefore their protein requirements in g/kg/d is lower. Currently, the protein requirement stated by the recent FAO report is 0.66 g of protein /kg/d for adults and 1.12 g of protein /kg/d for 0.5 year old infants (FAO, 2011a). Le Louer *et al.* (2014) reported severe malnutrition of young infants with inappropriate PBMS consumption. The children suffered from various diseases, like protein-calorie malnutrition and deficiencies of minerals and vitamins, with severe consequences. Fat content, shown in Table 3-3, was high for bovine milk at 3.28%. Almond-based samples from The good little Cook and Provamel as well as the carob/almond-BMS exceeded this level, while samples based on coconut, oat, rice and brown rice contained <1% fat. Besides the quantity of fat, the quality is of interest; Bovine milk is high in saturated fatty acids with about 2.5 g/100 g, whereas plants have generally a low content (Food Standards Agency, 2002).

Table 3-3 Composition in g/100 g and whiteness index [-] of plant-based milk substitutes and bovine milk.

Brand	Name	Protein	Fat	Ash	Starch	Whiteness Index
The good little Cook	Almond MLK	2.11±0.09 ^d	4.40±0.11 ^a	0.35±0.04 ^g	0.06±0.00 ^g	68.36±1.58 ^{efgh}
alpro	Almond original	0.41±0.02 ^{fg}	1.18±0.05 ^{fgh}	0.55±0.00 ^{cd}	0.06±0.00 ^g	72.57±0.09 ^{cd}
Provamel	Organic almond drink	0.95±0.39 ^e	3.69±0.11 ^{ab}	0.21±0.02 ^{ij}	0.07±0.01 ^{fg}	75.95±0.66 ^b
The good little Cook	Carob almond MLK	2.4±0.24 ^{cd}	3.35±1.73 ^{abc}	0.36±0.00 ^{ef}	0.07±0.01 ^g	51.57±0.18 ^l
Provamel	Organic cashew drink	0.87±0.10 ^e	2.50±0.10 ^{cde}	0.23±0.01 ^{hij}	0.73±0.07 ^c	65.57±0.52 ^{hi}
alpro	Coconut original	0.08±0.00 ^g	0.84±0.00 ^{gh}	0.52±0.01 ^d	0.13±0.02 ^{fg}	67.75±2.70 ^{fgh}
alpro	Hazelnut original	0.36±0.00 ^{fg}	1.56±0.05 ^{defg}	0.52±0.00 ^d	0.04±0.00 ^g	56.31±0.07 ^k
Braham & Murray	Hemp Milk Unsweetened	0.08±0.04 ^g	2.44±0.23 ^{cde}	0.42±0.01 ^e	0.05±0.01 ^g	68.49±0.02 ^{efgh}
Provamel	Organic macadamia drink	0.29±0.01 ^g	2.62±0.06 ^{bcd}	0.25±0.01 ^{hi}	0.21±0.11 ^{efg}	51.73±1.18 ^l
Oatly	Organic oat drink	0.70±0.19 ^{ef}	0.38±0.06 ^h	0.2±0.00 ^{ijk}	2.00±0.20 ^a	60.21±4.46 ^j

continued

Brand	Name	Protein	Fat	Ash	Starch	Whiteness Index
EcoMil	Quinoa drink	0.22±0.04 ^g	2.32±0.13 ^{cde}	0.17±0.01 ^{jk}	0.19±0.05 ^{efg}	71.35±0.20 ^{cde}
Vitariz	Organic rice drink natural	0.32±0.04 ^{fg}	0.85±0.06 ^{gh}	0.14±0.02 ^{kl}	0.54±0.04 ^d	66.49±3.94 ^{ghi}
Rude Health	Organic brown rice drink	0.07±0.00 ^g	0.95±0.04 ^{gh}	0.10±0.01 ^l	1.17±0.08 ^b	63.47±3.91 ⁱ
Provamel	Organic soya drink, calcium	2.72±0.06 ^c	2.11±0.04 ^{def}	0.61±0.04 ^{bc}	0.10±0.07 ^{fg}	70.34±0.04 ^{def}
Sojade	Plain UHT organic soya drink	3.70±0.03 ^a	2.04±0.11 ^{def}	0.34±0.02 ^{fg}	0.11±0.03 ^{fg}	74.49±0.01 ^{bc}
alpro	Soya organic, wholebean	3.16±0.32 ^b	1.77±0.06 ^{defg}	0.29±0.00 ^{gh}	0.10±0.01 ^{fg}	69.27±0.69 ^{efg}
alpro	Soya original	2.61±0.13 ^c	1.48±0.05 ^{efg}	0.99±0.08 ^a	0.08±0.01 ^{fg}	74.56±0.10 ^{bc}
Clona Dairy Product Ltd.	Fresh milk, pasteurised & homogenised	3.70±0.14 ^a	3.28±0.05 ^{bc}	0.62±0.01 ^b	0.06±0.01 ^g	81.89±0.01 ^a

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$).

Furthermore, the hemp-based sample claims to be a source of Omega-3 and 6. The soya-based product from alpro contained the highest ash content at 0.99%, as it contains added calcium. The other samples had lower values than bovine milk with 0.62% (Table 3-3). Bovine milk is an important food source of several minerals e.g. calcium, potassium, magnesium and iodine (FAO, 2013). PBMSs are often fortified with minerals to prevent deficiencies compared to cow's milk; seven of the samples contain calcium salts. On the other hand, pollution, like high arsenic levels in rice is a well-known problem, A study by Meharg *et al.* (2008) revealed that 19 out of 19 rice-BMSs exceeded the inorganic arsenic EU and US limits for drinking water standards.

The starch content for all the PBMSs was low and did not significantly differ among the samples evaluated (Table 3-3) with the exception of cashew-BMS and the cereal-BMSs, including oat, rice and brown rice where the starch detected was at 0.73, 2.00, 0.54 and 1.17%, respectively. Some of the PBMSs contain added sugars or sweeteners, which contributed to their main sugar and resulted in comparatively high total sugar contents. Samples based on cashew, macadamia, and quinoa were sweetened with agave syrup, the organic soya drink from Provamel contained apple concentrate. Both sweeteners are high in fructose, resulting in high fructose levels for those PBMSs (< 1.27%). Samples high in sucrose were sweetened with sucrose (Almond original, Hazelnut original, Soya original from alpro) or maple syrup (carob/almond-BMS) with high values above 2.88%. Another source of sugar is the starch hydrolysis step during processing. Products containing ingredients high in starch are naturally high in maltose and, or glucose. The oat-BMS contained high amounts of maltose at 3.34%, but the rice-PBMSs were high in both, maltose and glucose, and resulted in total amounts of sugar at 7.02 and 5.58% respectively. Bovine milk contained 3.33% lactose and 0.05% galactose only, whereas none of the BPMSs contained lactose or galactose.

Carbohydrates are digested and absorbed in the blood as glucose to provide energy. The blood glucose level affects the human metabolism greatly and is strictly controlled by peptide hormones like glucagon and insulin (Litwack, 2008). A way to quantify the effect of food on the blood sugar level is the GI; it represents the post-prandial uptake of glucose into the blood compared to a reference (Bell and Sears,

2003). The type of carbohydrate is the main factor accounting for the glycaemic response since all the carbohydrates follow different metabolic pathways to be transformed to glucose and enter the blood (Litwack, 2008). The glucose concentration correlated with the *in vitro* GI (0.80, $p < 0.001$). Hence, samples containing mainly glucose such as coconut- and rice-BMSs had a high GI (>96). The samples also contained maltose, sucrose, fructose and lactose. These sugars have a GI by itself of 105, 61, 19 and 46, respectively (Foster-Powell *et al.*, 2002). Generally, the sugar type governs the value of GI. Just the oat-BMS was an exception in this study. Even though it contained mainly maltose it resulted in a moderate GI of 59. This can be explained by the β -glucan content in oats, which is known to reduce the GI (Englyst *et al.*, 2003). The GI can be classified into three categories: values ≤ 55 are defined as low, 56–69 as medium, foods having a GI ≥ 70 are defined as high (Atkinson *et al.*, 2008). Bovine milk and 8 samples including the products based on almond from Provamel, carob/almond, cashew, macadamia, quinoa and soya (from Provamel, Sojade and alpro (wholegrain)) had low GI values. Five samples had medium GI values and both of the rice-based products as well as the coconut-BMS resulted in a high GI greater than 97 (Table 3-4). Literature values are not readily available for PBMSs, but values for soya-, rice- and quinoa-BMSs were found (Atkinson *et al.*, 2008; Foster-Powell *et al.*, 2002; Pineli *et al.*, 2015) and are in accordance with the values in this work. As recommended by the American Diabetes Association (American Diabetes Association, 2002) it is not the source or type of carbohydrates is decisive for a healthy diet, but the total amount, which effects the GI in foods. A good tool to measure this is the GL, indicating the effect of one food serving on the blood glucose level after consumption. The rice-BMSs, which had a high GI showed as well a high GL value (18.33 and 16.85) since they contained a lot of carbohydrates. These values are comparable to Coca-Cola or cakes (Foster-Powell *et al.*, 2002). However, considering the GL value, the rest of the samples showed low to moderate values. Bell and Sears (2003) reviewed the impact of GL on the human health. They came to the conclusion that a low GL diet reduces the risk for e.g. cardiovascular disease, obesity and diabetes. Therefore, attention should be brought to this value and some of these milk substitutes cannot be perceived as healthy, but should be handled as a treat.

Table 3-4 Sugar compositions (except lactose and galactose, stated in the text) in g/100 g, and glycaemic properties [-] of plant-based milk substitutes and bovine milk.

Brand	Name	Glucose	Fructose	Sucrose	Maltose	Sum of sugars	Glycaemic index	Glycaemic load
The good little Cook	Almond MLK	0.06±0.00 ^e	n.d.	0.52±0.02 ^d	n.d.	0.58±0.02 ⁱ	58.68±3.61 ^b _{cde}	0.94±0.05 ^g
alpro	Almond original	0.22±0.00 ^d _e	0.06±0.01 ^{fg}	3.42±0.03 ^a	n.d.	3.69±0.05 ^d _e	49.10±2.53 ^{ef}	4.60±0.30 ^e
Provamel	Organic almond drink	n.d.	n.d.	0.16±0.01 ^{gh}	n.d.	0.16±0.01 ⁱ	64.21±1.96 ^b	0.37±0.00 ^g
The good little Cook	Carob almond MLK	0.87±0.02 ^c	0.61±0.00 ^e	3.10±0.14 ^b	n.d.	4.58±0.16 ^c	54.33±1.10 ^b _{cdef}	6.32±0.25 ^d
Provamel	Organic cashew drink	0.49±0.03 ^c _{de}	1.96±0.06 ^c	.43±0.01 ^{de}	n.d.	2.87±0.09 ^{fg}	52.82±3.77 ^c _{def}	4.76±0.28 ^e
alpro	Coconut original	0.81±0.00 ^c _d	n.d.	n.d.	1.05±0.11 ^d	1.86±0.11 ^b	96.82±5.05 ^a	4.81±0.01 ^e
alpro	Hazelnut original	n.d.	n.d.	3.09±0.01 ^b	n.d.	3.09±0.01 ^{ef} _g	55.76±0.24 ^b _{cdef}	4.37±0.02 ^e
Braham & Murray	Hemp Milk Unsweetened	0.02±0.03 ^e	0.01±0.02 ^g	0.05±0.01 ^h	n.d.	0.09±0.04 ⁱ	59.94±1.28 ^b _c	0.21±0.04 ^g
Provamel	Organic macadamia drink	0.30±0.03 ^c _{de}	2.23±0.11 ^a _b	0.26±0.03 ^{fg}	n.d.	2.79±0.12 ^g _{ef}	49.47±0.57 ^d _{ef}	3.71±0.18 ^{ef}
Oatly	Organic oat drink	0.01±0.00 ^e	n.d.	n.d.	3.34±0.17 ^a	3.35±0.18 ^{ef} _{cd}	59.61±5.44 ^b	7.98±0.71 ^c

continued

Brand	Name	Glucose	Fructose	Sucrose	Maltose	Sum of sugars	Glycaemic index	Glycaemic load	Glycaemic index
EcoMil	Quinoa drink	0.43±0.03 ^c _{de}	2.34±0.13 ^a	n.d.	0.43±0.11 ^e	3.2±0.27 ^{ef} _g	53.28±0.70 ^{cd} _{ef}	4.51±0.24 ^e	53.28±0.70 ^{cde} _f
Vitariz	Organic rice drink natural	4.12±0.79 ^a	0.07±0.01 ^{fg}	n.d.	2.83±0.29 ^b	7.02±1.09 _a	97.74±6.81 ^a	18.33±1.28 ^a	97.74±6.81 ^a
Rude Health	Organic brown rice drink	3.07±0.06 _b	0.10±0.02 ^{fg}	n.d.	2.41±0.02 ^c	5.58±0.03 _b	99.96±5.75 ^a	16.85±1.02 ^b	99.96±5.75 ^a
Provamel	Organic soya drink, calcium	0.50±0.08 ^c _{de}	1.27±0.02 ^d	n.d.	0.66±0.02 ^e	2.43±0.05 _{efg}	47.53±4.07 ^f	3.01±0.23 ^f	47.53±4.07 ^f
Sojade	Plain UHT organic soya drink	0.52±0.58 ^c _{de}	n.d.	0.36±0.01 ^{ef}	n.d.	0.88±0.57 _i	54.02±8.76 ^{bc} _{def}	1.24±0.51 ^g	54.02±8.76 ^{bc} _{def}
alpro	Soya organic, wholebean	0.01±0.00 ^e	n.d.	0.35±0.02 ^{ef}	n.d.	0.36±0.02 _i	49.49±2.75 ^{def}	0.57±0.01 ^g	49.49±2.75 ^{def}
alpro	Soya original	0.15±0.05 ^e	0.06±0.02 ^{fg}	2.88±0.07 ^c	n.d.	3.09±0.11 _{efg}	61.50±3.75 ^{bc}	4.87±0.14 ^e	61.50±3.75 ^{bc}
Clona Dairy Product Ltd.	Fresh milk, pasteurised & homogenised	n.d.	n.d.	n.d.	n.d.	3.38±0.04 _{ef*}	46.93±0.53 ^f	4.03±0.02 ^e	46.93±0.53 ^f

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$). * Cow's milk consisted of 3.33% lactose and 0.05% galactose, none of the PBMSs contained lactose or galactose. n.d. refers to not detectable

Additionally, bovine milk is an important food source for vitamin A, D, B₁₂ and riboflavin (FAO, 2013). These results and other research showed, avoiding dairy products is resulting in a nutrient deficiency and does generally not result in a nutritionally equivalent diet (Fulgoni *et al.*, 2011; Millward and Garnett, 2010).

3.4.2 Physicochemical properties

Whiteness index

The WI is given in Table 3-3. The colour of food is one of the first properties observed by consumers, influencing choice and preference. The WI is one of the most important quality parameters for milk (Cadwallader, 2010). The WI for bovine milk is the highest with 81.89 and all PBMSs appeared darker ($p < 0.05$). Raw material and processing steps are influencing WI; however all samples appeared more or less dark and brown to yellow. The WI ranged from 52 for carob/almond- and macadamia-BMSs up to 75 for soya- (-original, alpro) BMS, which made all of the samples easily distinguishable from cow's milk.

Rheological behaviour

The apparent viscosity of bovine milk was low (3.15 mPa·s) and no significant differences could be found to most of the PBMSs (Table 3.-5). Products based on coconut and almond from Provamel had a viscosity of 47.80 and 26.23 mPa·s and showed pseudoplastic behaviour with a flow index of 0.40 and 0.56. Almond- (alpro), quinoa-, hazelnut-, hemp- and soya- (-original, alpro) based samples showed as well higher viscosities than bovine milk and pseudoplastic behaviour. All of them contained hydrocolloids like locust bean gum, carrageenan or xanthan gum (Table 3-1). Hydrocolloids increase the viscosity and have an impact on the flow behaviour (Saha and Bhattacharya, 2010). Only the soya- (original, alpro) based sample had a considerably low viscosity and newtonian flow behaviour, even though it contained gellan gum.

Particle size distribution

Considering the particle size measurements, volume mean diameters ($d_{4,3}$) showed no significant differences for all samples with low values varying from 0.60 to 10.51 μm , but quinoa- and cashew-BMSs, with values of 81.47 and 29.17 μm respectively. Considering the mean diameters, it should be noted that the $d_{4,3}$ is

sensitive to the presence of larger particles, and the $d_{3,2}$ parameter to smaller particles. Evaluating the $d_{3,2}$ mean diameter for the quinoa- and cashew-BMSs, it is evident that just a small amount of big particles was present in these samples, since they did not have the highest values. PBMSs are produced by disintegration of plant materials, which means that particle composition and size is not as uniform as in bovine milk, which had significantly the lowest $d_{3,2}$ and $d_{4,3}$ values (0.36 and 0.60 μm). The samples which were similar to bovine milk, with low $d_{4,3}$ (≤ 3.43 μm) values showed a monomodal particle size distribution. Only almond- (Provamel), cashew-, oat-, quinoa- and rice-BMSs showed a polydisperse distribution. Chu *et al.* (1996) found that polydispersity of particles in colloidal dispersions leads to destabilisation of the system. Indeed, it was found that the polydisperse samples had generally high separation rates (>27 %/h).

Suspension stability

Numerical data describing the stability of the beverages are presented in Table 3-5. Bovine milk was the most stable one with a separation rate of 3.87 %/h. Samples based on almond (alpro), macadamia, hemp, hazelnut and soya (Sojade and Provamel) showed considerable stabilities with values <10 %/h. Whereas the rest of the samples were unstable and some separated rapidly including almond- (The good little Cook), carob/almond-, macadamia- and brown rice-BMSs with values >50 %/h. The markedness and velocity of sedimentation or creaming depends also on the viscosity of the continuous phase and the density difference between particles and the continuous phase (Dickinson and Miller, 2001). The viscosity was high for samples containing stabilisers. Stabilisers improve the stability by simply increasing the viscosity of the continuous phase. Most of the samples containing this kind of additives (almond- (alpro), hazelnut- and hemp-BMSs) indeed showed a good stability. Denser particles sediment while the lighter ones cream on top of the liquid. Only the oat-BMS had a considerable creaming layer (2.48 mm). This sample showed as well a thick sedimentation layer (3.51 mm). Together with samples based on carob/almond and cashew, it was also the only sample, which had a significantly thicker sediment layer than bovine milk.

Table 3-5 Physicochemical properties of plant-based milk substitutes and bovine milk.

Brand	Name	Separation rate [%/h]	Sediment [mm]	Creaming [mm]	Viscosity [mPa·s]	Flow index [-]	d _{3,2} [μm]	d _{4,3} [μm]
The good little Cook	Almond MLK	52.42±2.48 ^a	0.00±0.00 ^c	0.68±0.21 ^{bc}	4.63±0.98 ^{gh}	0.82±0.11 ^{cde}	2.36±0.17 ^a	0.90±0.02 ^c
alpro	Almond original	1.35±0.55 ^k	0.00±0.00 ^c	0.00±0.00 ^c	19.08±1.98 ^{ef}	0.70±0.04 ^{efg}	1.10±0.01 ^{fg}	1.84±0.11 ^c
Provamel	Organic almond drink	30.17±4.97 ^{fgh}	1.72±0.76 ^{abc}	1.16±0.60 ^b	26.32±7.03 ^b	0.56±0.21 ^g	2.09±0.27 ^b	5.96±1.84 ^c
The good little Cook	Carob almond MLK	51.7±1.83 ^a	3.93±1.79 ^a	0.81±0.28 ^{bc}	3.87±0.85 ^h	0.98±0.04 ^{ab}	1.48±0.04 ^{de}	2.59±0.20 ^c
Provamel	Organic cashew drink	27.46±8.31 ^{gh}	4.22±2.91 ^a	0.98±0.18 ^{bc}	5.57±0.80 ^{gh}	0.97±0.03 ^{ab}	2.30±0.49 ^a	29.17±24.65 ^b
alpro	Coconut original	37.43±1.06 ^{def}	2.60±0.31 ^{abc}	0.60±0.01 ^{bc}	47.80±2.16 ^a	0.40±0.03 ^h	1.34±0.01 ^e	1.72±0.13 ^c
alpro	Hazelnut original	1.27±0.26 ^k	0.00±0.00 ^c	0.00±0.00 ^c	24.80±1.45 ^{bcd}	0.67±0.04 ^{fg}	1.52±0.07 ^d	2.21±0.11 ^c
Braham & Murray	Hemp Milk Unsweetened	4.44±3.98 ^{jk}	0.26±0.30 ^c	0.00±0.00 ^c	25.00±7.27 ^{bc}	0.73±0.05 ^{ef}	1.06±0.16 ^{fg}	1.51±0.09 ^c
Provamel	Organic macadamia drink	54.39±3.91 ^a	0.29±0.07 ^c	0.97±0.03 ^{bc}	2.22±0.31 ^h	1.01±0.03 ^a	1.77±0.02 ^c	3.43±0.08 ^c
Oatly	Organic oat drink	40.13±3.13 ^{cde}	3.51±0.60 ^{ab}	2.48±1.05 ^a	6.77±1.03 ^{gh}	0.89±0.02 ^{abcd}	1.7±0.07 ^c	3.83±0.53 ^c
EcoMil	Quinoa drink	32.01±5.55 ^{efg}	1.08±0.10 ^{bc}	0.64±0.19 ^{bc}	13.20±2.20 ^{ef}	0.76±0.07 ^{def}	1.12±0.02 ^f	81.47±39.81 ^a

continued

Brand	Name	Separation rate [%/h]	Sediment [mm]	Creaming [mm]	Viscosity [mPa·s]	Flow index [-]	d _{3,2} [μm]	d _{4,3} [μm]
Vitariz	Organic rice drink natural	42.83±1.23 ^b cd	0.36±0.23 ^c	0.74±0.16 ^{bc}	2.77±0.06 ^h	0.97±0.03 ^{ab}	0.88±0.09 ^{hi}	10.51±13.20 ^c
Rude Health	Organic brown rice drink	50.86±0.44 ^a b	2.15±0.02 ^{abc}	0.64±0.06 ^{bc}	2.21±0.02 ^h	1.02±0.01 ^a	0.63±0.00 ^j	0.72±0.05 ^c
Provamel	Organic soya drink, calcium	11.33±3.22 ^{ij}	2.65±2.40 ^{abc}	0.65±0.76 ^{bc}	7.58±4.84 ^{fgh}	0.91±0.10 ^{abc}	0.94±0.07 ^{ghi}	1.28±0.13 ^c
Sojade	Plain UHT organic soya drink	8.61±1.41 ^{ijk}	1.15±0.25 ^{bc}	0.00±0.00 ^c	3.49±0.10 ^h	1.01±0.02 ^a	0.80±0.00 ⁱ	0.99±0.01 ^c
alpro	Soya organic, wholebean	13.27±0.78 ⁱ	0.20±0.19 ^c	0.00±0.00 ^c	2.57±0.04 ^h	1.00±0.02 ^a	0.85±0.02 ⁱ	1.01±0.06 ^c
alpro	Soya original	22.56±3.88 ^h	0.60±0.37 ^c	0.91±0.45 ^{bc}	5.98±0.22 ^{gh}	0.92±0.01 ^{abc}	0.94±0.00 ^{ghi}	1.22±0.01 ^c
Clona Dairy Pr-oduct Ltd.	Fresh milk, pasteurised & homogenised	3.87±0.17 ^{jk}	0.60±0.05 ^c	0.70±0.27 ^{bc}	3.15±0.01 ^h	1.01±0.02 ^a	0.36±0.03 ^k	0.60±0.02 ^c

Values within a column that share a superscript are not significantly different from one another (p<0.05).

3.5 Conclusion

This study showed that PBMSs differ remarkably in nutritional and physicochemical properties. Depending on the raw material, some had very low protein contents and high glycaemic values. If these products are portrayed as cow's milk substitutes, the nutritional inferiority can cause severe illnesses. To the authors' knowledge, this paper presents the first assessment of many PBMSs, taking several nutritional values into account. Especially the determination of GI values gave new insights to evaluate the nutritional importance. Moreover, stability and rheology properties were poor and only products based on soya showed good performances without containing hydrocolloids. PBMSs have a reputation to be healthy and nutritionally valid (Organic Monitor, 2005; Mintel Group Ltd, 2016), but this study unveiled that most products lack in nutritional quality. Only soya-BMSs showed overall good results, comparable to cow's milk. Manufactures need to improve these, e.g. by choosing adequate raw materials as well as tailored and consumer-friendly processing technologies (i.e. the application of enzymes and/or fermentation technology), rather than adding low-cost fortifiers and additives like sweeteners and gums. More research is needed in this field to gain knowledge and to overcome issues regarding nutrition and stability. Further, the development of new milk alternatives that cause no adverse effects in humans and that have better nutritional, sensory and technological properties is necessary.

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***Chapter 4: Polyol-producing lactic acid bacteria isolated
from sourdough and their application to reduce sugar in
a quinoa-based milk substitute***

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4.1 Abstract

The interest for plant-based dairy substitutes is expanding rapidly and consumers are opting for nutritious and healthy dairy alternatives. The reduction of sugar using different exogenous enzymes in combination with lactic acid fermentation in a quinoa-based milk substitute was explored in this study. Different amylolytic enzymes were used to release sugar from the raw material, which were further metabolised to mannitol, due to fermentation with two heterofermentative lactic acid bacteria. Using these two biotechnological techniques enables the reduction of sugar, while also preserving some of the sweetness. *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 were isolated from sourdough. Both strains showed high viable cell counts with *L. citreum* TR116 reaching 8.4 and *L. brevis* TR055 9.3 log cfu/ml, and a reduction in pH to 3.7 and 3.5, respectively. When fructose was available, mannitol was produced in conjunction with acetic acid in addition to lactic acid. Due to these processes, the original glucose value was reduced from 50 mmol/100g to approximately 30 mmol/100g, which equates to a glucose reduction of 40%. In respect to mannitol production, both strains performed well: *L. citreum* TR116 showed a conversion factor of 1:1 from fructose to mannitol, while *L. brevis* TR055 showed a lower yield, with a conversion factor of 1:0.8. Glycaemic load was reduced by more than a third, bringing it down to the low range with a value of about 10. Overall, enzymatic modification in conjunction with mannitol-producing lactic acid bacteria shows great potential for further possible application in the development of nutritious and sugar reduced plant-based milk substitutes.

4.2 Introduction

Plant-based milk substitutes (PBMSs) are gaining popularity and the interest is expanding rapidly. Consumers are choosing dairy substitutes over dairy products for various reasons; obviously so in the case of individuals suffering from milk allergies and intolerance, but an increasing consumption is based on preference. In this regard, PBMSs can serve as a sustainable, ethical and nutritious option to meet the needs of consumers. Owing to this increasing interest, the market is expected to grow at a significant rate: MarketsandMarkets (2017) estimated the value of the dairy alternative market to be 7.37 Billion USD for 2016 and predicted a growth rate of 11.7% from 2017 on, reaching a forecasted market value of 14.36 Billion USD in 2022. Nevertheless, many studies reported several concerns about the nutritional value of some products (Jeske *et al.*, 2017 (Chapter 3); Sousa *et al.*, 2017; Katz *et al.*, 2005). In particular, the low protein content was found to be a major risk. Furthermore, PBMSs based on starchy raw materials, such as rice or quinoa contain high amounts of sugar due to hydrolysis of starch and release of maltose and/or glucose thereof. Sugar contents and *in vitro* glycaemic indices of commercial PBMSs were analysed, and rice-, and coconut-based products especially showed high values for the glycaemic indices with 97.74, and 96.82, respectively, with sugar content of 7.02 and 1.86 g/100g, respectively (Jeske *et al.*, 2017; Chapter 3).

High sugar consumption affects human health, being a major inducer for obesity and chronic diseases (Lustig *et al.*, 2012). The public awareness of this problem is increasing and consumer behaviour is changing: 64% of consumers in Ireland are concerned about their sugar intake (James Wilson, 2018), and similarly German consumers have reduced their sugar consumption by 48% (Mintel Press Team, 2017). For this reason, research and industry are investigating methods for sugar-reduction or use of sweeteners as alternatives to sugar. Mannitol, as one promising alternative, is a natural sugar alcohol, prevalent in several plants, fungi, yeast and bacteria (Wisselink *et al.*, 2002). It has a sweet taste, being perceived about 40% less sweet than sucrose, and its incorporation in food has several potential beneficial effects; health claims relating to mannitol include protection against tooth-decay and reduction of the glycaemic response, both due to mannitol not being absorbed in the human intestine, and thus exhibiting a low calorific value. Both claims are approved by the European Food Safety Authority (2011). Although an increasing amount of

consumers (17% in Germany) believe that plant-based yoghurt alternatives are healthier, the biggest challenge lies in the taste for these products (Mintel Press Team, 2017). In this regard, mannitol could improve the properties of these products, increasing both health benefits and flavour at the same time.

Industrially, mannitol is produced by catalytic hydrogenation of a glucose/fructose syrup, producing a mixture of sorbitol and mannitol. However, the yield is low and costs are high for this chemical process (Grembecka, 2015). As an alternative, lactic acid bacteria (LAB) can be used to produce mannitol in a more sustainable and efficient way. Heterofermentative LAB can reduce fructose directly to mannitol. It is catalysed by the enzyme mannitol-dehydrogenase and, metabolically, serves to regenerate NAD^+ (Wisselink *et al.*, 2002).

Fermented foods are attracting increased interest and recently much emphasis has been granted to their unique functional properties and contribution to the health of consumers. Their application has evolved from preserving food to understanding and exploiting metabolites, other than organic acids and antifungal compounds. Studies focus on compounds associated with health benefits and additional functional properties, such as mannitol or exopolysaccharides (Chilton *et al.*, 2015; Tamang *et al.*, 2016; Selhub *et al.*, 2014; Lynch *et al.*, 2018).

Further, new raw materials are explored as substrate for LAB fermentation and for the development of novel products. For instance, the ancient pseudocereal quinoa has received renewed interest, particularly in Western countries due to its high nutritional value (Arendt and Zannini, 2013). It is especially rich in protein and essential amino acids, contains adequate levels of important micronutrients such as minerals and vitamins, and significant amounts of other bioactive compounds, such as polyphenols (Arendt and Zannini, 2013; Alvarez-Jubete *et al.*, 2010). As a versatile substrate, quinoa has been used for different fermented products; Axel *et al.* (2015) improved the nutritional value and bread quality using quinoa flour as a base for sourdough with exceptionally high amounts of antifungal compounds; Zannini *et al.* (2018) developed a quinoa-based yoghurt, having a higher water holding capacity and viscosity than a chemically-acidified control, due to dextran exopolysaccharide excretion by an LAB strain. In addition, fermentation has been shown for many cereals and legumes to improve sensorial and textural properties (Peyer *et al.*, 2016)

and could be used as a tool to ameliorate grassy and bitter off-flavours, characteristic for quinoa.

In this study, a quinoa-based milk substitute (QBMS) was used as a substrate to study the production of mannitol by means of a two-step process including enzyme treatment, and fermentation with two heterofermentative LABs. The samples were treated with amylases and a glucose-isomerase, in order to generate fructose as a substrate, which was further metabolized to mannitol by the action of LAB. The aim was to study the potential for sugar-reduction using this two-step process.

4.3 Materials and methods

4.3.1 Materials, strains and culture conditions

Organic quinoa was obtained from Ziegler & Co. GmbH Naturprodukte (Wunsiedel, Germany). Chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise stated. The microorganisms *Leuconostoc citreum* TR116 and *Lactobacillus brevis* TR055 were isolated from yellow pea sourdough and teff sourdough, respectively, and belong to the culture collection of the Department of Biological Sciences, Cork Institute of Technology, Ireland. These cultures were selectively chosen for their ability to produce mannitol. The LAB isolates were maintained as frozen stocks in 40% (w/w) glycerol at -80 °C. The strains were routinely sub-cultured on de Man Rogosa and Sharp (MRS) agar under anaerobic conditions for 24 h at 30 °C.

4.3.2 Preparation of quinoa-based milk substitute

50 g organic quinoa flour and 350 g water were mixed in a semi-industrial blender (Kenwood Major Titanium, New Lane, Havant, UK) at maximum speed for 3 minutes. To each sample 250 mg α -amylase (Hitempase 2XP, Kerry, Carrigaline, Ireland), 300 μ L amyloglucosidase (Attenzuzyme, Novozymes), and 36.6 μ L protease (Flavourzyme, Novozymes) were added. The samples were mixed again for 30 s at lower speed. Additionally, 0.8 g glucose-isomerase were added to some of the samples (labelled as “iso”). All samples were kept in a stirring water bath at 60 °C for 24 h for enzyme action and cooled to 25 °C within 20 min (Lochner mashing device LP electronic, Berching, Germany). Samples were cooled on ice straight after, filtered with cheese clothes and homogenised (APV Homogenizer, SPX FLOW, Inc., Charlotte, USA) at 150 bar for the 1st stage, 30 bar for the 2nd stage. Finally, the samples were pasteurized in a water bath at 65 °C for 30 min.

4.3.3 Fermentation

Single colonies of each LAB strain were propagated twice in 10 mL MRS broth in anaerobic and static conditions for 24 h at 30 °C. Cultures were cultivated until the late exponential phase (ca. 14 h) and enumerated by performing a viable plate count in duplicate. After cell count determination, suspensions were prepared in the same manner for inoculation and harvested by centrifugation at 9000 g for 10 min at 4 °C

and washed twice with Ringer's solution. The inoculation was performed at 7 log cfu/mL directly into tempered QBMS samples. Fermentation was performed anaerobically, under static conditions at 30 °C for 24 h. Figure 4-1 depicts the enzymatic processing of quinoa starch, using the exogenous enzymes (α -amylase, γ -amylase, and glucose-isomerase) added at the before outlined part of the preparation of QBMS samples, and the endogenous enzyme mannitol-dehydrogenase, which is secreted by *Leuconostoc citreum* TR116 and *Lactobacillus brevis* TR055 during fermentation.

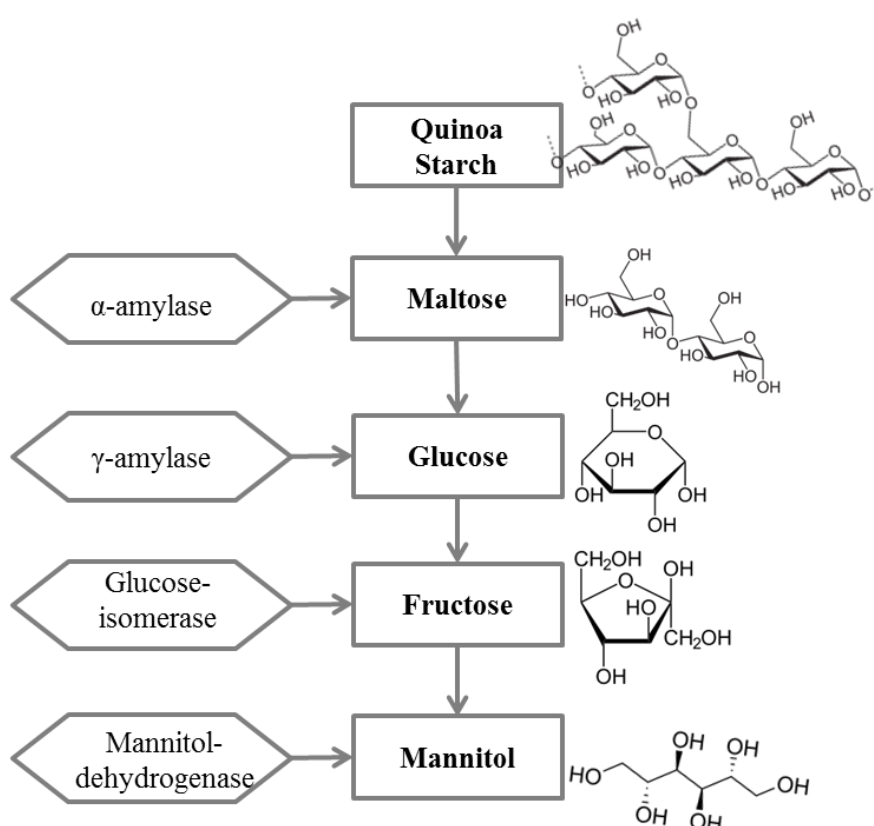


Figure 4-1 Enzymatic processing of quinoa starch with exogenous enzymes (α -amylase, γ -amylase, and glucose-isomerase), and endogenous enzymes, secreted by LAB (mannitol-dehydrogenase).

4.3.4 Compositional analysis

Compositional analyses were performed on the quinoa flour and unfermented samples. Total nitrogen content was determined according to the Kjeldahl method (MEBAK 1.5.2.1). Nitrogen content was converted into protein using the factor 5.75

according to Fujihara *et al.* (2008). Fat content was measured following the Soxhlet method. Ash content was determined in a muffle furnace by incineration (4 h, 600 °C), pre-heated in crucibles (1 h, 100 °C). The moisture content was determined by drying in an oven at 103 °C until constant mass was reached. Total starch was analysed using the enzyme kit K-TSTA supplied by Megazyme, Ireland.

4.3.5 Viable cell counts

Total cell counts of LAB were performed on MRS agar plates after incubation for 48 h under anaerobic conditions using Anaerocult A gas packs (Merck, Darmstadt, Germany) at 30 °C.

4.3.6 Measurement of titratable acidity, and pH

The total titratable acidity (TTA) was determined by suspending 5 g of sample in 45 mL distilled water and titrating against 0.1 N NaOH to pH 8.5 (Katina *et al.*, 2006). After 3 min, the pH was readjusted to 8.5. The TTA was expressed as the number of millilitres of NaOH used for titration. The pH was monitored using a commercial digital pH meter.

4.3.7 Determination of sugar and organic acids profiles

Samples were diluted with ultrapure water, filtered (0.2 µm) and analysed by high performance liquid chromatography using an Agilent Infinity 1260 HPLC System. For sugar analysis the system was equipped with a Waters Sugar-Pak, 300 x 6.5 mm HPLC column at 0.5 mL/min flow rate of 0.0001 mmol/L CaEDTA at 80 °C, using a refractive index detector (Agilent Technologies, Palo Alto, CA) for detection. Glucose, maltose, fructose, and mannitol were used as external standards. Results were reported in mmol/100g. Organic acids were determined using an Agilent Hi-Plex H, 7.7 x 300 mm, pack size 8 µm HPLC column with a 178 PL Hi-Plex Guard column mounted upstream at a flow rate of 0.5 mL/min of 0.005 mmol/L H₂SO₄, and a column temperature of 60 °C. Lactic acid, and acetic acid were used as external standards. Results were reported in mmol/100g.

4.3.8 Glycaemic index

In vitro determination of the glycaemic index (GI) was evaluated according to Magaletta & DiCataldo (2009) using a calculation designed by an artificial neural

network. A certain amount of sample (equivalent to 0.5 g of available carbohydrates, based on the results of sugar and starch analysis) was digested by a multi-enzyme preparation. The digestate was analysed for glucose, fructose, lactose, galactose, and maltitol with HPLC, described as above. These results, together with the results from the protein and fat determination, were used to feed the calculation:

$$\begin{aligned}
 GI = & 26.264529 - 1.048186 \cdot \text{Protein} [\%] - 0.248138 \cdot \text{Fat} [\%] \\
 & + 621.7824 \cdot \text{Glucose} [\%] - 52.7993 \cdot \text{Fructose} [\%] \\
 & - 233.67679 \cdot \text{Lactose} [\%] - 61.21071 \cdot \text{Galactose} [\%] \\
 & - 84.689245 \text{ Maltitol} [\%]
 \end{aligned}$$

Glycaemic load (GL) was calculated according to Atkinson *et al.* (2008):

$$GL = (GI \cdot \text{available carbohydrate (g) per portion})/100$$

The portion size was set to 250 g.

4.3.9 Physicochemical Properties

Rheological behaviour of the products was characterised using a controlled stress rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a sensor system of coaxial cylinders (C-CC27-T200/SS, Anton Paar GmbH, Austria). The shear stress (σ) was measured as a function of shear rate ($\dot{\gamma}$) ranging from 0.5 to 100 s⁻¹ within 500 s. The measurements were carried out at 10 °C. The apparent viscosity measured at 10 s⁻¹ is referred to as viscosity. Suspension stability was determined through phase separation analysis using an analytical centrifuge (LUMiSizer; LUM GmbH, Berlin, Germany). The instrumental parameters used were as follows: 1000 rpm for 30 min followed by 3000 rpm for 60 min at 24 °C. Separation rate in %/h was determined by plotting the % of transmission over the time. Syneresis of quinoa milk was analysed using a slight modification of the centrifugation method previously reported by Keogh and O’Kennedy (1998). Forty g of sample were centrifuged at 220 g for 10 min at 4 °C. The supernatant was poured off and weighed again. Syneresis was expressed as a %. Colour values were measured using the CIE L*a*b* colour system and obtained using illuminant D65. The instrument used was a

colorimeter (CR-400, Konica Minolta, Osaka, Japan). Colour of samples was characterised according to whiteness index (WI), defined as:

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

4.3.10 Statistics

All analyses were carried out at least in triplicate. Means were compared with one-way analysis of variance (ANOVA) and Tukey pairwise. The significance level was set to $\alpha = 0.05$.

4.4 Results and discussion

4.4.1 Compositional analysis

The composition of the quinoa flour used for the preparation of the samples given in % (w/w) was as follows: moisture: $9.6\% \pm 0.4$, protein: $14.64\% \pm 0.14$, ash: $2.59\% \pm 0.03$, fat: $7.24\% \pm 0.00$, and total starch (dry basis): $67.4\% \pm 3.55$. The prepared QBMS samples contained $0.84\% \pm 0.01$ protein, $0.29\% \pm 0.01$ ash, $0.33\% \pm 0.04$ fat and <1.8 mg/L starch. Due to hydrolysis of starch, the samples contained glucose with $9.09\% \pm 0.45$. The level of fructose during glucose-isomerase treatment at different time points is displayed in Figure 4-2. Fructose contents continued to increase, while finally the amount of glucose was reduced to $7.21\% \pm 0.09$ and fructose levels occurred at $1.55\% \pm 0.07$ after 24 hours.

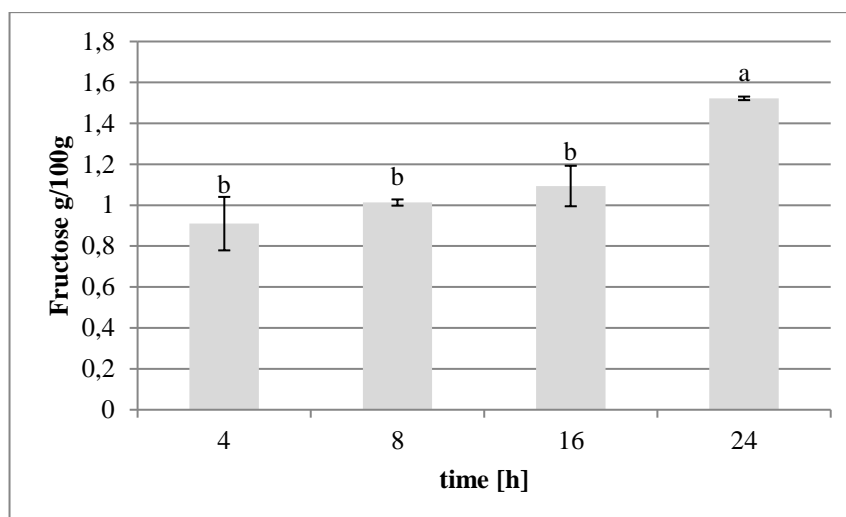


Figure 4-2 Concentration of fructose in quinoa-based milk substitutes over time during glucose-isomerase treatment. Values that share a label are not significantly different from one another ($p < 0.05$).

4.4.2 Cell growth and acidification properties

The values of viable cell counts, pH, total titratable acidity, and acid profile are presented in Table 4-1. The results showed that QBMS facilitated the growth of *L. citreum* TR116, as well as *L. brevis* TR055. The latter showed a more vigorous growth, reaching values of 9.35 log cfu/mL, while *L. citreum* TR116 reached cell counts of 8.48 log cfu/mL after 24 h incubation. Ruiz Rodríguez *et. al* (2016) found both strains to be autochthonous in spontaneously fermented sourdough produced

Table 4-1 Cell counts, pH, TTA, and organic acid values of unfermented (Unf.), isomerase treated samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 of quinoa-based milk substitutes.

	Cfu [log cfu/mL]	pH	TTA [mL]	Lactic acid [mmol/100g]	Acetic acid [mmol/100g]
Unf.	n.d.	5.37±0.08 ^b	1.70±0.01 ^e	n.d.	n.d.
Unf. Iso	n.d.	5.67±0.05 ^a	1.63±0.06 ^e	n.d.	n.d.
TR116	8.48±0.03 ^b	3.75±0.02 ^c	4.38±0.35 ^d	5.49±0.42 ^b	n.d.
TR116 Iso	8.42±0.26 ^b	3.64±0.03 ^{cd}	6.79±0.47 ^b	5.59±0.37 ^b	4.79±0.41 ^a
TR055	9.35±0.10 ^a	3.52±0.05 ^{de}	6.04±0.12 ^c	8.82±0.13 ^a	n.d.
TR055 Iso	9.24±0.09 ^a	3.45±0.02 ^{de}	8.68±0.27 ^a	8.95±0.08 ^a	4.12±0.23 ^a

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$); n.d.: not detectable, limit for cfu $< 3 \log$ cfu/mL, for lactic and acetic acid $< 1\text{mM}/100\text{g}$.

from quinoa. Both strains showed similar cell counts in QBMS, regardless of treatment with isomerase, i.e. the presence of fructose in the media had no impact on cell growth. However, the presence of fructose influenced acid production; TTA values increased for both strains in samples due to the treatment with glucose-isomerase, from 4.38 to 6.79 mL for *L. citreum* TR116, and from 6.04 to 8.68 mL for *L. brevis* TR055. The pH, however, dropped to 3.75 and 3.52, for *L. citreum* TR116 and *L. brevis* TR055, respectively and was not decreased considerably in QBMS treated with glucose-isomerase. A closer look at the acid profile revealed that in the absence of fructose, only lactic acid was produced (5.49 and 8.82 mmol/100g from *L. citreum* TR116, and *L. brevis* TR055, respectively), while in the presence of fructose, acetic acid was additionally produced from both strains (4.79 and 4.12 mmol/100g from *L. citreum* TR116 and *L. brevis* TR055, respectively). Heterofermentative LAB can generate additional ATP with the production of acetic acid from acetyl phosphate. However, this is only possible in the presence of fructose, which acts as an alternative electron acceptor, NAD^+ is regenerated via the reduction of fructose to mannitol, which would otherwise happen through the production of ethanol from acetyl phosphate (Wisselink *et al.*, 2002).

Table 4-2 shows the sugar composition with some stoichiometric parameters related to the sugar metabolism. Due to the glucose-isomerase treatment 8.58 mmol/100g fructose were produced from glucose. Furthermore, glucose was metabolised by both strains and approximately 9 mmol/100g glucose were consumed in all fermented samples; neither the glucose-isomerase treatment and changing carbohydrate composition, nor the bacteria itself had a considerable impact on this value. When fructose was present both LAB produced mannitol additionally. *L. citreum* TR116 produced 8.58 mmol/100g, while *L. brevis* TR055 produced less mannitol, at 7.18 mmol/100g. *L. citreum* TR116 metabolized fructose completely to mannitol, with a yield of 100%, while *L. brevis* TR055 achieved a yield of 84%. It was demonstrated previously that heterofermentative LAB can reduce fructose to mannitol with yields of up to 100%, when glucose and fructose were available (1:2) (Wisselink *et al.*, 2002).

Table 4-2 Sugar composition and stoichiometric parameters of unfermented (Unf.), isomerase treated samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 of quinoa-based milk substitutes.

	Glucose [mmol/100g]	Fructose [mmol/100g]	Maltose [mmol/100g]	Mannitol [mmol/100g]	Reduction of glucose [mmol/100g]	Mannitol yield on fructose
Unf.	50.44±2.49 ^a	n.d.	1.09±0.13 ^a	n.d.	-	-
Unf. Iso	39.99±0.48 ^b	8.58±0.40 ^a	0.86±0.07 ^{bc}	n.d.	10.44±2.27 ^b	-
TR116	39.92±2.00 ^b	n.d.	0.99±0.04 ^{ab}	n.d.	10.52±2.24 ^b	-
TR116 Iso	31.01±0.32 ^c	n.d.	0.67±0.04 ^c	8.58±0.10 ^a	19.43±2.29 ^a	100±5 ^a
TR055	42.38±2.58 ^b	n.d.	0.94±0.08 ^{ab}	n.d.	8.05±0.22 ^b	-
TR055 Iso	30.14±1.95 ^c	0.17±0.08 ^b	0.72±0.07 ^c	7.18±0.46 ^b	20.3±2.44 ^a	84±2 ^b

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$); n.d., not detectable, $< 0.5\text{mM}/100\text{g}$

L. citreum is known to produce mannitol, however other studies have found lower yield values of 89.3% or 70% from fructose (Otgonbayar *et al.*, 2011; Carvalho *et al.*, 2011). In these studies, the ratio of fructose to glucose was higher, and the strains used were grown in a different medium. On the other hand, *L. brevis* TR055 showed a lower ratio of fructose to mannitol with about 84%. Therefore, some fructose must have entered the phosphoketolase pathway instead of being metabolized to mannitol. Due to the combined reactions of glucose-isomerase and fermentation, a total amount of about 20 mmol/L glucose were removed and transformed into metabolites like organic acids, mannitol and other compounds. This bioprocess can be used to generate sour, fermented products, while at the same time not losing too much sweetness, since mannitol is produced. Glucose was reduced by approximately 40% (equivalent to 20 mmol/100g) through the action of the glucose-isomerase treatment and being used as a carbon source for bacterial growth. However, the sweetness of the product was only reduced by about 24% and 28% for samples treated with glucose-isomerase and fermented with *L. citreum* TR116 or *L. brevis* TR055, respectively, when considering literature values of the relative sweetness of glucose and mannitol (0.7 and 0.6, respectively, compared to sucrose) (Nutrients Review, 2016).

Furthermore, the glycaemic effect of the samples was determined with an *in vitro* method (Table 4-3). The digestion of carbohydrate-containing food products affects blood glucose levels, also known as the postprandial glycaemic effect. The GI is related to the type of carbohydrates and dependent on the rate of digestion (Wolever *et al.*, 2008). In fact, only glucose can be absorbed directly by the small intestine and used for energy generation; other sugars, such as fructose and galactose must be metabolised by the liver to glucose, or, in the case of sucrose, and most polysaccharides, must be hydrolysed into their constituent monosaccharides before being metabolised further. Therefore, the postprandial rate of increase in blood glucose levels is lower for those carbohydrates. In the case of mannitol, or other non-glycaemic carbohydrates such as dietary fibre and resistant starch, no effect on the blood glucose level can be observed, since these are not digested in the small intestine (Östman *et al.*, 2002). The GI of all samples was high, ranging from 64 to 76. Only the unfermented, glucose-isomerase-treated sample had a slightly lower GI, due to the conversion from glucose to fructose, which has a lower impact on the

blood sugar level (Foster-Powell *et al.*, 2002). Considering the GL on the other hand, considerable differences were observed. The GL relates the GI to a portion size, representing both quality and quantity of carbohydrates being consumed (Barclay *et al.*, 2008). Hence, results represent the impact on the blood sugar level after consuming 250 mL of sample. For the untreated sample, a GL of 16.22 was determined. With fermentation, only a slight, insignificant, reduction was obtained, to 14.40 and 14.09 for *L. citreum* TR116 and *L. brevis* TR055, respectively. A remarkable reduction of more than a third was obtained for both the glucose-isomerase treated, fermented samples, bringing the GL down to almost the low range (<10) (Venn and Green, 2007). *L. citreum* TR116 showed a value of 10.80 and *L. brevis* TR055 one of 10.43. The depletion of about 20 mmol/100g glucose and ultimate bioconversion into the non-glycaemic carbohydrate, mannitol, resulted in this substantial reduction of GL. Research studies strongly indicate a correlation between high GI and GL and increased risk of type 2 diabetes, breast cancer, gallbladder disease and heart disease, while low GI and GL diets show many health benefits i.e. weight control, protection against colon and breast cancer, obesity, cardiovascular disease, and diabetes (Brand-Miller *et al.*, 2009)

Table 4-3 *In vitro* glycaemic index, load, and reduction of glycaemic load of unfermented (Unf.), isomerase treated samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 of quinoa-based milk substitutes.

	Glycaemic index [-]	Glycaemic load [-]	Reduction of Glycaemic load [%]
Unf.	69.47±1.37 ^{bc}	16.22±0.70 ^a	-
Unf. Iso	64.57±1.11 ^c	14.12±0.51 ^a	9.89±1.90 ^b
TR116	76.51±0.22 ^a	14.40±0.72 ^a	14.68±4.08 ^b
TR116 Iso	73.04±1.61 ^a	10.80±0.22 ^a	34.45±2.91 ^a
TR055	70.82±0.60 ^{abc}	14.09±0.87 ^b	13.15±2.38 ^b
TR055 Iso	72.08±3.95 ^{ab}	10.43±0.58 ^b	36.71±0.64 ^a

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$)

4.4.3 Physicochemical properties

Samples were analysed for physicochemical properties to assess their characteristics as a beverage. The results are shown in Table 4-4. Due to the drop in pH during fermentation, the samples were destabilized, which is evident in the results for syneresis and separation rate; both values increased from 10.20 to 15.21% and from 13.34 to 21.86 %/h, respectively, due to fermentation of samples with *L. brevis* TR055. No considerable differences between the fermented samples were found. The pH after fermentation is close to the isoelectric point of quinoa proteins, being around 4 (Elsahaimy *et al.*, 2015), resulting in a low solubility and destabilisation. As seen in the measurements of rheology, the decrease of pH did not affect the viscosity and no significant differences were found between samples. Unlike other proteins, such as casein in bovine milk, quinoa proteins do not facilitate a network-forming matrix and gel strength is weak, as shown also by Mäkinen *et al.* (2014). However, in order to compensate for the lack of network forming properties of the quinoa proteins, Zannini *et al.* (2018) used an EPS-producing culture (*Weissella cibaria* MG1) to produce a quinoa-based yoghurt substitute. The resulting yoghurt showed increased viscosity ($> 0.5 \text{ Pa}\cdot\text{s}$), and improved water holding capacity, both due to the amounts of EPS produced. A combined fermentation with an EPS-producing strain could therefore overcome the rheological and suspension stability challenges, generating a product with multiple new functional properties. The samples showed very slight differences for the chromaticity and similar whiteness indices, ranging from 49.49 to 54.32. These values indicate a lower whiteness of the samples compared to bovine milk (81.89), but the values are similar to other commercial PBMSs (Jeske *et al.*, 2017; Chapter 3).

Table 4-4 Physicochemical properties of products of unfermented (Unf.), isomerase treated samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 of quinoa-based milk substitutes.

	Separation rate [%/h]	Viscosity [mPa·s]	Syneresis [%]	Whiteness Index [-]
Unf.	13.34±1.08 ^c	5.92±0.89 ^b	10.20±1.86 ^b	49.49±1.57 ^d
Unf. Iso	15.04±0.82 ^{bc}	5.83±0.48 ^b	11.68±1.96 ^b	51.18±1.23 ^{cd}
TR116	18.25±2.53 ^{ab}	5.99±1.04 ^b	15.13±1.60 ^a	52.06±1.43 ^{bc}
TR116 Iso	18.19±1.41 ^{ab}	8.14±1.41 ^a	16.20±1.05 ^a	53.25±1.94 ^{ab}
TR055	21.86±2.14 ^a	6.36±1.20 ^a	15.21±1.56 ^a	53.53±0.77 ^{ab}
TR055 Iso	15.04±1.02 ^c	7.06±0.81 ^a	16.50±0.63 ^a	54.32±1.50 ^a

Values within a column that share a superscript are not significantly different from one another (p < 0.05)

4.5 Conclusion

This study demonstrates a novel biotechnological processing approach to improve nutritional properties and meet consumer demands of PBMSs. The production of mannitol was examined in a quinoa-based milk substitute, using two LAB as starter cultures. It was shown that quinoa serves as a good substrate, facilitating the growth of *L. citreum* TR116 and *L. brevis* TR055 with high viability. The hydrolysis of starch, further conversion of glucose to fructose through the enzyme glucose-isomerase, and subsequent reduction of fructose to mannitol via fermentation, reduced the glucose content by 40% and GL by 35%. *L. citreum* TR116 and *L. brevis* TR055 could be used as novel functional starter cultures and this approach can be transferred to any kind of fermented food product, such as sourdough or beverages. This laboratory prototype could represent an example of novel PBMSs, characterised by improved nutritional and functional properties and also by a lower carbon and water footprint when compared to their dairy counterparts.

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***Chapter 5: Impact of protease and amylase treatment on
protein and product quality of a quinoa-based milk
substitute***

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5.1 Abstract

Plant proteins are often characterised by low solubilities and impaired functionalities e.g. emulsifying properties. In products like milk substitutes, these protein properties are of great importance to ensure good product quality. In this study proteolytic enzymes were used as a tool to increase protein solubility and alter their properties gently. A plant-based milk substitute based on quinoa was produced and treated with different enzymes. One α -amylase and three commercial proteases were selected: Hitempase 2XP, Profix 100L, Bioprotease N100L, and Flavourzyme 1000L. The protein solubility of the samples was initially low 48.02% and was improved with the increasing degree of hydrolysis up to a value of 75.82% with Profix. These results were supported by SDS-PAGE and circular dichroism analysis: especially Profix degraded the proteins extensively. Quality characteristics, such as foaming, and emulsifying properties were not influenced considerably by the protease treatment. The results of this study provide an in-depth understanding of the effects of different enzymes in a complex system of a plant-based milk substitute and contribute to the development of protein-based products.

5.2 Introduction

The market for plant-based milk substitutes (PBMSs) is growing fast and these products are becoming more popular all over the world. The Western European market in 2015 was valued 1.9 billion US\$ and is believed to grow by 50% within the next 5 years (Mintel Group Ltd, 2016). Initially, consumer demand for cow's milk alternatives increased as a need of people, suffering from lactose intolerance and cow's milk allergy. However, most people do not consume PBMSs for medical reasons nowadays but out of preference and lifestyle choices, including vegetarian, vegan or other alternative diets, as well as ethical considerations against the consumption of cow's milk (Mintel Group Ltd, 2016). Technologically, PBMSs are extracts of plant material in water, resembling cow's milk in appearance. Processing steps vary, but generally the following steps are applied: the raw material is either soaked and wet milled, or dry milled and the flour is extracted from water afterwards. Insoluble plant material is separated from the resulting slurry. It is noteworthy that this step may remove constituents like fibre or proteins. Standardisation and addition of other ingredients like oil, flavourings, sugar, and stabilizers may be employed, depending on the desired product. To improve the suspension and microbial stability, homogenisation and pasteurisation or ultra-high temperature treatment take place at the end of the process (Diarra *et al.*, 2005). PBMSs vary considerably in nutrient profiles, with some of the products having very low protein contents (Jeske *et al.*, 2017; Chapter 3). Yet, the protein quality and quantity is an important characteristic of PBMSs especially if they are consumed to replace cow's milk in the diet.

One crop, with promising properties is quinoa (*Chenopodium quinoa*): its protein content is remarkably high, with literature values ranging from 12 to 23% (Abugoch James, 2009; Arendt and Zannini, 2013), while having a well-balanced amino acid composition (Steffolani *et al.*, 2014). Not only the protein content of PBMSs poses challenges, but their colloidal stability or viscosity are also other issues, which are often overcome by the addition of emulsifiers or stabilisers. In cow's milk, proteins are responsible for many functional properties. Plant proteins are however very different from cow's milk proteins, e.g. being insoluble, or having lower emulsifying capabilities. Indeed, native quinoa proteins are stored in a stable form in the seeds and show low solubility (Mäkinen *et al.*, 2015). Solubility is among the most

important properties of food proteins (Day, 2013). It is generally accompanied by better functionality for most food applications (Nakai, 1983) and often a requirement for functional characteristics like emulsification (Day, 2013). Proteolytic enzymes are one of the tools for food scientists to amend proteins gently, and can be used to tailor their functionality (Panyam, 1996). Due to enzymatic hydrolysis, the molecular weight of proteins decreases, the amount of ionisable groups increases, and hydrophobic groups so far hidden in the inner core get exposed, which results in a change of properties (Panyam, 1996).

In this study, the effect of enzymatic treatment on protein quality in a beverage based on quinoa was investigated. As the functionality of proteins is often evaluated in model systems, even though food in general is a lot more complex, the assessment of hydrolysis in a PBMS provides new insight. The obtained product was characterised for important physiochemical characteristics. The findings will help to understand and predict product properties in the development of enzymatically modified products, which aim to meet the requirements of consumers, industry and environment.

5.3 *Materials and methods*

5.3.1 *Selection of enzymes*

Three different commercial enzymes were chosen to be applied and studied in the quinoa system due to their different composition and related mode of actions. Bioprotease N100L (Kerry Group, Ireland) is an enzyme obtained by a *Bacillus subtilis* strain (product specification, Kerry). *Bacillus subtilis* is known to produce a neutral metalloprotease and a serine protease (Aunstrup, 1983). Profix 100L (Kerry Group, Ireland), a purified papain preparation, consists of a wide range of cysteine proteases (product specification, Kerry). Both proteases work as endopeptidases. Flavourzyme (Novozyme A/S, Denmark) is an enzyme preparation from *Aspergillus oryzae*, containing an exopeptidase which releases amino acids from the N-terminal peptide bond. Additionally the α -amylase Hitempase 2XP (Kerry Group, Ireland) was used for dextrinization and liquefaction of starch.

Considering the impact of the protein content coming from the added enzyme preparations on the composition or any other properties of the samples, it is expected to be negligible. The protein contents of the enzyme preparations are reported by the manufacturers as follows: Profix 100L 9 g/100g, Bioprotease N100L 3 g/100g, Flavourzyme 17 g/100g, and Hitempase 2XP 8 g/100g. This adds up to less than 0.0003 g of added protein in 100 g of sample in combination 1 (highest dosages of all the 4 enzymes). Therefore the impact of proteases is assumed to have no significant contribution to the protein content of the samples.

5.3.2 *Preparation of quinoa-based milk substitute*

50 g organic quinoa flour (Ziegler & Co. GmbH Naturprodukte, Wunsiedel, Germany), and 350 g water were mixed in a semi-industrial blender (Kenwood Major Titanium, New Lane, Havant, UK) at maximum speed for 3 minutes. To each sample, except the blank sample, 62.5 mg α -amylase Hitempase 2XP (Kerry, Carrigaline, Ireland) per 100 g were added. The proteases Profix, Bioprotease and Flavourzyme were added in different concentrations, see Table 5-1. Combinations of proteases were applied under

the name of combination 1 (all proteases, highest dosage, respectively) and combination 2 (Profix and Bioprotease, highest concentration, respectively). The different concentrations were chosen based on pre-trials to see the spectrum of subtle to high impact of the proteases, starting off from the recommended dosages, as reported by the manufacturers.

Subsequently, the quinoa-based milk substitute (QBMS) samples were mixed again for 30 seconds at lower speed. The samples were heated to 50 °C for 180 minutes followed by cooling down to 25 °C (Lochner mashing device LP electronic, Berching, Germany). QBMS samples were homogenised (APV 1000 Homogenizer, SPX FLOW, Inc., Charlotte, USA) at 150 bar for the 1st stage, 30 bar for the 2nd stage, and pH was adjusted to 7 with 4 M NaOH. For all measurements except composition, stability, foaming, and emulsifying properties, QBMS samples were centrifuged at 3900 g for 5 minutes and the supernatants were analysed. Molecular weight and secondary structure measurements were performed with samples treated with the highest dose of each protease and the combinations.

Table 5-1 Dosages and respective multiples of recommended dosage for applied proteases.

Enzyme	Dosage [μL /100 mL QBMS]	Multiples of recommended dosage
Profix 100L	4, 40, 100, 200	1x, 10x, 25x, 50x
Bioprotease N100L	6.25, 62.5, 156.25	1x, 10x, 25x
Flavourzyme 1000L	9.15, 45.75, 91.5	1x, 5x, 10x

5.3.3 Compositional analysis

Total nitrogen content was determined according to the Kjeldahl method (MEBAK 1.5.2.1). Nitrogen content was converted into protein using the factor 5.75 according to Fujihara *et al.* (2008). Fat content was measured following the Soxhlet method. Ash content was determined in a muffle furnace by incineration (4 h, 600 °C), pre-heated in crucibles (1 h, 100 °C). The moisture content was received by drying them in an oven at 103 °C until

constant mass was reached. For sugar profile analysis, samples were clarified with Carrez reagents, diluted with water, centrifuged (5000 g, 10 minutes) and filtered (0.2 μm). Sugar profiles were analysed using an Infinity 1260 HPLC system equipped with a Hi-Plex H, 300 x 7.7 mm, 8 μm HPLC column (Agilent Technologies, Palo Alto, CA) at 1 mL/min flow rate of water and detected by a refractive index detector (Agilent Technologies, Palo Alto, CA). Sucrose, glucose, maltose and fructose were used as external standards. Values are reported in % (w/w) throughout the paper.

5.3.4 Degree of hydrolysis

Degree of hydrolysis (DH) was determined based on the reaction of primary amino groups with o-phthalaldehyde (Nielsen *et al.*, 2001). The absorbance of the formed compound was measured at 340 nm. Serine was used as a standard. To calculate DH from spectrophotometer readings the following equations were used:

$$\text{Serine} - \text{NH}_2 = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{serine}} - A_{\text{blank}})} \cdot 0.9516 \frac{\text{meqv}}{\text{L}} \cdot 0.1 \cdot X \cdot P$$

$$h = \frac{(\text{Serine} - \text{NH}_2 - \beta)}{\alpha}$$

$$\text{DH} = h / h_{\text{tot}} \cdot 100 \%$$

X represents sample volume, and P protein content in the sample's supernatant. The value of constants α and β were 1 and 0.4 respectively according to the method of Nielsen *et al.* (2001). While for h_{tot} 7.4 meqv/g was chosen, which is based on an calculation by Opazo-Navarrete *et al.* (2017) obtained specifically for quinoa. The measured DH for the blank sample (no enzyme treatment) (11.57) was set to zero.

5.3.5 Secondary structure

Information about the protein secondary structure was gained using a circular dichroism spectrophotometer (Chirascan, Applied Photophysics, Leatherhead, UK). The supernatants of QBMS samples were diluted with distilled water to achieve a concentration of 1 mg protein per mL solution based on the soluble protein content.

The spectra were recorded with a path length of 0.1 mm in the range of 180–260 nm, spectral resolution 1 nm, 1 s per point. The average of three spectra was obtained and a 5-point smoothing algorithm was applied. The values were corrected by setting samples containing only the respective enzyme(s) diluted with distilled water as a baseline.

5.3.6 SDS-PAGE

QBMS samples (diluted to 2 mg protein per mL solution) were analysed under reducing and non-reducing conditions according to the protocol of BioRad (2011) based on Laemmli (1970). Wells of 12% polyacrylamide Mini-PROTEAN® TGXTM Precast Protein Gels (Bio-Rad, Richmond, California) were loaded with 20 µL of sample and run at 120 V for 1 h at room temperature. Molecular weight markers (10–250 kDa) were run in parallel with the samples (Precision Plus Protein standards, All Blue, Bio-Rad, Richmond, California). Following electrophoresis, the bands were stained with the coomassie stain.

5.3.7 Protein solubility

The supernatants of each sample were analysed according to Kjeldahl as described above. Protein solubility was expressed as the percentage of protein content in the supernatant of the total protein content:

$$\text{Protein solubility [\%]} = \frac{\text{Protein content in supernatant [\%]}}{\text{Total protein content [\%]}} * 100$$

5.3.8 Surface hydrophobicity

Surface hydrophobicity (S_0) was measured according to Hayakawa and Nakai (1985) using 1-anilino-8-naphthalenesulfonate (ANS). The supernatants of QBMS samples were serially diluted with 0.01 M phosphate buffer (pH 7) with dilution factors ranging from 100 to 3200. Ten µL ANS (8.0 mM in 0.1 M phosphate buffer, pH 7) were mixed with 2 mL diluted sample. After 15 minutes in the dark fluorescence was measured ($\lambda_{\text{excitation}} = 390$ nm, $\lambda_{\text{emission}} = 470$ nm). The measured values were corrected by a blank measured without ANS, and standardized by a reading of methanol according to Wu, Hettiarachchy and Qi, (1998). The shown values represent the slopes ($R^2 \geq$

0.97) based on the plotting of corrected and standardized absorbance over the soluble protein concentration.

5.3.9 *Foaming properties*

Samples were frothed up using an Ultra-Turrax equipped with an S10N-10G dispersing element (Ika-Labortechnik, Janke and Kunkel GmbH, Staufen) at a high speed for 30 s. The height of the samples was measured over 1 hour. The foaming capacity was elaborated as sample expansion at 0 minutes, while foam stability was expressed as sample expansion after 60 minutes. Foam expansion was calculated according to the following equation:

$$\text{Foam expansion} = \left(\frac{\text{Increase of sample height}}{\text{Initial sample height}} \cdot 100 \right) - 100$$

5.3.10 *Suspension properties*

Stability was measured using an analytical centrifuge (LUMiSizer®, LUM GmbH, Berlin, Germany). The samples were treated at 1000 rpm for 30 minutes and subsequently at 3000 rpm for 60 minutes at 24 °C. The separation rate represents the slope in %·h⁻¹ and was determined by plotting the % of transmission over time, with an intercept set to 0. The sediment height and creaming [mm] were calculated by subtracting the position with ≤20% light transmission of the last profile from the cell bottom, or meniscus, respectively.

5.3.11 *Emulsifying properties*

In order to study the emulsifying properties lupin oil was added to the samples to reach a total fat content of 3.5% (w/w) to be comparable to full fat bovine milk. Samples were homogenised like described above. The pH was adjusted to 7 and the emulsifying properties of the samples were immediately determined using the LUMiSizer (L.U.M. GmbH, Germany) thereafter. Measurements were carried out with the same settings as for the suspension stability described above.

5.3.12 *Statistics*

All analyses were carried out at least in triplicate. Means were compared with one-way analysis of variance (ANOVA) and Tukey's pairwise

comparison test. The significance level was set to $\alpha = 0.05$. Linear correlation measurements of the results were performed using Pearson's correlation.

5.4 Results and discussion

5.4.1 Compositional analysis

The quinoa flour used for the preparation of the samples comprised of the following composition in g per 100 g: moisture: 9.6 ± 0.4 , protein: 14.64 ± 0.14 , ash: 2.59 ± 0.03 , fat: 7.24 ± 0.00 , total starch (dry basis): 67.4 ± 3.55 . Compositional analyses of the QBMS samples were not affected by protease treatment. Therefore, the following results refer to values obtained for samples treated solely with Hitempase. The prepared QBMS contained 1.01 ± 0.03 g per 100 g fat and 1.64 ± 0.01 g per 100 g protein, and contained 50% less protein and 71% less fat than cow's milk (Jeske *et al.*, 2017, Chapter 3). Compared to commercial plant-BMSs, the obtained QBMS contained more protein than 64% of PBMSs investigated by Jeske *et al.* (2017) (Chapter 3). Moreover, 76% of these tested substitutes had higher fat contents than the QBMS of this study. Water and ash contents were $88.97 \pm 0.14\%$ and $0.33 \pm 0.01\%$, respectively. Due to starch hydrolysis, the glucose content increased from $1.24 \pm 0.30\%$ to $5.55 \pm 0.33\%$ and the maltose content rose from $0.09 \pm 0.03\%$ to $1.04 \pm 0.08\%$ compared to a QBMS without Hitempase treatment. Neither sucrose nor fructose was found in any of the samples.

5.4.2 Structure and molecular changes

Degree of hydrolysis

Proteases cleave peptide bonds and have therefore a huge effect on all levels of protein structure. The DH displays changes regarding the primary structure, since it is measuring the degradation of the polypeptide chain. Results are shown in Table 5-2. Already the addition of Hitempase increased the value up to 3.97%. This could be due to the degradation of starch; the granules of quinoa starch are very small and can be packed in a tight complex with fibres and proteins, and additionally contain proteins within their membrane (Rayner *et al.*, 2012). Due to the starch degradation, proteins are released from interactions with the starch and become more accessible, and moreover, primary amino groups as seen during the considerable increase of the DH measured for samples only treated with Hitempase.

Table 5-2 Degree of Hydrolysis (DH), protein solubility, and surface hydrophobicity (S₀) results of QBMS samples.

Sample	Degree of Hydrolysis [%]	Protein solubility [%]	Surface Hydrophobicity [-]
Blank	-	48.02±0.77 ^a	19.01±0.94 ^a
Hitempase	3.97±0.98 ^a	56.93±1.13 ^b	25.08±1.54 ^{ab}
Profix 1x	5.29±1.81 ^a	58.39±2.12 ^{bc}	30.55±2.61 ^{bc}
Profix 10x	24.19±0.38 ^c	70.04±2.46 ^{efgh}	36.90±2.52 ^{cd}
Profix 25x	38.88±0.71 ^e	76.31±3.14 ⁱ	52.40±4.91 ^f
Profix 50x	43.79±0.78 ^{ef}	75.82±0.37 ^{hi}	55.75±5.10 ^f
Bioprotease 1x	14.78±1.20 ^b	64.37±1.10 ^{de}	36.39±2.04 ^{cd}
Bioprotease 10x	31.35±1.47 ^d	69.87±0.37 ^{efg}	48.77±3.34 ^{ef}
Bioprotease 25x	41.9±0.36 ^{ef}	70.37±0.51 ^{cd}	50.40±2.30 ^{ef}
Flavourzyme 1x	17.50±1.30 ^{bc}	62.22±0.52 ^{fgh}	32.48±1.19 ^{bc}
Flavourzyme 5x	23.36±30.29 ^e	64.02±2.55 ^{cd}	37.42±4.14 ^{cd}
Flavourzyme 10x	46.24±1.28 ^f	66.20±0.52 ^{def}	43.16±2.83 ^{de}
Combination 1	97.11±1.74 ^h	74.56±4.02 ^{ghi}	42.17±1.32 ^{de}
Combination 2	58.71±3.19 ^g	75.19±2.40 ^{ghi}	49.05±0.77 ^{ef}

Values within a column that share a superscript are not significantly different from one another (p < 0.05)

The use of proteases led to an increase of DH with increasing protease concentration. Flavourzyme and Bioprotease showed at the lowest added dosages a considerable increase, whereas Profix increased significantly just with higher dosage levels. All proteases reached a comparable DH with their highest dosage, being around 44%. The combination of all enzymes led to a remarkable DH of 97.11%, whereas the combination of only Profix and Bioprotease did not increase the DH to that extent (58.71%). A study by Clemente *et al.* (1999) based on chickpea hydrolysates showed similar results; flavourzyme was used in conjunction with Alcalase 2.4 L (consisting of a

serine peptidase, similar to Bioprotease); they found that both enzymes together were more successful to hydrolyse chickpea proteins. Flavourzyme consists of exoproteases, cleaving the terminal peptide bonds but leaves the internal structure of the protein intact. Profix and Bioprotease, on the other hand, consist of endoproteases, cleaving internal peptide bonds. However, the globular structure of the major protein in quinoa, chickpea or many other plant proteins possesses natural obstacles that can limit the action of proteases. The combination of Flavourzyme with Profix and Bioprotease facilitated the action of each protease to achieve a higher DH, then possible individually.

Circular dichroism

The secondary structure was studied using circular dichroism (CD). Due to different structural elements, the optical activity of amide groups is distinctive and results in characteristic CD spectra (Greenfield, 2007). As CD samples have to be of at least of 95% purity (Greenfield, 2007), the spectra for QBMS samples were analysed with the intention of comparing the curves rather than calculating a structural composition. The results for the CD measurements are shown in Figure 5-1. The y-axis shows ellipticity measured in millidegrees. The spectra of the blank, QBMS without the addition of any enzymes, is comparable to the ones investigated by Mäkinen *et al.* (2016) for quinoa protein isolates: a strong positive peak at 190–195 nm was exhibited followed by a broad negative peak with a minimum at 208 nm. They found that quinoa protein isolates were composed of α -helix (16.7%), β -sheet (30.4%), β -turn (17.2%) and random coil (35.7%). After the addition of the α -amylase Hitempase to the QBMS samples, the maximum of ellipticity was shifted to 185 nm and occurred much more strongly. This deduces proteins rearranged to a heavily α -helix based structure, which could be due to the aforementioned released proteins from interactions with starch. Furthermore, the proteases changed the secondary structure, due to the degradation to peptides. The application of Profix seemed to result in a complete loss of secondary structure, since the maxima and minima compared to the blank diminished and the crossover from positive and negative shifted to 190 nm. Similarly, the CD spectra of Bioprotease treated samples were considerably changed suggesting a loss in the secondary structure. Flavourzyme, on the other hand, seemed to have another impact on the protein structure: the positive maximum of ellipticity was shifted to a smaller wavelength,

the cross-over from positive to negative was shifted to a higher wavelength, passing it at 200 nm, followed by a less pronounced negative profile. Overall, the changes in the spectra show a loss in the secondary structures compared to the native quinoa protein, and depict the different effects of the enzymes on the protein structure.

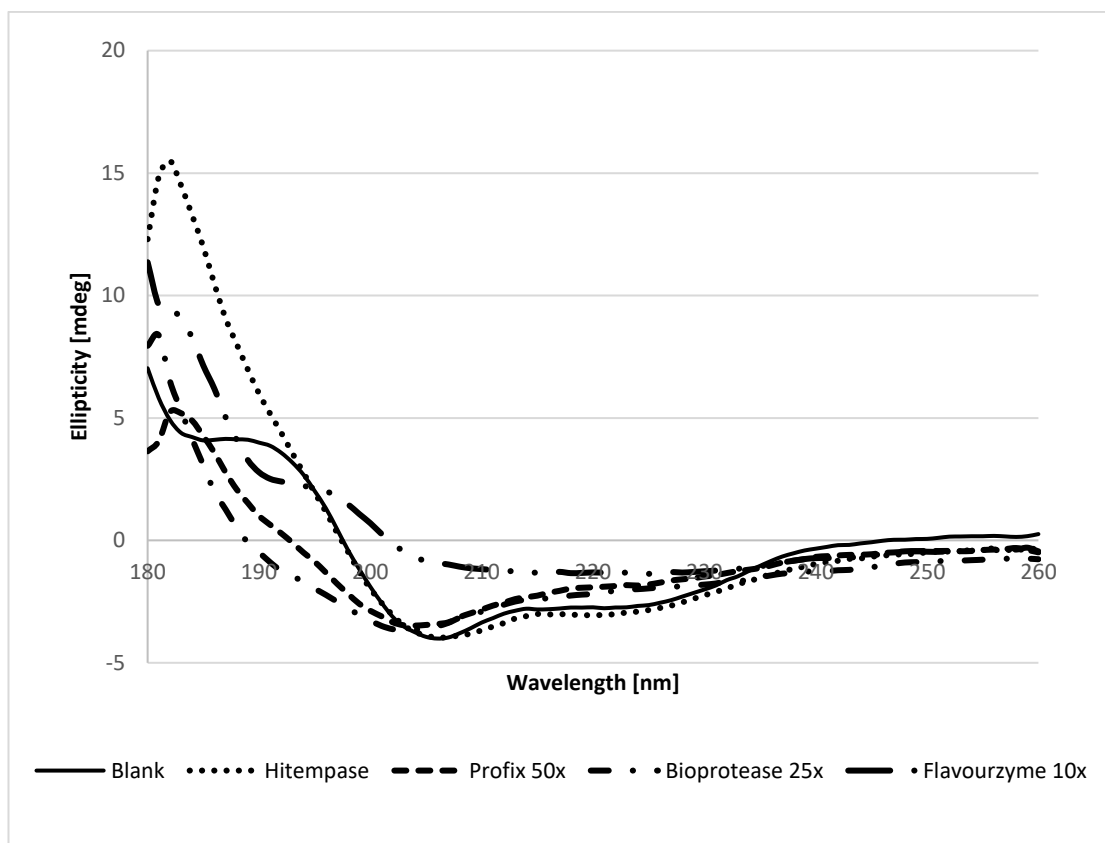


Figure 5-1 Circular dichroism spectra for QBMS samples.

SDS-PAGE

The molecular weight distribution was studied using SDS-PAGE. Figure 5-2 shows the gels of electrophoretic analysis under non-reducing and reducing conditions of QBMS samples. In the absence of DDT, major bands were found at ~49 and ~63 kDa (marked as “C”), whereas under reducing conditions these bands disappeared and bands at ~31, ~36 kDa, and ~22, ~23 kDa were detected, marked as “A” and “B”. In native quinoa protein, chenopodin subunits are connected by disulphide bridges, whereas under reducing conditions, acidic and basic subunits were found at 30–40 kDa and 20–25 kDa, respectively. Additionally, a chenopodin A–B can be observed at 50 kDa, which evaded the reducing conditions, and is marked as “E” in Figure 5-2. The

same pattern was found by Brinegar and Goundan (1993). With the treatment of different proteases, the molecular weight was decreased. In particular, for Profix and the enzyme combinations, most proteins were <25 kDa. Bioprotease and Flavourzyme on the other hand did not degrade the proteins to that extent;

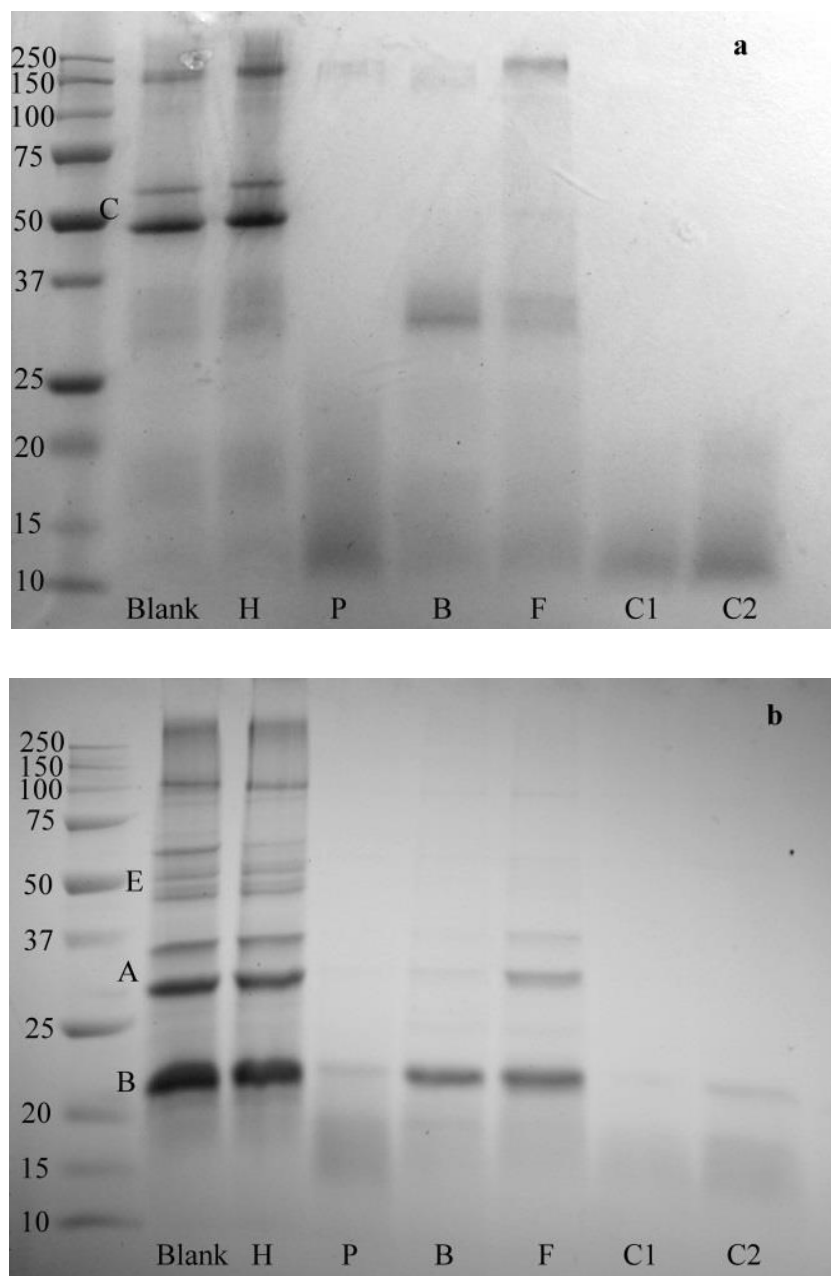


Figure 5-2 SDS-PAGE gels of QBMS, treated with different enzymes, under non-reducing (a) and reducing conditions with DTT (b), H=Hitempase, P=Profix 50x, B=Bioprotease 25x, F=Flavourzyme 10x, C1=combination 1, C2=combination 2.

for both of them the basic subunit of chenopodin (22 and 23 kDa) was detected, and for Flavourzyme, also the acidic subunit was clearly visible on the gels under reducing conditions, while the chenopodin mark was faded but noticeable indicating remnants of an intact higher structure. The α -amylase included in Hitempase did not alter the protein profile and no changes in the molecular weight were observed.

5.4.3 Physicochemical properties

One of the main changes caused by enzymatic hydrolysis is the alteration of the surface properties of proteins (Wang *et al.*, 2015). At large, owing to enzymatic cleavage, solubility increases with the amount of polar groups, while at the same time also hydrophobic sites which were hidden inside the core of the protein structure get exposed (Radha and Prakash, 2009), which has an impact on further properties and behaviours like foaming, and emulsifying properties.

5.4.3.1 Protein solubility

Solubility is one of the most important properties of proteins, since it is essential for many other functional properties, like emulsification, which in turn can contribute to a stable colloidal emulsion (Day, 2013). A high solubility is advantageous for the production of PBMSs also, since proteins are more stable in solution and the extraction of protein during the process increases. Applying proteases results in an increased solubility of proteins. Due to the breakdown of compact formations, hydrophilic parts get revealed, which are able to interact with water molecules (Nielsen, 1997). Table 5-2 shows the protein solubility of QBMS samples. The blank and the sample only containing Hitempase showed low solubilities with 48.02 and 56.93%. The highest protein solubility was obtained with Profix reaching 75.82% at the highest concentration, while the lowest dosage of Profix did not increase the protein solubility significantly compared to the Hitempase treated sample. These values reflect the degree of hydrolysis, showing the same trends. Indeed, these two measurements correlated (0.76, $p < 0.005$). Samples containing Flavourzyme and Bioprotease showed at the lowest concentrations a higher DH and also a considerable increase of protein solubility. However, at the highest concentrations the solubility was low for Flavourzyme, even though the DH was high. Clearly the working

mechanism of Flavourzyme, being an endoprotease, explains these results. Bioprotease and Profix, on the other side work internally at the core of the proteins, opening up the structure more. On that score, the DH is not increased to the highest extent, but the highest solubility is obtained. Protein solubility of the combinations were not significantly different from samples treated just with Profix, even though the DH was increased in the combinations. As seen on the SDS-PAGE gels, the molecular weight decreased the most for Profix treated samples followed by Bioprotease, and the least for Flavourzyme, which reflected in high protein solubilities for Profix and Bioprotease and the lowest for Flavourzyme.

5.4.3.2 Surface hydrophobicity

Strong correlations between solubility and S_0 were found (0.92, $p < 0.0005$). As shown in Table 5-2, S_0 of QBMSs increased after enzyme treatment. The initial value of the blank sample is low, but increases with the addition of Hitempase, like observed for solubility as well as DH. With increasing protease concentration, S_0 increases due to a more open, unravelled structure. This as a foundation explains the low S_0 obtained with Flavourzyme, whereas Profix and Bioprotease, which showed a huge decline of structure also showed higher values of hydrophobicity. Treatments with Bioprotease increased S_0 up to 50.40, while Profix treated samples even reached values of 55.75. Combining the enzymes did not lead to an increase of S_0 , but did not even reach the value of Profix treated samples.

5.4.3.3 Foaming properties

Foaming properties are generally related to the surface hydrophobicity of proteins, which migrate into an air–liquid interface. By doing so, the interfacial tension is reduced, improving or obtaining foam and emulsions (Nakai, 1983). Generally, foaming capacity decreased with the addition of proteases in this study and results are shown in Table 5-3. Blank and Hitempase treated samples showed the highest value with 44.93 and 46.38% respectively. The addition of Flavourzyme decreased the foaming capacity initially slowly but with increasing concentration, the capacity was the lowest for Flavourzyme with 28.99%. The foaming capacity of Bioprotease and Profix treated samples did not differ significantly regardless of the concentration. On the other hand, foaming stability showed no significant changes between the individual

proteases, compared to the Hitempase treated samples. The blank sample, without any enzymes showed the highest foaming stability, due to the starch, whereas the combinations of enzymes decreased the foaming stability to the lowest values. Unlike reported in other studies (Nakai, 1983; Aluko and Monu, 2003), the hydrolysis of proteins did not improve their ability to form interfacial layers around air bubbles. Even at a low DH, with Profix 1× for example, no improvement of foam capacity or stability was achieved. Proteins are an important stage in the vast system of food, giving a structure and acting as an important framework. However, interactions and other factors play a crucial role and may interfere with the role of proteins.

5.4.3.4 Suspension and emulsifying properties

As PBMSs are suspensions of disintegrated and dissolved plant material, suspension stability is often a problem. The sedimentation effect is common for these products and was observed by Durand, Franks and Hosken (2003) for oat- and rice-BMSs and for many other commercial plant-BMSs analysed by Jeske, Zannini and Arendt, (2017) (Chapter 3). Figure 5-3 depicts exemplary typical transmission profiles of different samples measured by using an analytical centrifuge. These graphics reveal velocity and markedness of separation. The profiles change from red to green during the measurement, thereby visualizing the process of separation. Sedimentation occurred for all samples during the measurement, which can be seen from the high transmission at the top of the sample cell for the last measurement (green line). Profiles of the α -amylase-treated sample (Hitempase) showed fairly steady separation over time and resulted in clearly separated sediments. The protease-treated samples showed different profiles and were more stable at the beginning of the measurement. Bioprotease-treated samples showed profiles, which indicate a separation of particles, both on the top, and on the bottom of the cuvette (compare orange profiles in Figure 5-3). Transmission profiles for Profix- and Flavourzyme treated samples evidenced a less clear separation when compared to the sample without proteases. However, in the end of the measurements, all samples showed dense sediments in the lower part of the cuvette and a clear supernatant above. The sediment heights at the end of the stability measurement of the QBMS samples were neither significantly different among different enzymes nor for different concentrations, as shown in Table 5-3. Only for the blank, a significantly

greater sediment height value was determined, probably due to big intact starch molecules. No creaming was detected, which is in accordance with the findings of Durand, Franks and Hosken (2003). Creaming was shown for cow's milk and a soya-BMS but was less for oat- and rice-BMSs since they contain less fat (3.3 and 3.4% compared to 2.0 and 1.1%, respectively). QBMS was similarly low in fat (1.01%). By adding the oil for the emulsifying measurements the separation rate and sediment height were decreased for almost all the samples significantly, while also a creaming layer was obtained. It is noteworthy that the different enzyme concentrations showed no significant impact on the creaming and sediment height, and just little differences were found concerning the separation rate without any trend. The markedness and velocity of sedimentation or creaming depend on the particle size, viscosity of the continuous phase, and the density difference between the particle and the continuous phase (Dickinson and Miller, 2001). Bernat *et al.* (2015) prevented the phase separation of almond- and hazelnut-BMSs by both homogenising at 1720 bar and low heat treatment, as this combination resulted in a reduction of particle size. Rosenthal *et al.* (2003) increased the product stability of a soya-BMS not merely by cellulase treatment but also by filtration. The sedimentation of the QBMS samples is probably driven by big particles of undissolved plant material, since no filtration step was applied and sedimentation occurred for all samples. However, samples treated with proteases showed different profiles and appeared to be more stable during the first step of centrifugation at a lower gravitational force. A combination of protease treatment and filtration or homogenisation at higher pressures seems therefore to be promising to improve the stability further.

Table 5-3 Foaming properties, and suspension stability results of QBMS samples.

Sample	Foaming Capacity [%]	Foaming Stability [%]	Separation rate [%/h]	Sediment height [mm]	Emulsifying Separation rate [%/h]	Emulsifying Sediment height [mm]	Emulsifying Creaming height [mm]
Blank	44.93±5.47 ^d	75.70±4.63 ^{ef}	52.29±0.29 ^a	6.65±0.08 ^c	49.13±2.73	4.44±0.11	1.20±0.62
Hitempase	46.38±3.32 ^d	67.08±2.31 ^{cdef}	61.62±0.10 ^e	4.69±0.16 ^{ab}	51.08±2.36	4.51±1.54	1.50±0.73
Profix 1x	36.23±2.51 ^{abc}	78.20±3.43 ^f	61.86±0.60 ^e	5.08±0.17 ^b	45.95±2.98	3.75±0.42	0.95±0.16
Profix 10x	40.22±1.26 ^{cd}	67.15±6.30 ^{cdef}	59.95±1.44 ^{de}	4.92±0.05 ^{ab}	46.16±2.89	3.87±0.51	1.21±0.61
Profix 25x	34.06±3.32 ^{abc}	64.19±5.01 ^{bcd}	59.28±0.63 ^{cde}	5.10±0.02 ^b	45.20±0.86	3.46±0.29	1.03±0.04
Profix 50x	39.13±2.17 ^{bcd}	61.24±3.41 ^{abcd}	56.62±1.29 ^{bc}	5.22±0.18 ^b	47.23±1.71	3.44±0.28	0.94±0.12
Bioprotease 1x	39.49±1.66 ^{bcd}	69.79±3.99 ^{def}	61.65±0.50 ^e	4.86±0.15 ^{ab}	49.87±1.09	3.68±0.33	1.35±0.39
Bioprotease 10x	39.13±0.00 ^{bcd}	61.67±2.00 ^{abcd}	53.12±1.41 ^a	4.87±0.26 ^{ab}	51.04±0.94	3.53±0.06	0.80±0.29
Bioprotease 25x	36.96±0.00 ^{bc}	65.79±3.28 ^{cde}	52.44±2.02 ^a	4.72±0.34 ^{ab}	49.00±0.99	4.19±0.09	1.00±0.13
Flavourzyme 1x	41.30±2.17 ^{cd}	71.34±3.35 ^{def}	60.06±0.76 ^{de}	4.94±0.14 ^{ab}	47.75±2.86	3.36±0.36	0.91±0.23
Flavourzyme 5x	36.96±3.77 ^{bc}	67.59±6.99 ^{def}	57.68±0.09 ^{bcd}	4.80±0.14 ^{ab}	48.50±3.25	3.42±0.07	0.96±0.13
Flavourzyme 10x	28.99±1.20 ^a	55.63±4.90 ^{abc}	53.30±1.89 ^a	5.06±0.09 ^b	50.84±0.56	3.43±0.07	1.13±0.43
Combination 1	32.61±2.17 ^{ab}	51.11±1.92 ^a	54.69±0.21 ^{ab}	4.41±0.52 ^a	46.69±0.27	4.56±1.43	0.96±0.26
Combination 2	32.61±2.51 ^{ab}	52.38±4.12 ^b	55.45±0.05 ^{ab}	4.70±0.17 ^{ab}	46.81±2.42	4.26±1.40	0.95±0.10

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$)

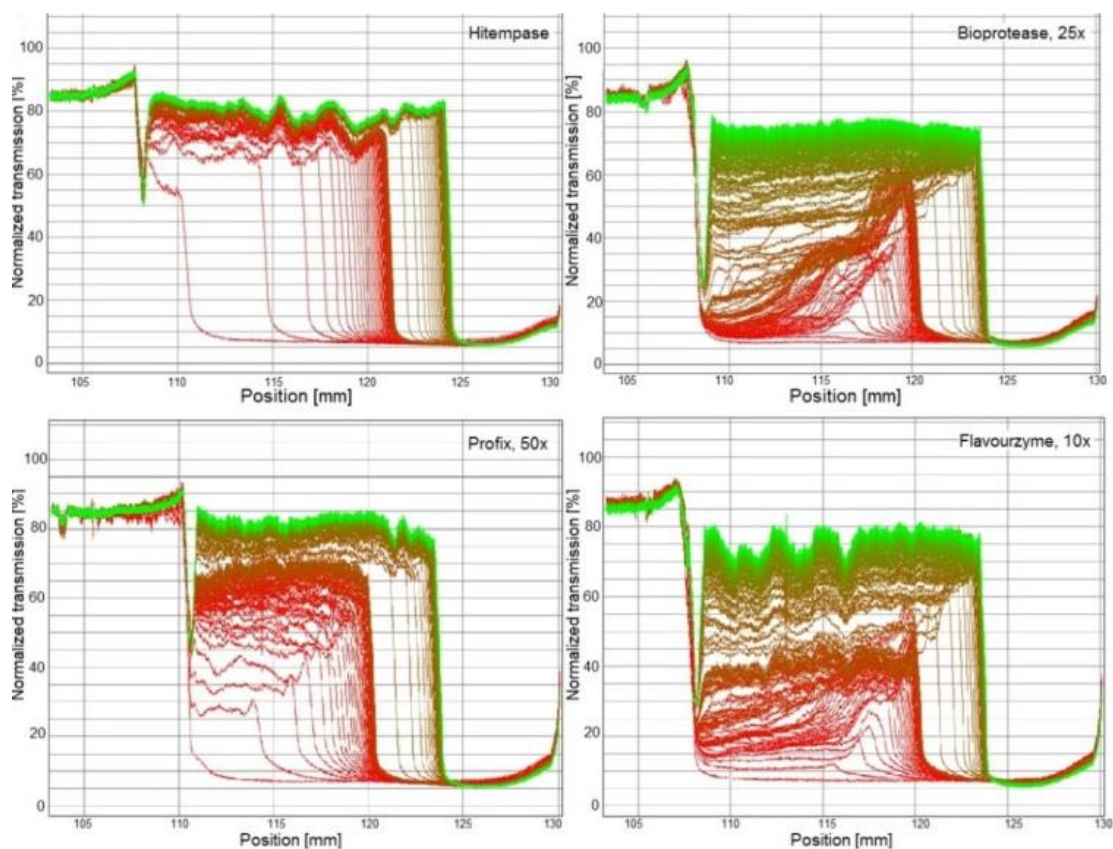


Figure 5-3 Transmission profiles of Hitempase-treated sample and samples treated with highest concentrations of Bioprotease, Profix and Flavourzyme.

5.5 Conclusion

The present study showed how amylolytic and proteolytic enzymes influence the product quality of a quinoa-BMS, as well as how important the choice and dosage of proteases are in order to achieve desired properties. While Flavourzyme showed the highest DH, changes in the secondary structure, and the molecular weight were not as high as with the others. Even at a lower DH, Profix and Bioprotease had a greater impact on the protein than Flavourzyme. Furthermore, this study showed that even by altering the protein properties by hydrolysis, the functionalities of the quinoa protein remained limited, as seen in the emulsifying or foaming properties. This publication adds to the knowledge available on plant protein modification and their properties in a plant-BMS. Jeske, Zannini and Arendt (2017) (Chapter 3) showed that the market for dairy substitutes is growing fast, but the product quality of most commercial products is poor. This is often overcome by the addition of additives like emulsifiers and stabilisers rather than exploring the full potential of the raw material and its compounds.

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Chapter 6: Membrane filtration and isoelectric precipitation technological approaches for the preparation of novel functional and sustainable protein isolates based on lentils

Alonso-Miravalles L. and Jeske S., Bez J., Detzel A., Busch M., Krueger M., Wriessnegger C.L., O'Mahony J.A., and Arendt E.K.

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6.1 Author Contributions

Stephanie Jeske and Loreto Alondo Miravales were the main authors for the paper, contributing to the conception and performance of all the laboratory work and experiments. Stephanie Jeske carried out the experiments on the composition, protein solubility, water holding capacity, protein secondary structure, hydrophobicity, sulfhydryl groups, foaming and gelation characteristics.

Jürgen Bez provided the protein isolates, which were produced at the Fraunhofer Institute for Process Engineering and Packaging, Freising, Germany. He also critically reviewed the final manuscript.

Andreas Detzel, Mirjam Busch, Martina Krüger, and Clara Larissa Wriessnegger conducted the life cycle assessment. They also critically reviewed the final manuscript.

Prof Elke K. Arendt and Dr. James A. O'Mahony helped with the conception of the paper as well as making contributions and suggestions throughout the experimental process and supervised the project. They also critically reviewed the final manuscript.

6.2 Abstract

Isoelectric precipitation and ultrafiltration processes were both investigated for their potential to produce protein products from brown lentils. Higher protein concentrations were obtained when ultrafiltration was used (>90%), whereas the lentil protein isolates obtained by isoelectric precipitation had significantly higher contents of dietary fibre and some minerals (i.e., sodium and phosphorus). They showed similar physical properties with similar particle sizes ($D_{4,3}$ ~30 μm) and approximately spherical shape as assessed by laser diffraction and scanning electron microscopy. Differences in the functional properties between the two ingredients were found as the isoelectric precipitated ingredient showed lower protein solubilities over the investigated pH range (from 3 to 9) which can be linked to the slightly higher hydrophobicity values (2688.7) and total sulfhydryl groups (23.9 $\mu\text{M/g}$ protein) found in this sample. In contrast, the protein ingredient obtained by ultrafiltration was superior with regard to its solubility (48.3%; pH 7), fat-binding capacity (2.24 g oil/g protein), water holding capacity (3.96 g water/g of protein) and foam-forming capacity (69.6%). Also, marked differences in the gelling properties were found as the ultrafiltrated lentil protein ingredient showed better ability to form a heat-induced gel with less protein concentration required (11% w/w). The assessment of the environmental performance showed that both LPIs exhibited promising properties especially when compared to traditional cow's milk proteins. Carbon footprints of LPIs were lower than cow's milk proteins and soya protein isolates, therefore, contributing to the reduction of greenhouse gases. The results obtained in this study suggest potential to design novel and sustainable plant-based food prototypes with target functional properties.

6.3 Introduction

The expected continued growth of the global population to 9.6 billion people by 2050 is creating a need to identify and develop solutions for the provision of high-quality food (United Nations, 2015; Henchion *et al.*, 2017). In addition, the high demand for healthy, sustainable and cost-effective food protein ingredients by consumers is driving the investigation of new and innovative protein sources (Day, 2013; Don, 2017). Agriculture is one of the main contributors to climate change, and cattle farming faces particular sustainability challenges (Poore and Nemecek, 2018). The conversion factor of feed protein to milk protein is about 14%, while the remaining 86% is “lost” for human nutrition (Shepon *et al.*, 2016). Plant-based protein ingredients can serve as an alternative to animal-derived protein, due to their contribution to environmental sustainability, their role in addressing food security challenges and their cost-effectiveness (Aiking, 2011). However, replacing animal-based protein ingredients with those of plant origin is not easy, as significant differences exist between ingredients from both sources in composition, taste, digestibility and techno-functional properties. Nevertheless, research is advancing and several plant ingredients have been applied in a wide range of products. For instance, using extrusion, soya protein was processed into a highly fibrous texture simulating that of meat (Lin *et al.*, 2002), bread quality and nutritional profile was improved with the addition of fermented faba bean (Coda *et al.*, 2017), and sensory evaluation of a strawberry flavoured lupin-based yogurt-like product showed good sensory properties (Jiménez-Martínez *et al.*, 2003b).

In that respect, legumes are gaining increased attention, as they contain high amounts of protein, typically ranging between 20 and 40%, and are rich sources of essential amino acids such as lysine (Duranti 2006; Boye *et al.* 2010). Traditionally, they are consumed as whole, split or milled products (Jarpa-Parra *et al.* 2015; Joshi *et al.*, 2017) and approximately 5,481,120 ha are harvested and 6,315,858 tonnes of lentils are produced globally each year (FAOSTAT, 2016). Lentil seeds are showing promising results for the preparation of protein flours, concentrates and isolates due to the lack of allergens and anti-nutritional compounds (e.g., isoflavones found in soya) and also as they are an affordable, sustainable and abundant raw material (Karaca *et al.*, 2011). Various techniques and approaches such as wet fractionation (e.g., ultrafiltration and isoelectric precipitation) are used to separate and concentrate

high levels of protein from other constituents (Arntfield and Maskus, 2011; Dijkstra *et al.*, 2003) in cereals and legumes. The physicochemical properties and functionality of these isolated protein ingredients are essential in the processing and formulation of food products, providing texture, taste and nutrition for a desirable and pleasurable product. These properties depend not only on the nature of the protein but also on the processing and isolation techniques used. Most studies focused on this subject have been conducted on dairy and soya (Nishinari *et al.* 2014; Fox *et al.* 2015), and increasingly also on legumes in recent years (Boye, Zare, *et al.*, 2010; Joshi *et al.*, 2011; Papalamprou *et al.*, 2009; Peng *et al.*, 2016; Sánchez-Vioque *et al.*, 1999). The results of these studies indicate that the methods applied for isolation affect the composition and the physicochemical characteristics of extracted protein ingredients.

The aim of this work was to produce novel lentil protein isolates using two different technological approaches and to study the techno-functional properties (e.g., solubility, emulsifying, gelling properties) and environmental sustainability (life cycle assessment) of the ingredients based on the same raw material. The results obtained in this study will provide much needed information about the sustainability of the two different approaches and potential applications of the resultant ingredients in the development of novel, healthy and sustainable food product formulations.

6.4 Materials and Methods

6.4.1 Raw materials and chemicals

For extraction of lentil proteins, brown lentils of commercial quality (*Lens culinaris* cv. *Itaca*), provided by Agroservice Spa (San Severino Marche, Italy), were used as raw material. All chemicals used were purchased from Sigma-Aldrich (St Louis, Missouri, USA), unless otherwise stated.

6.4.2 Preparation of protein isolates

Lentil seeds were dehulled in an underrunner disc sheller (Streckel & Schrader GmbH, Germany) and the kernels and hulls were separated in an air classifier (Turboplex, Hosokawa Alpine AG, Augsburg, Germany). Kernels were milled using an impact mill (UPZ, Hosokawa Alpine AG, Germany) to a mean particle size (D50) of 21 μm . For extraction of protein, lentil flour was suspended in water at pH 7.5 to extract the high molecular weight proteins. The insoluble dietary fibre and lentil starch were then separated from the soluble high molecular weight proteins by decanting. Lentil protein isolate (LPI) was recovered from the resulting protein extract either by isoelectric precipitation (IEP) or by ultrafiltration (UF), as shown in Figure 6-1. LPI-IEP was isolated from the aqueous protein extract by acid precipitation at pH 4.5, which coincides with minimum solubility of lentil proteins (Johnston *et al.*, 2015). Subsequently, the precipitated proteins were separated in a disc separator and the sediment was neutralized with 3 M NaOH, pasteurised (65 °C, 30 min) and spray dried (T_{in} : 180°C, T_{out} : 75°C) to obtain the protein isolate powder. LPI-UF was extracted at 50 °C using a polysulfone membrane with a molecular weight cut-off of 10 kDa followed by diafiltration with demineralized water (retentate:water 1:1.7) to enrich the protein content of the retentate. The resulting retentate was pasteurized (65 °C, 30 min) and spray dried (T_{in} : 180°C, T_{out} : 75°C). The protein isolates were stored at room temperature until further analysis.

6.4.3 Compositional analysis

Total nitrogen content of the LPIs was analysed according to the Kjeldahl method (MEBAK 1.5.2.1) using a nitrogen-to-protein conversion factor of 6.25. Fat content was measured following the Soxhlet method (AACC Method 30-25.01). Ash content was determined by dry ashing in a muffle furnace at 500 °C for 5 h (AOAC

923.03). Moisture was determined by oven drying at 103 °C for 5 h (AOAC 925.10). Total starch (AOAC Methods 996.11 and AACC Method 76-13.01) content was determined using an enzymatic kit (Megazyme, Bray, Co. Wicklow, Ireland). Minerals were analysed using inductively coupled plasma-optical emission spectrophotometry (Neubauer, 2008). The soluble and insoluble fibre content of the samples was analysed in accordance with the AOAC method 991.43.

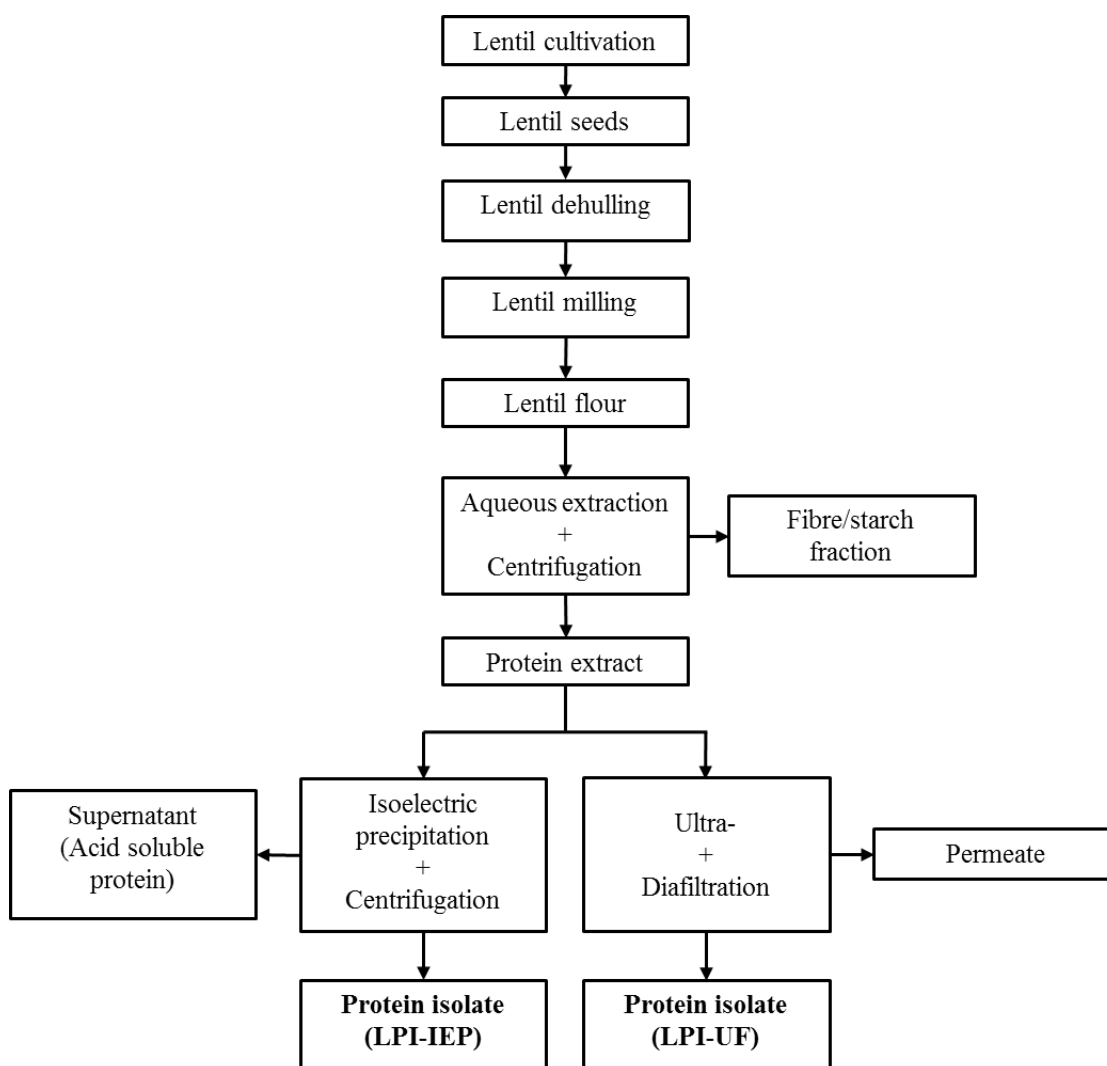


Figure 6-1 Preparation of lentil protein isolates from *Lens culinaris* cv. *Itaca* in pilot-scale.

6.4.4 SDS-PAGE

Protein profile was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using precast gels (Mini-PROTEAN TGX, Bio-Rad Laboratories, CA, USA) under non-reducing and reducing conditions as described by

Alonso-Miravalles & O'Mahony (2018). The sample loading buffer contained 65.8 mM Tris-HCl (pH 6.8), 26.3% (w/v) glycerol, 2.1% SDS and 0.01% bromophenol blue. The running buffer (10x Tris/Glycine/SDS, Bio-Rad Laboratories, CA, USA) had a composition of 25 mM Tris, 192 mM glycine and 0.1% SDS (w/v), pH 8.3. The staining solution used was Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, CA, USA). The target final protein concentration was 1 mg/mL and 8 μ L of sample solution was loaded into each well of the gel and the gels were run at a constant voltage of 150 V.

6.4.5 Protein secondary structure

Information about secondary structure of the proteins was obtained using circular dichroism (CD) spectrophotometry (Chirascan, Applied Photophysics, Leatherhead, UK). Protein solutions of 1 mg/mL were prepared in 10 mM sodium phosphate buffer (pH 7) and solubilized overnight at 4 °C using magnetic stirring at 250 rpm. Subsequently, samples were filtered (0.25 μ m) and the CD spectra was measured with a path length of 0.1 mm in the range 180-260 nm at a spectral resolution of 1 nm and data acquisition rate of 1 point/s. The average of three spectra was obtained and a 5-point smoothing algorithm was applied.

6.4.6 Scanning electron microscopy

Protein powders were mounted on aluminium stubs using double-sided adhesive carbon tape, and sputter coated with a 5 nm layer of gold/palladium (Au:Pd = 80:20) using a Quorum Q150R ES Sputter Coating Unit (Quorum Technologies Ltd., Sussex, UK). The coated samples were loaded into a sample tube and examined using a JSM-5510 scanning electron microscope (JEOL Ltd, Tokyo, Japan), operated at an accelerating voltage of 5 kV.

6.4.7 Particle size distribution

Particle size distribution of protein dispersions was measured using static laser light diffraction (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK). For the preparation of samples, the protein isolate powders were mixed with ultrapure water at a concentration of 1% protein (w/v), pH adjusted to 7, and stirred overnight at 4 °C. The refractive index of protein was set at 1.45 (Johnston *et al.*, 2015) and the absorption and dispersant refractive indices used were 0.1 and 1.33,

respectively. LPI dispersions, equilibrated at 22 °C, were introduced into the dispersing unit using ultrapure water as dispersant until a laser obscuration of 12% was achieved.

6.4.8 Surface hydrophobicity

Surface hydrophobicity (S_0) of protein particles was measured according to Hayakawa and Nakai (1985) using 1-anilino-8-naphthalenesulfonate (ANS) with slight modifications as described by Karaca *et al.* (2011). Protein solutions were serially diluted with 10 mM phosphate buffer (pH 7) ranging from 0.0006–0.015% (w/v). ANS (10 μ L; 8.0 mM in 0.1 M phosphate buffer, pH 7) were mixed with 2 mL of diluted sample and left in darkness for 15 min. Fluorescence was measured ($\lambda_{\text{excitation}}$ 390 nm, $\lambda_{\text{emission}}$ 470 nm) and corrected by a blank measured without ANS. The results are presented as the slopes ($R^2 \geq 0.98$) of the absorbance versus protein concentration.

6.4.9 Sulfhydryl groups

Sulfhydryl groups were determined using Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid) according to the method of Van der Plancken *et al.* (2005). The protein samples were diluted to 2 mg/mL with 10 mM phosphate buffer (pH 7) for free sulfhydryl groups, while for total sulfhydryl groups a buffer containing 6 M urea and 0.5 M SDS was used. Ellman's reagent (80 μ L) was added to 2.5 mL of diluted sample and absorbance was measured at 412 nm after 15 min. For the reagent blank, the protein samples were replaced by the sodium phosphate buffer and mixed with 80 μ L of Ellman's reagent. Sulfhydryl groups were quantified as follows:

$$\mu\text{m SH/g protein} = (A_{412} - A_{412B}) \cdot \frac{1,000,000}{\epsilon} \cdot C$$

where A_{412} is the absorbance at 412 nm, A_{412B} is the absorbance at 412 nm for the blank, ϵ is the extinction coefficient, which was taken as 13,600 $\text{M}^{-1} \text{cm}^{-1}$, and C is the protein concentration in mg/mL of the diluted sample.

6.4.10 Protein solubility

The solubility of proteins as influenced by pH, was determined by adjusting the pH of protein dispersions from 3.0 to 8.0 at 0.5 unit intervals using 0.1 and 1 M HCl

or NaOH. Protein samples (1% w/v) were hydrated at 4 °C. The pH was re-adjusted before measurements. Samples were centrifuged at 5,000 g for 30 min. The protein contents of the supernatants were analysed using the Kjeldahl method as described in Section 2.3. The results were expressed as % of the total protein content.

6.4.11 Zeta potential

The zeta potential of protein solutions at the same pH values as for protein solubility analysis were determined using a Zetasizer nano-Z (Malvern Instruments Ltd; UK). Samples were prepared as described for the protein solubility, excluding the centrifugation step, and diluted with ultrapure water to a concentration of 0.1% (w/v) and pH was readjusted. The measurement was performed using an automatic voltage selection and zeta potential was calculated using the Smoluchowski model. Refractive and absorption indices of 1.45 and 0.001 were used, respectively.

6.4.12 Water holding capacity

Analysis of water holding capacity (WHC) of proteins was determined according to AACC method 56-30.01 with some modifications. Samples ($1.000 \text{ g} \pm 0.005 \text{ g}$) were mixed with 30 mL of distilled water using an Ultra-Turrax equipped with a S10N-5G dispersing element (Ika-aborstechnik, Janke and Kunkel GmbH, Staufen, Germany) for 15 s and then shaken for 30 min at 1,000 rpm using a platform shaker (UNI MAX 1010, Heidolph, Schwabach, Germany). Subsequently, the mixture was centrifuged at 2,000 g for 10 min. WHC was expressed as grams of water retained per gram of protein isolate.

6.4.13 Fat absorption capacity

Fat absorption capacity (FAC) was determined following the method described by Boye *et al.* (2010) with slight modifications. Powder (1 g) and sunflower oil (6 g) were weighted into a 15 mL centrifuge tube (Sarstedt, Nümbrecht, Germany), mixed with a vortex for 3 min and centrifuged at 4,000 g for 30 min. The oil was removed from the tube carefully and weighed again. FAC was expressed as grams of fat retained per gram of protein isolate.

6.4.14 Foaming properties

Protein dispersions (20 mL) with a protein concentration ranging from 0.1 to 3.3% (w/v) in ultrapure water were frothed using an Ultra-Turrax equipped with a S10N-10G dispersing element (Ika-Labortechnik, Janke and Kunkel GmbH, Staufen) at high speed for 30 s. The height of the sample (liquid and foam phase) was measured over 60 min. The foaming capacity was taken as sample expansion at 0 min, while foam stability was expressed as sample expansion after 60 min. Foam expansion was calculated according to the following equation:

Foam expansion

$$= \frac{\text{Sample height after foaming} - \text{Initial sample height}}{\text{Initial sample height}} \cdot 100$$

6.4.15 Emulsifying properties

Protein solutions (1%, w/v) were hydrated with ultrapure water using a magnetic stirrer at 250 rpm overnight at 4 °C and pH 7. The next day samples were adjusted to room temperature and the pH was re-adjusted if necessary and pre-emulsions were prepared as follows: 20 mL of sunflower oil was added to 180 mL of 1% protein (w/v) solution and homogenized for 3 min at 10,000 rpm using an ultraturrax (T 25 digital Ultra-Turrax, Staufen, Germany). Emulsifying activity (EAI) and stability (ESI) indices were determined using the method described by Pearce and Kinsella (1978), with slight modifications. In brief, 250 µL emulsion were taken from the bottom of the homogenized sample after 0 and 120 min and diluted (1:100, v/v) in 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance at a wavelength of 500 nm was read using a spectrophotometer. EAI and ESI were calculated using the following equations:

$$EAI \left(\frac{m^2}{g} \right) = \frac{2 \cdot 2.303 \cdot A_0 \cdot DF}{C \cdot \theta \cdot l}$$

$$ESI (min) = \frac{A_0}{A_0 - A_{120}} \cdot 120$$

where DF is the dilution factor (100), C is the initial concentration of protein (g/m³), θ is the fraction of oil used to form the emulsion (0.1), l, is the cuvette length (m) and

A_0 and A_{120} are the absorbance of the diluted emulsion at 0 and 120 min, respectively.

6.4.16 Heat induced gelation characteristics

Least gelling concentration

The least gelling concentration (LGC) is defined as the lowest concentration required to form a self-supporting gel. The LGC test was performed according to the method of Sathe *et al.* (1982) with some modification. LPI dispersions ranging from 6 to 16% (w/v) were prepared in 0.01 M phosphate buffer at pH 7.0. These suspensions in 15 mL test tubes (Sarstedt, Nümbrecht, Germany) were heated in a water bath at 90 °C for 30 min, after which they were cooled rapidly under running water and stored at 4 °C overnight. LGC was determined visually as the minimum concentration of protein at which the contents of the tube did not flow.

Texture profile analysis

Texture profile analysis (TPA) of LPI gels was performed using a TA.XT Plus™ texture analyser (Stable Microsystems Ltd., Crawley, UK) to determine their mechanical properties. Protein gels (25%; w/v) were prepared by heating LPI dispersions as described above. Gels were cut into small cylinders of 8.2 mm in diameter and 8.0 mm in height. The gel pieces were compressed twice to 30% of their original height at a constant speed of 0.3 mm/s using a cylindrical probe with 20 mm diameter. The TPA parameters of hardness, cohesiveness, adhesiveness, gumminess and springiness were calculated according to the definitions of Bourne (2002).

6.4.17 Life cycle assessment

Environmental performance of LPIs was examined by means of life cycle assessment (LCA) using Umberto 5.5 software. LCA is carried out as an attributional cradle-to-gate LCA and includes the individual processes associated with LPIs shown in Figure 6-1. Impact assessment methods are based on Umweltbundesamt Berlin (2016).

6.4.18 Statistical analysis

All analyses were carried out in triplicate, with exception of analyses of fibre and minerals, which are performed following a validated method and therefore analysed just once and reported without standard deviation. All other data generated was subjected to student's T-test to determine statistically significant differences ($p < 0.05$) between mean values for the different samples, at a 95% confidence level. The statistical program used was Excel (Microsoft Office 365 ProPlus, version 1809).

6.5 Results and Discussion

6.5.1 Compositional analysis

The macro- and micro-nutrient composition of the LPIs is shown in Table 6-1. The protein content of LPI-UF (93.7%) was significantly higher ($p < 0.05$) than that obtained for LPI-IEP (85.1%). The reason for this higher protein content can be explained by the ultrafiltration process, where specific pore sized membranes are used leading to higher protein levels in the final ingredient (Boye *et al.* 2010). Additionally, by diafiltration more soluble substances (e.g. sugars, minerals) permeate the membrane thereby further purifying the protein. Regarding the fat content, no significant differences ($p < 0.05$) were found between LPI-UF (4.40%) and LPI-IEP (4.49%). The ash content for LPI-IEP (5.46%) was significantly higher than for LPI-UF (3.51%) which was expected since, with the former approach, NaOH and HCl are used to solubilize and precipitate the proteins (Arntfield and Maskus, 2011); this can be seen in the determined sodium content of LPI-IEP. Interestingly, high values of magnesium and calcium were obtained in LPI-UF. An explanation for these high values might be the retention of these minerals in the retentate along with the protein during the UF process. These differences in the mineral profile can play an important role in the functionality of these protein ingredients such as the solubility, emulsifying and gelling properties (Foegeding and Davis, 2011). For example, in dairy proteins, especially caseins, calcium plays an important role in determining their gelation behaviour, facilitating linkages between proteins (Farrell *et al.*, 2002). Also, other authors have studied the effect of calcium on the gelation properties of a soya drink, finding coagulation of soya proteins when the ionic calcium concentration was increased (Pathomrungsinyounggul *et al.* 2010). The fibre content, mostly soluble dietary fibre, was higher in the LPI-IEP (1.8%) than in LPI-UF (<0.1%). A reason for the higher fibre content in LPI-IEP could be that a part of the fibres were precipitated together with the protein and/or were only partially removed by the centrifugation step; the lower protein content of LPI-IEP is an indicator of this.

Table 6-1 Macro- and micro-nutrient composition of lentil protein isolates obtained by ultrafiltration (LPI-UF) or isoelectric precipitation (LPI-IEP).

Composition [g/100 g]	LPI-UF	LPI-IEP
Protein	93.7 ± 0.34 ^a	85.13 ± 0.76 ^b
Fat	4.40 ± 0.13 ^a	4.49 ± 0.37 ^a
Starch	*N.D.	*N.D.
Moisture	5.63 ± 0.02 ^a	4.87 ± 0.08 ^b
Ash	3.51 ± 0.11 ^a	5.46 ± 0.04 ^b
Insoluble dietary fibre	<0.1	<0.1
Soluble dietary fibre	<0.1	1.8
Minerals (mg/kg)		
Chlorine	2.0	2.4
Sodium	1300	11000
Zinc	57	48
Calcium	2200	710
Magnesium	2300	750
Iron	150	170
Phosphorous	6100	9400

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$). *N.D. = Not Detected

6.5.2 Structural properties

SDS-PAGE

SDS-PAGE analyses under non-reducing and reducing conditions of the two LPIs are shown in Figure 6-2. Both samples showed similar protein profiles, with several common bands under non-reducing and reducing conditions. Proteins with molecular weight (MW) of ~50, ~37 and ~20 kDa under non-reducing conditions were observed. The bands at MW ~50 kDa may correspond to vicilin subunits, which composes a 7S trimeric protein, one of the major globulins, together with legumin found in many pulses. Each trimer of vicilin has a MW of 150 kDa without disulphide bridging (Dagorn- Scaviner, Gueguen, & Lefebvre, 1987; Oomah *et al.*, 2011). The bands at 37 and 25 kDa correspond to the acidic and basic subunits of

legumin, in accordance with previous studies (Ladjal-Ettoumi *et al.*, 2016; Barbana and Boye, 2011). Legumin, an 11S globulin, is an hexameric protein formed by subunits with MW ~60 kDa, which consist of an acidic (~40 kDa) and a basic (~20 kDa) subunit linked by disulphide bonding (Jarpa-Parra *et al.*, 2015; Joshi *et al.*, 2011). Under reducing conditions, similar profiles were observed, although bands at 37 and 25 kDa were slightly more intense, with the disappearance of some high MW bands at ~50 kDa. This can be correlated with the dissociation of legumin into its acidic (MW ~40 kDa) and basic (~20 kDa) subunits by the dissociation of the disulphide bond when a reducing agent (DTT) is applied.

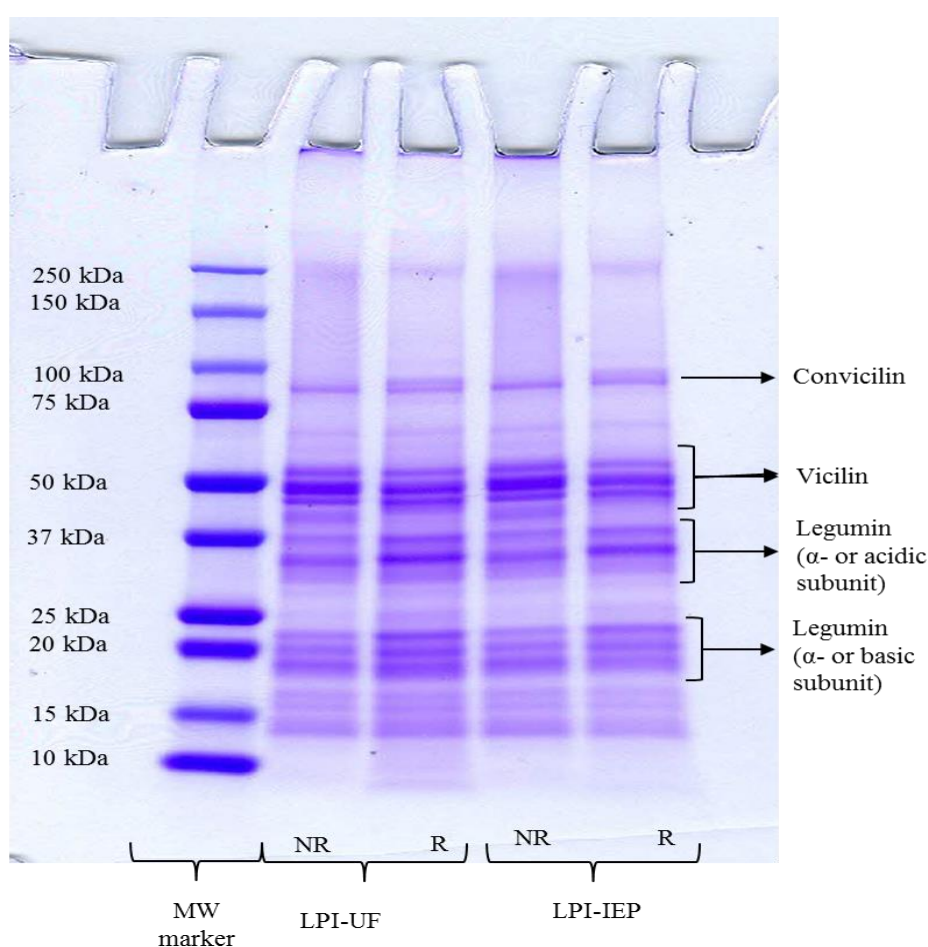


Figure 6-2 Representative sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of lentil protein isolates obtained by ultrafiltration (LPI-UF) and isoelectric precipitation (LPI-IEP) under non-reducing (NR) and reducing (R) conditions. The first lane of the gel contains the molecular weight marker.

Secondary structure

Furthermore, far-UV CD spectroscopic measurements were performed to gain information about the secondary structure of LPIs. Amide groups are optically active and absorb circular polarized far-UV light. Depending on their conformation, i.e., their secondary structure, characteristic CD spectra are obtained (Greenfield, 2007). As shown in Figure 6-3, both LPIs exhibited a positive peak at 185 nm, and a broad negative peak with a minimum at 208 nm, indicating a defined secondary structure of α -helix (Greenfield, 2007). Only slight differences can be observed in the spectra. Similar spectra for lentil flour and isolated proteins using IEP were found by Aryee and Boye (2015), indicating that secondary structure conformational changes were limited during the extraction of the proteins.

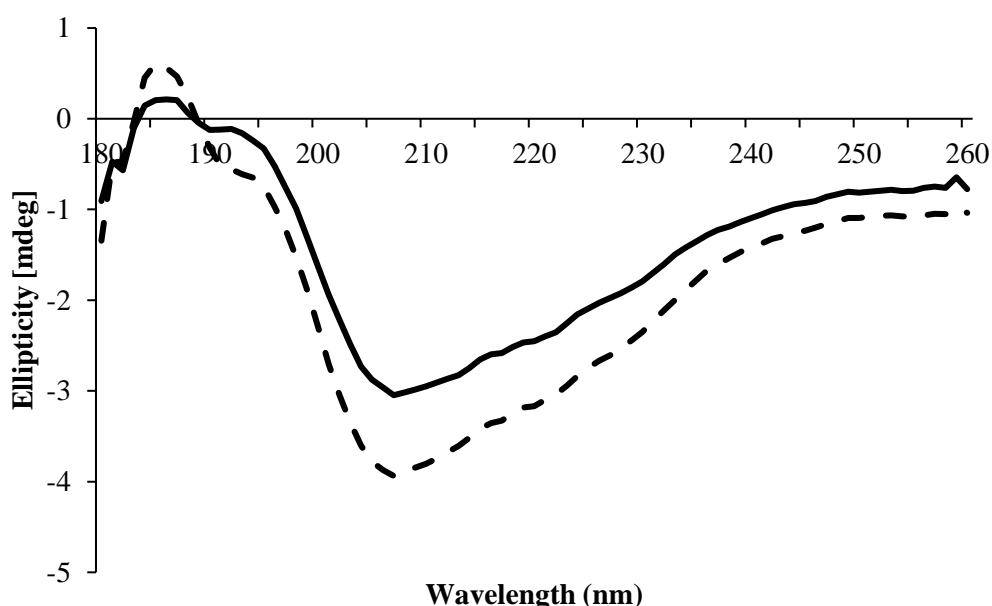


Figure 6-3 Far-UV circular dichroism (CD) spectra (smoothened curve) of lentil protein isolates obtained by ultrafiltration (solid) or isoelectric precipitation (dashed line).

Scanning electron microscopy

Representative micrographs of the LPI powders are given in Figure 6-4. In general, a heterogeneous mixture of rounded particles with smooth, shrivelled, hollow and wrinkled surfaces were observed in both LPIs. These features are typical for spray dried powders and have been attributed to rapid evaporation of water during the spray-drying process (Kim *et al.*, 2009; Kelly *et al.*, 2015; Amagliani *et*

al., 2016). Joshi *et al.* (2011) also observed similar folded and wrinkled surfaces in LPI powders obtained by spray drying. The sizes of the powder particles, as seen from the scale bars, were generally between 10 and 50 μm . LPI-IEP and LPI-UF showed similar powder characteristics, although the LPI-IEP primary particles are in a closer arrangement than the LPI-UF particles where the powder particles seem more dispersed.

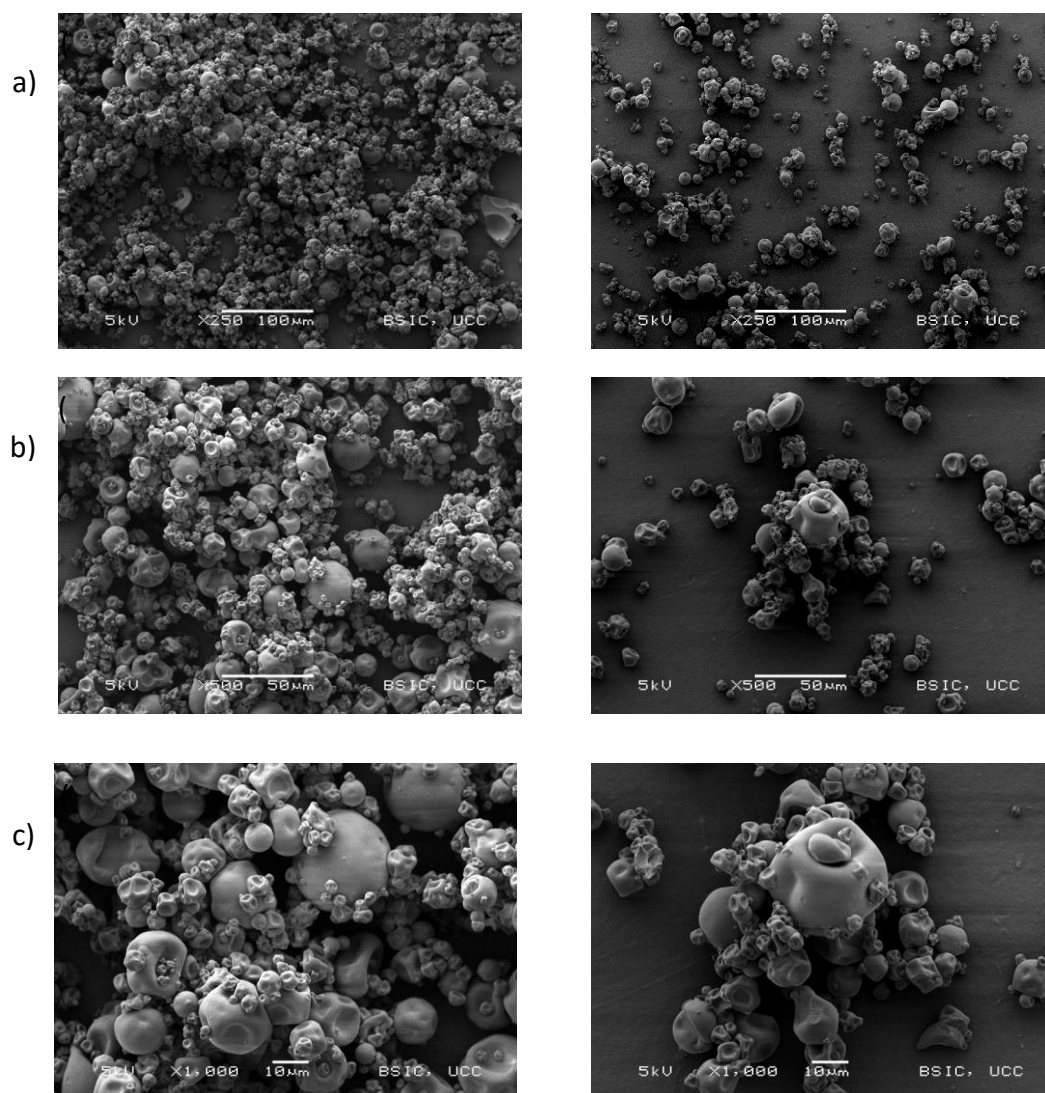


Figure 6-4 Scanning electron micrographs of isoelectric precipitated (column 1) and ultrafiltrated (column 2) lentil protein isolate powder ingredients. Magnification of row (a) 250, (b) 500 and (c) 1000. Scale bars 10 μm .

Particle size

These particle size observations obtained by SEM can be correlated with the particle size distribution (PSD) determined using laser diffraction (Figure 6-5). Both LPIs showed a monomodal size distribution with a size range of 10 to 100 μm . The volume-weighted mean particle diameter ($D_{4,3}$) of LPI-UF and LPI-IEP were 32.8 μm and 29.4 μm , respectively. The LPI-IEP also had significantly lower values for surface-weighted mean particle diameter ($D_{3,2}$), D_v (50) and D_v (90) (Table 6-2). Similar profiles were observed by Crowley *et al.* (2015) in high-protein (90%) milk protein concentrates after 24 h of rehydration, with particle sizes ranging from 10 to 100 μm , classifying them as large and poorly-dispersible particles.

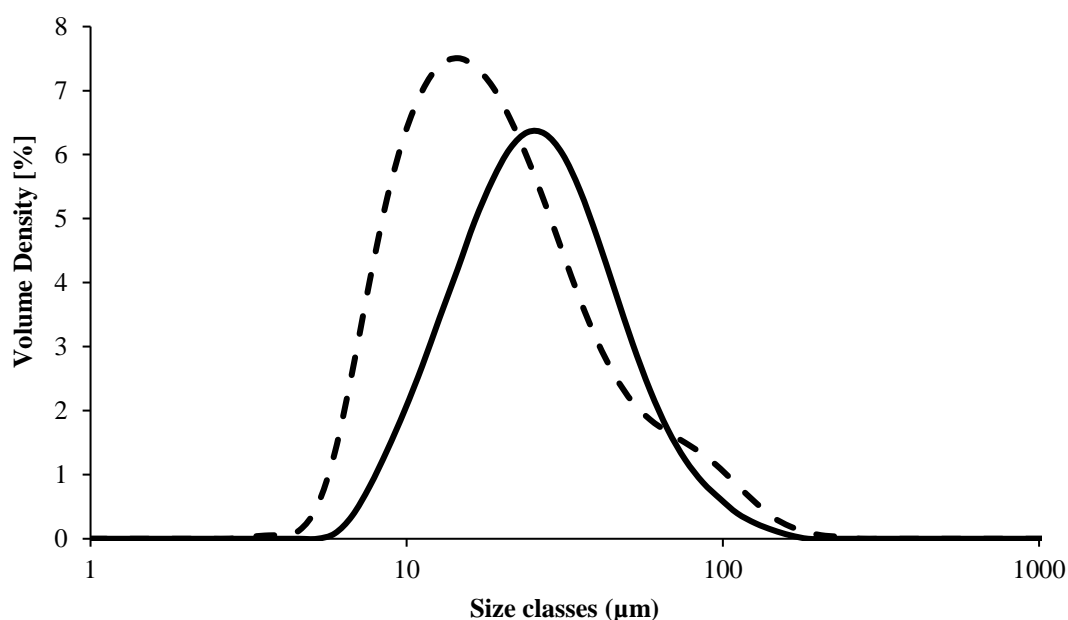


Figure 6-5 Particle size distribution of 1 % (w/v) ultrafiltrated (solid) and isoelectric precipitated (dashed line) lentil protein solutions obtained in deionised water at 25 °C.

Table 6-2 Particle size distribution parameters of 1% (w/v) protein solutions, surface hydrophobicity, sulfhydryl groups, water and oil holding capacity of lentil protein isolates obtained by ultrafiltration (LPI-UF) and isoelectric precipitation (LPI-IEP).

	LPI-UF	LPI-IEP
Particle size distribution [μm]		
D _{4,3}	32.8 \pm 3.21 ^a	29.4 \pm 0.64 ^a
D _{3,2}	23.3 \pm 0.91 ^a	18.1 \pm 1.37 ^b
Dv (10)	12.5 \pm 0.26 ^a	9.02 \pm 0.24 ^b
Dv (50)	26.9 \pm 1.31 ^a	19.0 \pm 1.41 ^b
Dv (90)	62.8 \pm 9.03 ^a	56.6 \pm 5.81 ^a
Surface Hydrophobicity	2411 \pm 49.5 ^a	2688 \pm 92.8 ^b
Free Sulfhydryl groups [$\mu\text{M/g}$ protein]	5.88 \pm 0.01 ^a	6.04 \pm 0.58 ^a
Total Sulfhydryl groups [$\mu\text{M/g}$ protein]	22.5 \pm 0.15 ^a	23.9 \pm 1.42 ^a
Water holding capacity [g water/g protein]	3.96 \pm 0.2 ^a	2.60 \pm 0.11 ^b
Fat holding capacity [g oil/g protein]	2.24 \pm 0.16 ^a	2.09 \pm 0.23 ^a

Values within a column that share a superscript are not significantly different from one another

Surface hydrophobicity and sulfhydryl groups

Hydrophobic groups exposed to the surface of the proteins enable hydrophobic interactions, and adsorption to interfaces; hence having an influence on many properties, such as emulsification and foaming (Kato and Nakai, 1980). These values are shown in Table 6-2. The LPI-IEP had a significantly higher surface hydrophobicity with a value of 2688 in comparison to LPI-UF with a value of 2411. However, the differences were not major, but significantly different, indicating that the extraction method had no major impact on the surface hydrophobicity of the proteins. Comparable studies found a value of 2200 for legumin-like proteins isolated from lentils (Jarpa-Parra *et al.*, 2015), while Joshi *et al.* (2012) found a considerably higher value of 568 determined for mg/mL, which translates to 5680 using the same protein concentration units as in this study.

Results of sulfhydryl groups measured as free and total are shown in Table 6-2. The concentration of free and total sulfhydryl groups were found to be higher for LPI-IEP, with 6.04 and 23.9 $\mu\text{mol/g}$ protein, respectively, compared to 5.88 and 22.5

$\mu\text{mol/g}$ protein, respectively for LPI-UF. Literature considering the sulfhydryl groups of lentil proteins is scarce and diverse; Li & Lee (2000) reported disulphide contents of $0.31 \mu\text{mol/g}$, and free sulfhydryl groups of $0.032 \mu\text{mol/g}$, being considerably lower than the values found in this study. On the other hand, Ladjal-Ettoumi *et al.* (2016) found comparable values; they reported $16.1 \mu\text{mol/g}$ and $31.0 \mu\text{mol/g}$ for free and total sulfhydryl groups, respectively. In both cases, the relatively low amount of free sulfhydryl groups indicated the formation of aggregates, being characteristic for globular proteins, and can be linked also to the relatively large particle size. In general, both LPIs showed similar values for hydrophobicity and also sulfhydryl groups. However, LPI-IEP showed a trend with significant difference ($p < 0.05$) to higher values, indicating a slightly more open structure with higher surface active groups.

6.5.3 Functional properties

Protein solubility and zeta potential

Both isolates showed similar solubility and zeta potential values across the pH range, as shown in Figure 6-6, achieving the highest solubility at acidic and alkaline pH values. Similarly, at the extreme low and high pH ranges, the lentil protein particles showed the highest positive and negative charge, respectively. LPI-IEP showed lower solubility values across the pH range compared with LPI-UF. This may be explained by the removal of soluble proteins in the supernatant during the extraction process and, therefore, more insoluble protein fraction is present in LPI-IEP (Figure 6-1). High solubility values and positive charge (+ 30 mV) were observed at pH 3, followed by minimum solubility and a net charge of 0 mV for both isolates at pH 4.5, indicating that the isoelectric point was reached. Solubility was higher again at pH 6 and pH 6.5 for LPI-UF and LPI-IEP, reaching a value of 43% for both isolates at pH 7 and the surface charge decreased, reaching values between -20 and -30 mV. Karaca *et al.* (2011), found similar values for surface charge (-22.6 mV) for LPI-IEP at pH 7. The highest solubility was obtained for the LPI-UF at pH 9, at 54.7%, while LPI-IEP reached 50.18%. LPI-UF showed also a higher solubility at acidic conditions with a value of 39.4% at pH 3.5. The LPI-IEP exhibited lower solubility at lower pH's, having a value of 11.9% at pH 3.5. The general profiles of the observed solubility and zeta potential curves are characteristic for lentil proteins,

as previously reported by Boye *et al.* (2010), who studied the solubility of different plant-based protein isolates finding high solubilities for lentil and pea proteins in comparison with chickpea. Lee *et al.* (2003) analysed the protein solubility of commercial soya products including flours, concentrates and isolates and found generally lower solubility at low pH's. Solubility is one of the most important properties of proteins, influencing for example the ability to form and stabilise foams, emulsions and gels.

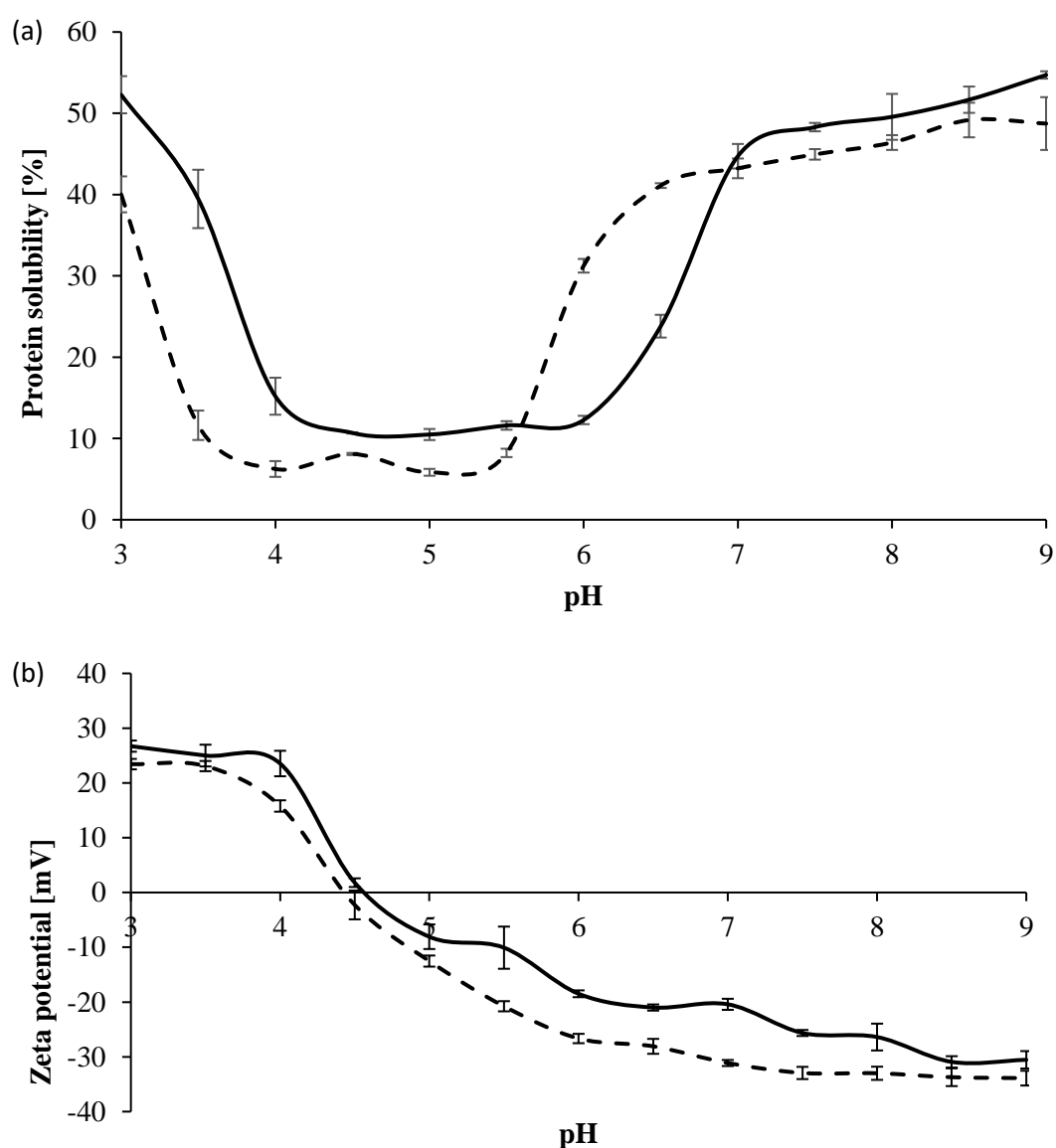


Figure 6-6 Protein solubility (a) and zeta potential (b) values at different pH ranges of lentil protein isolates obtained by ultrafiltration (solid) and isoelectric precipitation (dashed line).

Although insoluble proteins can be used in meat preparations, highly soluble proteins provide the most versatility for substitution and extension of animal proteins (Boland *et al.*, 2013).

Foaming properties

Foaming is, in many product applications, a desired property of proteins, providing structure and stability. The foaming properties of LPIs as a function of protein concentration are shown in Table 6-3. The foaming capacity was low for both isolates at 0.1% (w/v) at 9.42 and 6.52% for LPI-UF and LPI-IEP, respectively. With increasing concentration, the foaming capacity increased, reaching 69.5 and 57.2% for 3.3% (w/v) LPI-UF and LPI-IEP, respectively. Likewise, the foam stability increased from 0% for both LPIs to 44.9% and 39.1% for LPI-UF and LPI-IEP, respectively. The LPI-UF showed significantly better foaming properties at the high protein concentrations compared to the LPI-IEP. In addition, other studies found that protein isolates of various sources prepared by UF were superior to those obtained by precipitation, especially in terms of protein solubility and foaming characteristics (Fuhrmeister and Meuser, 2003; Boye, Aksay, *et al.*, 2010). The results obtained show a high ability of lentil proteins to create foam with high stability, indicating its potential for application in food processing. Compared to other commercial proteins from potato (36.9%), pea (10.6%), carob (17.2%), lupin (13.9%) and soya (36.4%) analysed by Horstmann *et al.* (2017) for their application in bread, both LPIs showed better foam capacities; even potato protein, known to have good foaming ability, showed lower values. These results underline the great prospect of LPIs being used in bakery products, ice cream or other dairy formulations, where foaming properties are desired.

Table 6-3 Foaming properties of protein solutions of lentil protein isolates obtained by ultrafiltration (LPI-UF) and isoelectric precipitation (LPI-IEP).

Protein concentration [w/v]	Foam capacity [%]		Foaming stability after 60 min [%]	
	LPI-UF	LPI-IEP	LPI-UF	LPI-IEP
0.1	9.42 ± 1.26 ^a	6.52 ± 0.00 ^b	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
0.5	18.1 ± 1.26 ^a	18.8 ± 1.26 ^a	6.52 ± 5.75 ^a	5.80 ± 3.32 ^a
1.0	33.3 ± 2.51 ^a	33.3 ± 11.2 ^a	15.9 ± 4.53 ^a	12.3 ± 1.26 ^a
3.0	58.7 ± 9.48 ^a	51.4 ± 6.28 ^a	43.5 ± 5.75 ^a	31.8 ± 5.47 ^a
3.3	69.6 ± 3.77 ^a	57.2 ± 5.47 ^b	44.9 ± 1.26 ^a	39.1 ± 5.75 ^a

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$).

Emulsifying properties

Proteins can act as an emulsifier by forming a film/skin around oil droplets dispersed in an aqueous medium, thereby stabilizing emulsions and preventing structural changes such as coalescence, creaming, flocculation or sedimentation (Boye *et al.*, 2010). The emulsifying activity (EAI) and emulsifying stability index (ESI) of LPI-UF and LPI-IEP are shown in Table 6-4. EAI and ESI are two indices often used to evaluate the emulsifying properties of proteins. ESI values were found to be higher for LPI-UF (63.8 min), compared to LPI-IEP (51.0 min). The EAI values were quite similar for both LPIs; however, higher values were found for LPI-IEP (16.5 m²/g) in comparison to LPI-UF (14.3 m²/g). These higher values for LPI-IEP can be related to the higher surface hydrophobicity compared to LPI-UF. The EAI values were lower in comparison to other studies where different protein-to-fat ratios or high-pressure homogenisation were applied (Boye *et al.*, 2010; Avramenko *et al.*, 2013). For example, Joshi *et al.* (2012) found that the EAI increased 3-fold when the concentration of protein increased from 10 mg/mL to 30 mg/mL, whereas in this study the protein concentration was maintained at 10 mg/mL. In addition, high pressure homogenisation, as applied to cow's milk, can help to unfold globulins (which are known for having high MW and compact structures) and enable them to migrate to the interface in order to form a stable emulsion (Karaca *et al.*, 2011). These factors can be taken into consideration for further studies in order to enhance the emulsification properties.

Water and fat holding capacity

Water and fat holding capacity (WHC and FHC) of proteins are important functionalities, since they influence structure, mouth feel and flavour retention of food formulations. The ability of protein to retain oil or water can be important in food applications, such as ground meat formulations, doughnuts and bakery products. Values are shown in Table 6-2. Significant differences were found for the WHC between the two different LPIs, showing a higher value of 3.96 g/g for the LPI-UF, compared to 2.60 g/g for LPI-IEP. Compared to other studies, both isolates showed a relatively high WHC; Boye *et al.* (2010) reported values of 0.6 and 2.7 g/g for protein concentrates isolated from several legumes. However, the authors found no considerable effect of the preparation method, possibly due to the comparatively low protein contents of their samples. Horstmann *et al.* (2017), found values ranging from 0.0 g/g for a potato and soy, and up to 2.66 g/g for a pea protein ingredient. They associated the protein content to be negatively correlated with the WHC, i.e., other constituents affect the values to a substantial degree. In contrast, in this study it was found that a higher protein content correlated with a higher WHC.

Results for FHC showed also significantly higher values for LPI-UF (2.24 g/g) in comparison to LPI-IEP (2.09 g/g). The value obtained for LPI-UF is comparable to that obtained by Boye *et al.* (2010) for red lentil protein with a FAC of 2.26 g oil/g protein. In addition, this author found the highest FAC value for LPI in comparison with yellow pea and kabuli chickpeas proteins.

Gelation characteristics

Heat induced gelation occurs when proteins aggregate form a three-dimensional network. The ability to do so depends on the state and surface conformation of the proteins, e.g., free sulfhydryl groups, hydrophobicity, charge and correspondingly the electrostatic interaction between proteins, and their ability to associate to form a continuous network throughout the matrix (Kinsella, 1982). LGC was measured as an indicator of the gelation capacity. The LPI-UF formed a gel, resisting flow when inverted, at a concentration of 11% (w/v), whereas for the LPI-IEP 16% was needed (Table 6-4). The lower concentration needed for a firm gel to be formed by the LPI-UF may be associated with the higher protein solubility, which is known to be an important factor in gel formation (Joshi *et al.*, 2011). Likewise, Boye *et al.* (2010)

Table 6-4 Emulsifying and gelling properties of protein solutions of lentil protein isolates obtained by ultrafiltration (LPI-UF) and isoelectric precipitation (LPI-IEP).

	LPI-UF	LPI-IEP
Emulsifying properties		
Emulsifying activity [m^2/g]	14.3 ± 1.22^a	16.5 ± 0.03^b
Emulsifying stability [min]	63.8 ± 6.70^a	51.0 ± 0.96^b
Least gelation concentration [% w/v]	11.0 ± 0.00^a	16.0 ± 0.00^b
Texture profile analysis of LPI gels		
Hardness [mN]	2055 ± 114^a	669 ± 20.2^b
Adhesiveness [mN/s]	-98.7 ± 9.02^a	-83.7 ± 1.53^b
Springiness [%]	0.47 ± 0.08^a	0.32 ± 0.04^a
Cohesiveness [%]	0.30 ± 0.01^a	0.30 ± 0.02^a
Resilience [%]	0.05 ± 0.01^a	0.03 ± 0.00^a
Gumminess	623 ± 53.7^a	210 ± 4.36^b
Chewiness	257 ± 83.1^a	76.3 ± 7.09^b

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$).

found that various legume proteins isolated by UF have lower LGC in comparison to IEP methods. They found comparable values, with 10% for LPI prepared by UF and 12% for isolates prepared by IEP.

The LPI-UF also formed a much stronger gel, which is evident in the values obtained from the TPA test. The hardness for LPI-UF was three-fold higher than that of LPI-IEP. Likewise, gumminess and chewiness were also significantly higher for LPI-UF than for LPI-IEP gels. On the other hand, other parameters, such as adhesiveness, springiness, resilience and cohesiveness were not significantly different. The higher gelling properties of LPI-UF may be linked to the higher calcium levels, which has been shown to enhance hydrophobic coagulation of heat treated milk and soya proteins (Pathomrungsriyounggul *et al.*, 2010). The ability to form strong gels upon heating is a desirable functionality in bakery products, when heat is applied to the dough, its viscosity increases, which gives stability to expanding gas cells, resulting in a higher gas retention during baking and a higher desirable specific volume of the product (Zhou *et al.*, 2018). Further, in non-

traditional ways, also meat, yoghurt and cheese alternatives may be produced from heat-set gels, facilitating the product with a gel-like matrix.

6.5.4 Life cycle assessment

Environmental performance of LPI obtained by IEP and UF was examined by means of life cycle assessment (Table 6-5). Indicators such as aquatic eutrophication, photochemical oxidant formation, stratospheric ozone depletion, phosphorus use and land use showed lower potential environmental impacts for LPI-UF in comparison to LPI-IEP. For the remaining indicators studied, the ranking was switched. Especially the contribution of the lentil cultivation stage affects the outcome of these indicators. An overview on main contributors is exemplarily given in Figure 6-7 for four indicators - the remaining indicators followed one out of those four illustrated result contribution patterns. The higher the contribution of the lentil cultivation stage, the more important is the protein yield advantage from LPI-UF, as less lentil seeds were required per kg protein isolated. On the other hand, lower process energy was required for processing of LPI-IEP, which leads to lower potential environmental impacts for the remaining indicators including climate change. The net nitrogen benefit due to air nitrogen fixation by lentil plants (as they are legumes) in the growth phase is up to 20% of the total environmental impact depending on the indicator for both LPIs. Further, LPIs showed promising carbon footprints within the portfolio of soya-based and cow's milk-based protein isolate food ingredients: The production of both LPIs potentially releases a quarter of carbon dioxide equivalents (3.5 to 4.2 kg CO²-e/kg) than caseinate or whey protein production (19 kg CO²-e/kg and 20 kg CO²-e/kg, respectively) as examined in an attributional LCA by Thrane *et al.*, (2017). Compared to soya protein isolate, depending on the literature source chosen, LPIs showed similar (Thrane *et al.*, 2017) or up to 4-fold lower values (Berardy *et al.*, 2015) for their potential release of carbon dioxide equivalents.

Table 6-5 Environmental impact profile of lentil protein isolates per kg isolate (LPI), prepared by ultrafiltration (LPI-UF) and isoelectric precipitation (LPI-IEP).

	LPI- IEP	LPI-UF
<i>Environmental impact potentials (LCA):</i>		
Climate Change (kg CO ² -e/kg PI)	3.53	4.17
Aquatic Eutrophication (g PO ₄ -e/kg PI)	111	103
Terrestrial Eutrophication (g PO ₄ -e/kg PI)	1.57	1.77
Acidification (g SO ₂ -e/kg PI)	14.5	18.2
Photochemical Oxidant Formation (g O ₃ -e/kg PI)	2.22	2.17
Fine Particulate Matter (g PM _{2.5} -e/kg PI)	11.9	14.9
Stratospheric Ozone Depletion (mg CFC11-e/ PI)	58.2	55.1
<i>Additional indicators at the inventory level (LCI):</i>		
Phosphorus Use (g/kg PI)	245	229
Cumulative Energy Demand, non-renewable (MJ/kg PI)	45	59
Blue Water (process) (kg/kg PI)	42	49
Land Use (m ² /kg PI)	57	53

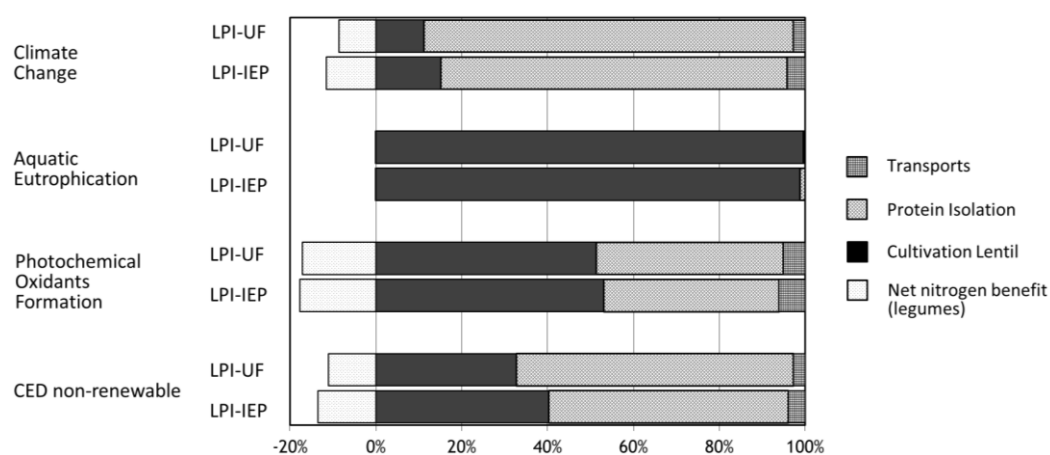


Figure 6-7 Contributions of main life cycle steps to environmental impact profiles of lentil protein isolates prepared by ultrafiltration (LPI-UF) and isoelectric precipitation (LPI-IEP). CED = Cumulative primary energy demand.

The environmental impact profiles of LPIs were also compared with traditional cow's milk protein as illustrated in Figure 6-8. Two different scenarios for the production of cow's milk protein were taken into consideration: the environmental impact, high or low, of the milk protein was set up depending on the theoretical amount of protein that is fed to the cow, i.e. cow feed per kg milk and share of concentrate versus silage feed components within the feed mix were the parameters set to low and high for those ranges. Indicator results of LPIs are lower (and thus favourable) or equal for all of the examined indicators except the land use indicator. The latter is related to comparatively high agricultural yields of feed crops in comparison with relatively low yields for lentils. It should be noted that feed crops have undergone long-time optimisation of agricultural practices in order to reach relatively high yields. Lentils on the other hand have not been cultivated in comparable amounts on global scale than animal feed crops. Therefore, related optimization might take place along with increased interest in lentils in the future. Overall, the environmental impact of both LPIs was low, contributing e.g. to a reduction of greenhouse gases compared to cow's milk protein.

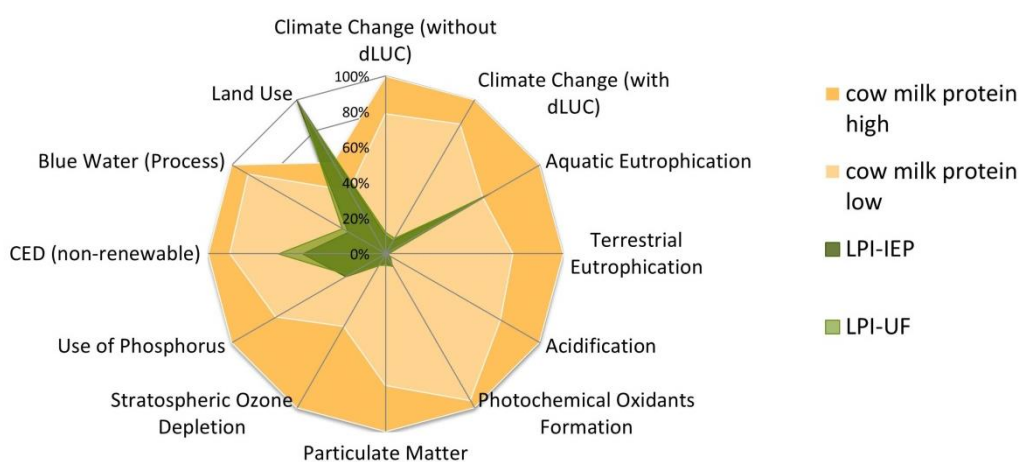


Figure 6-8 Comparison of environmental impact profiles of lentil protein isolates versus cow's milk protein ranges, lentil protein isolates are prepared by ultrafiltration (LPI-UF) and isoelectric precipitation (LPI-IEP). Highest result is set to 100%. dLUC = direct land-use change.

6.6 Conclusion

Various physical and functional properties of two LPI isolated by IEP and UF were investigated, indicating that they could contribute different desirable attributes to a wide range of food products. The results suggest that, in general, UF resulted in a product with better functional properties, such as higher protein solubility, WHC, greater gelling and foaming properties and emulsion stability. Differences in functional properties between the isolates under investigation were attributed to differences in the extraction methods, resulting in different compositions. Both isolates contained high levels of protein; however, LPI prepared by UF contained significantly higher values of protein, calcium and magnesium, whereas LPI prepared by IEP had higher levels of other minor constituents such as fibre, sodium and phosphorous. The life cycle assessment showed that the two main drivers for the environmental impact of LPIs were the cultivation stage and the protein isolation process. Overall, both LPIs exhibited promising environmental performance, especially if compared to traditional cow's milk proteins. These favourable functional, nutritional and environmental properties of LPIs could be exploited in the preparation and development of diverse food products and may also be suitable for the substitution of soya or animal derived proteins. Further studies are required to investigate protein functionality and applicability of these in food systems as well as life cycle assessments of the food products thereof.

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***Chapter 7: Formation, stability and sensory
characteristics of a lentil-based milk substitute as affected
by homogenisation and pasteurisation***

Jeske S., Bez J., Arendt E.K. and Zannini E.

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7.1 Abstract

This study aimed to investigate the suitability of lentil protein and emulsions thereof for the formulation of a milk substitute. The effect of high-pressure homogenisation and heat treatments on functional and physico-chemical properties of lentil protein solutions (3.3% w/w) and the emulsions, containing fat level similar to commercial cow's milk, was studied. Dynamic high-pressure treatments of 180 and 900 bar greatly affected physical and structural properties of the lentil proteins: the particle size was reduced by 100-fold to 129.00 nm for samples homogenised at 900 bar, leading to an almost complete dispersion. Surface properties of lentil protein changed, as shown in an increase of hydrophobicity and decrease of free sulfhydryl groups, while changes in secondary structure and aggregation did not develop. Little impact was observed of the heat-treatment at 65 or 85 °C, however, colour changed from a faint pink hue to be more white in appearance. The obtained emulsions exhibited good colloidal stability at both homogenisation pressures, while overall product quality was best when treated at 900 bar. Sensory analyses showed the formulated lentil-based milk substitute had textural and organoleptic profiles comparable to commercial plant-based milk substitutes, including soya-based products. Lentil protein isolates showed great potential to be used formulating milk substitutes with a high protein content, similar to cow's milk.

7.2 Introduction

Cow's milk is one of the most complete nutrient sources in the human diet and dairy products are widely consumed around the world. Since it is designed to be the sole food source for calves, it is not surprising that it provides all the major nutrients like fat, carbohydrates and proteins to our diet, while also being a good source of minerals and vitamins (FAO, 2013). Yet, the consumption of cow's milk is decreasing. In fact, sales of cow's milk have fallen by 15% in the US since 2012, while the demand for plant-based milk substitutes (PBMSs) has grown by 61% within the same time period, reaching 2.11 billion US\$ in 2017 (Mintel Group Ltd., 2018b). The trend is driven by the desire of consumers for new flavours and taste, followed by health reasons and concerns about environment and animal welfare (Mintel Group Ltd., 2018a). However, the market is still in its infancy and currently only soya-based milk substitutes (BMSs) are catering as balanced alternatives to cow's milk based on nutrients (Vanga and Raghavan, 2018; Jeske *et al.*, 2017; Chapter 3; Sousa *et al.*, 2017). However, soya may not be the most sustainable alternative, as it does not grow in cold climates and relies on long supply chains (Riaz, 2006; Nadathur *et al.*, 2017). Similar to soya beans, lentils belong to the legume family (*fabaceae*) and are a traditional staple food in many temperate climates, like Canada or Europe (Joshi *et al.*, 2017). Lentils contain high amounts of protein between 20.6 and 31.4% with promising properties, as it has high digestibility (~83%) with high nutritional value (good Leu/Ile and Leu/Lys ratios) and provides a wide variety of functional properties (Jarpa-Parra, 2018).

The isolation and application of proteins inherits some advantages: anti-nutrients and compounds causing off-flavours can be removed and the protein isolate can be specifically applied to administer techno-functional properties. Lentil proteins have been investigated for their functionality and exhibit promising properties as emulsifiers (Ma *et al.*, 2016; Joshi *et al.*, 2012; Johnston *et al.*, 2015). Proteins can stabilise emulsions as they are able to interact with both oil and water, due to their amphiphilic properties: their polar and nonpolar regions (i.e. hydrophilic and hydrophobic amino acid residues) align at the oil-water interface to form stabilising films by providing electrostatic and steric repulsion between droplets, and by reducing interfacial tension between the two phases (Dickinson, 1994). In the process of creating emulsions homogenisation is one crucial step. With increasing pressure,

the oil droplet size decreases. Furthermore, this treatment may also alter specific features and the spatial structure of proteins, which may affect their emulsifying properties, since these strongly depend on the proteins structure and surface properties (Kinsella, 1982). The impact of dynamic high-pressure treatment on proteins has still scarcely been studied. However, as shown for lupin and whey protein isolates high-pressure homogenisation is likely to improve interfacial and other functional properties (Bader *et al.*, 2011; Bouaouina *et al.*, 2006). While the mechanism of emulsion formation has been studied intensively by many researchers (Kinsella, 1982; Dickinson and Miller, 2001), the newly gained knowledge about protein modification may help to design improved emulsion or also protein ingredients with specific altered properties for other applications.

Recent work from Primožic *et al.* (2018, 2017) examined the emulsifying property of lentil protein isolates at pH 3 and demonstrated its potential as an emulsifier in nano-emulsions. However, at higher protein concentrations the emulsion was unstable, and aggregates were formed leading to emulsion gels. Further, most research focuses on high oil load emulsions (Joshi *et al.*, 2012; Can *et al.*, 2011; Ma *et al.*, 2016) but few studies have looked at applicable systems with the aim to develop milk alternatives, comprising stable high protein contents. In this regard, there is still a missing body of research bringing the knowledge together.

This study aims to evaluate the formation of emulsions for the development of a novel lentil-BMS, containing protein (3.3% w/w) and fat contents (3.3 or 1.5% w/w) similar to commercial cow's milk. The impact of dynamic pressure and pasteurisation treatments on lentil proteins, as on the prepared emulsion thereof was studied in terms of functional properties and product properties, respectively. This provides a thorough understanding of the process and final product.

7.3 *Materials and methods*

Lentil protein isolates (LPI) were provided by Fraunhofer (Institute for Process Engineering and Packaging, Freising, Germany) as described in Chapter 6. In short, brown lentils of commercial quality (*Lens culinaris* cv. *Itaca*), provided by Agroservice Spa (San Severino Marche, Italy) were used as raw material and extracted using ultrafiltration. Protein content of the final LPI was 93.7 g/100g. Chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise stated.

7.3.1 *Preparation of protein stabilised emulsions*

Solutions of LPI were prepared using a stirring and an ultraturrax device (Janke & Kunkel IKA Labortechnik). The solution was heated to 50 °C and pH was adjusted to 7 and hydrated for 1 hour. The solution was stirred at 70 rpm and ultraturraxed at 4.600 rpm for 10 min. Then, sunflower oil was mixed with the protein suspension for 10 min using the stirrer and ultraturrax. The final emulsion contained 3.3% or 1.5% (w/w) oil, and in both cases 3.3% (w/w) protein. To further reduce the particle size, the emulsions were homogenised with a two-stage high pressure homogeniser at 180 bar (150 bar and 30 bar), or 900 bar (750 bar and 150 bar) (APV-2000, SPX FLOW Inc., Charlotte, USA). To ensure microbial stability, samples were subjected to pasteurisation. A low temperature at 65 °C for 30 min and a higher temperature at 85 °C for 2 min (to simulate high-temperature short-time processing) were chosen and applied in a stirring water bath (Lochner mashing device LP electronic, Berching, Germany). Samples were refrigerated (4 °C) and measured on the same day of preparation. Further, samples were kept for 21 days to assess storage stability, supplemented with sodium azide (0.02% (w/w)) to evade microbial spoilage.

7.3.2 *Preparation of protein solutions*

In order to study the impact of processing on the proteins, solutions of 3.3% (w/w) protein were prepared in the same manner without the addition of oil.

7.3.3 *Surface hydrophobicity*

Surface hydrophobicity was measured according to Hayakawa & Nakai (1985) measuring the fluorescence intensity using 1-anilino-8-naphthalenesulfonate (ANS)

with some modifications. Protein solutions were serially diluted with 0.01 M phosphate buffer (pH 7) ranging from 0.0006 – 0.015% (w/v). Ten μL ANS (8.0 mM in 0.1 M phosphate buffer, pH 7) were mixed with 2 mL diluted sample and left in darkness for 15 min. Fluorescence was measured ($\lambda_{\text{excitation}} = 390 \text{ nm}$, $\lambda_{\text{emission}} = 470 \text{ nm}$) and corrected by a blank measured without ANS. The values represent the slopes ($R^2 \geq 0.98$) calculated by linear regression analysis and used as an index of the protein surface hydrophobicity.

7.3.4 Total sulfhydryl groups

Total sulfhydryl groups were determined with Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid) according to the method of Van der Plancken *et al.* (2005). The protein samples were diluted to 2 mg/mL with 0.01 M phosphate buffer (pH 7) containing 6 M urea and 0.5 M SDS. Eighty μL of Ellman's reagent were added to 2.5 mL of diluted samples. The absorbance was measured at 412 nm after 15 min. For the reagent blank, the protein samples were replaced by the sodium phosphate buffer mixed with 80 μL of Ellman's reagent. SH contents were calculated as follows:

$$\mu\text{m SH/g protein} = (A_{412} - A_{412B}) \cdot \frac{1000000}{\epsilon} \cdot C$$

where A_{412} is the absorbance at 412 nm, A_{412B} is the absorbance at 412 nm for the blank, ϵ is the extinction coefficient, which was taken as $13,600 \text{ M}^{-1} \text{ cm}^{-1}$, and C is the sample concentration in mg/mL.

7.3.5 Protein solubility

Protein solubility was adapted from the method of the International Dairy Federation, (1995). Protein contents of whole samples and supernatants (centrifuged at 3000 g for 10 minute) were determined using the Kjeldahl method (MEBAK 1.5.2.1) and nitrogen content was converted into protein using the factor 6.25. Protein solubility was expressed as percentage of protein content in the supernatant of total protein content:

$$\text{Protein solubility [\%]} = \frac{\text{Supernatant protein content [\%]}}{\text{Total protein content [\%]}} \cdot 100$$

7.3.6 *Secondary structure*

Far-UV circular dichroism (CD) measurements of protein solutions were obtained using a circular dichroism spectrophotometer (Chirascan, Applied Photophysics, Leatherhead, UK). Protein solutions of 1 mg/mL were prepared in ultrapure water. CD spectra were measured with a path length of 0.1 mm in the range 180-260 nm, spectral resolution 1 nm, 1 s/point. The average of three spectra was obtained and a 5-point smoothing algorithm was applied after correction for the water baseline.

7.3.7 *Particle size distribution*

Particle size distribution (PSD) of untreated protein dispersions was measured using a laser light diffraction unit (Mastersizer 3000, Malvern Instruments Ltd, Malvern, UK). A polydisperse model with particle and dispersant refractive index of 1.336 and 1.33, respectively, and absorption of 0.1 were selected for data analysis. Samples were introduced to the mixing chamber and dispersed in ultrapure water until a laser obscuration of 12% was reached and three readings were taken. Size measurements are presented as volume weighted mean particle diameter ($d_{4,3}$). Due to the shift to smaller sizes with homogenisation particle size and oil droplet distribution of protein solutions and emulsions were determined by dynamic light scattering using a Malvern Zetasizer Nano ZS system (Malvern Instruments Ltd., Malvern, Worcestershire, UK), equipped with a 633 nm laser. All solutions and emulsions were diluted with ultrapure water (1:250) before analysis.

7.3.8 *Accelerated physical stability*

Stability was measured using an analytical centrifuge (LUMiSizer®, LUM GmbH, Berlin, Germany). The samples were treated at 1000 rpm for 30 minutes and subsequently at 3000 rpm for 60 minutes at 24 °C. The separation rate represents the slope in $\% \cdot h^{-1}$ and was determined by plotting the % of transmission over time. The creaming, sediment height and bottom clearance in mm were observed by subtracting the position with $\leq 20\%$ (or $\geq 20\%$ for the bottom clearance) light transmission of the last profile from the meniscus or cell bottom, respectively.

7.3.9 Rheological behaviour

The rheological behaviour of the products was characterised using a controlled stress rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a sensor system of coaxial cylinders (C-CC27-T200/SS, Anton Paar GmbH, Austria). The shear stress was measured as a function of shear rate ranging from 0.5 to 100 s⁻¹ within 500 s. The power law model was fitted to the experimental points to determine K and the flow behaviour index (n). The measurements were carried out at 10 °C. The apparent viscosity measured at 10 s⁻¹ is referred to as viscosity.

7.3.10 Heat stability

For the determination of heat stability 2.5 mL of samples were placed in glass tubes (10 mm x 130 mm, AGB Scientific, Dublin, Ireland), sealed with silicone bungs, immersed in an oil bath thermostatically controlled at 140 °C (Elbanton BV, Kerkdriel, The Netherlands), with continuous rocking at motor speed setting 3. The heat coagulation time (HCT) was examined visually and taken as the time in minutes that elapsed between placing the sample in the oil bath and the onset of coagulation.

7.3.11 Confocal laser scanning microscopy

The microstructural analysis of emulsions was performed using a confocal laser scanning microscope (CLSM) (Olympus FV1000, incorporating an IX81 inverted microscope Germany). A saturated solution of Nile blue (Sigma-Aldrich, Wicklow, Ireland; 500 µL) was used to label both protein and lipid in 1 mL of sample. Samples were observed using a 100× oil immersion objectives, an Ar laser operating at an excitation wavelength of 488 nm with emission detected between 500 and 530 nm and a He-Ne laser operating at an excitation wavelength of 633 nm with emission detected between 565 and 615 nm for oil and protein observation, respectively (Auty *et al.*, 2018). At least three specimens of each sample were observed to obtain representative micrographs of samples.

7.3.12 Colour

The colour values were measured using the CIE L*a*b* colour system and obtained using illuminant D65. The instrument used was a colorimeter (CR-400, Konica Minolta, Osaka, Japan). Colour of samples was characterised according to whiteness index (WI):

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

7.3.13 Sensory affective testing

Sensory acceptance testing (SAT) was performed on the emulsion containing 3.3% oil, treated at 900 bar and 85 °C, and five commercial PBMSs based on soya, almond, oat, rice, and hemp, purchased from a local health store. This lentil-based emulsion was chosen, since it performed best in the physico-chemical tests completed beforehand, however sucrose (2.5% w/w) and salt (0.08% w/w) were added to the formulation, to improve the taste. SAT was conducted according to the methods of Stone *et al.* (2012) using a total of 30 untrained assessors (n = 30, 53% female, ages ranged from 20 to 55). SAT took place over two separate days, evaluating the samples in duplicates, and was carried out in the panel booths of the sensory science laboratory, food science building, University College Cork according to international standards (ISO 11136, 2014). The refrigerated samples (20 mL) were assigned a randomised three-digit code and presented to the panellists under white light. Participants used a 9-point hedonic scale (appearance, aroma, mouth feel, flavour, overall; like extremely - dislike extremely) to indicate their degree of liking of the six PBMSs in each session. After the sensory assessment, panellists were asked to fill out a questionnaire on demographic information, habits and motivation for milk and milk substitute consumption.

7.3.14 Statistics

All analyses were carried out at least in triplicate, unless otherwise stated. Means were compared using one-way analysis of variance (ANOVA) and Tukey's post hoc-test using Minitab release 16 (Minitab Inc. State College, Pa., USA). The level of significance was determined at $p < 0.05$. Linear correlation measurements of results were performed using Pearson's correlation.

7.4 Results and Discussion

7.4.1 Effect of homogenisation and heat treatment on protein solubility, surface properties and secondary structure

The effect of homogenisation pressures and heat treatments on solubility, surface hydrophobicity and total sulfhydryl groups of lentil protein dispersions (3.3% w/w) is shown in Figure 7-1. The protein solubility increased due to homogenisation considerably from 54.34% to 96.40% and 98.85% at homogenisation pressures of 180 bar and 900 bar, respectively. The increase in protein solubility might be due to the improved dispersion and hydration of the smaller protein particles (Bader *et al.*, 2011). This can also be seen in the reduced particle size and CLSM pictures. Subsequent thermal treatment did not change the solubilities considerably. However, thermal treatments lead to an increase of surface hydrophobicity and decrease in total sulfhydryl groups, revealing changes in the protein conformation. Surface hydrophobicity increased significantly with homogenisation pressure from 1959.8 to 2831.3 and 3124.3 for 180 bar and 900 bar treatment, respectively. However, heat treatments increased the values further to 3355.6 and 3811.2 for 65 and 85 °C treated samples, respectively for protein dispersions homogenised at 180 bar. A similar trend was observed for 900 bar treated samples. Surface hydrophobicity is the predominant factor assessed by many studies as it is important for functionality of food proteins. Correlations have been found to interfacial tension and emulsifying activity. It enables the protein to interact between phases, may it be water and oil for emulsions, or water and air for foams. Indeed, this study also showed this correlation between hydrophobicity and the separation rate (-0.772 , $p < 0.001$). The exposure of hydrophobic groups to the surface by partially unfolding of protein molecules, can make them more flexible and facilitate their faster adsorption into the oil-interfaces (Beverung *et al.*, 1999).

Total sulfhydryl groups were found to be highest for untreated protein dispersions. No significant differences were found for samples homogenised at 180 bar, while 900 bar homogenisation showed a considerable decrease from 34.02 down to 31.08 $\mu\text{M/g}$ protein. However, temperature showed to have a big impact and significantly decreased the total sulfhydryl groups to 27.60, and 24.22 $\mu\text{M/g}$ protein for protein solutions homogenised at 180, and 900 bar, respectively after a heat

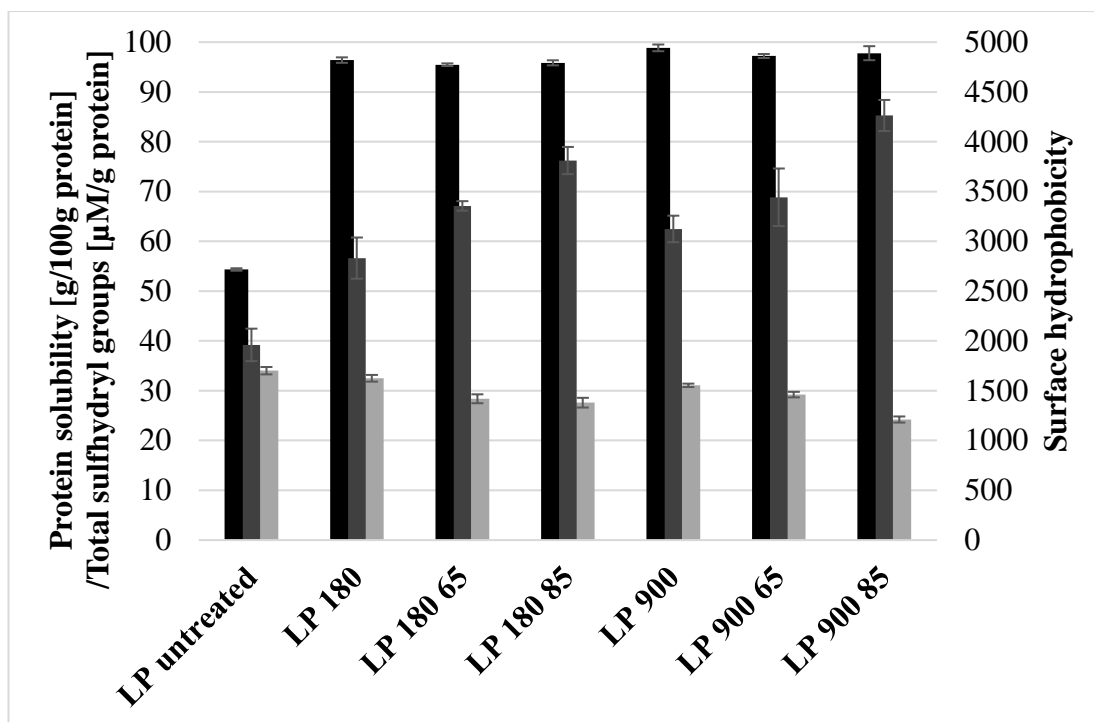


Figure 7-1 Protein solubility (black), total sulfhydryl groups (light grey) and surface hydrophobicity (dark grey) of lentil protein dispersions treated at 180 and 900 bar and 65 and 85 °C.

treatment of 85 °C for 2 min. The decrease of sulfhydryl groups suggests that new disulphide bridges were formed post heating. Disulphide bonds play an important role in the formation of aggregates and gel network structures. For instance, the formation of self-standing gels upon heating of soya or whey proteins in the manufacture of tofu, or ricotta is mainly driven by the formation of disulphide bonds (Saio *et al.*, 1971; Berghout *et al.*, 2015). However, also emulsion stability may be improved; once formed, film stability may be enhanced by disulphide bond formation as shown by Dickinson (1986). Further, the decrease of disulphide bonds also indicated a flexibility of LP to alter its structure, which is also relevant in the formation of stable interfacial layers.

The secondary structure of the proteins as analysed by far-UV CD spectroscopy is shown in Figure 7-2. The homogenised and pasteurised protein solutions all exhibit a similar profile with a respective negative and positive band above and below 200 nm. The observed patterns are characteristic of α -helical structures.

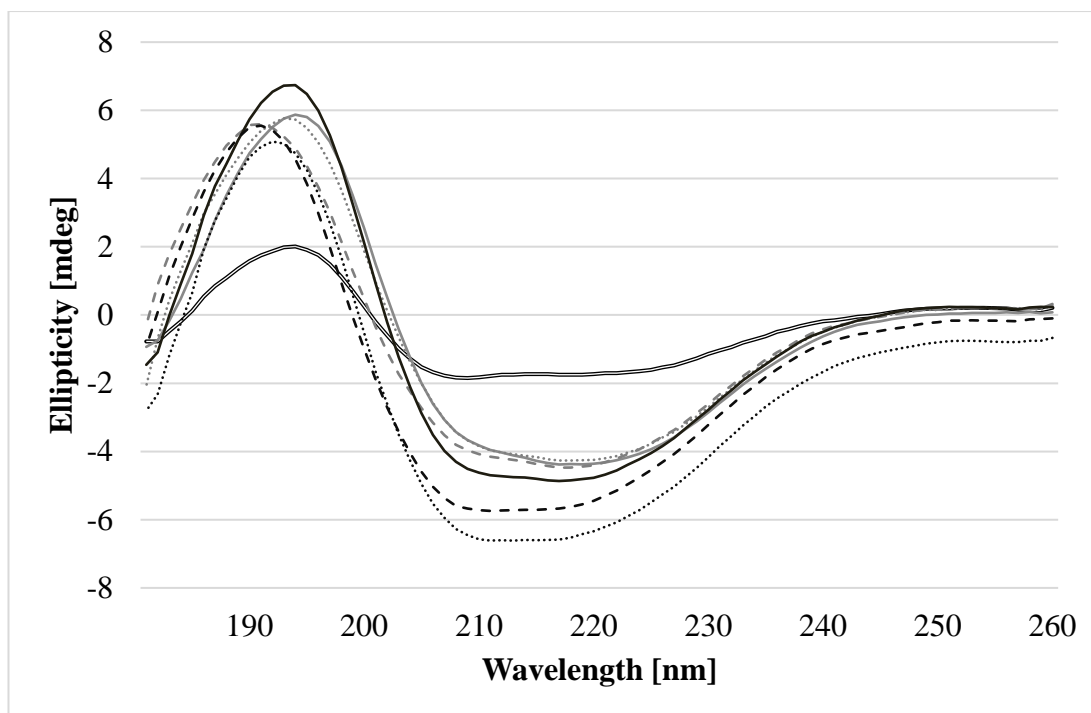


Figure 7-2 Circular dichroism spectra of lentil protein untreated (double lined), homogenised at 180 (grey), or 900 bar (black) and pasteurized at 65 °C (dotted), 85 °C (dashed), or not heat-treated (solid).

While only slight shifts across the spectra appeared and overall the same shape of the spectra was maintained, structural changes may have occurred but suggested no major difference arose. In regard to the untreated sample, these spectra did differ. However, it needs to be noted, that only 54% of the protein were soluble. This might also be the reason why less pronounced bands were obtained; Nevertheless, the same pattern of bands can be observed, indicating the same secondary structure of the untreated protein dispersion. Aryee and Boye (2015) observed similar spectra for lentil protein. However, they observed after cooking lentils for 30 min at 95 °C, the spectra changed and a loss in secondary structure conformational was shown. Heat treatments in this study did not show such changes, neither did the homogenisation pressures.

7.4.2 Particle and droplet size distribution

The initial particle size of lentil protein dispersions was measured using a static light-scattering system. The dispersion consisted predominantly of large and poorly-dispersible particles being about 28 μm big with a broad distribution with a span of

1.7 mm. With the application of homogenisation, the protein changed drastically, as already seen in the protein solubility. The particle size was reduced by more than 100-fold. Therefore, samples were analysed using the dynamic light-scattering system and values using this method are reported as z-average presented in Table 7-1. The particle size was shifted down to 219.19 nm for protein solutions homogenised at 180 bar, and further down to 129.00 nm for samples homogenised at 900 bar. As indicated by the polydispersity index, a narrow and monomodal distribution was achieved by both pressure treatments. Further, no significant changes ($p < 0.05$) in these parameters were found when applying the different heat treatments. This decrease in particle size may be the main reason for improved solubility: increasing the surface of protein particles improved the hydration possibilities (Sathe *et al.*, 2018). Both pressure and heat treatments are known to cause denaturation of proteins. As apparent from the values of surface hydrophobicity and sulfhydryl groups, changes in the structure of proteins occurred. However, as evident from the reduction of particle size and high protein solubility, this did not lead to extensive protein-protein interactions, and aggregation. In contrary, Joshi *et al.* (2012) assumed LPI aggregated upon heat treatment at 80 °C for 10 min, which lead to inferior emulsifying properties. Particle size does not deduce such in this study, which may be due to the lower protein concentration. Similarly, the effect of high pressure homogenisation on lupin protein isolates did not show the formation of large aggregates at pressures up to 1000 bar, but a reduction of the particle size distribution to 0.1 to 10 μm (Bader *et al.*, 2011).

With the addition of sunflower oil, emulsions were prepared by applying the same homogenisation pressure, and pasteurisation treatments. At a homogenisation pressure of 180 bar, mean particle/droplet sizes of 371.78 nm and 447.82 nm using 1.5% and 3.3% of sunflower oil, respectively, were achieved. The polydispersity index was rather high for these samples (<0.3), indicating a polydisperse distribution. From the PSD of the mere protein solutions it can be concluded that the smaller particles are mainly proteins, whereas fat droplets constitute the biggest particles. The size of oil droplets was successfully decreased with the homogenisation processing at 900 bar. Mean particle/droplet sizes of 205.12 and 223.36 nm were achieved for 1.5% and 3.3% oil emulsions, respectively.

Table 7-1 Effect of homogenisation pressure (180 or 900 bar) and heat treatment (65 or 85 °C) of lentil protein dispersions (LP) and lentil protein stabilised emulsions (LPE) containing 1.5 or 3.3% fat, measured on day 0 and after 21 days of storage on average particle (Z-Average) and polydispersity index.

Samples	Particle size [nm]	Polydispersity index
LP 180	219.19±3.75 ^{fgh}	0.20±0.01 ^{defg}
LP 180 65	220.91±8.50 ^{fgh}	0.21±0.03 ^{de}
LP 180 85	211.63±7.04 ^{fgh}	0.20±0.02 ^{def}
LP 900	129.00±2.55 ^k	0.18±0.01 ^{defgh}
LP 900 65	132.98±4.67 ^k	0.19±0.03 ^{defg}
LP 900 85	128.79±1.96 ^k	0.18±0.01 ^{defgh}
LPE 180 65 1.5%	371.78±29.00 ^c	0.37±0.08 ^{ab}
LPE 180 85 1.5%	340.07±7.56 ^{de}	0.32±0.03 ^{ab}
LPE 900 65 1.5%	205.12±5.81 ^{hij}	0.15±0.03 ^{fgh}
LPE 900 85 1.5%	208.73±12.95 ^{ghi}	0.16±0.04 ^{efgh}
LPE 180 65 3.3%	447.82±11.41 ^a	0.30±0.05 ^{bc}
LPE 180 85 3.3%	430.57±16.64 ^{ab}	0.34±0.05 ^{ab}
LPE 900 65 3.3%	223.36±9.05 ^{fg}	0.13±0.03 ^h
LPE 900 85 3.3%	223.33±11.6 ^{fg}	0.13±0.03 ^h
LPE 180 65 1.5% 21 days	343.85±14.72 ^d	0.37±0.06 ^a
LPE 180 85 1.5% 21 days	322.68±15.79 ^e	0.37±0.06 ^a
LPE 900 65 1.5% 21 days	193.52±2.64 ^{ij}	0.14±0.01 ^{fgh}
LPE 900 85 1.5% 21 days	190.61±2.90 ^j	0.13±0.02 ^{gh}
LPE 180 65 3.3% 21 days	415.81±28.70 ^b	0.34±0.11 ^{ab}
LPE 180 85 3.3% 21 days	381.21±6.49 ^c	0.24±0.05 ^{cd}
LPE 900 65 3.3% 21 days	226.91±20.11 ^f	0.14±0.03 ^{fgh}
LPE 900 85 3.3% 21 days	227.39±8.85 ^f	0.16±0.06 ^{efgh}

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$)

Samples treated at 900 bar showed a narrower distribution due to the size reduction of the oil droplets, as expressed in the decrease of the polydispersity index (≤ 0.15). Additionally, it may also be concluded that more of protein was absorbed in the interface of oil/water, due to the increased surface area of the oil droplets.

No significant differences in the PSD were found when applying the different heat treatments. Further, during storage, no considerable differences were found, and in all but one case the differences were not significant. Oil droplets were successfully stabilised by electrostatic repulsive forces of the protein film, which protected droplets against flocculation and coalescence during storage and upon heating.

Primožic *et al.* (2017) studied the characteristics of 5% oil-in-water emulsions stabilised with LPI at pH 3 as a function of protein concentration, using a high-pressure homogeniser at 1378 bar. Similar to the present study, nano-emulsions with an oil droplet size of 0.163 μm at a protein concentration of 3% (w/w) were achieved. However, these emulsions were considerably unstable during storage, and protein aggregates were formed with particle sizes of approximately 10 μm . At a protein concentration of 5% even a strong gel was formed. Joshi *et al.* (2012) studied the emulsion characteristics of LPI-stabilized emulsions at pH 7, but with higher oil-loads. With a pressure of 500 bar, they achieved similar droplet sizes of 398 nm containing 10% (v/v) oil and 3% (w/v) protein. These results show that processing conditions are vital, and especially the pH within a food system is impacting the characteristics and functionality.

7.4.3 Rheology

The influence of homogenisation and pasteurisation on the rheological parameters (K , n and η) is shown in Table 7-2. With the application of homogenisation the viscosity of protein dispersion decreased as smaller and more homogenous particles were produced. The untreated protein dispersion showed a viscosity of 3.35 mPa·s, with a newtonian behaviour ($n = 0.92$), which was reduced to about 2 mPa·s for all protein solutions, as changing homogenisation pressure, and pasteurisation temperature showed no significant impact on the viscosity. The flow behaviour index (n) was slightly shifted to values above 1, indicating a slight shear thickening behaviour. Emulsions exhibited no differences in flow behaviour, while viscosity was slightly increased. The incorporation of 3.3% oil at low

Table 7-2 Effect of homogenisation pressure (180 or 900 bar) and heat treatment (65 or 85 °C) of lentil protein dispersions (LP) and lentil protein stabilised emulsions (LPE) containing 1.5 or 3.3% fat, measured on day 0 and after 21 days of storage on consistency index (K), flow behaviour index (n) apparent viscosity (at shear rate of 10 s⁻¹) and heat stability.

Samples	K	n	Viscosity [Pa·s]	Heat Stability [min]
LP untreated	5.09±2.74 ^a	0.92±0.16 ^c	3.35±0.03 ^a	8.28±0.25 ^k
LP 180	1.90±0.13 ^b	1.09±0.02 ^{abc}	2.31±0.04 ^{ghij}	10.00±0.87 ^{ij}
LP 180 65	1.45±0.22 ^b	1.18±0.05 ^{ab}	2.20±0.09 ^{hij}	9.22±0.19 ^{jk}
LP 180 85	2.00±0.21 ^b	1.09±0.04 ^{abc}	2.48±0.05 ^{efgh}	10.75±0.22 ^{hi}
LP 900	1.50±0.14 ^b	1.12±0.03 ^{ab}	1.98±0.04 ^j	9.11±0.19 ^{jk}
LP 900 65	1.59±0.12 ^b	1.11±0.03 ^{ab}	2.06±0.02 ^{ij}	8.83±0.29 ^{jk}
LP 900 85	1.73±0.16 ^b	1.10±0.03 ^{abc}	2.17±0.03 ^{hij}	9.47±0.21 ^{jk}
LPE 180 65 1.5%	2.13±0.07 ^b	1.10±0.00 ^{abc}	2.63±0.06 ^{defg}	14.17±0.29 ^{cde}
LPE 180 85 1.5%	2.14±0.15 ^b	1.10±0.02 ^{abc}	2.70±0.10 ^{def}	14.22±0.39 ^{cde}
LPE 900 65 1.5%	1.84±0.26 ^b	1.11±0.06 ^{abc}	2.33±0.05 ^{ghi}	13.28±0.35 ^e
LPE 900 85 1.5%	1.95±0.10 ^b	1.09±0.02 ^{abc}	2.41±0.04 ^{fgh}	13.08±0.14 ^{ef}
LPE 180 65 3.3%	2.60±0.57 ^b	1.06±0.07 ^{abc}	3.07±0.19 ^{ab}	17.41±0.80 ^a
LPE 180 85 3.3%	2.67±0.62 ^b	1.07±0.09 ^{abc}	3.22±0.08 ^a	16.15±0.66 ^b
LPE 900 65 3.3%	2.11±0.30 ^b	1.08±0.05 ^{ab}	2.66±0.13 ^{def}	14.13±0.49 ^{cde}
LPE 900 85 3.3%	2.63±0.36 ^b	1.04±0.04 ^{bc}	2.83±0.18 ^{cd}	13.91±0.42 ^{cde}
LPE 180 65 1.5% 21	2.55±0.33 ^b	1.05±0.04 ^{abc}	2.82±0.10 ^{bcd}	12.05±0.08 ^{fg}
LPE 180 85 1.5% 21	2.36±0.27 ^b	1.07±0.04 ^{abc}	2.78±0.10 ^{cde}	11.97±0.13 ^g
LPE 900 65 1.5% 21	1.35±0.23 ^b	1.21±0.06 ^a	2.15±0.09 ^{hij}	11.27±0.31 ^{gh}
LPE 900 85 1.5% 21	1.84±0.14 ^b	1.11±0.02 ^{ab}	2.35±0.08 ^{ghi}	11.64±0.07 ^{gh}
LPE 180 65 3.3% 21	2.46±0.34 ^b	1.08±0.04 ^{abc}	3.06±0.15 ^{abc}	14.68±0.50 ^c
LPE 180 85 3.3% 21	3.02±0.55 ^b	1.05±0.07 ^{abc}	3.21±0.08 ^a	14.4±0.32 ^{cd}
LPE 900 65 3.3% 21	2.12±0.50 ^b	1.09±0.09 ^{abc}	2.61±0.13 ^{defg}	13.53±0.17 ^{de}
LPE 900 85 3.3% 21	2.35±0.38 ^b	1.07±0.06 ^{abc}	2.81±0.06 ^{bcd}	13.61±0.09 ^e

Values within a column that share a superscript are not significantly different from one another (p < 0.05)

homogenisation pressure of 180 resulted in the highest viscosity of 3.07 mPa·s. Cow's milk shows a similar viscosity with 3.15 mPa·s (Jeske *et al.*, 2017; Chapter 3), suggesting a comparable mouth feel when swallowing. Pasteurisation treatments did not cause significant changes ($p < 0.05$) in the consistency index, or apparent viscosity of samples which indicates that no changes in the component arrangement were induced by thermal treatment. Further, no changes were observed after a storage time of 21 days.

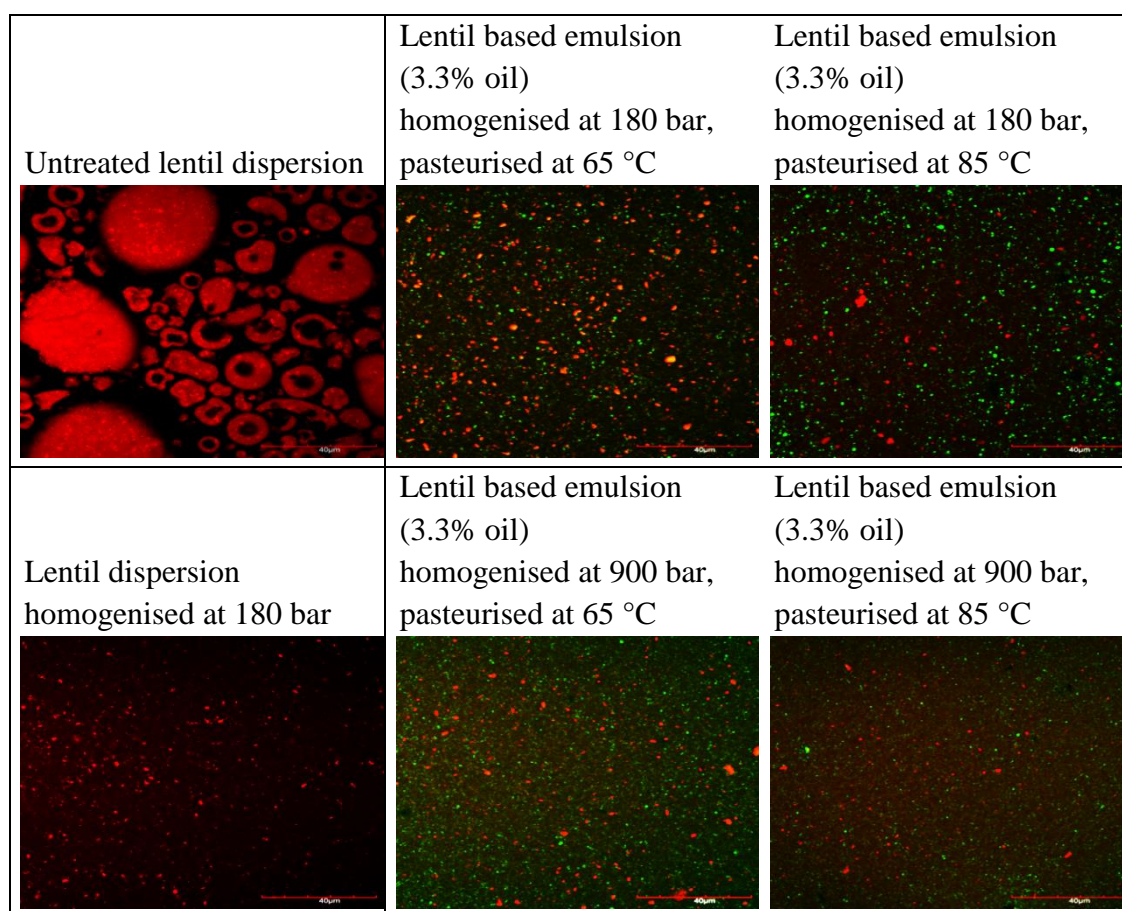
7.4.4 Heat stability

Suspensions of LPI exhibited a heat coagulation time (HCT) of 8.28 min (Table 7-2) in an oil bath at 140 °C. The homogenisation pressure and pasteurisation temperature seemed not to affect heat stability considerably. Heat coagulation is caused by hydrophobic interactions, and covalent cross-linking (disulphides), induced by the denaturation of protein (Nakai, 1983). Even the pasteurised protein solutions, which already had more exposed hydrophobic groups and underwent a reduction of total sulfhydryl groups seemed to be relatively insusceptible to coagulation. Thermal stability of soya protein isolate, at pH 7 and 3.6 % (w/v) was reported to be around 20 min (Ryan *et al.*, 2008), while high concentrated milk protein, exhibited a low HCT of < 2 min at pH 7 and a suspension concentration of 3.50% (w/w) (Crowley *et al.*, 2014). The prepared emulsion showed higher heat stabilities than the mere protein solutions itself. Emulsions with 3.3% fat, homogenised at 180 bar and pasteurised at 65 °C were the most stable and coagulated after 17.41 min. The other samples showed similar HCT, being around 14 min. Generally, it can be concluded that proteins were stabilised and protected against heat induced coagulation to a certain extent. While proteins are in the interface of oil, their hydrophobic groups are aligned into the oil phase, thus, no interactions are forced between the exposed hydrophobic sites of protein molecules. However, with increasing degree of denaturation, proteins may lose their ability to stabilise the interface and eventually the system gets unstable, leading to the exposure of hydrophobic groups and aggregation occurs. With storage the heat stability decreased, only not significantly for 900 bar treated 3.3% oil emulsions. This could be an indication of an onset of instability of the protein in solution or in the oil interface. However, particle size measurements indicated no aggregation,

therefore it can be assumed, that little changes in the protein conformation affected the heat stability.

7.4.5 Confocal laser scanning microscopy

Selected micrographs, as obtained by CLSM are displayed in Figure 7-3. Untreated LPI dispersions were constituted by a heterogeneous mixture of small and larger rounded, or broken pieces of particles with freckled, smooth surfaces. As observed in the particle size, much smaller particles are found in the homogenised samples. The proteins were distributed very homogeneously, without any kind of formation. As indicated by particle size distribution and viscosity already, the heat treatment did not affect the component arrangement, also when observing the emulsions. Oil droplets were distributed homogeneously and did get reduced with increased homogenisation pressure.



continued

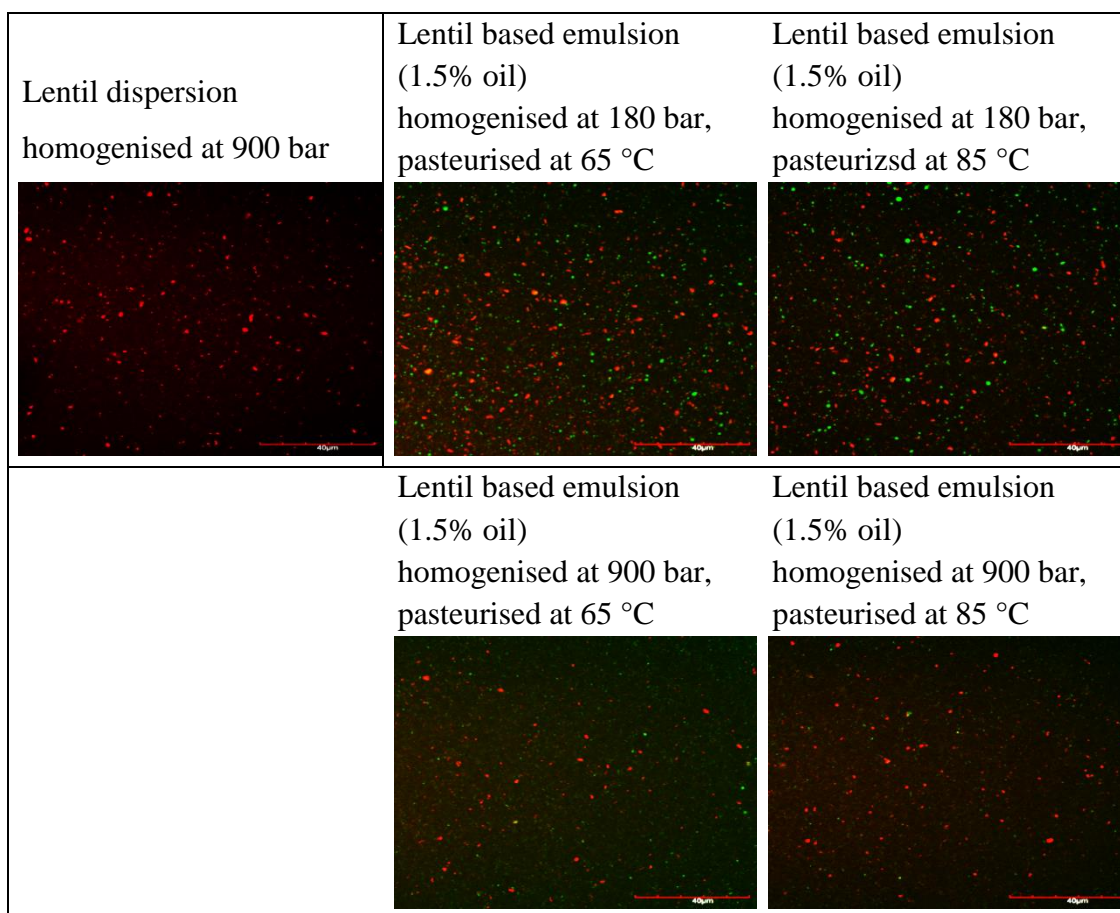


Figure 7-3 Confocal laser scanning microscopy images of lentil protein dispersions and emulsion. Scale bar 40 μm .

7.4.6 Colour

As it was isolated from brown lentils, LPI contained some pigments, exhibiting a faint light pink colour also in solution. Pictures of protein dispersions and emulsion are shown in Figure 7-4 and values of instrumental colour analysis are presented in Table 7-3. This light pink colour of the LPI powder is expressed in the a^* value (4.12 for the untreated LPI dispersion). Most interestingly, with heat treatment at 85 °C, these values were reduced and shifted away from red, appearing more neutral (-0.25 for LPI homogenised at 180 bar, pasteurised at 85 °C). The colour compounds in lentils, such as anthocyanins, are sensitive to heat treatments (Sadilova, Carle, & Stintzing, 2007). This pigment degradation is clearly an advantage for the preparation of emulsions, aiming to imitate the white appearance of cow's milk. Further, with the incorporation of oil, the lightness of the samples was increased to more than 70 from initially 46.46 for the untreated LPI dispersion. The dynamic

high-pressure treatment at 900 bar increased the lightness the most, with values reaching 77, as the number and size of oil droplets is increasing the scattering of light. Both the degradation of colour compounds and light scattering is presented in the WI. A value of 76.78 was reached for the emulsion containing 3.3% (w/w) fat treated at 900 bar and 85 °C, which is relatively comparable to cow's milk with a whiteness index of 81.89 (Jeske *et al.*, 2017; Chapter 3).

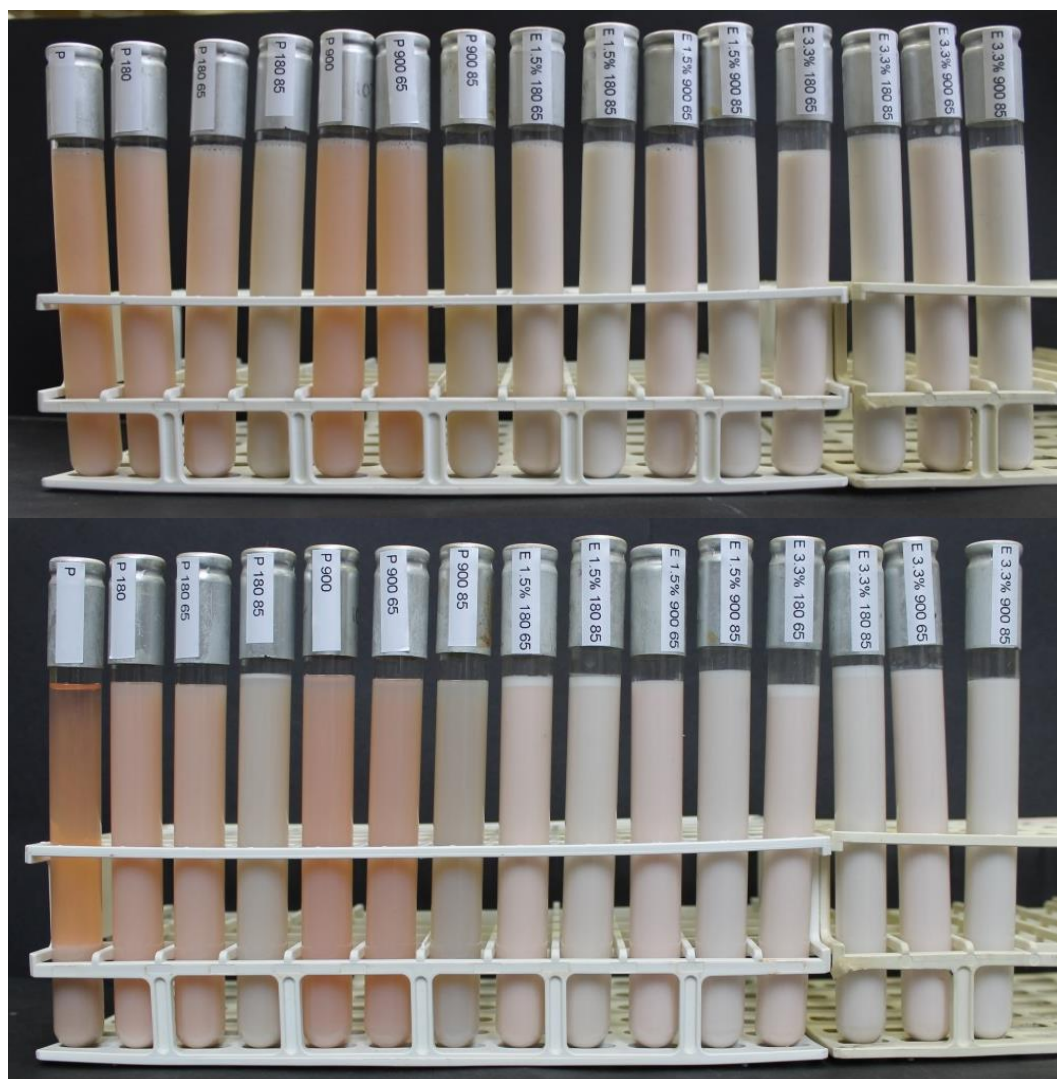


Figure 7-4 Lentil protein dispersions (to the left) and lentil protein stabilised emulsion containing 1.5 or 3.3% fat (to the right), measured on day 0 (first row) and after 21 days of storage (second row).

Table 7-3 Effect of homogenisation pressure (180 or 900 bar) and heat treatment (65 or 85 °C) of lentil protein dispersions (LP) and lentil protein stabilized emulsion (LPE) containing 1.5 or 3.3% fat, measured on day 0 and after 21 days of storage on CIE L*a*b* and whiteness index.

Samples	L*	a*	b*	Whiteness Index
LP untreated	46.46±0.40 ^h	4.12±0.04 ^d	1.75±0.23 ^m	46.27±0.40 ^l
LP 180	55.53±0.29 ^e	4.73±0.13 ^{bc}	4.30±0.24 ^{jk}	55.07±0.28 ⁱ
LP 180 65	56.98±0.12 ^d	3.86±0.12 ^e	4.15±0.23 ^k	65.60±0.10 ^h
LP 180 85	58.65±0.61 ^c	-0.25±0.12 ^l	2.64±0.24 ^l	58.57±0.6 ^g
LP 900	47.73±0.59 ^g	3.41±0.11 ^f	-0.32±0.17 ⁿ	47.62±0.59 ^k
LP 900 65	48.75±0.33 ^g	2.58±0.10 ^g	-0.71±0.16 ⁿ	48.68±0.33 ^k
LP 900 85	50.16±0.84 ^f	-1.28±0.05 ^m	-2.34±0.18 ^o	50.09±0.85 ^j
LPE 180 65 1.5%	72.53±1.01 ^b	5.54±0.12 ^a	8.88±0.23 ^a	70.60±1.03 ^f
LPE 180 85 1.5%	72.28±0.38 ^b	1.90±0.05 ^h	7.52±0.18 ^{cd}	71.21±0.38 ^{ef}
LPE 900 65 1.5%	72.53±0.64 ^b	4.56±0.10 ^c	6.01±0.17 ^h	71.51±0.62 ^{def}
LPE 900 85 1.5%	73.25±0.53 ^b	1.14±0.07 ^k	4.76±0.29 ^{ij}	72.81±0.54 ^c
LPE 180 65 3.3%	76.90±0.12 ^a	4.83±0.03 ^b	8.13±0.04 ^b	75.04±0.12 ^b
LPE 180 85 3.3%	76.71±0.61 ^a	1.71±0.06 ^{hi}	6.98±0.20 ^{ef}	75.62±0.55 ^{ab}
LPE 900 65 3.3%	76.28±0.96 ^a	4.26±0.11 ^d	6.20±0.22 ^{gh}	75.12±0.99 ^b
LPE 900 85 3.3%	77.38±0.49 ^a	1.40±0.20 ^j	5.01±0.53 ⁱ	76.78±0.39 ^a
LPE 180 65 1.5% 21	73.03±0.32 ^b	5.65±0.09 ^a	8.66±0.06 ^a	71.12±0.33 ^f
LPE 180 85 1.5% 21	73.39±0.38 ^b	1.92±0.10 ^h	7.19±0.03 ^{de}	72.37±0.38 ^{cde}
LPE 900 65 1.5% 21	72.64±0.26 ^b	4.81±0.02 ^b	5.98±0.07 ^h	71.58±0.25 ^{def}
LPE 900 85 1.5% 21	73.01±0.28 ^b	1.16±0.07 ^k	4.40±0.09 ^{jk}	72.63±0.26 ^{cd}
LPE 180 65 3.3% 21	76.92±0.76 ^a	4.84±0.02 ^b	7.84±0.15 ^{bc}	75.15±0.73 ^b
LPE 180 85 3.3% 21	77.03±0.51 ^a	1.66±0.07 ^h	6.51±0.24 ^{fg}	76.07±0.49 ^{ab}
LPE 900 65 3.3% 21	77.19±0.57 ^a	4.34±0.21 ^d	6.10±0.30 ^{gh}	75.99±0.43 ^{ab}
LPE 900 85 3.3% 21	77.23±0.72 ^a	1.33±0.08 ^{jk}	4.68±0.44 ^{ij}	76.71±0.61 ^a

Values within a column that share a superscript are not significantly different from one another (p < 0.05)

7.4.7 Accelerated physical stability

Physical stability was analysed with an analytical centrifuge, measuring light transmission across the entire length of the sample over time. Figure 7-5 shows exemplary typical stability graphs, and numerical data of the separation rate of protein dispersions and emulsions obtained as affected by homogenisation and thermal treatments. It can be seen that the untreated LPI dispersions were highly unstable. The transmission increased quickly throughout the whole length of the sample during the measurement (red profile being the initial transmission to green the last profile) and a thick sediment layer of 6.08 mm at the bottom of the cuvette was formed. Due to homogenisation, the LP became more stable in solution; Transmission profiles of 180 bar treated LPI dispersions changed slowly and only during the second step of centrifugation at higher g forces a higher transmission, with a slight slope from top to bottom can be seen. This indicates a particle movement to the bottom of the cuvette, which formed a sediment layer of 0.18 mm. Homogenisation of 900 bar treatments lead to an increase of the initial transmission. This is due to a decrease of opacity, since the protein particles were reduced in size and solubilised. During accelerated gravitation, the transmission profiles increased slowly throughout the samples, showing the same trend as the 180 bar treated samples. Temperature treatments showed no significant effect on the stability.

Emulsions were characterised by an initial transmission very close to zero throughout the cuvette, meaning no light was passing through the sample due to homogeneous distribution of oil droplets and protein particles. During accelerated gravitation, the transmission at the bottom of the cuvette progressively increased, indicating a movement of particles and droplets to the top, in the opposite direction as the protein particles moved. The addition of oil and formation of oil droplets improved the stability of the proteins in the solution, by diffusing at the interface of the oil droplets. Essentially, the LP and the oil stabilised each other. Overall, both homogenisation pressures produced stable emulsions, but 3.3% fat emulsions homogenised at 900 bar were the most stable, having the lowest separation rate of about 2 %/h.

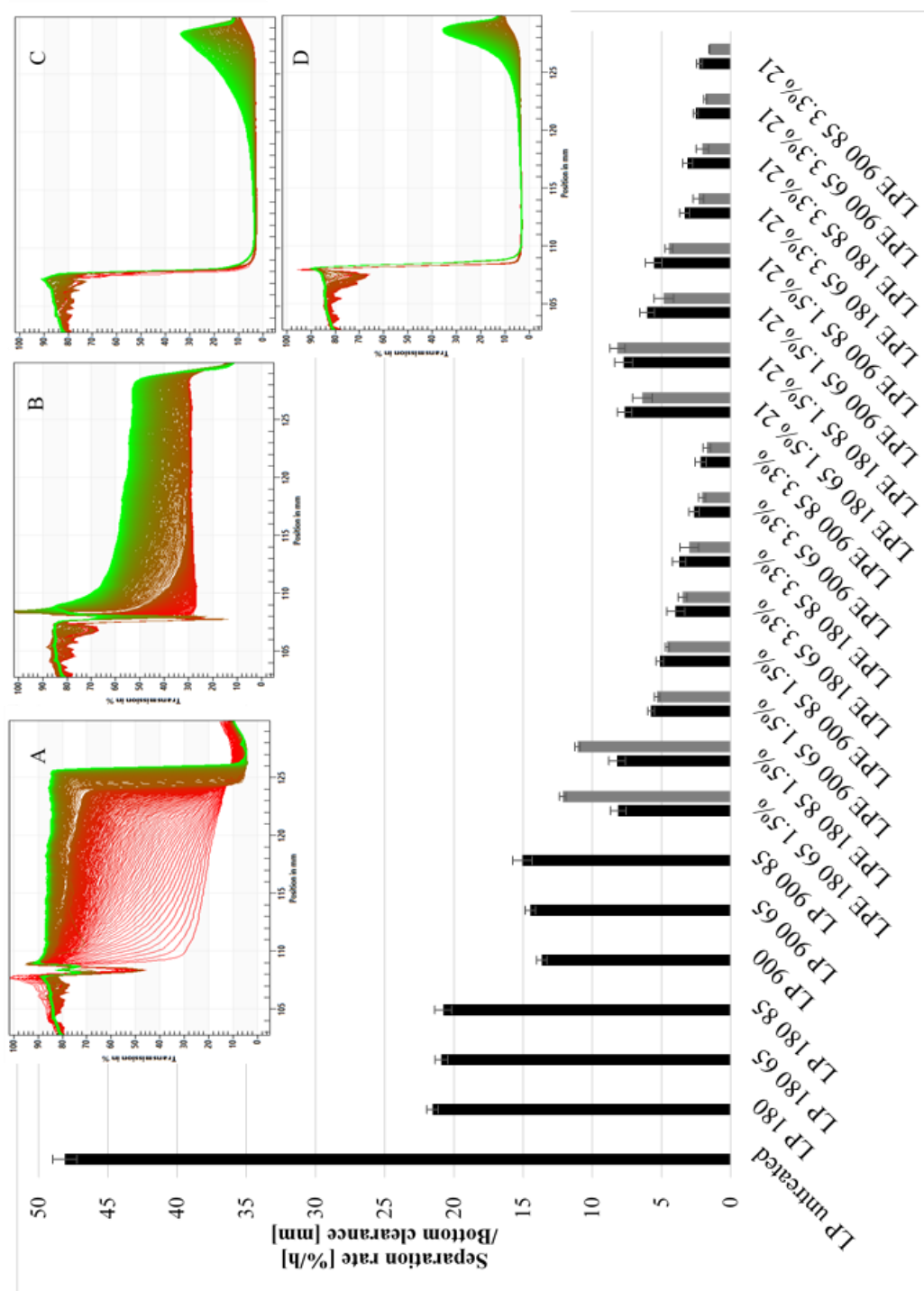


Figure 7-5 Separation rate (black) and bottom clearance (grey) of lentil protein (LP) dispersions and lentil protein stabilised emulsion (LPE) containing 1.5 or 3.3% fat, measured on day 0 and after 21 days of storage as effected by of homogenisation pressure (180 or 900 bar) and heat treatment (65 or 85 °C) with selected transmission profiles A) untreated lentil protein dispersions, B) lentil protein dispersions homogenised at 180 bar, lentil stabilised emulsion, containing 3.3% fat, pasteurised at 65 °C, homogenised at C) 180 bar and D) 900 bar.

Colloidal stability is one of the main issues for PBMSs. A wide range of commercial PBMSs analysed in a previous study (Jeske *et al.* 2017, Chapter 3) showed high separation rates for almost all products due to insoluble plant material, flocculation and fat separation. Cow's milk on the other hand, as a stable emulsion, showed a separation rate of 3.87 %/h. For all LPI stabilised emulsions comparable separation rates were found while the 900 bar treated sample heated to 85 °C was even more stable (2 %/h), exhibiting a lower separation rate than cow's milk.

After a storage time of 21 days, emulsions were still as stable. Only changes were found regarding the bottom clearance for emulsions homogenised at 180 bar. These samples showed to have slightly less particles moving to the top of the cuvette during accelerated gravitation. However, the separation rate did not change over storage, indicating the same colloidal stability overall.

7.4.8 Sensory affective testing

The sensory ratings of selected commercial PBMSs and the lentil emulsion, containing 3.3% oil and additionally 2.5% sucrose and 0.08% (w/w) salt, are presented in Table 7-4. Bovine milk was deliberately not included, and consumers were informed that only PBMSs were displayed, to avoid a comparison to bovine milk, and concentrate on "liking". The hedonic ratings were similar for all the samples and "slightly liked" overall, except for the hemp-BMS. This is probably due to the untrained assessors, of which just 36.7% consumed PBMSs at least weekly, while 20% of them claimed to never drink it. While the appearance of hemp-BMS was liked most, possibly due to the bright white colour similar to cow's milk, it scored the lowest for all the other attributes and was overall "disliked slightly". No significant differences were found between the lentil-BMS and the other commercial PBMSs. Only in terms of appearance, the lentil sample scored lowest together with soya and oat, probably due to the off-white colour. Some of the commercial PBMSs contained additives, like stabilisers, and flavours, which improve the sensory. Already this simple formulation of a lentil-BMS showed promising results and further incorporating of some ingredients could improve the sensory perception of this lentil-BMS. While taste is one of the most important reasons for consumers to buy a product (60% of the panellists claimed they would consume more often PBMSs, if the taste improved), also 40% look for proven health benefits. For

instance, the produced lentil-BMS could be advertised as “high in protein”, according to the European law, since the protein provides 25% of the energy (European Parliament and Council of the European Union, 2006), while most of the PBMS contain <0.5 % protein (Jeske *et al.*, 2017; Chapter 3).

Table 7-4 Sensory acceptance testing of commercial plant-based milk substitutes and a lentil-based formulation homogenised at 900 bar, pasteurised at 85 °C evaluated on a 9-point hedonic scale.

Samples	Appearance	Aroma	Mouthfeel	Flavour	Overall
Oat-BMS	5.12±1.80 ^d	6.02±1.54 ^{ab}	6.50±1.41 ^a	6.15±1.75 ^a	6.18±1.63 ^a
Rice-BMS	6.23±1.66 ^{bc}	6.00±1.56 ^{ab}	6.42±1.34 ^a	5.90±2.06 ^a	6.00±1.80 ^a
Hemp-BMS	7.48±1.11 ^a	5.42±1.84 ^b	4.90±2.02 ³	3.88±1.98 ^b	4.45±1.98 ^b
Almond-BMS	6.82±1.61 ^{ab}	6.48±1.72 ^a	5.92±1.69 ^a	5.42±1.86 ^a	5.75±1.59 ^a
Soya-BMS	5.27±1.73 ^d	6.02±1.47 ^{ab}	6.05±1.82 ^a	5.43±1.88 ^a	5.62±1.65 ^a
Lentil-BMS (LPE 900 85 3.3%)	5.48±1.82 ^{cd}	5.77±1.83 ^{ab}	6.20±1.42 ^a	5.27±1.89 ^a	5.53±1.51 ^a

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$)

7.5 Conclusion

Lentil proteins have been studied for their functional properties and ability to stabilise emulsions. With the application of high-pressure homogenisation lentil proteins were solubilised to a major extent and sunflower oil was successfully emulsified. With a homogenisation pressure of 900 bar and a heat treatment of 85 °C highly stable nano-emulsion were generated with great colloidal stability, and appearance and viscosity similar to cow's milk. Sensory testing also proved the great potential of lentil protein-based emulsions as novel products, since the textural and organoleptic attributes compared well to commercial PBMSs, including soya-based products. The produced lentil-BMS possessed great functional and nutritional properties, providing valuable protein to the diet. Further work on the formulation and processing of related products like yoghurt will be performed to meet the growing demand of consumers of such dairy alternatives.

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Chapter 8: General discussion

8.1 General discussion

The dairy alternatives market has seen rising levels of interest in recent years, and the market is developing fast. Especially the range of plant-based milk substitutes (PBMSs) has expanded considerably and grew by 20% between 2012-2016 with products based on different nuts, seeds, legumes, and cereal grains (Green, 2017). Even though the array of PBMSs is very versatile (compare Table 3-1), they are all essentially produced by dissolving and disintegrating plant material to obtain a watery extract. Compounds such as oil, proteins, carbohydrates and other minor composites are dissolved or dispersed to generate a colloidal liquid system (Diarra *et al.*, 2005). The increasing consumer demand is based on lifestyle changes, interest in alternative diets and the health benefits associated with plant-based diets in general, making PBMSs not only desirable anymore for those with allergies or intolerances. In **Chapter 2**, a literature review was presented which explored the heritage and current status of PBMSs and also assessed leading trends, nutritional properties, consumer demands, legislation, and sustainability of dairy alternatives. Further, the current market situation was explored by analysing some of the key products driving the market. A detailed study of the physicochemical and nutritional properties of commercial products was conducted in **Chapter 3**. PBMSs appear similar to bovine milk and are used to replace it in the diet. However, essential differences are found when comparing commercial PBMSs, which differ remarkably in nutritional and physicochemical properties. Only soya-based milk substitutes (BMSs) showed overall good results with comparable characteristics to cow's milk. Half of the samples analysed contained less than 0.5% of protein, and some also contained high amounts of sugar and showed high values for *in vitro* glycaemic properties. As explained in **Chapter 2**, processing techniques are fundamentally different and depend highly on the starting material. Rice-BMSs on the one hand, being based on a starchy cereal containing low amounts of protein (Nadathur *et al.*, 2017) are produced by hydrolysis of the starch. Therefore, the yield of simple sugars is high (5.58 - 7.02 g/100g) and the protein content is correspondingly low (0.07 - 0.32 g/100g) in rice-BMSs. Soya-BMSs, on the other hand are based on a legume, which is one of the major oil and protein crops globally, but does not contain a lot of carbohydrates (Thrane *et al.*, 2017). Hence, the composition of soya-BMSs is low in sugar (0.88 - 0.36 g/100g), while higher amounts of protein (2.61 - 3.70 g/100g) and oils (1.48 - 3.28 g/100g) are extracted (compare Table 3.3). Also, stability and

rheology properties of commercial PBMSs were generally poor and again soya-BMSs were superior. They were the only products which showed good performances without containing hydrocolloids. The work presented in **Chapter 3** showed clearly that the product quality needs to improve in terms of nutritional and physicochemical properties. Based on these findings, the following studies were designed to target specific challenges using processing techniques, enzymology and microbiology. Quinoa and lentils were selected as raw materials for their specific composition and techno-functional properties and were used to showcase different opportunities for the processing and development of PBMSs.

Quinoa was chosen for its high nutritional value and in particular for its exceptional quality of protein (which was discussed in **Chapter 5**). Furthermore, quinoa contains a substantial amount of starch (60%) similar to rice (Nadathur *et al.*, 2017). Due to hydrolysis of this starch, the obtained quinoa-BMS contained high amounts of sugar (9.09 g/100g of glucose), similar to rice-BMS. In **Chapter 4**, fermentation in combination with different exogenous enzymes was used to process a quinoa-BMS in order to reduce the sugar content. Fermentation has long been used as a natural way of preserving and improving nutritional and sensorial attributes in many products. Lactic acid bacteria (LAB) are involved in numerous fermentation processes due to their ability to release a broad range of functional metabolites and have long been used in the dairy industry (Caplice and Fitzgerald, 1999). One such functional metabolite is mannitol. Its sweet taste, but low calorific value, is making it an interesting compound for the food industry. In this study, specific LAB strains were selected for their ability to metabolise fructose to mannitol. In order to provide the LAB with fructose as a substrate, different amylolytic enzymes (α -amylase, amyloglucosidase and glucose isomerase) were applied to modify the carbohydrate composition. *Leuconostoc citreum* TR116 and *Lactobacillus brevis* TR055 showed high viability and grew well in the quinoa-BMS as a substrate. The fermentation process was examined with regards to its effect on general product quality and especially assessed for the process of sugar reduction and related *in vitro* glycaemic properties. Due to the enzymatic treatment and 24 hours of fermentation with *Leuconostoc citreum* TR116 the glucose content was reduced by 40% and the glycaemic load by 35% compared to the untreated sample. Furthermore, it was estimated that the sweetness was only reduced by 24%, due to the production of

mannitol. This study demonstrated that the thorough design of combined biotechnological techniques offers elegant ways to improve nutritional properties and meet consumer demands of PBMS.

Further work will be needed to evaluate and improve the sensory properties and processing to develop healthy and functional products. The excretion of mannitol goes hand in hand with the production of acetic acid, which can result in a vinegar-like and more pungent sourness than lactic acid (Burdock, 2009). Therefore, the balance between organic acids and also sugars and mannitol needs to be determined for a harmonious organoleptic perception. Another group of metabolites that attracted much attention recently are exopolysaccharides (EPS). Due to their molecular structure EPS can provide texture and prevent physical instability and phase separation in a liquid media. Quinoa proteins do not easily form self-supporting cold setting gels upon acidification like casein during the production of yoghurt (Mäkinen *et al.*, 2015). Therefore, EPS can contribute to the formation of a stronger three-dimensional network during the fermentation process. Yoghurt substitutes with good textural and organoleptic properties based on different plant materials were produced by EPS excreting LAB strains (Lorusso *et al.*, 2018; Zannini *et al.*, 2018; Mårtensson *et al.*, 2001). The combination of functional LAB strains providing both texture and taste (i.e. EPS and mannitol) would be an interesting approach for the development of new clean-label products.

As previously broached, the functionality (e.g. gelling properties) of plant proteins can be limited. Generally, plant proteins show low solubilities and are therefore difficult to extract into a water-based beverage (Day, 2013). Unlike cow's milk, which is engineered for perfect nutrition of calves, seeds are made to endure time and biotic as well as abiotic stress factors. Therefore, seed storage proteins are stored in a stable form. Only during germination do proteins and other constituents become accessible for the awakening metabolism of the plant with the help of plant hormones and released enzymes (Koornneef *et al.*, 2002). Emulating the approach of nature, the application of specific digestive enzymes is an inexpensive and effective tool for controlled catalysis and production of hydrolysates (Clemente, 2000). In **Chapter 4** the application of different commercial amylolytic and proteolytic

enzymes was investigated in the production of a quinoa-BMS and its effect on protein and product quality parameters.

The initial protein solubility found in the quinoa-BMS was low, with 48.02%. During processing steps, such as filtration or decanting, the insoluble part might be lost. (However, as shown in **Chapter 7**, homogenisation as a processing step increased the solubility of lentil proteins to a major extent.) Three different commercial proteases with different modes of action were added to a whole grain quinoa flour in order to increase the protein solubility. The results showed that endopeptidases (Profix and Bioprotease), which cleave bonds inside the peptide chain and unravel the protein structure to a greater extent (as observed in the SDS-PAGE and circular dichroism), had a bigger impact on the protein solubility. The enzyme Profix increased the protein solubility the most (75.82%) with increasing degree of hydrolysis (DH). Considering the product properties like foaming, colloidal stability or the emulsifying properties, little effect of the proteases was observed. However, it can be concluded that the other constituents had a major impact on these properties also. Sedimentation in the quinoa-BMS was caused by big particles of undissolved plant material, since no filtration step was applied. As pointed out in **Chapter 3**, the product quality of most commercial products is poor. This is often overcome by the addition of additives like emulsifiers and stabilisers rather than exploring the full potential of the raw material and its compounds. However, understanding the nature of sedimentation and its composition would help to stabilise the products. Quinoa contains substantial amounts of insoluble fibre, about 5.37 g/100g, which could be one cause of the instability (Lamothe *et al.*, 2015; Hager *et al.*, 2012). Different enzymes that target fibres, such as cellulases or pectinase, could be applied to break down these compounds. This approach was used by Rosenthal *et al.* (2003) and successfully improved the physical stability and sensory attributes of a soya-BMS. Another compound causing the formation of poorly soluble compounds is phytic acid. It can form chelates with metal ions such as calcium, magnesium, zinc, and iron and can also interact with amino acids, proteins and starch, which may affect the bioavailability of these nutrients negatively. Quinoa contains high amounts of phytate (the salt of phytic acid) with 1.34 g/100 g (Hager *et al.*, 2012). The hydrolysis of phytate could potentially help to improve the physical stability, while it has been applied to various products already and proved to increase

the *in vitro* digestibility (Liener, 2003). Endogenous phytase is produced during germination by the seeds themselves or can be released *in-situ* by LAB fermentation.

In general, the change of the nutritional value of the proteins as affected by enzymatic hydrolysis needs to be assessed. The same should be considered for physical treatments like homogenisation and heat treatment (**Chapter 7**) as these also modify the protein considerably. Protein hydrolysates are of high interest for specific formulations, such as high-energy supplements, or hypoallergenic infant formulas for special dietetic or therapeutic needs (Clemente, 2000). Further, hydrolysis, or general modification of proteins, are widely used to improve nutritional properties like digestibility (Koopman *et al.*, 2009). It has also been reported that allergenicity can be decreased with such processing, for example for chickpea protein hydrolysates (Clemente, Vioque, Sanchez-Vioque, *et al.*, 1999), or for proteins in an almond-BMS homogenised at ultra-high pressure (Briviba *et al.*, 2016).

Furthermore, the assessment of sensory characteristics of hydrolysed proteins is an important aspect in food applications. Released short peptides chains may cause bitter off-flavours due to their structure-activity and exposure of interior hydrophobic amino acid side chains (Maehashi and Huang, 2009). Flavourzyme was identified to not cause such bitterness, as it releases single amino acid residues with low levels of hydrophobic residues (Nilsang *et al.*, 2005; Seo *et al.*, 2008). Therefore, Flavourzyme showed the most favourable characteristics, increasing the solubility considerably, improving the sensory, and was chosen to be applied in **Chapter 4**, to release amino acids for fermentation.

Overall, it was found that the assessment of quinoa proteins within the complex system of its whole grain is difficult to conduct, and that other constituents like starch and fibre are governing the product properties to a major extent. The use of extracted protein ingredients enables the formulation of a product with desired nutritional and functional characteristics and does not rely solely on the composition and functionality of the raw material. This approach of product development and research was followed in **Chapters 6 and 7**, where lentil protein isolates (LPIs) were studied and applied to formulate a lentil-BMS. Obtained by two different isolation techniques, LPIs were characterised and compared for their techno-functional properties. Both isoelectric precipitation (IEP) and ultrafiltration (UF) resulted in

high protein concentrations. However, LPI prepared by UF contained higher levels of protein (93.7 g/100g), less soluble fibre, but higher amounts of calcium and magnesium. LPI-UF resulted also in a product with greater functional properties like higher solubility (44.72% at pH 7), water holding capacity, greater gelling and foaming properties as well as emulsion stability. This was of especially great interest for the application in **Chapter 7**. The ability of LPI-UF to stabilise emulsions with similar fat (3.3 and 1.5%) and protein (3.3%) contents to commercial cow's milk was evaluated. The purpose of this study was to explore the development of a product, containing the same macronutrients as cow's milk, since the nutrient density was one of the main pitfalls of commercial products pointed out in **Chapters 2 and 3**. Many studies already assessed the formation of emulsions containing a higher fat-load and less protein, since they were aiming for other product types such as salad dressings and creams (Ma *et al.*, 2016; Primozic *et al.*, 2018; Can *et al.*, 2011; Jarpa-Parra *et al.*, 2015). The composition of a higher protein to fat ratio can be critical, since the oil-interface may be saturated, leaving more protein in the water-phase, causing a dry mouthfeel and colloidal instability. This and other product characteristics of LPI-stabilized emulsions were evaluated as affected by different dynamic pressures (180 and 900 bar) and pasteurisation treatments (65 °C for 30 min, and 85 °C for 2 min). Valuable knowledge was gained about the impact on the proteins by applying the same treatments to mere protein solutions. The solubility of the LPI-UF was increased to a major extent after the homogenisation at both pressures (96.40 and 98.85% for homogenisation at 180 and 900 bar, respectively). Generally, dynamic-high pressure treatments could be a promising tool to disrupt and improve colloidal stability of a whole range of compounds. Kubo, Augusto and Cristianini (2013) demonstrated the effectiveness of high-pressure homogenisation to disrupt suspended pulp particles and improve stability to sedimentation in tomato juice. It would therefore be interesting to apply this to samples used in **Chapters 3 and 4** based on whole grain flour of quinoa.

Highly stable nano-emulsion stabilized by LPI-UF were generated, with appearance and viscosity similar to cow's milk. Samples homogenised at 900 bar and heat-treated at 85 °C were found to be more stable against phase separation than cow's milk. Dynamic-high pressure and heat treatments affected the surface properties of the lentil protein, i.e. an increase of hydrophobicity and decrease of free

sulfhydryl groups was observed. However, due to the pH adjusted to 7 and thus far from the isoelectric point of pH 4.5, the proteins remained stable in solutions and were less susceptible to induced aggregation than observed at a lower pH, as found by Primožic *et al.* (2018). The ability of LPI-UF to stabilise emulsions shows great potential in the food and beverage industry, with potential applications in cosmetics and pharmaceutical production (Mason *et al.*, 2006).

Furthermore, a life cycle assessment was conducted to evaluate the environmental impact of LPIs and revealed promising performances for both isolation processes, especially when compared to traditional cow's milk proteins. For example, it was anticipated that the production of both LPIs releases 4-fold less carbon dioxide equivalents into the atmosphere (3.5 to 4.2 kg CO²-e/kg) than the formation of caseinate or whey protein (19 kg CO²-e/kg and 20 kg CO²-e/kg, respectively) (Thrane *et al.*, 2017). It would also be interesting to study the environmental impact of the formulated PBMS to assess the whole production path.

Overall, the functional, nutritional and environmental properties of LPIs showed promising characteristics. With the availability of protein isolates, tremendous amount of possibilities are unveiled for product formulations such as pasta, bread and infant formulae and may also be suitable for the substitution of soya or animal derived proteins. Sensory affective testing of the formulated lentil-BMS showed good results and textural and organoleptic attributes compared well to commercial PBMSs. All in all, the produced lentil-BMS possessed great functional, organoleptic and nutritional properties. As a novel PBMS it would provide valuable protein to the diet. Further work on the formulation will be needed regarding the micronutrients, since cow's milk is also an important source of dietary calcium, iodine, or vitamin B12 (FAO, 2013).

A possible way to counteract the individual drawbacks of either quinoa- or lentil-BMSs would be to combine the two ingredients used in this thesis. Quinoa flour could serve as a base, supplying mainly carbohydrates, high quality proteins and valuable compounds like minerals and fibres. The main quantities of protein and the colloidal stability would be provided by the emulsion stabilised by LPI. Hence, a functional beverage with a balanced nutritional value could be formulated, addressing the challenges pointed out in **Chapters 2 and 3**.

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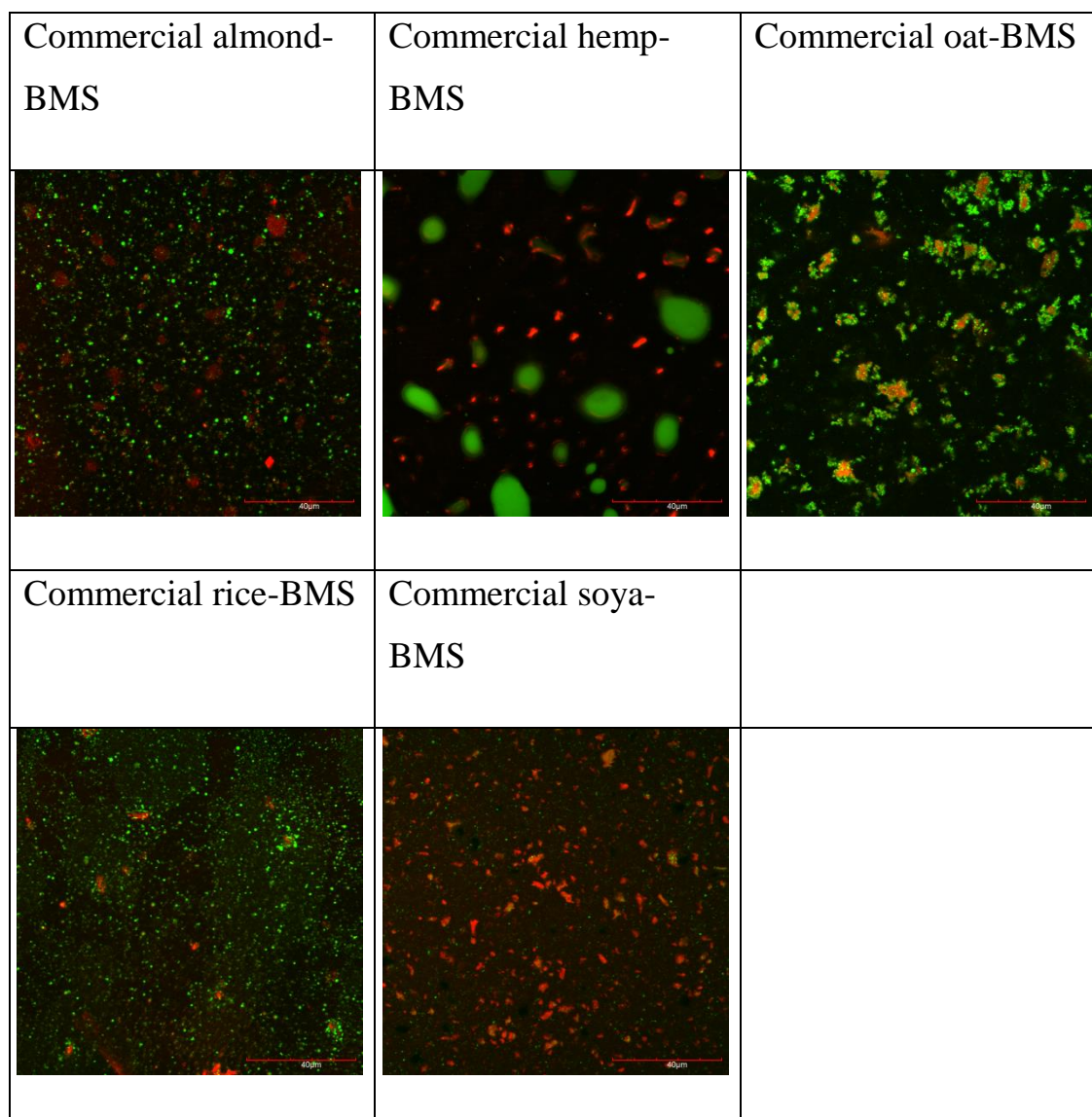
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Appendix

Appendix A- Additional figures

Appendix A-1. Confocal laser scanning microscopy images of commercial PBMS. The microstructural analysis of emulsions was performed using a confocal laser scanning microscope according to the method described in 7.3.11. Proteins are stained red, fat is stained green. Scale bar 40 μm .

Appendix B- Publications and presentations

First author publications

Jeske, S., Zannini, E., Arendt, E.K. (2017) Evaluation of Physicochemical and Glycaemic Properties of Commercial Plant-Based Milk Substitutes. *Plant Foods for Human Nutrition*. 72(1), 26–33.

Jeske, S., Zannini, E., Arendt, E.K. (2018) Past, present and future: The strength of plant-based dairy substitutes based on gluten-free raw materials. *Food Research International*. 110, 42–51.

Jeske, S., Zannini, E., Cronin, M.F., Arendt, E.K. (2018) Impact of protease and amylase treatment on proteins and the product quality of a quinoa-based milk substitute. *Food and Function*. 9(6), 3500–3508.

Jeske, S., Zannini, E., Lynch, K.M., Coffey, A., Arendt, E.K. (2018) Polyol-producing lactic acid bacteria isolated from sourdough and their application to reduce sugar in a quinoa-based milk substitute. *International Journal of Food Microbiology*. 286(April), 31–36.

Other publications

Zannini, E., Jeske, S., Lynch, K.M., Arendt, E.K. (2018) Development of novel quinoa-based yoghurt fermented with dextran producer *Weissella cibaria* MG1. *International Journal of Food Microbiology*. 268, 19–26.

Publications in Progress

Alonso-Miravalles L. and Jeske S., Bez J., Detzel A., Busch M., Krueger M., Wriessnegger C.L., O'Mahony J.A., and Arendt E.K. Membrane filtration and isoelectric precipitation technological approaches for the preparation of novel functional and sustainable protein isolates based on lentils

Jeske S. Bez J. Arendt E.K., Zannini E. Formation, stability, and sensory characteristics of a lentil-based milk substitute as affected by homogenisation and pasteurisation.

Oral presentations

Jeske S., Zannini E. and Arendt E. K. (2016). Evaluation of Physicochemical and Glycaemic Properties of Commercial Plant-Based Milk Substitutes. 15th European Young Cereal Scientists and Technologists Workshop, Bergamo, Italy, April 2016. Awarded for best oral presentation prize

Jeske S., Zannini E. and Arendt E. K. (2017). The strength and challenges of plant-based dairy substitutes based on gluten-free raw materials. 4th International Symposium on Gluten-Free Cereal Products and Beverages, Cork, Ireland, October 2016.

Jeske S., Zannini E. and Arendt E. K. (2016). Evaluation of Physicochemical and Glycaemic Properties of Commercial Plant-Based Milk Substitutes. 46th Annual Food Science and Technology Conference, Dublin, Ireland, December 2017.

Jeske S., Zannini E., Lynch K. M., Coffey A. and Arendt E. K. (2018). Polyol-producing lactic acid bacteria isolated from sourdough and their application to reduce sugar in a quinoa-based milk substitute. 7th International Symposium on Sourdough, Cork, Ireland, June 2018.

Jeske S., Zannini E. and Arendt E. K. (2017). Understanding and Innovating Plant-Based Milk Substitutes. 3rd Edition of International Conference on Agriculture & Food Chemistry, Rome, Italy, July 2018.

Poster presentations

Jeske S., Zannini E., Cronin M. F. and Arendt E. K. (2018). Impact of protease and amylase treatment on proteins and the product quality of a quinoa-based milk substitute. 4th International Symposium on Gluten-Free Cereal Products and Beverages, Cork, Ireland, October 2016.