

Functional properties of protein fractions from gentle membrane separation of green biomass (legume grass) compared to traditional animal- and plant-based proteins

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ABSTRACT

Legume grasses are rich in the functional protein ribulose-1,5-bisphosphate carboxylase/oxygenase, making legume grass concentrates (LGC) a promising ingredient for plant-based foods. To facilitate their industrial adoption, it is essential to understand their performance against established ingredients. In this study, LGCs were produced using multi-stage filtration without heat or acid, followed by freeze (LGC-F) or spray-drying (LGC-S). The two LGCs were compared to common protein ingredients (whey, egg white, soy, and pea). LGCs contained ~60% protein (w/w), with LGC-F proteins remaining native, while LGC-S were denatured. LGCs exhibited high solubility (>90%) at pH > 6, comparable to whey and egg white. Moreover, LGCs formed gels at just 1.5% protein (w/w), while egg white required 5%. The foaming capacity of LGCs was similar to that of whey and egg white-based foams, although slightly less stable. LGC emulsions displayed small oil droplets; however, stability was compromised due to droplet growth within 14 days, similar to soy and pea emulsions. Importantly, the drying method did not impair the functionality of the LGC; hence, from an industrial perspective, spray drying could be an efficient method for large-scale production. Overall, the functionality of LGC aligns more closely with egg white and whey than with plant ingredients.

1. Introduction

Humanity is at a crucial juncture where decisive actions are needed to mitigate further environmental degradation. In this global context, the food industry and its stakeholders are making gradual efforts to enhance sustainability. These efforts are manifested in various ways, including the optimisation of processes, the repurposing of side streams, and the increased availability of plant-based foods, particularly protein ingredients (Prasanna et al., 2024). However, the growing consumption of plant-based proteins poses environmental risks if these ingredients are sourced exclusively from monocultures (Macdiarmid, 2022). Therefore, achieving a genuine green transition requires diversifying the sources of plant-based proteins, making the exploration and study of new materials a critical priority.

Among these new materials, green leaves, such as perennial grasses and legume-grass blends, i.e., ryegrass (*Lolium perenne*), and red

(*Trifolium pratense*) and white (*Trifolium repens*) grass-clover have garnered increased attention as a sustainable source of plant proteins (Hadidi et al., 2023a). These perennial forage crops, as well as other green leaves, primarily contain the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). RuBisCO plays a crucial role in photosynthesis; thus is found in most green terrestrial and marine plants, photosynthetic bacteria, and eukaryotic algae, making it the most plentiful protein on the planet (Di Stefano et al., 2018). RuBisCO is a globular protein comprised of eight small and eight large subunits, forming a hexadecameric quaternary structure with a molecular weight of approximately 550 kDa (Hadidi et al., 2023a). Furthermore, its amino acid composition is highly nutritional, containing up to 60 % essential amino acids, with a variability among species of less than 3 % (Anoop et al., 2023). This high nutritional value, combined with its abundance, makes RuBisCO a promising candidate for extraction and application in plant-based food products. Consequently, exploring grass extracts as a

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source of plant-based proteins presents a valuable opportunity for advancing sustainable food production.

The transition of RuBisCO-rich extracts into a widespread food ingredient is progressing steadily. For any ingredient to become of industrial relevance, its functionality as well as its extraction processes must be thoroughly tested and refined. Regarding functionality, some research efforts have focused on evaluating the solubility, gelling, emulsifying, and foaming properties of protein concentrates derived primarily from green crops such as alfalfa (Hojilla-Evangelista et al., 2017; Knuckles and Kohler, 1982), mangold and kale (Nynäs et al., 2023), sugar beet leaves (Martin et al., 2019), duckweed (Nieuwland et al., 2021; Tan et al., 2022; Zhou et al., 2022), and grasses (Pérez-Vila et al., 2024b). These studies consistently report performance comparable to whey and soy protein isolates. However, the functionality of these protein concentrates is highly dependent on the processing methods used for their extraction (Martin et al., 2014). A common extraction process involves crushing the green material with water and separating it into green juice and pulp. The green juice is then heated to up to 55 °C to precipitate chlorophyll, followed by isoelectric precipitation of the protein with the use of acids, centrifugation, and drying into powder (Jwanny et al., 1993; Martin et al., 2019, 2014). Variations and improvements to this procedure have been explored, including the use of additives such as sodium phosphate buffer (Delahaije et al., 2022), polyvinylpolypyrrolidone, calcium chloride, metabisulfite (Martin et al., 2019, 2014), and sodium sulphite (Tanambell et al., 2022), as well as additional finishing techniques like ultracentrifugation (Tanambell et al., 2022) and microfiltration (Nieuwland et al., 2021).

However, most of these efforts have been exclusively carried out at laboratory scales, limiting practical applications for obtaining grass protein concentrates as food-grade ingredients that contribute to diversifying the offer of plant-based materials. Filtration techniques present an alternative for separating and concentrating grass proteins efficiently and cleanly. Nonetheless, conventional membrane filtration of grass protein extracts often involves extensive pretreatment steps that can potentially impact protein functionality and lead to low yields (Damborg et al., 2020; Lamsal et al., 2007; Nynäs et al., 2024). Apart from isolated instances like the studies by Ruiz et al. (2018) and Zhang et al. (2015) on membrane filtration parameters for RuBisCO concentration, to the best of our knowledge, no study has explored the techno-functionality of protein ingredients recovered from a completely cold-based membrane filtration system with industrial potential for producing a RuBisCO-rich ingredient suitable for human consumption. (Lübeck et al., 2025; Mattsson et al., 2025)

Consequently, this research aimed to study a protein ingredient extracted from a mixture of legume-grass species, namely, legume-grass protein concentrate (LGC), using a novel and mild semi-industrial production system, without heating or acid precipitation (Lübeck et al., 2025; Mattsson et al., 2025), followed by either spray or freeze drying. The material underwent evaluation for characteristics and functional performance against a range of conventional animal proteins (whey protein isolate and egg white) and plant proteins (soy and pea protein isolates). This study focused on investigating the techno-functional properties of the resulting LGCs and assessing their potential utility compared to established ingredients, thereby solidifying their role as a viable option for the green transition of the food industry. Moreover, this study evaluated the potential influence of the drying process on the functional attributes of the product.

2. Methods

2.1. Raw material

2.1.1. LGC production

The LGC was produced at a demonstrator scale biorefining process (20 kg of raw material) from a mix of ryegrass, *X Festulolium*, Timothy, and white clover, harvested in July 2022 in Suldrup, Denmark, as

described by Mattson et al. (2025). Briefly, the biorefining process was carried out as follows. First, freshly harvested legume grass leaves (LG) were shredded while suspended in a stabilising solution to inhibit browning reactions and protein degradation. The stabilised LG suspension was then screw-pressed to extract a protein-containing green juice and a fibrous pulp fraction. After pre-filtration with bag filters (down to 1 µm), the LG juice was separated using a two-step crossflow membrane filtration approach. In the first crossflow filtration stage, the goal was to obtain the permeable proteins, including the desired RuBisCO, while retaining unwanted compounds responsible for the green colour and grassy flavour, as well as any microorganisms. The retentate from the first filtration stage was a concentrated mass of particulate matter, suitable for feed applications. The first stage permeate was then filtered and concentrated in a second membrane step. In this second stage, the membrane allowed for the transmission of lower molecular weight compounds (e.g. salts and peptides) while retaining much of the extracted protein. This protein-rich retentate was then washed in a diafiltration step, further removing low molecular weight compounds (including the stabilising solution) from the final concentrate and thereby increasing the purity.

2.1.2. Drying

The drying of the liquid LGC was carried out either by spray-drying or freeze-drying. The spray-drying process was done in a pilot-scale device (Gea Niro Mobile Minor R&D Soeborg, Denmark) operating in a co-current configuration. Atomisation was carried out using a two-fluid nozzle with an atomising air pressure of 1.5 bar and an atomising air flow of approximately 60 kg/h of compressed air. The process temperatures were kept at an inlet temperature of 161 °C and an outlet temperature of 81 °C with a liquid feed rate of 1.5 kg/h. For freeze-drying, a benchtop device was employed (Christ Gamma 1–16 LSC, Osterode am Harz, Germany) for 120 h. Once drying was completed, the powders were sealed in plastic containers and stored at room temperature (~20 °C) until further use. The resulting fractions were designated LGC-S and LGC-F, corresponding to spray drying and freeze drying, respectively.

2.1.3. Commercial protein materials

Commercial protein ingredients (powders) were evaluated to benchmark the functionality of the LGCs. Egg white (EW) was purchased from Zumub S.A. (Lisbon, Portugal), whey protein isolate (WPI) and soy protein isolate (SPI) were purchased from Sports Supplements Limited (Essex, United Kingdom), and pea protein isolate was obtained from Emsland Group (Emlichheim, Germany).

All analyses that follow were carried out on solutions prepared at the described concentrations, without filtration, thereby using both soluble and insoluble fractions of the ingredients.

2.2. Molecular characterisation

2.2.1. Electrophoresis (SDS-PAGE)

The gel electrophoresis was performed to determine the protein fraction patterns. For the extraction of proteins, 40 mg of LGC-S or LGC-F were mixed with 600 µL of a 5 % SDS solution at pH 8. This solution was mixed in a mixer mill and centrifuged at 22,000 × g for 5 min. Then, the supernatant was taken, and its protein concentration was adjusted to 1.54 mg/mL. Under reduced conditions, an aliquot of 65 µL of protein supernatant was mixed with 25 µL of 4 × lithium dodecyl sulphate buffer and 10 µL of 1 M reducing agent dithiothreitol (DTT), and the mixture was heated at 80 °C for 10 min in a thermomixer operating at 350 rpm. The non-reduced samples for gel electrophoresis were prepared the same as above, only that 10 µL of Milli-Q water was added to replace DTT to reach a total volume of 100 µL. The reduced and non-reduced samples were loaded on a 4–12 % Bis-Tris gel placed inside an electrophoresis device with a 2-(N-morpholino) ethanesulfonic acid running buffer. The marker used corresponded to a pre-stained protein

standard ranging from 3.5 to 260 kDa. The electrophoresis ran for around 40 min at 200 V. Once the separation concluded, the gel was taken out of the cassette and placed in a plastic container together with a 0.02 % (w/V) Coomassie brilliant blue solution to cover the gel. The container was stained with continuous shaking overnight. Next, the solution was discarded, and the gel was shaken again with MilliQ water for 30 min. After this time, the water was changed, and this process was repeated twice. The gel was then scanned, and the identification of the bands/proteins was done with the software GelApp (APDLab, Singapore) and based on values reported in the literature. A pure RuBisCO standard from spinach (*Spinacia oleracea*) (R800–5UN, Sigma Aldrich, St. Louis, MO, USA) was used as reference following the same protocol.

2.2.2. Fourier transform -Infrared spectroscopy (FT-IR)

Fourier transform infrared analysis of the LGC ingredients was performed qualitatively as carried out by Pérez-Vila, et al. (2024a). The measurements were performed with an FT-IR spectrometer (Bruker Vertex 70, Billerica, MA, USA) with an ATR accessory in the range of 4000–400 cm⁻¹ with a resolution of 4 cm⁻¹ and a total of 128 scans. The data obtained was analysed by the software Bruker OPUS 7.2 (Billerica, MA, USA) without normalisation. A RuBisCO standard (same as above) was used for reference.

2.2.3. Differential scanning calorimetry (DSC)

The denaturation of the proteins in the LGC ingredients was determined with a differential scanning calorimeter (820, Mettler Toledo, Schwerzenbach, Switzerland). Approximately 15 mg of the LGCs were hydrated to a concentration of 50 %. An aliquot (20–25 mg) of this solution was transferred to a 40 µL aluminium DSC crucible (ME 27,331) that was hermetically sealed. An empty crucible was used as a reference. The heating scan was carried out at a rate of 10 °C/min in the range of 25 to 100 °C, and the peak temperature of the endothermic peak was taken as corresponding to the denaturation temperature of the protein.

2.3. LGC characterisation

The pH of the LGC and the commercial protein ingredients was measured with a benchtop pH meter (Mettler Toledo FiveEasy F20 Greifensee, Switzerland). The conductivity and zeta potential were analysed by dynamic light scattering in a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, United Kingdom) using the supernatant (after centrifugation at 15,000 g for 10 min) of a 0.1 % (w/w) powder solution prepared in deionised water. The particle size distribution was obtained with a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, United Kingdom) from a solution containing 1 % (w/w) powder and with a refractive index of 1.45 for particles and 1.33 for water. The protein content of each sample was determined by the Dumas method with a Rapid MAX-N Exceed (Elementar, Langenselbold, Germany) using 6.25 as the nitrogen conversion factor. Moisture was measured by placing 0.5 g of powder into a moisture analyser (Mettler Toledo HB43-S, Greifensee, Switzerland) operating at a temperature of 105 °C. The total ash content was determined following the gravimetric method AOAC 930.05 (AOAC, 2012), which consisted of subjecting 1 g of sample to 550 °C for 8 h and assessing the weight difference before and after combustion.

2.4. Functionality assessment

2.4.1. Protein solubility

Protein solubility of the LGC and the commercial protein ingredients was measured in a pH range from 3 to 8 based on the work of Knuckles and Kohler (1982). Briefly, the pH of a 1 % (w/w) powder solution was adjusted to 8 and then decreased with HCl (0.1 or 1 M) until reaching pH 3. One mL of solution was taken at the appropriate pH and transferred to a 1.5 mL Eppendorf tube. Subsequently, the samples were centrifuged at

15,000 x g for 10 min, and the protein content of the supernatant was determined by the Bradford method. For this procedure, 10 µL of the corresponding supernatant were mixed with 300 µL of Coomassie Reagent (Pierce Bradford Plus, Thermo Fisher Scientific, Waltham, MA, USA) in microplate wells. The plate was shaken for 30 s and then incubated for 10 min at room temperature (~20 °C). The absorbance was subsequently measured at 595 nm. A calibration curve prepared with Bovine Serum Albumin served to calculate the protein concentration of the samples. The protein solubility was calculated as the percentage of protein content in the supernatant to the protein content in the powder.

2.4.2. Least gelling concentration

The least gelling concentration was assessed as the minimum amount of protein in solution that is necessary to form a self-supporting gel that does not flow when inverted, based on the method carried out by Yu et al. (2024). For this, aqueous solutions of varied protein contents that included 0.1, 0.5, and from 1 to 20 % (w/w) in 1 % increments, at native pH (see Table 1 for values) were prepared. The solutions were placed in plastic tubes covered with a lid to prevent evaporation and heated for 1 h at 95 °C in a water bath. Next, the tubes were cooled under running water and then stored at 5 °C overnight. On the next day, each sample was inverted to assess the formation of a gel.

2.4.3. Foam properties

The foaming properties of the LGC and the commercial protein ingredients were determined with a dynamic foam analyser (DFA 100, Kruss, Hamburg, Germany) coupled with a sparging attachment, based on the work carried out by Qin et al. (2025). Briefly, 50 mL of a 1 % (w/w) powder solution at native pH (see Table 1 for values) were introduced into the glass cylindrical column (height 25 cm and inner diameter 4 cm) of the device. The solution was injected from the bottom with air at 0.3 L/min for 30 s. The device assessed the changes in the height of the foam and liquid phases as well as the foam structure at 1 frame per second. From this analysis, the foaming capacity (defined as the ratio of foam formed to the amount of gas injected), foam stability (height evolution during 15 min), foam half-life (time at which the maximum foam volume reached 50 % of its initial value), and structure of the bubbles (taken by the built-in camera of the device) were obtained. Additionally, the D_(3,2) Sauter mean diameter of the bubbles was calculated with the built-in software ADVANCE (Kruss GmbH, Hamburg, Germany).

Table 1

Composition and characteristics of the LGC and the commercial protein ingredients. LGC-S: legume grass concentrate spray-dried, LGC-F: legume grass concentrate freeze-dried. EW: egg white, WPI: whey protein isolate, PPI: pea protein isolate, SPI: soy protein isolate. Significant differences in the means ($p < 0.05$) are expressed by different superscripts across rows.

	LGC-S	LGC-F	EW	WPI	PPI	SPI
Protein (g/100 g (d.b))	58.53 ± 3.09 ^a	59.97 ± 0.79 ^a	88.25 ± 0.14 ^a	93.58 ± 0.48 ^a	84.91 ± 0.17 ^a	86.63 ± 0.10 ^a
Ash (g/100 g (d.b))	3.88 ± 0.02 ^d	3.96 ± 0.03 ^{cd}	2.67 ± 0.01 ^e	1.92 ± 0.00 ^f	3.69 ± 0.01 ^d	4.52 ± 0.02 ^b
pH	8.20 ± 0.01 ^a	8.36 ± 0.01 ^b	7.44 ± 0.03 ^d	7.32 ± 0.01 ^e	7.75 ± 0.01 ^c	7.47 ± 0.06 ^d
ζ-potential (mV)	-14.31 ± 0.83 ^b	-12.81 ± 0.69 ^a	-15.91 ± 0.62 ^c	-30.10 ± 1.01 ^e	-31.33 ± 1.27 ^e	-17.16 ± 0.84 ^c
Particle size D _(4,3) (µm)	10.55 ± 0.81 ^e	93.94 ± 2.77 ^b	30.86 ± 2.37 ^d	82.45 ± 2.58 ^c	80.09 ± 0.79 ^c	149.28 ± 4.00 ^a
Colour	Light brown	Light brown	Light beige	Milky white	Light beige	Light beige

2.4.4. Emulsion properties

The emulsion properties of the LGC ingredients were determined by oil-in-water (o/w) emulsions. The o/w emulsions were prepared by adding an amount of rapeseed oil (Brökelmann + Co., Ölzmühle und Verwaltungs-GmbH, Hamm, Germany) at a ratio of 9:1 (solution: oil) to a 1 % (w/w) powder aqueous solution at native pH (see Table 1 for values). They were then homogenised with a T-10 Basic Ultra-Turrax homogeniser (IKA Works, Inc., Staufen, Germany) for 2 min at 10,000 rpm. These coarse emulsions were then passed twice through a benchtop homogeniser (Niro-Soavi NS 1001 L Panda, Gea Group, Düsseldorf, Germany) operating at 200 bars. Following the method of Tan et al. (2022), the fine emulsions were immediately characterised by light scattering with the use of a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK) using a refractive index of 1.475 for the oil and 1.330 for water, with an absorption of 0.01. The droplet size distribution as well as the volume-weighted mean diameter ($D_{[4,3]}$) were recorded on day 0 and after 14 days of refrigerated storage (5 °C). A visual inspection was carried out daily, and pictures were taken on Day 0 and Day 14.

2.4. Statistical analysis

Statistical analysis was performed using SPSS (24.0; SPSS Inc., Chicago, IL, USA). The results are presented as mean \pm standard deviation.

A one-way ANOVA was used to analyse the significant differences among means, and a Tukey post-hoc test was used to assess statistical significance ($p < 0.05$).

3. Results and discussion

3.1. Protein characterisation

The protein profiles of the LGC ingredients under non-reduced and reduced conditions are presented in Fig. 1A. The molecular weights of the detected protein bands in the LGC-S and LGC-F gels correspond to those of the RuBisCO standard, hence confirming the presence of RuBisCO in the LGC ingredients. Native RuBisCO is composed of eight large subunits of 55 kDa and eight small subunits of 12.5 kDa (Hadidi et al., 2023b). Both the large and small subunits were identified in LGC-S and LGC-F at slightly above 50 kDa and near 15 kDa, respectively. These results are in agreement with isolated RuBisCO from different sources such as alfalfa (Hojilla-Evangelista et al., 2017; Lamsal et al., 2007), spinach (Martin et al., 2014), sugar beet leaves (Martin et al., 2019), beetroot leaves, kale, marigold (Nynäs et al., 2023), duckweed (Tan et al., 2023), perennial ryegrass (Pérez-Vila et al., 2024a), and quinoa leaves (Pérez-Vila et al., 2024c). The other faint bands in the gels have been suggested to result from proteolysis, fraction II proteins, residual

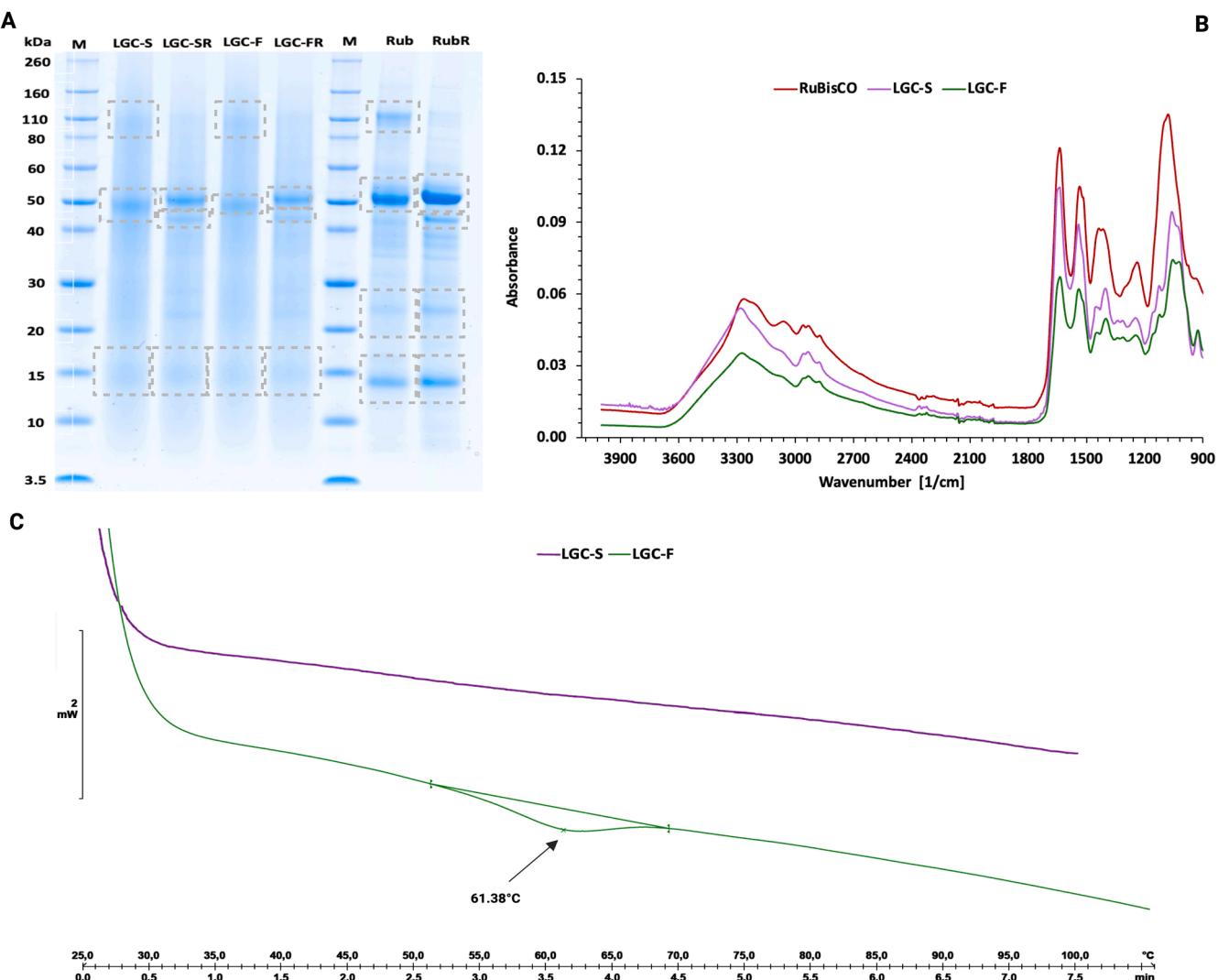


Fig. 1. Protein characteristics of the LGCs. (A) SDS-PAGE. The lanes correspond to the marker (1 and 6), non-reduced LCGs (2 and 4), reduced LGCs (3 and 5), and reduced and non-reduced RuBisCO standard (7 and 8). The dashed boxes highlight the bands identified in the gel (B) FT-IR spectra of LGCs and RuBisCO standard. (C) Thermograms of LGC-F (light green) and LGC-S (dark green).

chloroplasts, and intermediate products of the de novo synthesis of RuBisCO in the plant tissue (Lamsal et al., 2007; Tan et al., 2023; Pérez-Vila et al., 2024c). The comparison between the reduced and non-reduced profiles shows a faint band around 110 kDa in the non-reduced gels. This band is hypothesized to represent aggregates formed by two large subunits (each 50 kDa) stabilized by disulfide bonds, hence disappearing under the reduced condition. The different drying methods did not affect the protein profiles of the LGCs.

FT-IR spectroscopy is a reliable method to characterise the structure of proteins (Gallagher, 2009). Fig. 1B displays the spectra of the LGCs alongside that of pure RuBisCO, suggesting, though not conclusively, its presence in both LGCs ingredients. Proteins extracted from grass material should contain mostly RuBisCO, while other minor proteins such as glycoproteins are expected to remain bound to the cell walls and thus not be a major component of the protein grass extracts (Nieuwland et al., 2021). The FT-IR spectra show peaks in both LGC-S and LGC-F at wavenumbers close to the RuBisCO standard, only differing in the intensities of the peaks. These protein peaks correspond to the amide I between 1600–1700 cm⁻¹, amide II at 1540–1570 cm⁻¹, and amide A around 3300 cm⁻¹ representing C = O stretching, NH in-plane bending with CN stretching, and the Fermi resonances between amide II and NH stretching, respectively (Tatulian, 2013). There are limited FT-IR-based studies of RuBisCO-rich ingredients, one on perennial ryegrass extracts by Pérez-Vila, et al. (2024a), and one by Petrović et al. (2024) on pumpkin leaf proteins. Both groups of researchers reported peaks in the same regions as the ones identified in our study.

The state of the proteins in the LGCs was determined by DSC, and the resulting thermograms are shown in Fig. 1C. The denaturation temperature (T_d) was found to be 61.38 °C for the protein present in LGC-F. This T_d is in agreement with the T_d of about 61 °C for duckweed protein isolates (Nieuwland et al., 2021), but slightly lower than the 64.9 °C reported for a pure RuBisCO protein isolate from spinach (Martin et al., 2014). The finding of the endothermic peak around 61 °C confirms the native state of RuBisCO in the LGC-F ingredient likely due to the gentle freeze-drying. Each subunit of RuBisCO likely has a distinct T_d , but their tight binding causes them to denature as a unit, resulting in a relatively broad exothermic peak in the DSC thermogram. Alternatively, the T_d of the individual subunits are within this temperature range causing the exotherms to overlap, hence appearing within a single denaturation exotherm. The T_d of RuBisCO varies according to the leaves/grass material, but generally slightly higher values have been found such as 70 °C and 66 °C for duckweed (Tan et al., 2022; Zhou et al., 2022), 64.9 °C for spinach (Martin et al., 2014), 76.3 °C and 67.5 °C for alfalfa (Tomimatsu, 1980; Béghin et al., 1993). These variations are attributed to the raw materials since slight differences in amino acid composition, conformation, and compounds bound to RuBisCO have an impact on its thermostability. Nonetheless, the T_d of RuBisCO in the LGC-F ingredients is lower than that of the main proteins in commonly used ingredients, such as β -lactoglobulin in whey protein isolate (84.0 °C) (Park and Lund, 1984), ovalbumin in egg white (78–80 °C) (Photchanachai et al., 2002), legumin in pea protein isolate (88 °C), and glycinin in soy protein isolates (87 °C) (O'Kane et al., 2004). The comparatively low denaturation temperature of RuBisCO in LGCs not only reflects its lower thermal stability relative to conventional proteins but also explains its susceptibility to denaturation during mild processing, contributing to the distinct functional behaviour of these ingredients.

The spray-drying process involves an inlet temperature of 161 °C and an outlet temperature of 81 °C, hence exceeding the T_d of RuBisCO, resulting in protein denaturation. As seen in Fig. 1, the endothermic peak is absent in the thermogram of LGC-S, indicating thermal denaturation of RuBisCO.

3.2. LGC characterisation

The composition of the different protein ingredients is presented in Table 1. The protein content of LGC-S and LGC-F was below 60 g/100 g

powder, making it comparable to commercial protein concentrates but significantly lower than that of the commercial protein isolates used in this study, which range from 84.91 ± 0.17 for PPI up to 93.58 ± 0.48 for WPI. Moreover, the amount of protein in LGC-S and LGC-F was not significantly affected by the drying process. The protein content of RuBisCO-rich extracts has been reported to be dependent on various factors, including the extraction process, harvesting time, stage of plant development, and source of the green material (Pérez-Vila et al., 2022). Nevertheless, the protein levels in our LGCs fall within the ranges reported in the literature from protein extracts from various sources, spanning from 35 g/100 g in alfalfa (Lamsal et al., 2007) to approximately 97 g/100 g in edible vegetable leaves (Famuwagun et al., 2020). The ash content of both LGCs is similar, around 4 g/100 g, and comparable with the other plant protein ingredients (PPI and SPI), though it is slightly higher than the 2.5–3.2 g/100 g found in duckweed isolates (Tan et al., 2022). Conversely, WPI showed the lowest amounts of ash, likely due to the processing; WPI is highly purified and demineralised, resulting in a product primarily composed of protein (Pires et al., 2021). The pH of the commercial ingredients is marginally higher than neutral, while the pH of the LGCs is more basic. These relatively high pH values can be attributed to the pH of the local water used during extraction, which is commonly fairly alkaline (Schröder et al., 2015). In terms of visual colour, the LGC-S and LGC-F appeared light brown, thereby differing from the commercial ingredients that are light beige or milky white. The brownish appearance of the LGCs is probably due to the remaining, and potentially bound, polyphenols, in agreement with other RuBisCO extracts from the biorefining of grass and leaf materials (Tan et al., 2022).

Furthermore, the protein ingredients were characterised by the ζ -potential (Table 1). The ζ -potential denotes the surface charge at the edge of a particle's double layer, reflecting the protein's tendency to attract or repel its surrounding medium, itself, and other particles. High ζ -potentials, whether positive or negative, indicate strong electrostatic repulsion, which can prevent aggregation and influence behaviour in colloidal systems (Feng et al., 2020). As seen, all the proteins were negatively charged. The lowest ζ -potentials, around -30 mV, were observed for PPI and WPI, whereas the highest values were obtained for LGC-F, LGC-S, EW, and SPI, all above -20 mV. The particle size distribution of the ingredients is presented in Fig. 2, with the corresponding volume-weighted mean ($D_{[4,3]}$) shown in Table 1. To the best of our knowledge, there are no published results on the particle size distribution of RuBisCO-containing powder ingredients; hence, for the first time, the effect of the drying process on particle sizes is reported. Different particle size distributions were obtained for the two LGCs. LGC-S displayed a bimodal distribution, where the first peak is located around 0.19 μ m and the second peak around 6.72 μ m. The distribution of LGC-F was, on the other hand, unimodal with a peak at 86.36 μ m. The different particle size distributions resulted in significantly different $D_{[4,3]}$: 10.55 μ m for LGC-S and 93.94 μ m for LGC-F (Table 1). The markedly higher $D_{[4,3]}$ obtained for LGC-F reflects the predominant presence of large particles in this powder (LGC-F peak: 88.36 μ m and LGC-S peak: 6.72 μ m). It should be noted that the average particle size, $D_{[4,3]}$, based on a bimodal distribution, must be interpreted with caution due to its sensitivity to overestimation by the presence of large particles and since all the ingredients analysed likely contained soluble and insoluble fractions. Nonetheless, the difference in distribution patterns emphasises the influence of the drying process. Generally, freeze-drying is considered a gentle drying method that preserves the native conformation and size of proteins. This was confirmed by DSC analysis, which showed that RuBisCO remained in its native state in the LGC-F ingredient, resulting in the observed unimodal size distribution. In contrast, spray-drying, which involves higher temperatures, generally facilitates protein denaturation and aggregation, leading to the formation of larger particles. Although the heat applied during spray-drying disrupted the integrity of RuBisCO, causing its denaturation (c.f., DSC results), this drying process unexpectedly resulted in smaller particles in the LGC-S

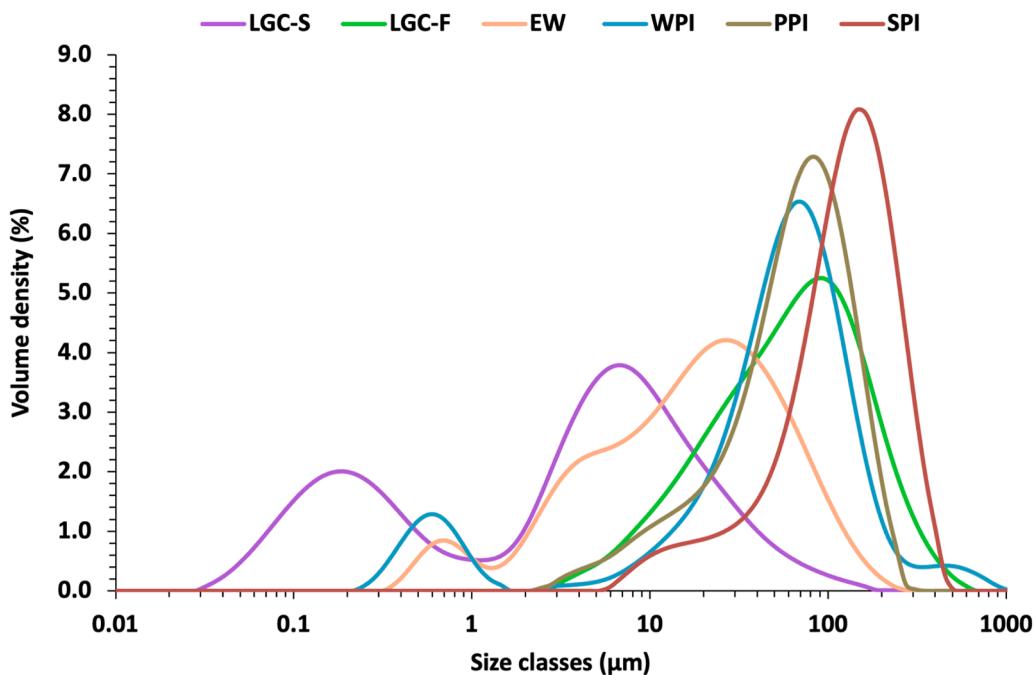


Fig. 2. Particle size distribution of the LGCs and the commercial protein ingredients.

ingredient compared to the gently dried LGC-F. It is suggested that spray-drying led to the dissociation of the native RuBisCO, followed by the denaturation of its subunits. The relatively low outlet temperature of 81 °C likely prevented extensive aggregation of the unfolded subunits,

instead forming small aggregates, resulting in the bimodal particle size range observed for LGC-S. Moreover, other residual materials may be impacted differently due to the drying method and contribute to the different particle distributions of LGC-S and LGC-F. Similar findings,

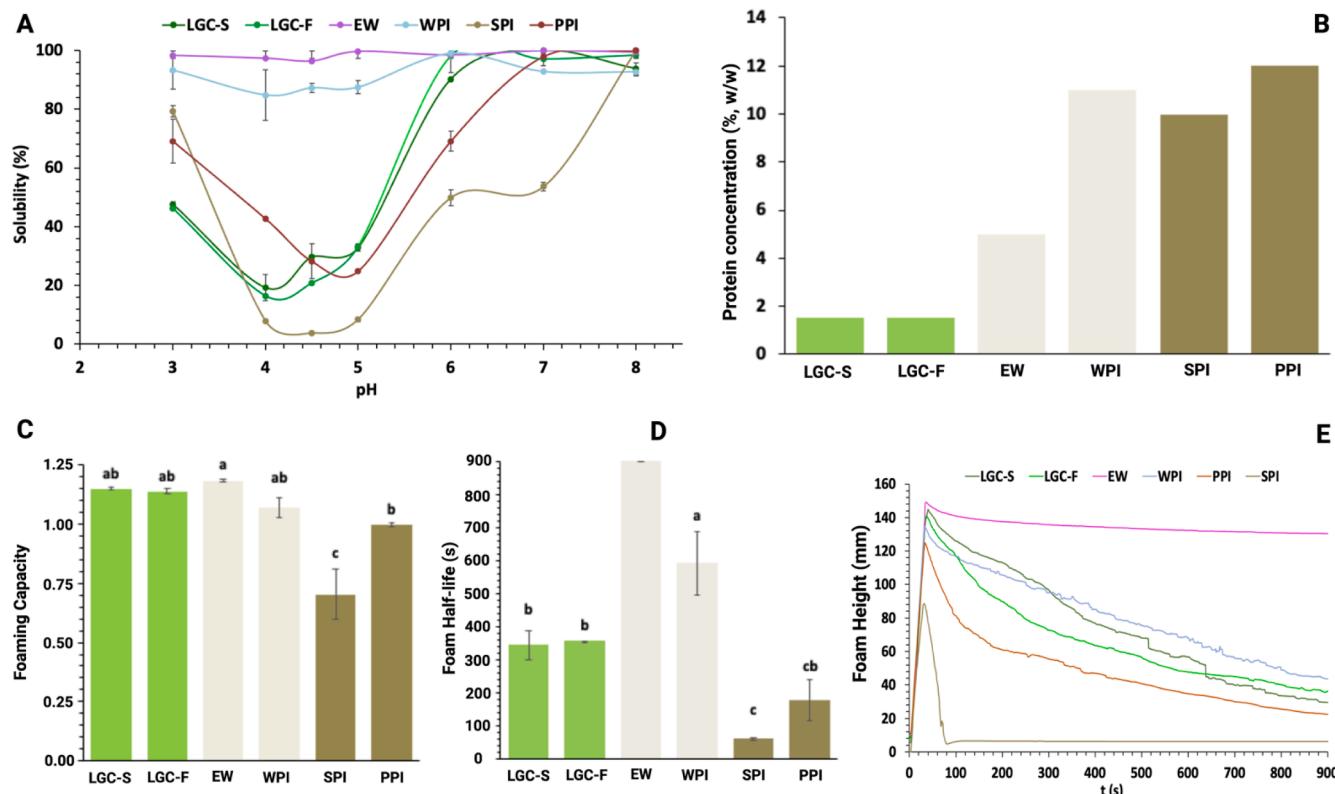


Fig. 3. Functional properties of the LGCs and the commercial protein ingredients. (A) Protein solubility at pH from 3 to 8 based on 1 % w/w powder solution. (B) Least gelling concentration (% w/w) (C) Foaming capacity, (D) Foam half-life expressed in seconds. For EW, the column represents a value of more than 900 s, given that this sample did not reach a half-life within the measured period of 15 min. EW was not included in the statistical analysis. (E) Foaming stability (decay of foam height) during 15 min of a 1 % w/w powder solution. Significant differences in the means ($p < 0.05$) are expressed by different superscripts.

where the particle size of high-protein ingredients was larger in freeze-dried samples compared to spray-dried ones, have been reported by Zhao et al. (2013) for rice dreg protein, Brishti et al. (2020) for mung bean protein isolate, and Chen et al. (2021) for pea processing water ingredients. These authors attributed the smaller particle size in spray-dried samples to the combined effects of heat treatment and atomisation, which contribute to the breakdown of larger particles and aggregates. In contrast, the particle size distributions of the commercial ingredients of this study were characterised by a main peak around 100 μm but significantly different $D_{[4,3]}$. The proteins in PPI and SPI are likely not in their native state but instead present as large aggregates, a characteristic outcome of the spray-drying process (Burger et al., 2022b), while EW demonstrated a very broad polymodal distribution (ranging from 0.77 to 27.37 μm), resulting in much smaller $D_{[4,3]}$.

3.3. Functionality assessment

The functional properties of the LGC ingredients were assessed and compared with the commercial animal (WPI, EW) and plant protein (SPI, PPI) ingredients to establish a benchmark of their performance.

3.3.1. Protein solubility

The protein solubility of the LGCs and the commercial protein ingredients was measured as a function of pH from 3 to 8 (Fig. 3A). Within this food-relevant pH range, both the LGC and the SPI and PPI ingredients showed a U-shaped solubility curve, with high solubility at acidic and basic pH and low solubility around their respective isoelectric point (pI). This profile is typical for legume- and cereal-derived proteins, which generally have surface characteristics that lead to a zero net charge around pH 4.5–5, resulting in minimal solubility. The RuBisCO-rich ingredients in this study exhibited solubility behaviour comparable to that of well-known plant protein ingredients. However, the pI of the RuBisCO protein itself, rather than that of the full ingredient, has been reported to be slightly lower, around pH 3.6–4.0 (Nynäs et al., 2023). In contrast, the LGCs in this study showed minimum solubility closer to pH 4 (Fig. 3A), indicating a shift towards a more acidic range when compared to plant-based proteins such as soy (SPI) and pea (PPI) protein isolates. Furthermore, the higher solubility at pH 3 for SPI (79.33 %) and PPI (69.12 %) compared to the LGCs' solubility of 47.58 % for LGC-S and 46.20 % for LGC-F is, therefore, due to the proximity to RuBisCO's pI. Unlike the conventional plant proteins, i.e., SPI and PPI ingredients, the LGC ingredients exhibited high solubility above 95 % at pH 6 and remained high at pH 7 and 8. A similar behaviour was observed for perennial ryegrass protein concentrates (Pérez-Vila et al., 2024a, 2024b) and the RuBisCO-rich quinoa leaves protein extracts (Pérez-Vila et al., 2024c) with solubility values above 90 % at neutral pH and below 30 % at pH 3 that outperformed SPI while displaying a classical U-shape curve. Achieving such high solubility is the result of the removal of interfering compounds like chloroplasts and polyphenols, which can be carried out either by a mild heating step, as in the case of Pérez-Vila and colleagues (2024a, 2024b, 2024c), or by a non-thermal two-stage filtration process, as used in our study. The presence of interfering substances, such as quinones, phytochemicals, and fats, which can be co-extracted with RuBisCO, has been identified as a factor contributing to the variation in solubility observed in grass protein ingredients (Nynäs et al., 2023; Tanambell et al., 2022) as well as the ratio of RuBisCO to other less soluble proteins that compose the ingredients (Pérez-Vila et al., 2024c).

An interesting observation is that, despite considerably different particle size distributions, hence different $D_{[4,3]}$ values of LGC-S and LGC-F, their solubility profiles are similar. Likely, the molecular conformation of the protein particles in the LGC-S appeared to confer surface characteristics that promoted high solubility at neutral to alkaline pH. The restraint in aggregation, and consequently the enhanced solubility, is often attributed to interactions involving hydrophilic amino acids on the protein's surface (LaClair and Etzel, 2010).

Moreover, the increased contact area between the smaller particles of LGC-S with water likely contributed to the improved solubility. As well-known and expected, WPI and EW exhibited high and stable solubility across the entire pH range.

3.3.2. Least gelling concentration

The least gelling concentration of an ingredient corresponds to the lowest amount of protein necessary to form a heat-induced self-supporting gel that does not flow when inverted (Ma et al., 2022). The results, displayed in Fig. 3B, revealed that both LGC-S and LGC-F outperformed the commercial protein ingredients, with a least gelling concentration of as low as 1.5 % (w/w). In comparison, EW required a concentration of 5 % (w/w), while the other ingredients needed concentrations above 10 % (w/w). This diverse gelling behaviour can be partly explained by the differences in the dominant types of linkages formed during gelation. RuBisCO gels are reported to depend on hydrogen bonds, hydrophobic interactions, and electrostatic interactions, whereas covalent interactions (disulfide bonds) are suggested to play a major role in the formation of WPI, EW, and SPI gels (Foegeding and Davis, 2011; Martin et al., 2019). Furthermore, the low T_d of RuBisCO in the LGC-F ingredient allows denaturation to occur already during heating up to 95 °C. This results in extensive exposure of its buried interaction sites, facilitating the formation of the gel network at low concentration. Likely, the small aggregates in LGC-S are still prone to heat-induced structural changes rendering them, as well as the unfolded subunits, susceptible to gelation. The markedly higher gelling concentration of WPI, SPI, and PPI may be explained by the impact of powder processing conditions. These ingredients are likely produced by spray-drying at high temperatures, causing protein denaturation and extensive aggregation, which makes them less predisposed to gelling (Tarone et al., 2013). The remarkable gelling ability of RuBisCO and its superiority when compared to conventional sources has been documented previously, regardless of the source and processing applied (Knuckles and Kohler, 1982; Martin et al., 2019, 2014). It is worth noting that, although the pH of the protein ingredient solutions varied only slightly, from 7.32 to 8.36 (Table 1), this may still have influenced their gelling behaviour. A follow-up study investigating the effects of food-relevant pH conditions is currently underway to build upon the findings presented here.

3.3.3. Foaming properties

The foaming properties of the LGCs and the commercial protein ingredients were evaluated using a dynamic foam analyser, allowing accurate measurements compared to commonly used techniques that rely on whipping and a visual registration of foam volume. It can be seen in Fig. 3C that the foaming capacity of both LGCs is similar to the animal-based ingredients and PPI. Foam formation relies on the capacity of proteins to decrease the interfacial tension between the air and liquid interface while simultaneously forming a film that effectively covers the bubble. This process is highly dependent on protein surface activity, interfacial stiffness, adsorption rate of the protein at the interface, and molecular flexibility (Foegeding and Davis, 2011; Hu and Meng, 2024; Shen et al., 2024). The flexibility of molecules enables rapid conformational changes in response to environmental modifications, such as the forces involved during foam formation (Amagliani et al., 2021). Comparing foam capacity in Fig. 3C, it appears that the proteins in the LGCs, predominantly composed of RuBisCO, exhibit a surface activity and molecular flexibility similar to β -lactoglobulin, the major functional protein in WPI, and ovalbumin, the major functional protein in EW. Nonetheless, this statement warrants further exploration at the molecular level in order to explore this possibility.

Foaming is a property where proteins extracted from grass or leaves have shown notable performance. Regardless of their botanical origin, these materials have been demonstrated to be superior or at least comparable to those derived from animal sources with foaming capacities at native or neutral pH of 300 to 1000 % for alfalfa (Knuckles and

Kohler, 1982; Nynäs et al., 2023) and 160 % for sugar beet leaves protein powders (Martin et al., 2019). Nonetheless, comparing results across different studies is challenging due to substantial variations in experimental conditions.

Figs. 3D and 3E show, respectively, the foam half-life and the foam stability, recorded as the decay in foam height for 15 min. As expected, the foams of EW and WPI were very stable with minimal height decrease throughout the measurement time, whereas the SPI and PPI foams collapsed quickly (~ 2 and 3 min, respectively). Furthermore, the half-life of the EW foam could not be recorded, given that by the end of the measurement, it did not reach half of its volume; thus, underscoring its high stability. Foams made with the two LGCs were not as stable as the EW and WPI foams, yet were much more stable and displayed a higher foam half-life than the two plant-based ingredients, especially SPI. Despite LGCs' high foam capacity, thus initially achieving a high foam volume, the protein-protein and protein-air/liquid interactions at the interface were not as robust as those of WPI and EW to withstand destabilisation mechanisms, such as coalescence and disproportionation that lead to foam collapse (Foegeding and Davis, 2011; Hu and Meng, 2024). It is noted that the foam capacity and stability of the two LGC ingredients are similar; hence, the foaming properties appear independent of the drying process.

Additionally, the bubble structures at the initial stabilisation time (~ 50 s) and the conclusion of the measurement period (900 s), together with the Sauter mean bubble diameter values ($D_{(3,2)}$), are displayed in Fig. 4. Initially, all samples except SPI featured relatively small and evenly distributed bubbles. By the end of the measurement period, only the EW-stabilised bubbles maintained a distribution close to the initial period with minimal growth. In contrast, LGC-stabilised foams exhibited more pronounced bubble growth compared to EW, while unexpectedly, they were smaller than the bubbles in the WPI-stabilised foam. For SPI and PPI, no images were obtainable at 900 s because the foam structure collapsed before the end of the measurement, further highlighting their

subpar foaming properties. Although comparison among studies is challenging due to inconsistent evaluation methods, the good stability of LGCs' foams, comparable to animal proteins and superior to plant proteins, has also been reported by other authors (Knuckles and Kohler, 1982; Martin et al., 2019; Nynäs et al., 2023; Mattsson et al., 2025). Ma et al. (2025) observed comparably high foamability of RuBisCO-rich fractions obtained by ultrafiltration or alkaline-acid precipitation from spinach, despite their different molecular conformation. The ultrafiltration fraction consisted of native, small particles (particle size distribution peak at 13.1 nm), while the alkaline-acid precipitation fraction consisted of denatured, aggregated particles (particle size distribution peak at 20.5 nm) having also distinctly different surface characteristics of ζ -potential and hydrophobicity. As a result, each fraction displayed different interfacial behaviours where the aggregated particles adsorbed more slowly to the air-water interface but formed stiffer interfacial films, while the native fraction showed faster adsorption with the formation of more flexible films. Interestingly, although the mechanisms were found to be different, the foaming capacities of both denatured and native fractions were comparable. These findings, together with our results, underline that RuBisCO and, therefore, RuBisCO-rich ingredients, can be good foaming agents in a wide range of states and not exclusively in a native, unaggregated form.

It is worth noting that all foams were prepared from a 1 % (w/w) powder solution, to mimic common industrial practices, hence emphasising the better foaming properties of the LGC ingredients as these contained less protein compared to the commercial protein ingredients (c.f., Table 1). Despite having lower protein concentrations, both LGC ingredients performed well, making them promising candidates for developing foam-based products with plant-based materials. As protein concentration is closely linked to the foaming performance of an ingredient (Amagliani et al., 2021), this warrants further investigation of concentration dependency for the foaming abilities of LGCs.

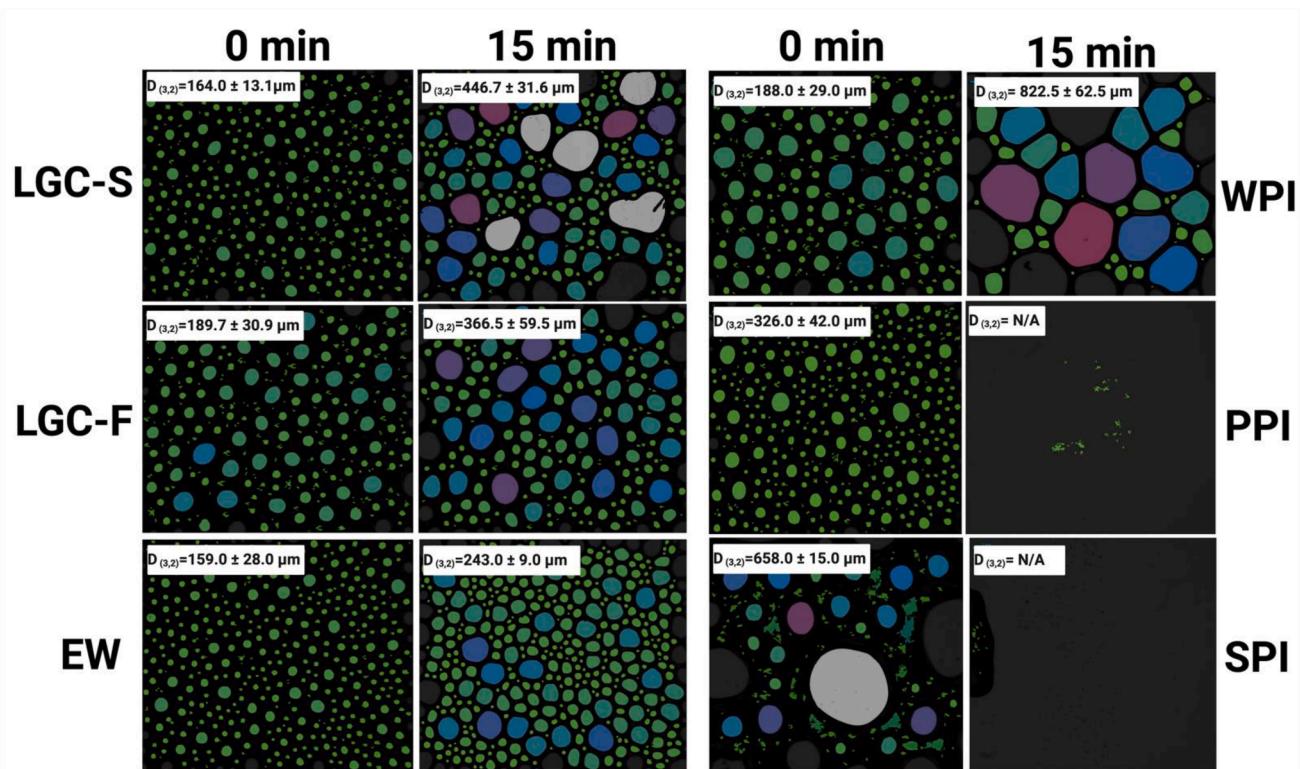


Fig. 4. Bubble structure of foams from 1 % powder (w/w) solutions of LGCs and the commercial protein ingredients at the time of stabilisation (labelled 0 min, though 50 s in practice) and after 15 min. The $D_{(3,2)}$ Sauter mean diameter is displayed on the upper left side of each frame.

3.3.4. Emulsion properties

Emulsions containing 10 % rapeseed oil were produced from 1 % w/w solutions of the two LGC and the commercial ingredients. Fig. 5A shows the droplet size distribution of the fresh emulsions prepared with the different protein ingredients which display a significant influence of the protein ingredient on the oil droplet sizes. The droplet size distribution of the EW and WPI emulsions clearly showed the presence of smaller oil droplets, resulting in the smallest average droplet diameters, $D_{[4,3]}$, both below 2 μm (Table 2). In contrast, the emulsions made with the plant protein ingredients, the two LGCs, PPI, and SPI, had larger $D_{[4,3]}$ values, which nearly doubled those of WPI and EW (Fig. 5A and Table 2). Furthermore, the freshly prepared LGC-S and LGC-F emulsions had a creamy homogenous opaque appearance (Fig. 5B), irrespective of their differences in particle size, due to the light scattering effect by the oil droplets. Conversely, the EW, WPI, PPI, and SPI emulsions were white in appearance, Fig. 5B, while the LGCs-based emulsions were slightly beige due to the light brown colour of the LGC powder. Nevertheless, their visual appearance is fairly similar to the other emulsions, implying the wide potential of LGC ingredients for food applications like dispersed systems.

Consequently, it was noted that the high-pressure homogenisation affected the protein or protein aggregates in the different ingredients, as seen when comparing the particle size of ingredient powder (Table 1) and oil droplet size (Table 2). Upon emulsion preparation, the homogenization process can disrupt native protein and aggregates, resulting in smaller protein subunits and/or particles (Burger et al., 2022a). The oil droplet size is dependent on the concentration and ability of the proteins to stabilise them; therefore, the superior emulsification performance of WPI and EW is explained by their ability to rapidly migrate to the interface, unfold, and quickly adsorb, forming a viscoelastic film around the newly formed droplets (Kim et al., 2020; Nishinari et al., 2014). Due to the different nature of the three plant-based ingredients, LGCs, PPI, and SPI, the mechanism of forming smaller oil droplets inside the homogeniser likely differs. The large protein aggregates in PPI and SPI must be disrupted into smaller fractions and/or individual proteins with a size appropriate for diffusion to the oil interface and enough surface activity to cover the oil droplets (Gharibzahedi and Smith, 2020; Luo et al., 2022). For the plant-based protein ingredients, the disruptive energy during high-pressure homogenisation may not have been sufficient to produce small, flexible protein units; thus, bulky protein units might have remained in the ingredient which hindered its ability to migrate to the interface, hence resulting in the formation of large droplets (compared to EW and WPI) (Tan et al., 2022). The particle size distribution of the fresh PPI and SPI emulsions (Fig. 5A) is fairly similar, thus the related $D_{[4,3]}$ are not significantly different (Table 2). Regarding the LGCs, despite their organizational structural differences (LGC-S, small aggregates, and LGC-F, native protein), the homogenisation provided emulsions with similar-sized droplets of 3.99 and 3.67 μm , respectively. In agreement with our results, comparable droplet sizes of $3.53 \pm 0.01 \mu\text{m}$ were found by Pérez-Vila, et al. (2024b) in emulsions prepared with 1 % (w/w) protein extracts of perennial ryegrass and 10 % of sunflower oil (w/w).

Furthermore, a critical factor for the emulsifying efficiency is the amount of protein available for covering the oil droplets. A higher number of available proteins in the water phase may imply that a bigger oil-water surface area could be covered during homogenization, thereby smaller droplets could be formed, as seen for EW and WPI. Likely, there was enough protein available at the interface to coat the freshly formed droplets to produce small ones (ranging from 0.25–20 μm , Fig. 5A) for both LGC-S and LGC-F. However, a concentration-efficiency relation of grass/leaf proteins has been found to be much higher. Martin et al. (2019) showed that 1 % protein (w/w) is the minimum concentration to obtain an emulsifying behaviour (with 10 % oil) similar to WPI, while Tan et al. (2022) found a higher concentration, 4 % protein (w/w) also with 10 % oil, as needed to obtain the smallest droplet size possible (< 0.35 μm). As previously mentioned, in this study, the LGC solutions were

evaluated at a concentration of 1 % (w/w) powder, not protein.

Fig. 5 and Table 2 show that all emulsions underwent an increase in droplet size during 14 days of storage. This increase was particularly notable in emulsions prepared with the SPI ingredient, followed by the PPI and the two LGC emulsions, whereas the instability was minimal in emulsions produced with WPI and EW. The mean droplet size in the SPI emulsion increased from 2.77 to 27.38 μm , and for the most stable EW emulsion, the $D_{[4,3]}$ value only increased from 1.63 to 1.73 μm during the storage period. After emulsification, the interfacial viscoelastic film formed via protein interactions surrounds the oil droplet and stabilises the emulsion by steric forces and/or charge repulsion. In the case of SPI-based emulsions, the small droplets were likely initially stabilised by large SPI aggregates adsorbing at their surfaces. However, the repulsive forces between the protein layers of neighbouring droplets were not strong enough to prevent coalescence, which, together with possible depletion flocculation caused by excess non-adsorbed protein in the continuous phase, contributed to an increase in the $D_{[4,3]}$ size measured.

Both LGC-S and LGC-F appeared stable since only small changes in the particle size distribution were observed (Fig. 5A) giving a small increase in the calculated $D_{[4,3]}$, from around 4 to around 5 μm (Table 2), during storage. However, it is noted that the particle size distribution measurement was conducted on the remaining emulsion phase. Visual inspection revealed that both LGC emulsions exhibited phase separation (Fig. 5B) with creaming caused by oil droplet enlargement through destabilisation processes such as coalescence, flocculation, or Oswalt ripening. Due to differences in the density of oil and water, these larger oil droplets settled at the top of the emulsion. The LG proteins and particles in the LGC-F and LGC-S ingredients of this study formed less stable emulsions which contrasts with findings from other studies where grass/leaf protein concentrates were reported to form and stabilize emulsions effectively (Hojilla-Evangelista et al., 2017; Knuckles and Kohler, 1982; Pérez-Vile, et al., 2024b; Sheen and Sheen, 1985).

These discrepancies may be attributed to various factors across studies, including differences in oil volume fraction and measurement methods. Additionally, the extraction process could impact the functional properties of the LGC proteins, as residual chloroplast material or fibres may interfere with protein-protein interactions. In this study, the two-stage filtration process resulted in LGCs that are complex ingredients containing multiple compounds alongside the protein. These additional compounds could have interfered with and altered the emulsifying capacity of the concentrate, warranting further investigation in future studies.

3.3.5. Effects of the drying process on the functionality

Overall, it was shown that the drying process did not affect the functional properties of the two LGC ingredients. This suggests that the proteins, mainly RuBisCO, in the LGCs perform similarly regardless of the drying process applied for production even though resulting in different molecular structures. We propose that the spray-drying process likely dissociate and denature RuBisCO subunits followed by a structural reassembly into small-sized particles with high surface activity and functionality comparable to its native counterpart from the milder freeze-drying process. The nature of the spray-drying process minimizes protein degradation due to the short contact time between the droplet and the hot air, resulting in less extensive damage compared to other more intensive heating methods like oven or roller drying (Feyzi et al., 2018; Kelly and Fox, 2016). A similar behaviour for RuBisCO-rich concentrates isolated from alfalfa was found by Knuckles and Kohler (1982). The authors subjected this ingredient to various spray-drying outlet temperatures, as well as freeze-drying, revealing that those materials dried at or below 85 °C displayed similar functionality as that of the freeze-dried samples. This finding, validated by our experiments, is valuable because spray-drying, unlike freeze-drying, can be efficiently performed on an industrial scale without compromising material quality or significantly increasing production costs.

It is noted that our analyses are based on 1 % powder suspensions to

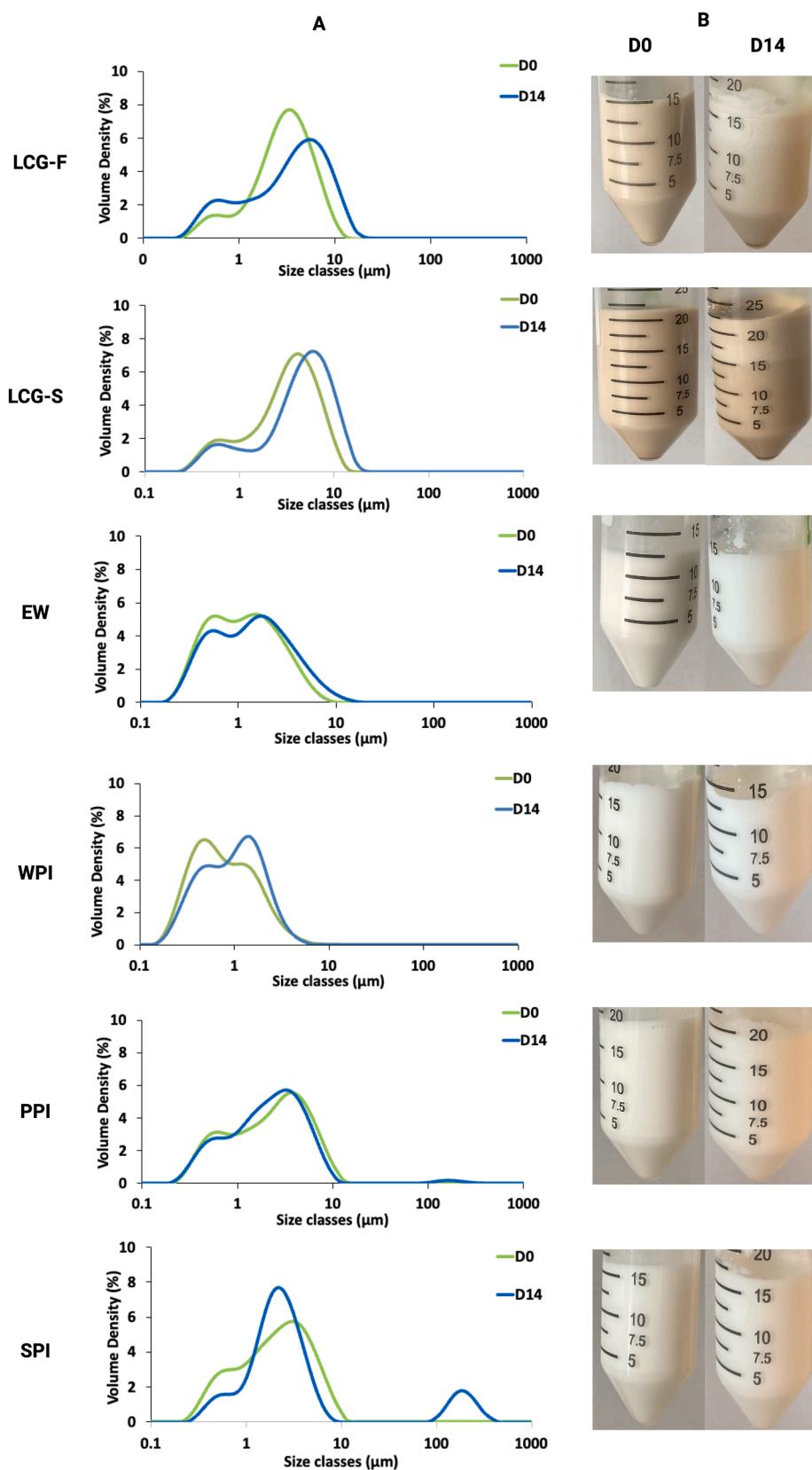


Fig. 5. Emulsion properties of the LCGs and the commercial protein ingredients. (A) Droplet size distribution of the emulsions measured immediately after emulsification (D0) and at 14 days of storage at 5 °C. (B) Photos of the emulsions taken at the corresponding times.

Table 2

Volume-weighted mean diameter ($D_{[4,3]}$) of the droplets of the emulsions prepared with the LGC and the commercial protein ingredients. LGC-S: legume grass concentrate spray-dried, LGC-F: legume grass concentrate freeze-dried. EW: egg white, WPI: whey protein isolate, PPI: pea protein isolate, SPI: soy protein isolate. The sizes were measured at 0 (D0) and 14 days (D14). The significant differences ($p < 0.05$) between means are expressed by different superscripts across the columns.

	$D_{[4,3]}$ at D0 (μm)	$D_{[4,3]}$ at D14 (μm)
LGC-S	$3.99 \pm 0.32^{\text{b}}$	$5.56 \pm 0.49^{\text{c}}$
LGC-F	$3.67 \pm 0.15^{\text{bc}}$	$4.70 \pm 0.45^{\text{c}}$
EW	$1.63 \pm 0.05^{\text{cd}}$	$1.71 \pm 0.14^{\text{c}}$
WPI	$1.09 \pm 0.04^{\text{d}}$	$1.26 \pm 0.07^{\text{c}}$
PPI	$3.13 \pm 0.92^{\text{bcd}}$	$4.60 \pm 3.00^{\text{c}}$
SPI	$2.77 \pm 0.27^{\text{bcd}}$	$27.38 \pm 9.08^{\text{b}}$

align the content of dry matter rather than 1 % protein suspensions in order to evaluate and compare the functional properties of the ingredients as a whole. Though restricting direct comparisons with other studies (protein-based), this provides basic theoretical knowledge and expands the application potential of LG ingredients.

4. Conclusion

The protein concentrates, LGC-S and LGC-F, from the abundant, yet underutilized legume grass blends, obtained through a new industrially oriented mild filtration process, contained around 60 % crude protein including the characteristic RuBisCO protein. The drying method, freeze-drying or spray-drying, of the extracted concentrates affected both the state of the proteins and particle size considerably; however, it did not affect their functional properties. The freeze-dried LGC consisted of native protein, yet arranged as large particles, while the spray-dried LGC contained denatured proteins, with surprisingly small particles. Compared with other commonly used protein ingredients, both LGCs exhibited good water solubility at very low, neutral, and high pH values, with a similar U-shape dependency to that of soy and pea proteins, although being as soluble as animal proteins at neutral and slightly basic pH values. The gelling ability of the LGCs was remarkably superior to the commercial protein ingredients, with a low least gelling concentration of 1.5 % protein (w/w). The foaming capacity of the LGCs was comparable to that of animal-based (whey and egg white) proteins, although the stability of their foams was less than the whey and egg white-based foams, while still much more stable than soy and pea protein foams. Their emulsifying properties were less satisfactory compared to those of established good emulsifying agents such as whey and egg white proteins. The freshly prepared LGC emulsions had a creamy homogenous opaque appearance with small oil droplets; however, stability was hampered by prominent droplet growth that led to creaming, similar to the behaviour of the soy and pea-stabilised emulsions. These functionality results showed that protein concentrates from legume grass blends, rich in RuBisCO, exhibited interesting techno-functional properties, with some features resembling animal proteins and others akin to plant proteins. Additionally, it was demonstrated that a relatively low outlet temperature of the spray-drying process does not negatively impact the functionality of the LGC, providing valuable insight for transitioning to the industrial production of grass protein ingredients. In conclusion, this study has demonstrated that LGCs possess unique characteristics that warrant further exploration. Future studies will focus on manipulating the extrinsic characteristics of these concentrates to better understand how this novel protein material would behave in the complexity of a food matrix, as well as the possibility to analyse exclusively the soluble fractions of these ingredients and contrast with the material as a whole.

Ethical statement - studies in humans and animals

The present manuscript does not deal with research carried out on

humans or animals; therefore, an Ethical Statement is not provided.

CRediT authorship contribution statement

Esteban Echeverria-Jaramillo: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anders Kjær Jørgensen:** Writing – review & editing, Resources. **Mads K Jørgensen:** Writing – review & editing, Resources, Conceptualization. **Tuve Mattsson:** Writing – review & editing, Resources. **Simon G Echers:** Writing – review & editing. **Peter S Lübeck:** Writing – review & editing, Resources. **Mette Lübeck:** Writing – review & editing, Resources, Funding acquisition. **Vibeke Orlien:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Vibeke Orlien reports financial support was provided by Green Development and Demonstration Program (GUDP) from The Danish Agrifish Agency, and the Ministry of Environment and Food of Denmark. Peter Lubeck reports a relationship with BiomassProtein ApS that includes: board membership. Peter Lubeck, Tuve Mattsson, Anders Jørgensen, Simon Echer, and Mette Lubeck have the patent #WO2025/133209: Method for Producing a food-grade protein product and/or feed protein product from plant material. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data can be made available upon request to the corresponding author.

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