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1. Introduction and objectives

1.1 Plant-based milk substitutes

A growing number of consumers opt for plant-based milk substitutes for medical reasons or as a lifestyle choice. Medical reasons include lactose intolerance, with a worldwide population prevalence of 75%, and cow's milk allergy (Mäkinen et al., 2016). Also, in countries where animal-based milk is scarce and expensive, plant-based milk substitutes serve as a more affordable option. However, many of these products have sensory characteristics objectionable to the mainstream western palate. Technologically, plant milk substitutes are suspensions of dissolved and disintegrated plant material in water, resembling cow's milk in appearance. They are manufactured by extracting the plant material in water, separating the liquid, and formulating the final product. Homogenization and thermal treatments are necessary to improve the suspension and microbial stabilities of commercial products. These can be consumed as such or be further processed into fermented dairy-type foods such as yoghurt or cheese-like products. The nutritional properties depend on the plant source, processing, and fortification. As some products have extremely low protein and calcium contents, consumer awareness is important when plant milk substitutes are used to replace cow's milk in the diet, e.g. in the case of dairy intolerances. If formulated into palatable and nutritionally adequate products, plant-based substitutes can offer a sustainable alternative to dairy products.

1.2 Infant formula

Foods for infants and young children collectively represent a wide range of products from the age of 0 months to 3 years. Infant formula (infant milk) or first-age infant formula (0 to 6 months) is an industrially-produced, highly formulated, nutritionally complete, human milk substitute, designed for infant consumption during the first months of life through to the introduction of appropriate complementary feeding (Koletzko et al., 2010). The protein sources commonlyused in infant nutritional products are dairy based (i.e., whey and casein). However, alternative sources to dairy proteins are necessary in the formulation of some infant nutritional products, mainly due to allergies and intolerances of the infants to human or cow's milk. These include cow's milk protein allergy or intolerance and lactose intolerance. Furthermore, the increase of people with vegetarian diet and/or environmental concerns are also causing the increased utilisation of plant proteins in a wide range of food product formulations. Soy-based protein is the main plant-based protein source currently used for non-dairy infant formula products. However, proneness to soybean allergy has

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been found in babies and children, and in addition, soybean production is not sustainable. As such, alternative plant-based and hypoallergenic formulas are in increasing demand.

According to EFSA (2014) and the European Regulation (EU) 2016/127, the only source of plant protein considered safe and suitable for use in infant and follow-on formulae up to date is soy protein. Therefore, the use of other protein sources in infant and follow on-formulae and/or the introduction of new technologies need clinical evaluation and their safety and suitability should be established in the target population prior to their general use in infant and follow-on formulae.

The objective of this task was to evaluate different ingredients provided by our Protein2Food partner (Fraunhofer Institute – WP2). Ingredients such as protein-rich crops (e.g. quinoa, amaranth and buckwheat) and protein isolates (e.g. lupin and lentil isolates) were tested in terms of nutritional quality and technological performance, in order to find the most optimum plant-based protein to be used in a first-age (0 to 6 months) infant formula prototypes (Figure 1). The steps carried out included nutritional composition, functional properties, mineral interactions and development of the formulation at laboratory scale and, afterwards, in pilot scale.

Figure 1. Process followed for the development of infant formula.

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2. Activities for solving the task(s)

The activities undertaken in this study to solve the tasks have been divided according to the different prototypes, due to the differences in activities that these products entailed.

2.1 Plant-based milk substitutes (UCC)

- **Selection of recipe and processing conditions for dairy alternatives (beverages, fermented products)**

UCC carried out several trials using different raw materials including whole seeds, flours and milling fractions. Based on the evaluation of the functional and processing properties, whole pulses were excluded from further trials due to anti-nutrient and high fibre contents of the hulls. Flours of dehulled faba beans and dehulled lentils (provided from FRAUNHOFER), as well as commercial chickpea, lupin, and quinoa flour were used for further investigation. These ingredients were used to create extracts in water following the processing depicted in Figure 1 shown for a quinoa-based milk substitute. Furthermore, lentil protein isolates (provided by FRAUNHOFER) were used to construct emulsion with similar nutritional composition to cow's milk. Figure 2 and 3 depicts the processing steps for quinoa and lentil-based prototypes from the raw materials to the storage of the end-products.

Figure 2. Flow chart of the production of a quinoa-based milk substitute

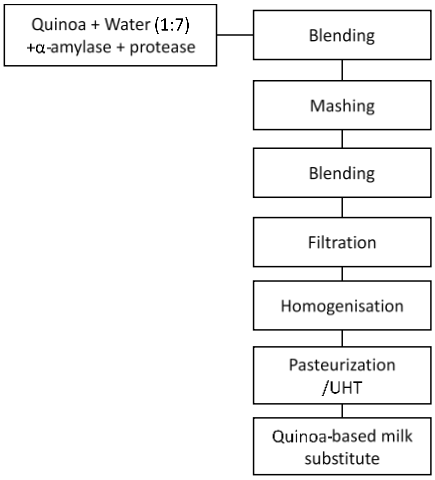
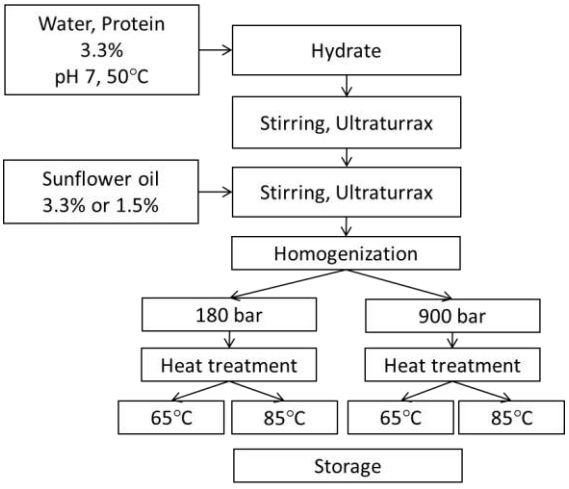


Figure 3. Flow chart of the production of a lentil-based milk substitute



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- **Selection of amylases and processing steps to improve the extractability and viscosity of plant-based milk substitute**

Quinoa flour was used as a reference to investigate and adjust processing conditions. Since legume and quinoa flour is high in starch, the efficiency of 4 α-amylases (Hitempase 2XP) have been tested on the Rapid Visco Analyzer (RVA) using different concentrations (1/10th, 1x and 10x of recommended dosage). Further, the impact of hydrolysation temperature, filtration, and the amount of raw material was analysed using also the RVA to create a product with a low viscosity.

- **Selection of proteases and processing steps to improve the protein extractability of plant-based milk substitutes**

Three commercial proteases were selected to assess their impact on protein and product properties in the quinoa-based milk substitute: Hitempase 2XP, Profix 100L, Bioprotease N100L, and Flavourzyme 1000L. Further, to study the impact of pH on the protein extractability, the protein solubility as a function of pH was analysed in a quinoa-based milk substitute. pH was adjusted before the heat treatment for starch hydrolysis.

- **Selection of germination processing steps to improve the extractability and viscosity of plant-based milk substitutes**

Response surface methodology (RSM) was used to investigate the influence of the three malting parameters, i.e. degree of steeping, germination time, and temperature on the quality of malt from lentil (Lens culinaris Medik.). Each predictor variable was tested at three levels. Germination times of 2, 3, and 4 days were chosen, degrees of steeping were chosen at 51, 53, and 55%, and germination temperatures were 15, 20, and 25°C. A series of malt quality parameters were investigated including α-amylase activity, extract, gelatinisation temperature, Kolbach Index, free amino nitrogen (FAN) and malting loss. Additionally, the impact on phytic acid and tannin was determined. Furthermore, the use and application of lentil malt for beverages was discussed.

- **Selection of lactic acid bacteria and processing steps to produce fermented plant based dairy substitutes**

UCC has been involved in screening and selecting lactic acid bacteria (LAB) which promote several characteristics in a product, such as flavor, texture or also stability. Three different LAB´s have been selected for their ability to improve texture, via the production of exopolysaccharides (EPS) (*Weisella cibaria* MG1), and to improve the flavor, due to the

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production of mannitol (*Leuconostoc citreum* TR116, and Lacto*bacillus brevis* TR055). Both applications have been developed and characterized for key properties, such as sugar composition, EPS content, acid production viscosity, and proneness for syneresis, and their microstructure.

- **Selection of processing steps to improve quality of lentil-based emulsion**

The effect of high-pressure homogenisation and heat treatments on functional and physico-chemical properties of lentil protein stabilized emulsions was studied for the formulation of a milk substitute. The products were characterized for their particle size, polydispersity index, stability index, and their microstructure.

2.2 Infant formula (UCC)

- **Evaluation of the allergenicity of the raw materials**

The first step was to carry out an evaluation of the allergenicity of the ingredients in order to exclude the ingredients with allergenicity potential. For this purpose, the Regulation (EU) No 1169/2011 and the Scientific Opinion on the evaluation of allergenic foods and food ingredients (EFSA, 2014) were revised.

- **Evaluation of the nutritional composition, physico-chemical and functional properties**

One of the objectives in task 3.5.4. (development of plant-based infant foods) is analysing the nutritional composition of the ingredients, which will facilitate in developing the most appropriate model nutritional formulations. These results have provided us with much-needed fundamental information for the further formulation. For this reason, an evaluation of flours, protein-rich flours and protein isolates, provided by our project partner Fraunhofer Institute (Work Package 2), were analysed.

❖ **Evaluation of flours and protein-rich flours**

A wide range of flours, protein-rich flours and high protein content powders were evaluated. These included quinoa wholegrain flour (QWGF), quinoa dehulled flour (QDF), quinoa protein-rich flour (QPRF), amaranth wholegrain flour (AWGF), amaranth protein-rich flour (APRF), buckwheat dehulled flour (BDF) and buckwheat protein-rich flour (BPRF), rice (RF) and maize flour (MF), and rice protein-rich flour (RPRF).

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- **Nutritional composition**

Moisture, ash, fat, protein, starch and fibre contents of samples were determined according to the standard methods of the Association of Analytical Chemists (AOAC, 2010).

- **Electrophoretic protein profile analysis**

The protein profile was assessed by sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) using precast gels (Mini-PROTEAN TGX, Bio-Rad Laboratories, CA, USA).

- **Rheological properties**

Pasting properties were studied using an AR-G2 controlled-stress rheometer equipped with a starch pasting cell (AR-G2; TA Instruments Ltd., Waters LLC, Leatherhead, UK).

- **Microstructural analysis**

Microstructural properties were examined using a JSM-5510 scanning electron microscope (JEOL Ltd, Tokyo, Japan), operated at an accelerating voltage of 5 kV.

- **Particle size distribution**

Particle size distribution of the powders was determined by laser diffraction using a Malvern Mastersizer 3000 with Aero S dry dispersion unit (Malvern Instruments, Worcestershire, UK).

- **Flow properties and colour**

Flowability and compressibility index (CI) of the powders were analysed using a Brookfield Powder Flow Tester (Brookfield Engineering Laboratories, Inc., Midleboro, MA, US). Colour of the powders was determined by measuring the CIELAB coordinates (L*, a* and b*) with a Chroma Meter CR-400 (Konica Minolta Sensing, Inc., Japan), equipped with a granular materials attachment CR-A50.

- **Evaluation of lentil protein isolate**

The nutritional composition, protein profile, microstructure and particle size distribution analysis were carried on the lentil protein isolates. Furthermore, other analysis were also carried out in this protein isolate to understand its suitability in infant nutritional products.

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- **Protein solubility at different pH**

The solubility of proteins influenced by pH, was determined by adjusting the pH of protein dispersions from 3.0 to 8.0, at 0.5 units 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. 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).

- **Emulsifying capacity and stability**

Emulsifying activity (EAI) and stability (ESI) indices were determined using the method described by Pearce and Kinsella (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:

$$(3)\ EAI\ \left(\frac{m^2}{g}\right) = \frac{2 \cdot 2.303 \cdot A_0 \cdot DF}{C \cdot \theta \cdot 10000}$$

$$(4)\ ESI\ (min) = \frac{A_0}{A_0 - A_{120}} \cdot 120$$

where DF is the dilution factor (100), C is the initial concentration of protein (0.01 g/mL), θ is the fraction of oil used to form the emulsion (0.1), and A_0 and A_{120} are the absorbance of the diluted emulsion at 0 and 120 min, respectively.

- **Selection of raw material for further processing**

An evaluation of all the analysed properties was carried out and one of the ingredients of our project partners (WP2) was selected for further processing.

- **Formulation and optimization of infant formula process with lentil protein isolate at laboratory scale**

The infant formula was formulated following the Commission Delegated Regulation (EU) 2016/127 for the macronutrient composition (protein, carbohydrate and fat). The typical process for infant formula with slight modifications was carried out (Figure 4). The amino-acids and minerals were also analysed in order to know in which ones the formulation needs to be supplemented.

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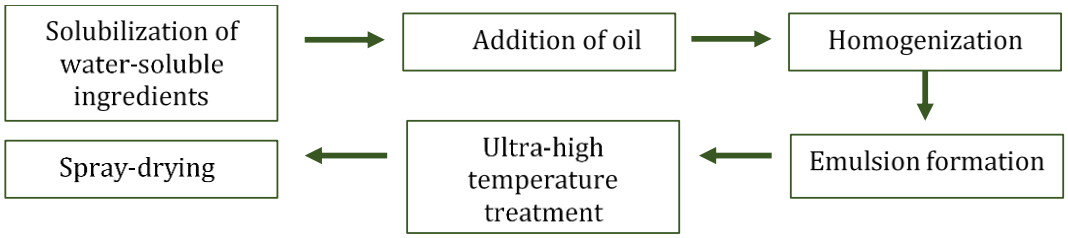

Figure 4. Diagram for the process followed to obtain first-age infant formula

- **Effect of mineral fortification on infant formula**

The infant formula was fortified with different levels of Ca^{2+} in order to understand the effect of mineral fortification on the infant formula, some properties analysed included heat stability and particle size distribution of the formula system.

- **Pilot scale processing and spray drying**

After optimizing the process and knowing the effect of minerals, a big scale process in the pilot plant facility at University College Cork was carried on. A powder formulation that could be reconstituted in water with the right amino-acid balance was obtained.

- **Evaluation of infant formula prototype**

Microscopy, solubility and microbiological analysis of the formulation were carried on. The infant formula was distributed to the different project partners (University of Copenhagen, Novolyze and Institute of Food Research Polish Academy of Sciences) for further nutritional analysis (antioxidants, *in vivo* and *in vitro* protein digestibility).

3. Results

3.1 Plant-based milk substitutes (UCC)

- **Selection of amylases and processing steps to improve the extractability and viscosity of plant-based milk substitute**

Quinoa was used as a role model and several tests were performed to optimise process parameters: Since quinoa is a product high in starch, the efficiency of 4 α-amylases (Hitempase 2XP) have been tested on the Rapid Visco Analyzer (RVA) using different concentrations (1/10th, 1x and 10x of recommended dosage). Hitempase 2XP seemed to decrease viscosity significantly even at low concentration and was therefore chosen for further use. Further, the impact of hydrolysation temperature was analysed. It was found

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that a heat treatment of 65 °C resulted in a lower viscosity than 75 °C. Filtration showed to decrease the viscosity considerably. Based on these results, the following trials were based on samples treated with the α-amylase Hitempase 2XP (500 mg/100g of quinoa) at 65 °C and filtration subsequently. Further, it was found that 12.5% of quinoa flour led to a beverage, which shows similar viscosities as cow's milk of 3.15 mPas•s (Figures 5-8). To improve the suspension and microbial stability, homogenisation and pasteurisation or ultra-high temperature treatment took place at the end of the process. Regarding the pasteurisation temperature, a low heat treatment is found favourable to prevent structural changes of the constituents. Therefore, 65 °C for 30 min was chosen to insure the microbial stability.

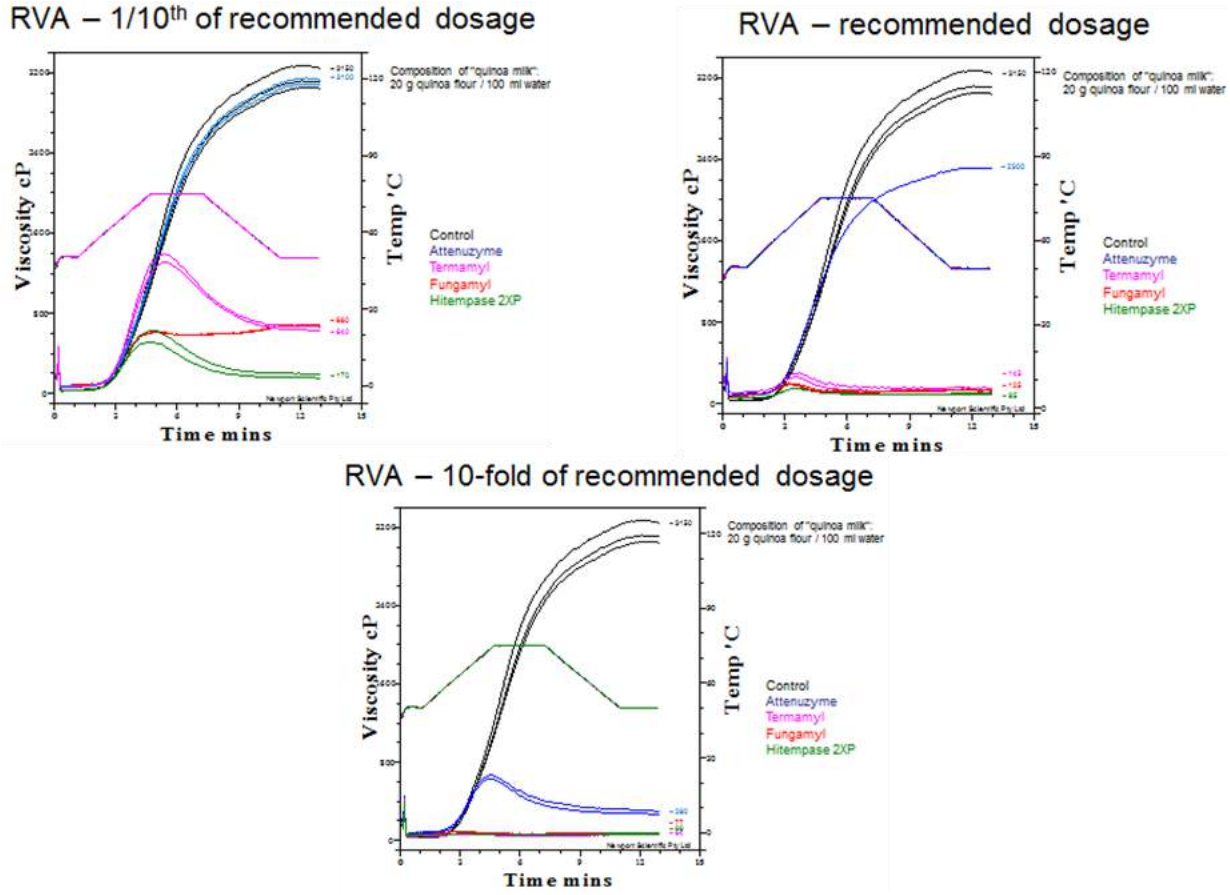

Figure 5. RVA graphs of quinoa-based milk substitutes treated with different amylases.

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Figure 6. Impact of different concentrations on viscosity 300 g/l vs 400 g/l; dark green: viscosity of 300 g/L quinoa flour, light green: viscosity of 400g/L quinoa flour; both were treated at 65 °C and filtered

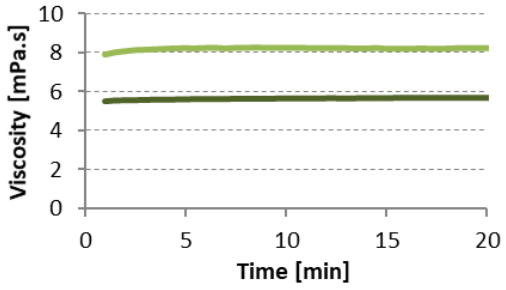


Figure 7. Impact of filtering on viscosity; dark green: unfiltered, light green: filtered. Both samples contain 300 g/L quinoa flour and were treated at 65 °C

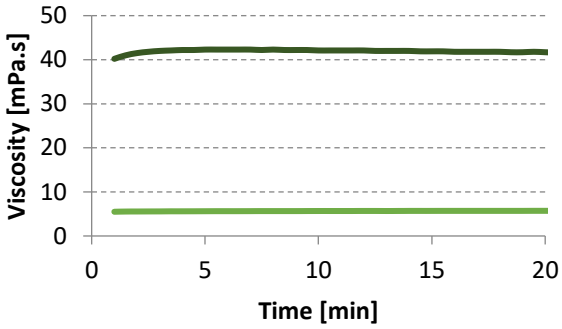
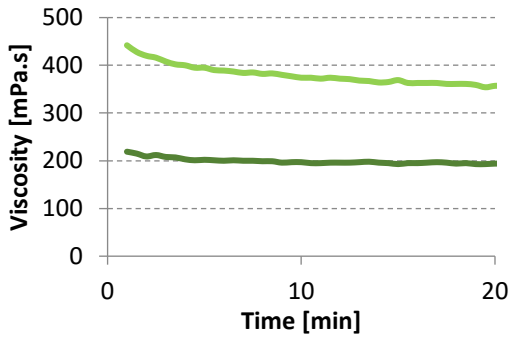


Figure 8. Effect of different mashing temperatures on viscosity; dark green: viscosity of 65 °C, light green: viscosity of 75 °C; both samples contained 400 g/L quinoa flour and were not filtered



- **Selection of proteases and processing steps to improve the protein extractability of plant-based milk substitutes**

The influence of different commercial proteases on protein properties, protein solubility and product quality in a quinoa-based beverage was studied. Three commercial enzymes were selected: Profix 100L, Bioprotease N100L, and Flavourzyme 1000L. Solubility is among the most important property concerning functionality of food proteins and it is, in general, accompanied by better functionality for most food applications and often a requirement for functional characteristics like emulsification. The protein solubility was initially low (48,02%) and was improved by increasing protease concentration (75.82% and

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70.37%, for Profix and Bioprotease, respectively) (Table 1). SDS-PAGE and circular dichroism analysis revealed the degree of hydrolysis; especially Profix degraded the proteins extensively (Figure 9).

Table 1. Degree of hydrolysis, protein solubility, and surface hydrophobicity of quinoa-based milk substitutes treated with different enzymes

Sample	Degree of Hydrolysis [%]	Protein solubility [%]	Surface Hydro-phobicity [-]
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}

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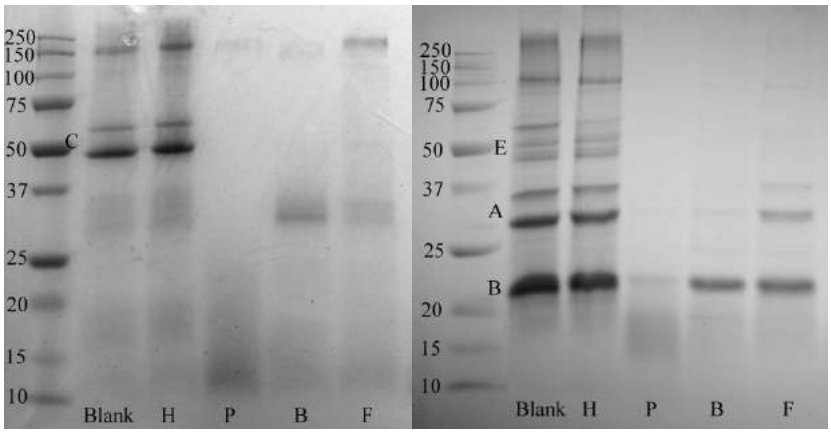

Figure 9. SDS-PAGE gels of QBMS, treated with different enzymes, under non-reducing (left) and reducing conditions with DTT (right); H=Hitempase, P=Profix 50x, B=Bioprotease 25x, F=Flavourzyme 10x, C1=combination 1, C2=combination 2.

Flavourzyme with a dosage of 91,5 µL/100g quinoa-based milk substitute was chosen for further trials due to its ability to solubilize the protein, while it did not hydrolyze the protein extensively. Maintaining a certain structure enables a protein also to maintain more of its functionality (e.g. foaming and emulsification properties). Furthermore, the impact of pH was found to be un-significant (Table 2). Therefore, no pH adjustment will be considered for the preparation of such samples.

Table 2. Protein solubility as a function of pH (adjusted before extraction) in quinoa based milk substitute

pH	Protein solubility [g/100g]
6.0	68.41 ± 1.24
6.5	65.17 ± 0.70
7.0	63.47 ± 1.05
7.5	65.79 ± 0.00
8.0	67.10 ± 0.00

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- **Formulation of milk substitutes based on legume and quinoa flour**

Based on the beforehand generated results, a comparative study was conducted using also different legume flours. The same basic procedure applied for quinoa was used to produce beverages using legumes. However, filtration was done before the α-amylase and heat treatment, due to the higher fibre content. After the heat treatment, the fibre swelled and therefore, it was not possible to filter the beverages anymore and a very viscous slurry was obtained. In summary, the samples were produced as follows: flours were blended with a semi-industrial blender at medium speed for 5 min consisting: 350 g water, 50 g flour, 0.250 g α-amylase (Hitempase 2XP, 0.05 %) and 366 µL protease (Flavourzyme). For the legume-based samples, coarse particles were filtered through cheesecloth after blending (while the quinoa-based samples were filtered after the starch hydrolysis). The obtained solutions were mashed for 120 min at 65 °C and cooled for 20 min down to 20 °C. The samples were homogenized in a two-step homogenizer, applying 150 bar during the first step and 30 bar during the second.

Lupin-based samples showed the highest protein content, while having the highest protein solubility also (Table 3 and 4). Regarding the physicochemical properties, the colour observed was different for all samples. Figure 10 shows a picture of the produced samples. Chickpea and lupin-based samples had a yellow hue, while lentil-based samples were coloured red and faba bean-based samples were coloured green, resulting also in lower whitening indices. Quinoa lupin and chickpea-based samples showed the highest whiteness index. Particle size analysis showed similar results for the volume-weighted mean particle diameter (D) [3,2] values, while only lentil showed considerable high values for D[4,3]. The separation rate reveals the stability of these samples, which was found to be low for all samples. All samples separated relatively quickly, ending up with a considerable sediment layer and a creaming layer on top.

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Figure 10. From right to left: Chickpea, lentil, faba bean and lupin-based milk substitutes

Table 3. Composition in g/100g (protein, fat, sugar) for PBMS (chickpea, lentil, faba bean, lupin and quinoa) and bovine milk

.	Protein [%]	Protein solubility [%]	Fat [%]	Sum of sugars [%]	pH
Chickpea	2.26±0	37.39±0.02	0.56±0.01	1±0.15	6.41±0.06
Lentil	2.69±0.09	44.07±0.06	0.22±0.02	0.99±0.17	6.2±0.05
Faba Bean	3.53±0.04	50.86±0.17	0.39±0.06	0.66±0.03	6.34±0.05
Lupin	3.76±0.03	67.29±0.09	0.86±0.01	0.35±0.06	7.16±0.03
Quinoa	1.64 ± 0.01	48.02±0.77a	1.01 ± 0.03	6.59±0.33	6.28 ± 0.02
Bovine Milk	3.7±0.14	100.11±4.7	3.28±0.05	3.38±0.04	6.79±0.01

Table 4. Physicochemical properties for PBMS (chickpea, lentil, faba bean, lupin and quinoa) and bovine milk

Legumes	Whitening Index	D [3.2] [µm]	D [4.3] [µm]	Viscosity[mPa·s]	Separation rate [%/h]
Chickpea	65.16±0.28	1.45±0.01	4.02±0.03	7.74 ± 0.02	45.06±2.81
Lentil	61.63±0.55	1.63±0.02	32.28±2.22	9.25 ± 0.01	39.23±6.58
Faba Bean	48.67±0.19	1.77±0.03	6.48±0.28	5.84 ± 0.00	54.42±1.82
Lupin	65.21±0.22	1.69±0.1	8.74±2.04	9.48 ± 0.01	39.04±4.31
Quinoa	65.23±0.78	1.13±0.03	6.73±0.21	2.96 ± 0.00	42.29 ± 0.29

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Bovine Milk	81.89±0.01	0.36±0.03	0.6±0.02	3.15 ± 0.01	3.87±0.17

Based on these results, and the difficulties of processing of the legume flours, quinoa was chosen to formulate a prototype, being further used also for the fermented products (Figure 11). On the other hand, to improve the processing of legumes, the application of malting technologies was applied in order to investigate and improve these products. Furthermore, legumes are especially interesting due to their high protein content. Hence, lentil protein isolates were used to generate emulsions instead, to leverage on the full potential of the proteins.

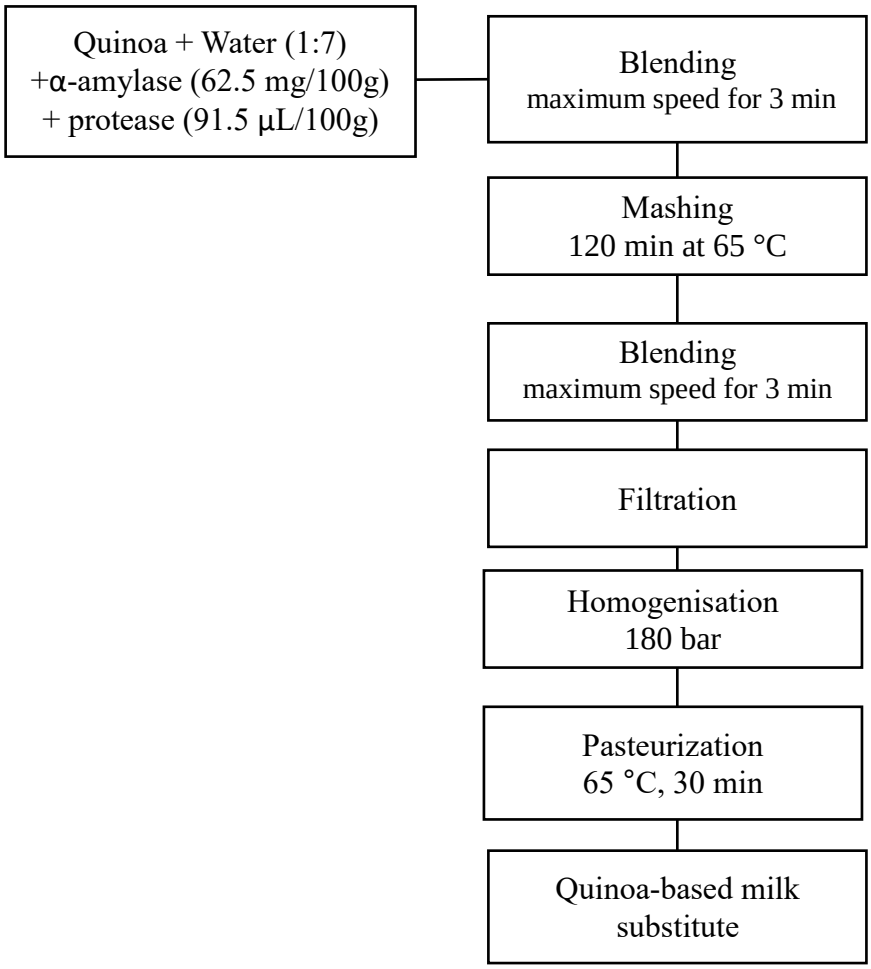

Figure 11. Flow chart of the developed prototype "quinoa-based milk substitute"

- **Selection of germination processing steps to improve the extractability and viscosity of plant-based milk substitutes**

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

The optimal malting program was achieved after the fourth day of germination, 55% degree of steeping, and a 24°C steeping and germination temperature (Figure 12). The obtained values were 65.8% extract, 1.32CU/g α-amylase activity, 200mg/l FAN, 69.2°C gelatinisation temperature, 32% Kolbach Index, 7.1mg/g phytic acid, 3.2mg/g tannins, and 15% malting loss (Table 5). The values of the optimised lentil malt were within the range of what was calculated via a central composite design and response surface methodology Response Surface Methodology (RSM) (Bruns et al., 2006). Response surface methodology was used to investigate the malting quality of lentil (lens culinaris medik.). This study clearly showed that RSM is a good method for testing the malting conditions for unknown maltable grains such as legumes.

Table 5. Results of the measured malting parameters of optimised lentil malt in comparison to raw lentils.

attributes	unit	raw lentils	lentil malt (opt.)
α-Amylase activity	CU/g	1.16 ± 0.1	1.32 ± 0.04
extract 50%/50%	% d.m.	70 ± 1.3	74.6 ± 0.6
gelatinisation temperature	°C	68.5 ± 0.4	69.2 ± 0.3
FAN	mg/l	107 ± 14	200 ± 17
Kolbach Index	%	28	32 ± 1
phytic Acid	mg/g	8.3 ± 0.07	7.1 ± 0.4
tannins	mg/g	4.77 ± 0.42	3.21 ± 0.09
malting loss	%	n.a.	15

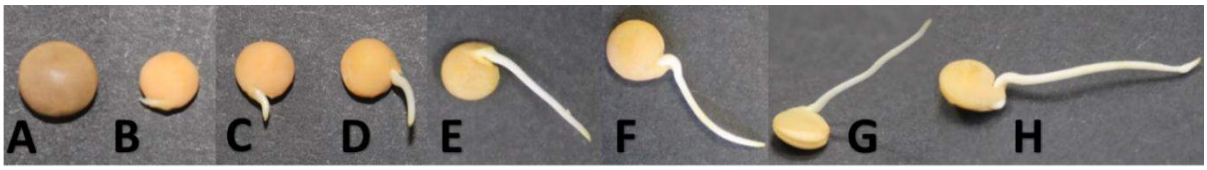

Figure 12. Germination stages of lentils after 12 (A), 24 (B), 36 (C), 48 (D), 60 (E), 72 (F), 84 (H) and 96 hours (G) at 24°C and 55% moisture content

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

The results showed clearly how malting improved the properties of lentils in terms of antinutrients (phytic acid, tannins). Further, this processing may be beneficial for the extraction, since enzymolytic activity might be found. This work proved that malting of lentils or other legumes is a possible approach to develop new beverages.

- **Selection of lactic acid bacteria and processing steps to produce fermented plant based dairy substitutes**

The developed quinoa-based milk substitute was used for further fermentation for prototyping plant-based alternative dairy products. Two different studies were conducted in order to promote different metabolites: EPS for a structured product for yoghurt-like products, and mannitol for a sugar reduced product, which could be applied also in smoothies.

❖ **EPS promoting fermentation**

In the dairy industry it is typically heteropolysaccharide-producing LAB that are used for fermentation. However, in this study, the homopolysacharide-producing strain *Weissella cibaria* MG1 has been investigated for its impact on quinoa yoghurt-like production. The process was based on the developed recipe of the "quinoa-based milk substitute". Further, the product was pasteurized (110 °C for 10 min) and inoculated with 1 $\times 10^7$ cfu/ml of *W. cibaria* MG1. To enable dextran production by *W. cibaria* MG1, sucrose was added in concentrations of 10% (w/v). The products were fermented at 30 °C for 24 hours. Research activities involved the analysis of fermentation characteristics, microstructure, viable cell count, pH, titratable acidity, viscosity and EPS production during storage.

The research outcomes showed that *W. cibaria* MG1 can grow well in wholemeal quinoa base (> 10^9 cfu/ml), and positively structuring the resulting yoghurt with high amounts of EPS (40 g/ml) if sucrose was added to the product (Table 6). The importance of the sugar composition was found to be key to produce texture: sucrose is used by the LAB to build homopolysacharide, whereas without sucrose no EPS can be produced (0.0 mg/L). This can also be appreciated in the microstructural changes Figure 13. Generally, *W. cibaria* MG1 showed satisfactory technology properties (see Table 13) and great potential for further possible application in the development of high viscosity fermented wholemeal quinoa yoghurt.

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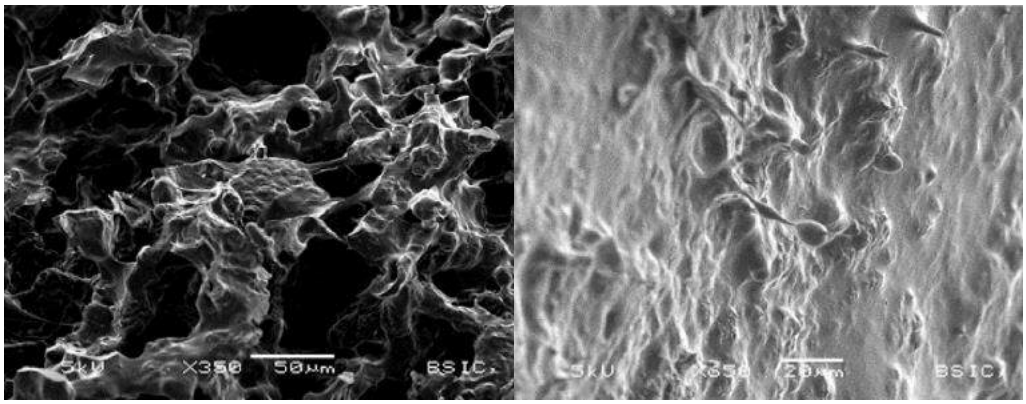

Figure. 13. Scanning electron micrographs of wholemeal quinoa chemically acidified yoghurt control (left) and wholemeal quinoa yoghurt fermented with *W. cibaria* MG1 (right) enriched with 10% sucrose

Table 6. Physicochemical characteristics of *W. cibaria* MG1 fermented wholemeal quinoa milk.

Parameters Time (h)	**0**	**24**
Cell counts (cfu/ml)	$1.07\times 10^{7} \pm 0.1 \times 10^{7}$	$1.31 \times 10^{9} \pm 0.3 \times 10^{9}$
pH	6.28 ± 0.02	5.18 ± 0.01
TTA (mL of NaOH)	0.2 ± 0.01	2.8 ± 0.00
WHC (%)	38.15 ± 0.12	100 ± 0.00
Sugar profile		
Maltose	31.00 ± 0.06	0.0 ± 0.00
Sucrose	110.00 ± 0.02	0.0 ± 0.00
Acid profile		
Lactic	0.0 ± 0.00	17.46 ± 0.18
Acetic	0.0 ± 0.00	0.0 ± 0.00
EPS amount (mg/l)	0.0 ± 0.00	40.00 ± 0.6
Viscosity (Pa s)	0.04 ± 0.00	0.57 ± 0.02

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❖ **Mannitol promoting fermentation**

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. The same basic recipe developed for the "quinoa-based milk substitute" was applied;. In addition, 300 µL amyloglucosidase (Attenuzyme, Novozymes) and 0.8 g glucose-isomerase were also added prior the mashing step. The enzymatic treatment is depicted in figure 14 for further details. Using these two biotechnological techniques enables the reduction of sugar, while also preserving some of the sweetness. Fermentation was carried out by inoculating at 7 log cfu/mL directly into tempered quinoa-based milk substitute. Fermentation was performed anaerobically, under static conditions at 30 °C for 24 h. Both, *Leuconostoc citreum* TR116 and *Lactobacillus brevis* TR055, are able to reduce fructose directly to mannitol, a sugar alcohol, which is perceived as sweet but without caloric value. Both strains showed high viable cell counts with *Leuconostoc citreum* TR116 > 8.4 and *Lactobacillus brevis* TR055 > 9.3 log cfu/mL, and a reduction in pH to 3.7 and 3.5, respectively. 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/100 g to approximately 30 mmol/100 g, which equates to a glucose reduction of 40%. Furthermore, the 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. These products are proposed also to be used in smoothie-like products to improve the organoleptic perception and nutritional value of such products.

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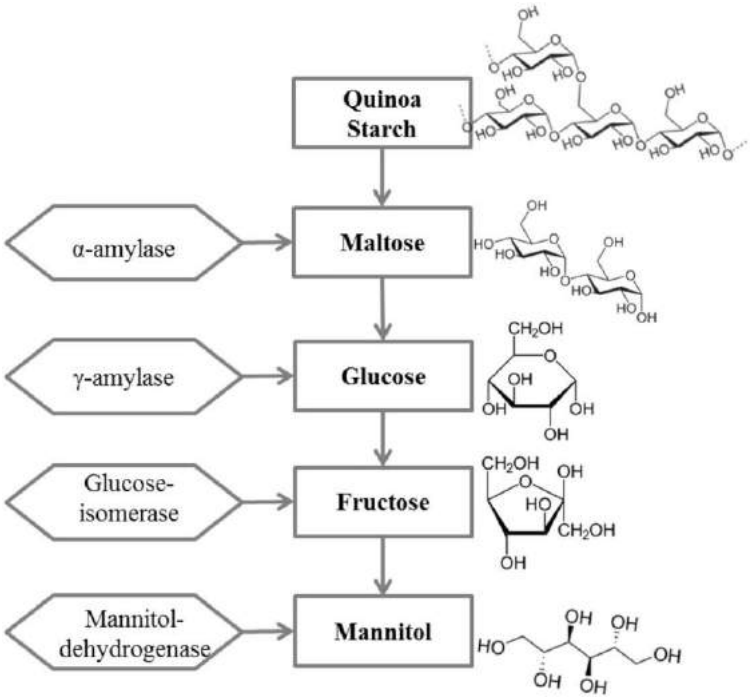

Figure 14. Enzymatic processing of quinoa starch with exogenous enzymes (α-amylase, γ-amylase, and glucose-isomerase), and endogenous enzymes, secreted by LAB (mannitol-dehydrogenase).

- **Selection of processing steps to improve quality of lentil-based emulsion**

Lentil proteins were studied for their functional properties and ability to stabilise emulsions with the application of high-pressure homogenisation. The 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, a highly stable nano-emulsion was generated with a great colloidal stability, appearance and viscosity similar to cow's milk (Figure 15). Table 7 shows the results of particle size and polydispersity; a measure for the homogeneity of particle size distribution and an indicator of stability. Sensory testing also proved the great potential of lentil protein-based emulsions as novel products, since the textural and organoleptic attributes of these emulsions compared well to commercial PBMSs, including soya-based products (Figure 16, table 8). Overall, the product quality was considered the best when treated at 900 bar and a pasteurization temperature of 85 °C. The produced lentil-BMS possessed great functional and nutritional properties, providing valuable protein to the diet.

Table 7. Effect of homogenisation pressure (180 or 900 bar) and heat treatment (65 or 85 °C) of lentil protein dispersions and lentil protein (LP) stabilised emulsion (LPE),

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containing 1.5 or 3.3% fat, measured on day 0 and after 21 days of storage on average particle size (Z-Average) and polydispersity index.

	Particle size [nm]	Polydispersity index	Stability Index
LPE 180 65 1.5%	371.78±29.00^{c}	0.37±0.08^{ab}	8.13±0.57^{d}
LPE 180 85 1.5%	340.07±7.56^{de}	0.32±0.03^{ab}	8.22±0.59^{d}
LPE 900 65 1.5%	205.12±5.81^{hij}	0.15±0.03^{fgh}	5.76±0.24^{e}
LPE 900 85 1.5%	208.73±12.95^{ghi}	0.16±0.04^{efgh}	5.13±0.27^{e}
LPE 180 65 3.3%	447.82±11.41^{a}	0.30±0.05^{bc}	3.99±0.64^{f}
LPE 180 85 3.3%	430.57±16.64^{ab}	0.34±0.05^{ab}	3.73±0.48^{f}
LPE 900 65 3.3%	223.36±9.05^{fg}	0.13±0.03^{h}	2.65±0.37^{gh}
LPE 900 85 3.3%	223.33±11.6^{fg}	0.13±0.03^{h}	2.19±0.4^{h}

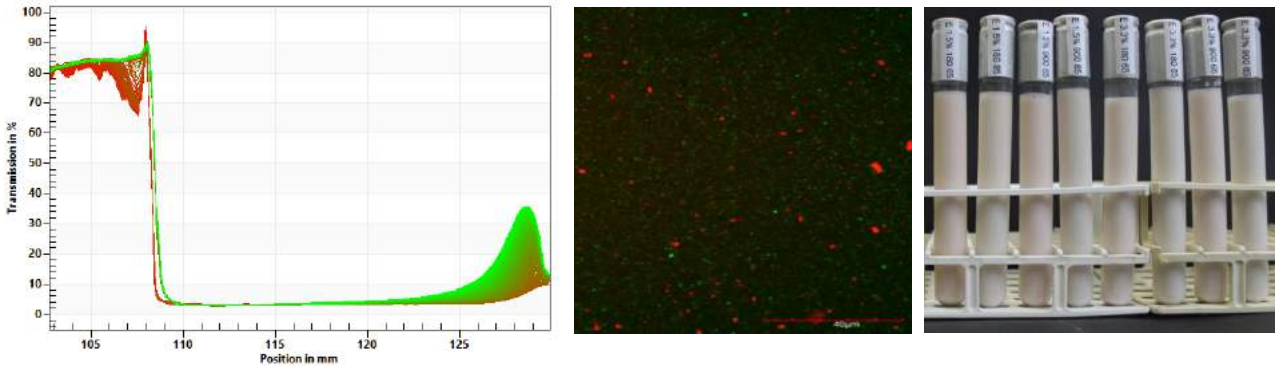

Figure 15. Transmission profile (left) and micrograph obtained with a confocal laser microscope of lentil stabilized emulsion (middle), containing 3.3% fat, pasteurized at 65 °C and homogenized at 900 bar, and a photograph of lentil protein stabilised emulsion containing 1.5 or 3.3% fat (right).

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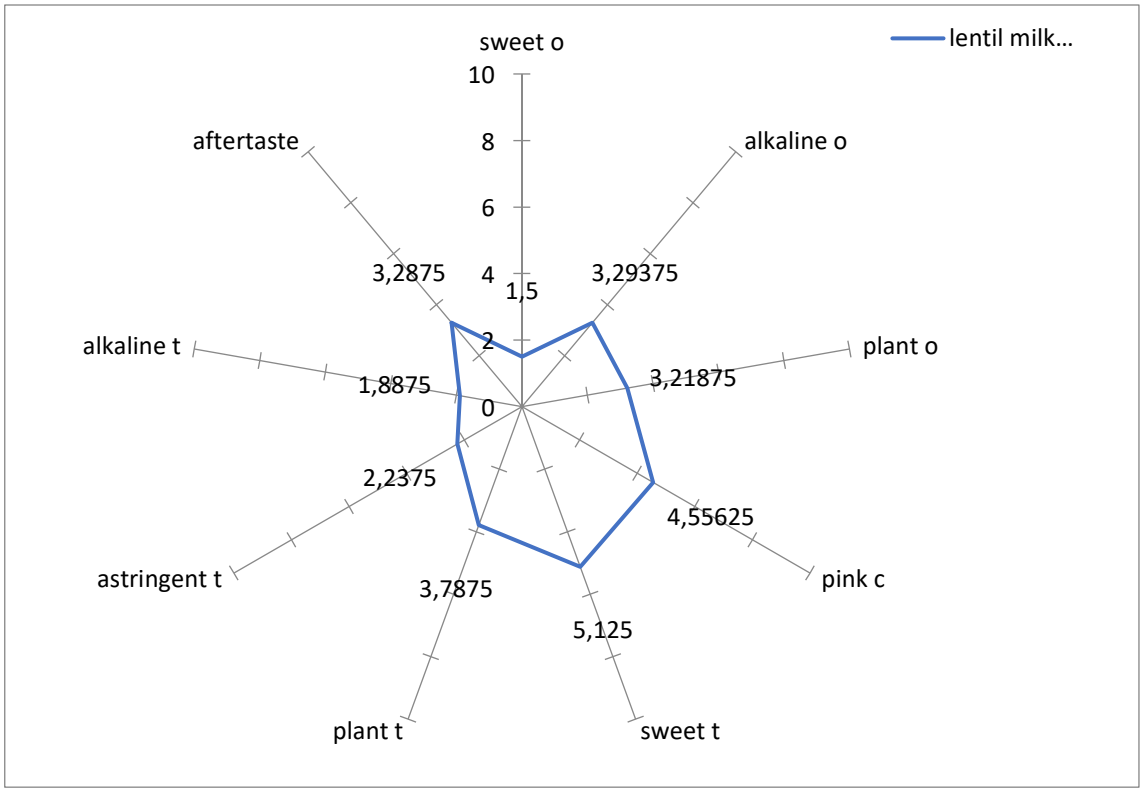

Figure 16. Descriptive sensory profile of lentil-based milk substitute as obtained by PAS

Table 8. 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.

	Appearance	Aroma	Mouthfeel	Flavour	Overall
Commercial 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}
Commercial Rice-BMS	6.23±1.66^{bc}	6.00±1.56^{ab}	6.42±1.34^{a}	5.90±2.06^{a}	6.00±1.8^{a}
Commercial 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}
Commercial 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}
Commercial 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 ($p < 0.05$) (Table 8).

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3.2 Infant formula

- **Evaluation of the allergenicity of the raw materials**

The allergenicity evaluation of the ingredients is shown in Table 9. As a conclusion, the lupin was eliminated from the scope of the project as it is considered an allergen.

Table 9. Evaluation of allergenicity potential of the raw materials

	Classification	Dairy protein-free	Lactose-free	Gluten-free	Allergen
Quinoa	Pseudocereal	Yes	Yes	Yes	No
Buckwheat	Pseudocereal	Yes	Yes	Yes	No
Amaranth	Pseudocereal	Yes	Yes	Yes	No
Lupin	Legume	Yes	Yes	Yes	Yes
Chickpea	Legume	Yes	Yes	Yes	No
Fava bean	Legume	Yes	Yes	Yes	No
Lentil	Legume	Yes	Yes	Yes	No
Grass pea	Legume	Yes	Yes	Yes	No

- **Evaluation of the nutritional composition, physicochemical and functional properties**

❖ **Evaluation of flours and protein-rich flours**

The characterization of flours and protein-rich flours was carried out using the following analysis, in order to understand their suitability for first-age infant nutritional products.

➢ **Nutritional composition**

From the analysis performed can be concluded that the protein-rich flours had higher levels of ash, fat and fibre than the flours that had higher levels of starch. The content of high levels

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of starch and fibre is not beneficial for the development of first-age infant nutritional products, as this produces high viscosity during processing (Table 10).

Table 10. Nutritional composition of flours and protein-rich flours of quinoa wholegrain flour (QWGF), quinoa dehulled flour (QDF), quinoa protein-rich flour (QPRF), buckwheat dehulled flour (BDF), buckwheat protein-rich flour (BPRF), amaranth wholegrain flour (AWGF), amaranth protein-rich flour (APRF), rice flour (RF), rice protein concentrate (RPC) and maize flour (MF).

	Moisture	Ash	Protein	Fat	Carbohydrate	Starch	Damaged starch	Total Fibre
	% w/w						% total starch	
Quinoa								
QWGF	9.01	2.30	13.1	6.54	69.0	60.0	10.6 ±	11.4
QDF	8.86	1.80	15.7	5.36	68.3	50.5	11.7	9.75
QPRF	5.25	3.60	33.3	12.8	45.0	21.4	10.4	18.8
Amaranth								
AWGF	8.94	2.40	14.6	6.04	68.1	52.8	12.2	11.3
APRF	7.76	6.86	38.6	16.6	30.2	20.3	2.61	24.0
Buckwheat								
BDF	8.75	1.51	14.2	2.77	72.8	61.6	1.52	10.3
BPRF	6.86	3.05	20.5	4.76	64.8	47.3	2.22	19.0
Rice								
RF	8.89	0.85	8.22	0.71	81.3	78.5	10.7	4.06
RPC	6.24	3.42	75.0	0.79	14.6	6.50	88.3	5.83

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Maize								
MF	12.2	0.74	6.42	1.66	79.0	76.0	7.21	3.77

- **Electrophoretic protein profile analysis**

All samples, except maize, showed common bands ~50 kDa (Figure 17). This band corresponds to the globulin and glutelin fraction in pseudocereals and rice, respectively. For quinoa samples (QWGF, QDF and QPRF) bands at ~50 kDa correspond to the 11S globulin fraction or chenopodin. Chenopodin consists of ~49 and 57 kDa subunits that are associated into a hexamer by non-covalent interactions. Amaranth samples (AWGF and APRF), same way as quinoa, showed a band at ~50 kDa, which corresponds to the hexameric 11S globulin or amarantin. This major band might also be correlated to another glutelin-type protein, which has similar molecular characteristics to those of amaranth 11S globulin. Buckwheat samples showed a main band ~50 kDa, which might correspond to the major storage protein of buckwheat, the 13S legume-like globulin, and the minor storage protein, the trimer 8S vicilin-like globulin. Rice samples also showed a major band ~50 kDa, which corresponds to the glutelin precursor. Low MW bands (~10-15 kDa) could be observed in the three gels for all quinoa, amaranth and buckwheat samples. This might be related to the albumin fraction, which is abundant in pseudocereals. For rice samples, the band showed at 13 kDa and was reported previously as the prolamin fraction.

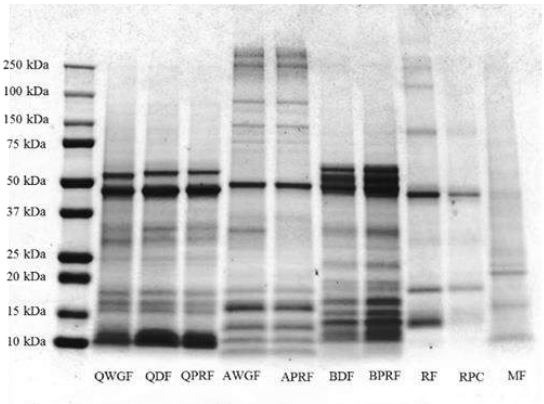

Figure 17. Representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of quinoa wholegrain flour (QWGF), quinoa dehulled flour (QDF), quinoa protein-rich flour (QPRF), buckwheat dehulled flour (BDF), buckwheat protein-rich flour (BPRF), amaranth wholegrain flour (AWGF), amaranth protein-rich flour (APRF), rice flour (RF), rice protein concentrate (RPC) and maize flour (MF). The first lane of each gel corresponds to the molecular weight marker.

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- **Rheological properties**

The protein-rich flours showed lower viscosity than the regular flours. However, same pattern was observed in the regular flours in respect to the initial, peak and final viscosities, but with a decrease in overall viscosity (Figure 18a - b). The lower viscosity of protein-rich flours can be explained by the lower content of starch in the protein-rich samples and the higher content in dietary fibre. Water binding capacity of the dietary fibre is greatly improved by the presence of high amounts of hydroxyl groups. This can also be related to a reduction in water availability, which could cause a reduction in viscosity and pasting properties. Also, the protein-rich fractions are rich in ash, protein and fat, which might affect the functionality of starch and change the viscosity profiles. It can be observed how buckwheat has significantly higher viscosity in comparison to quinoa and amaranth (Figure 18a and b).

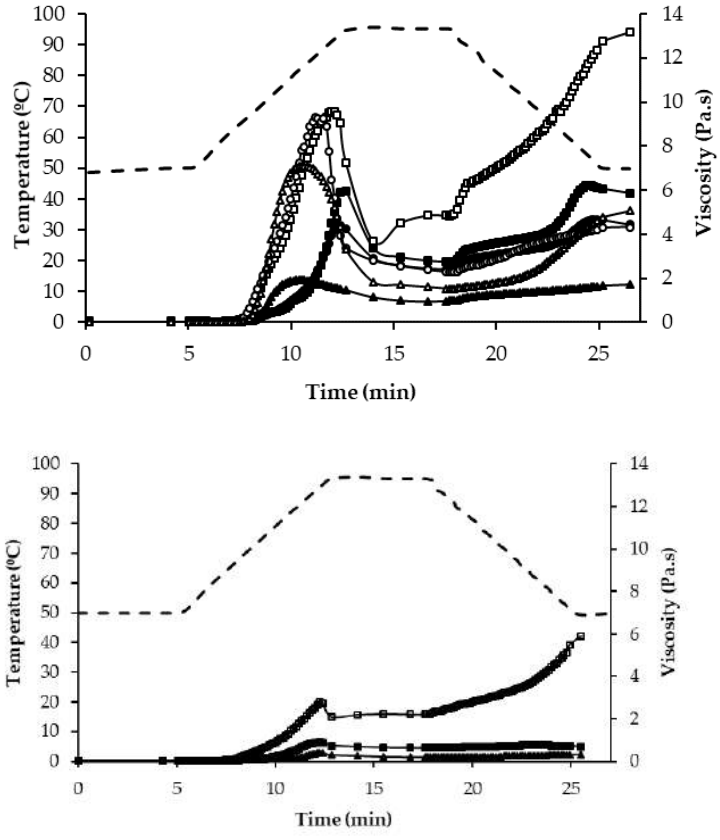

Figure 18a (left). Temperature (dashed line) and viscosity (symbols) at various stages of the pasting regime of regular flours: quinoa wholegrain flour ( ), quinoa dehulled flour ( ), amaranth wholegrain flour ( ), buckwheat dehulled flour (BDF) ( ), rice flour (RF) ( ) and maize flour ( ) dispersions.

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Figure 18b (right). Viscosity (symbols) at various stages of the pasting regime of protein enriched flours: quinoa protein-rich flour ( ), amaranth protein-rich flour ( ), buckwheat protein-rich flour ( ).

- **Microstructural analysis**

The flour and protein-rich flour powder particles were large with irregular shape and rough surface (Figure 19 - 20). Different sizes, shapes and structures were observed among the samples. Regarding the starch granules, quinoa samples presented the smallest sized granules (1-1.20 μm) among all samples and showed a polygonal shape. The quinoa protein-rich flour (QPRF) showed granules covered and linked to other types of substances. This embryo-rich fraction is rich in protein, fibre and fat, which suggests that the starch granules are embedded in a matrix formed by these compounds. Amaranth samples, AWGF and APRF, showed circular granules with a size of ~2.5-3 μm. Amaranth seed is one of the few sources of small-granule starch, typically 1 to 3 μm in diameter, with a regular granule size. The starch granules in APRF seem to be also embedded in a matrix as in QPRF. Buckwheat starch granules showed the largest size (5 to 7.5 μm) among the pseudocereal samples with spherical and polygonal structures. The small size of the starch granules of some pseudocerals, such as quinoa, can offer advantages in respect to product formulation, as they have emulsifying and stabilizing capacity in oil within water emulsions.

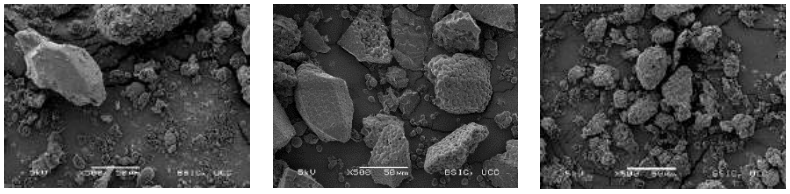

Figure 19. Examples of quinoa, amaranth and buckwheat particles from left to right, respectivley (magnification and scale bar x500 and 50 μm)

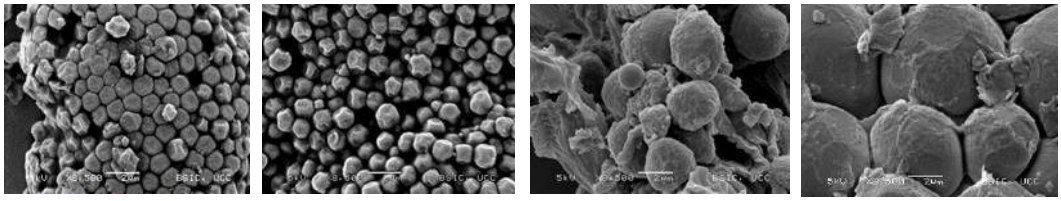

Figure 20. Examples of quinoa (image 1 and 2), amaranth (image 3) and buckwheat (image 4) starch granules

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- **Particle size distribution**

In general, a wide range of particle size distributions (from 72 to 215 μm for D[4,3] (volume-weighted mean particle diameter) were recorded. Having most of the flours and protein-rich flours big particle size distribution (>100 μm for D[4,3]). Greater particle size distribution of powders can create challenges during powder handling. The smaller particles tend to fill the inter-granular spaces of the larger particles, thus increasing the surface contact and cohesion between flour particles. The flours and protein-rich flours showed similar structures with irregular, non-homogeneous shapes and sizes and rough surfaces (Table 11).

Table 11. Particle size distribution of flours and protein-rich flours.

	D[4,3]	D[3,2]	Dv(10)	Dv(50)	Dv(90)	Span	SSA
	---------- μm----------						(m^2/kg)
Flours							
QWGF	82.7	16.3	6.45	27.8	244	8.62	367
QDF	202	65.9	27.3	177	417	2.20	91.0
AWGF	191	59.5	22.9	163	398	2.30	100
BDF	126	43.8	20.2	92.3	272	2.75	137
RF	95.7	42.1	17.8	77.5	196	2.27	142
MF	173	49.1	15.5	144	384	2.55	122
Protein-rich flours							
QPRF	134	33.6	11.8	95.8	323	3.15	178
APRF	215	69.2	28.7	202	433	2.05	86.7
BPRF	96.5	22.1	7.01	72.6	237	3.02	271
RPRF	72.0	55.6	34.1	67.2	118	1.25	408

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- **Flowability and colour**

Data for flow index, flow classification and compressibility index (CI) of the powders are provided in Table 12. If a powder has a flow index greater than 10 it is considered free-flowing. Powders with flow index of 10-4 are considered easy-flowing, whereas cohesive, very cohesive, and non-flowing powders have flow indices less than 4, 2 and 1, respectively. Of the eleven powders investigated, two were classified as very cohesive, four as cohesive and the remaining five as easy flowing. Among the protein concentrates and isolates, RPRF (0.79% fat), was classified as easy-flowing, while the protein-rich flours (QPRF, BPRF and APRF) were classified as cohesive. Protein-rich flours have higher fat contents than regular flours, and higher levels of surface fat are known to have a major influence on powder flowability.

Table 12. Colour and flowability of flour and protein-rich flours

	Colour space values			a_w	Flow index	Flow classification	CI
	L*	a*	b*				(%)
Flours							
QWGF	70.1	0.60	9.94	0.46	1.96	Very cohesive/Cohesive	49.5
QDF	61.4	0.29	13.0	0.46	4.35	Easy-flowing	33.9
AWGF	66.2	0.66	11.8	0.44	3.33	Cohesive	39.7
BDF	67.1	0.25	8.26	0.46	4.76	Easy-flowing	29.4
MF	70.0	1.85	24.0	0.62	2.70	Cohesive	28.0
RF	72.5	0.75	6.33	0.46	3.45	Cohesive/easy-flowing	28.8
Protein-rich flours							

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QPRF	62.5	0.83	14.6	0.28	2.08	Very cohesive/cohesive	51.4
APRF	62.8	0.83	14.4	0.41	3.45	Cohesive	45.7
BPRF	67.7	0.16	8.61	0.33	2.44	Cohesive	42.6
RPRF	64.0	-0.06	13.1	0.46	9.90	Easy-flowing	27.8

CI = Compressibility index

a_w = Water activity

L value measures brightness, with values ranging from 0 (black) to 100 (white), a* value measures degree of redness (positive values) or greenness (negative values), and b* value measures degree of yellowness (positive values) or blueness (negative values).*

- **Evaluation of lentil protein isolate**

  - **Nutritional composition**

The lentil protein isolate showed no starch and low levels of dietary fibre (Table 13). The high protein content makes it suitable for the production of first-age infant formula.

Table 13. Nutritional composition of lentil protein isolated by isoelectric precipitation (LPI-IEP)

Composition [g/100 g]	**LPI-IEP**
Protein	85.1
Fat	4.49
Starch	*N.D.
Moisture	4.87
Ash	5.46
Insoluble dietary fibre	<0.1
Soluble dietary fibre	1.8

  - **Electrophoretic protein profile analysis**

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The main proteins detected by SDS-PAGE were vicilin and legumin (Figure 21). In previous studies, these proteins were found to have good emulsifying capacity. For this reason, these proteins can be adequate for the formulation of first-age infant nutritional products.

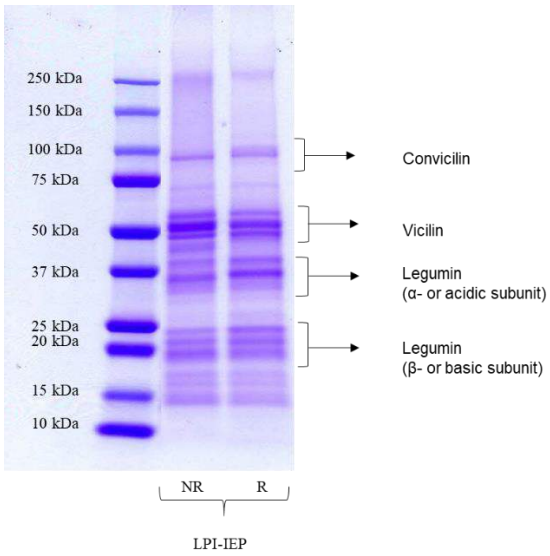

Figure 21. Sodium dodecyl sulphate electrophoresis of lentil protein isolates by isoelectric precipitation (LPI-IEP) under non-reducing (NR) and reducing (R) conditions

- **Microstructure**

The particles of the lentil protein isolate were round and small in comparison with the flours and protein-rich flours (Figure 22). This makes the isolate suitable for handling and the rehydration properties of the protein powder suitable for further formulation.

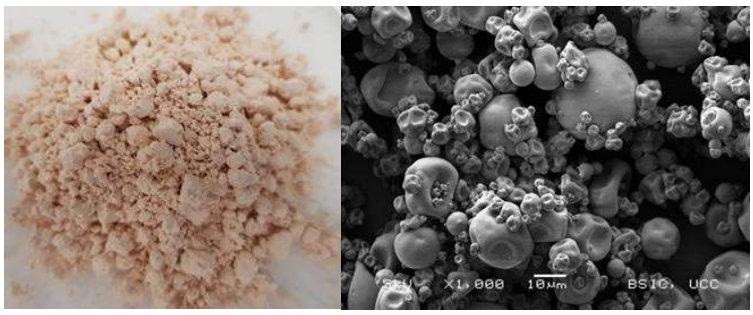

Figure 22. Scanning electron microscope of lentil protein isolate, isolated by isoelectric precipitation.

- **Protein solubility and effect of homogenization**

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The protein solubility of the lentil protein isolate was studied. Before homogenization the protein solubility was ~50% (pH range: 7 – 9). After homogenization the solubility is greatly improved and the solution is stable even after 24 h (Figure 23).

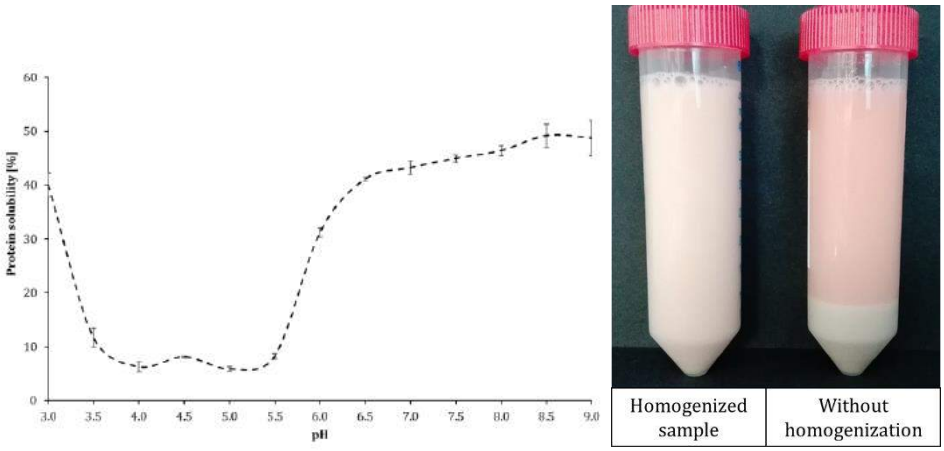

Figure 23. Protein solubility of lentil protein isolate at different pHs (left figure) and effect of homogenization on lentil proteins (right figure).

- **Emulsifying properties**

The emulsifying properties are shown in Table 14. The good emulsifying properties of lentil proteins were found beneficial for the development of firs-age nutritional products.

Table 14. Emulsifying properties of lentil protein isolate

	LPI-IEP
Emulsifying properties	
Emulsifying activity [m^2/g]	16.5
Emulsifying stability [min]	51.0

- **Selection of raw material for further processing**

Lentil protein isolate (produced by isoelectric precipitation) was chosen to develop the first-age infant formulation due to its nutritional composition and functional properties after homogenization.

The flours and protein-rich flours were excluded due to:

- Presence of other compounds such as starch and fibre that can affect negatively during the processing of the first-age infant product.
- Their high viscosity during heat treatment

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- The big particles size distribution and cohesive behaviour can affect negatively on the solubility properties

- **Formulation and optimization of infant formula process with lentil protein isolate at laboratory scale**

❖ **Formulation**

The infant formula was formulated following the Commission Delegated Regulation (EU) 2016/127 for the macronutrient composition (protein, carbohydrate and fat) (Table 15). Lentil protein, maltodextrin DE 17 and sunflower oil were selected as sources of protein, carbohydrate and lipid, respectively (Table 16 a-b and figure 24).

Table 15. Maximum and minimum values for infant formula composition received from Commission Delegated Regulation (EU) 2016/2017.

Commission Delegated Regulation (EU) 2016/127		
	Minimum	**Maximum**
Energy	60 kcal / 100 mL	70 kcal / 100 mL
Protein		
Cow's milk	1.80 g / 100 kcal	2.50 g / 100 kcal
Soya protein	2.25 g / 100 kcal	2.80 g / 100 kcal
Protein hydrolysates	1.86 g /100 kcal	2.80 g / 100 kcal
Lipids	4.4 g / 100 kcal	6.00 g / 100 kcal
Carbohydrates	9 g / 100 kcal	14 g / 100 kcal

Concentrated system for spray-drying	
Lentil Protein (g)	4.75
Cysteine (mg)	30.4
Methionine (mg)	3.55
Tryptophan (mg)	15.2

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Sunflower oil (g)	8.23
Maltodextrins (g)	16.9
Energy (kcal)	161
Total solids (TS)	30

Table 16a. Composition of concentrated formula before spray drying

Figure 24. Summarized diagram for infant formula production

Table 16b. Macronutrient composition of infant formula powder and reconstituted infant formula.

	Infant Powder Composition	Infant Formula Reconstituted (12%)	Per 100 kcal
Protein (g)	15	1.8	2.8
Lipids (g)	27	3.3	5.1
Carbohydrates (g)	55	6.8	10.6
Energy (kcal)	519	64.6	100
Total solids	97	12	18.5

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The formula was fortified with cysteine, methionine and tryptophan as these were the amino acids lacking in the formulation as seen in Figure 25. The amino acid composition of the lentil-based formula was compared to that of breast milk.

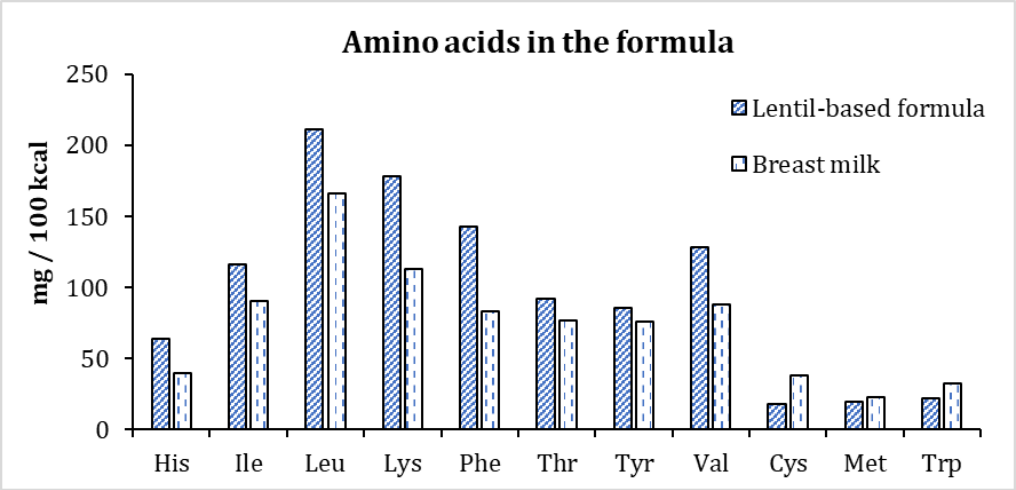

Figure 25. Amino acids in the lentil-based formula before supplementation in comparison with breast milk

❖ Laboratory-scale optimization

The concentrated emulsion system for spray-drying was developed using the process shown in Figures 26 - 27.

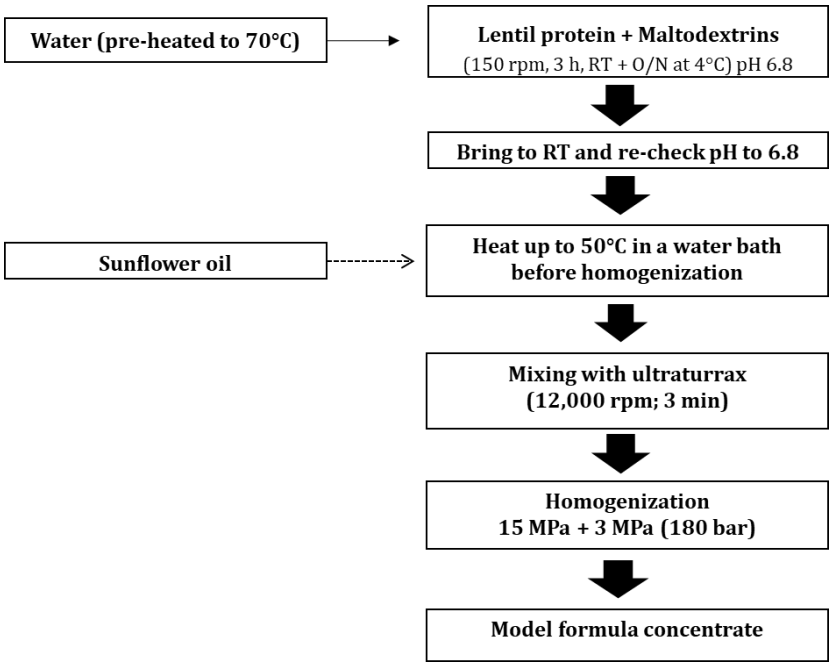

Figure 26. Diagram for obtaining the model formula concentrate before spray-drying.

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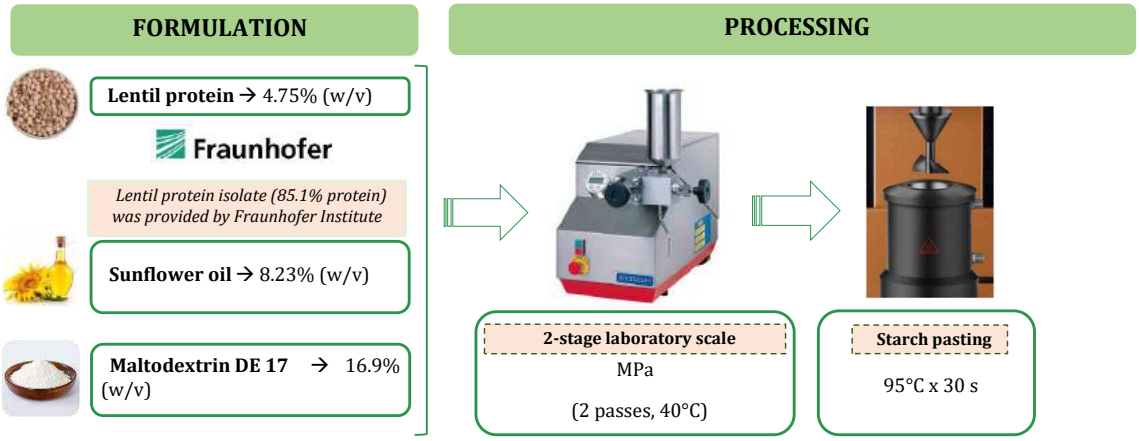

Figure 27. Process to test the heat stability of the model formula before spray drying.

The lentil emulsion system, after production was heated up to 95°C for 30 s and even 1 hour in a starch pasting cell, showed no increase in viscosity. The lentil-based emulsion system was heated to 140°C in an oil bath and was stable for ~4.5 minutes, with no increase in particle size were observed. This means that the formula is suitable for Ultra High Temperature (UHT) treatment.

- **Effect of mineral fortification on infant formula**

The infant formula was fortified with different levels of Ca^{2+} (from 0 to 6 mM) (Table 17), in order to understand the effect of mineral fortification on some emulsion properties, such as heat stability and particle size distribution.

Table 17. Mineral composition of the model formula before supplementation

	EU legislation for 66.5 kcal - 100 mL		Label claim			mg/100 mL	
	Minimum	Maximum	Commercial 1 67 kcal/100 mL	Commercial 2 (66 kcal/100 mL)	Commercial 3 (66 kcal/100 mL)	Average commercial products	Current Formula
Sodium (mg/100 mL)	16.6	39.8	16	17	17	16.7	29.4
Potassium (mg/100 mL)	53.1	106.2	65	72	72	69.7	12.7
Chloride (mg/100 mL)	39.8	106.2	43	46	46	45.0	0.16
Calcium (mg/100 mL)	33.2	93	42	55	55	50.7	1.65
Phosphorus (mg/100 mL)	19.9	66.4	24	31	31	28.7	19.6
Magnesium (mg/100 mL)	3.3	10	4.5	5.1	5.1	4.90	1.72
Iron (mg/100 mL)	0.3	1.3	0.8	0.5	0.5	0.60	0.37
Zinc (mg/100 mL)	0.5	0.8	0.6	0.51	0.51	0.50	0.11
Copper (µg/100 mL)	39.8	66.4	30	0.04	0.04	10.0	0.06
Iodine (µg/100 mL)	10	19.3	10	12	12	11.3	-
Selenium (µg/100 mL)	2	5.7	1.4	1.7	1.7	1.60	-
Manganese (µg/100 mL)	0.7	66.4	10	8	8	8.67	0.03
Molybdenum (µg/100 mL)	0	0	-	-	-	-	-
Fluoride (µg/100 mL)	0	66.4	3	<3	<3	<3	-

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The fortification in Ca^{2+} at different levels was performed observing a de-stabilization of the formula after heating at 95°C, as seen in Figure 28. Coagulation is observed at 10 mM levels.

Figure 28. Effect of mineral fortification with Ca^{2+} (from left to right: 0, 2, 3, 4, 5, 6, 10 mM) after heating the model formula during 95 °C, 30 s)

- **Pilot scale processing and spray drying**

After optimizing the process and learning the effect of mineral addition, a larger scale process in the pilot plant facility at UCC was carried out. A powder formulation with the right macro-nutrients (protein, carbohydrate and lipids) composition and amino-acid balance were obtained.

The typical process for infant formula with slight modifications was carried out as seen in Figures 29 and 30.

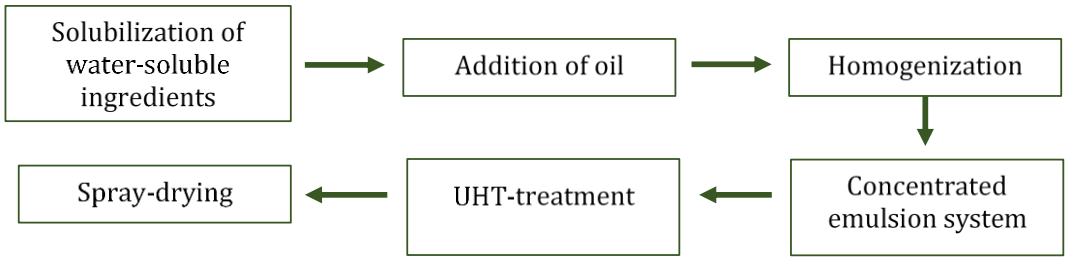

Figure 29. Diagram for producing lentil-based infant formula in the pilot plant

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Figure 30. Infant formula production in the pilot plant

The process consisted of the following steps as seen in Figure 30 The water-soluble ingredients (lentil protein and maltodextrin) were solubilized in water with high shear and left to disperse overnight at 5°C. Afterwards, the product was brought to 50°C and the sunflower oil was added. A homogenization step (2 passes) and UHT treatment (140°C, 2 min) of the concentrated system was performed in order to reduce the microbiological load. As a final step, spray-drying the concentrated emulsion system was carried out in order to obtain the powder infant formula (Figure 31).

Figure 31. Lentil-based infant formula: powder and reconstituted product developed at UCC

- **Evaluation of infant formula prototype**

The infant formula was distributed to several project partners for nutritional (antioxidants, *in vivo* and *in vitro* protein digestibility) and microbiological analysis (University of Copenhagen, Novolyze and Institute of Food Research Polish Academy of Sciences).

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4. Conclusion and next steps

4.1 Plant-based dairy substitutes (UCC)

Two different lab-scale prototypes have been developed for plant-based milk substitutes. Both prototypes were designed to be suitable for different raw materials. Whole grain quinoa has been used to develop a carbohydrate rich product, while lentils have been used to produce a protein rich product. Especially the quinoa-based milk substitute was promising to be used for fermentation, due to the high amount of fermentable sugars that can be used as a substrate by the selected LAB as back-bone for the *in-situ* production of EPS, which can act as natural thickening. By using LAB metabolic abilities, it is possible to shape and modulate the sensory properties of the new developed plant-base dairy substitute to match the preferences of the western consumers. Furthermore, two concepts have been developed aiming for texture on one side and sugar reduction and sweetness on the other side. Additionally, 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, 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 source of protein to a diet.

4.2 Infant formula (UCC)

Lentil protein isolate, provided by our project partner Fraunhofer Institute (WP2), was identified the best ingredient for developing an infant formulation by not using dairy or soy ingredients, which are most commonly used in these types of formulations. The flours and protein-rich flours contain other components such as starch and fibres, which can have a negative effect in the development of first-age infant nutritional products (breast milk substitutes). The lentil protein isolate was found to have good functional properties (e.g., emulsifying capacity and stability), which is an essential requirement for first-age infant nutritional products. Furthermore, the formulation was really heat stable to UHT processing, ensuring therefore a good microbial quality of the product. A powder infant formula that meets the macronutrient and amino acids recommendations by the European Union, was developed using spray-drying to obtain the powder. A process was developed

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that could also be applied for other plant protein sources. The formula had good reconstitution properties in water, is allergen-free, contains the right balance of amino acids, is easy to store and transport and could be a potential replacer of soya proteins. Further steps are, however, needed to enrich the micronutrients (especially minerals) in the formula, without affecting the technological properties of the formulation.

5. Delays and difficulties

The mineral fortification of the formula couldn't be achieved as the minerals had a negative effect on the formulation as demonstrated in the report. Further investigations are therefore needed to overcome this challenge. Furthermore, the protein isolates were produced at later stages on the project than the protein-rich flours and flours. This delayed had an impact on the timing for the development of the infant formulation.

6. Impact and outreach

Plant-based milk prototype, developed by UCC, has been validated in lab-scale while the infant-food formula prototype, likewise developed by UCC, has been validated also at pilot-scale reaching the TRL5 (technology readiness level 5). The processing condition and optimised formulation could represent a key connection point for the food and beverage industry. They can use such information as background, for developing/validating prototypes. This can assist the pre-commercial industrial scale and the developed prototypes to move forward the TRL from 5 to 6/7.

Both plant-based milk and infant-food formula prototypes address the main goal of the P2F project. As both prototypes apply nutritious plant based raw materials, they provide great potential in helping to identify European-based alternative plant proteins, with high nutrition/sensory and technological functionality as well as enhanced environmental sustainability.

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