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Residual barley proteins in brewers' spent grains: Quantitative composition and implications for food ingredient applications☆

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ABSTRACT

Brewer's spent grain (BSG) is the major side-stream from beer production with millions of tons produced each year. The direct use of BSG as a food ingredient has been limited due to subpar protein techno-functionality following extraction. As such, BSG is primarily used for low-value purposes and can be considered an underutilized resource. BSG has a protein content of up to 30 %, making it a readily available and high potential feedstock for valorization into a functional food ingredient through approaches such as enzymatic hydrolysis. However, little attention has previously been given to the protein-level composition of BSG, which is essential for developing hydrolysis strategies to improve functionality in an efficient and targeted manner. Here, we present an in-depth quantitative characterization of the BSG proteome and investigate dynamic proteome changes during malting and mashing, the initial phases of beer production. We show dynamic and selective changes in the proteome across the different process steps, where 29 % of reproducibly identified proteins display differential abundance. BSG was found to have a significantly higher proportion of intracellular protein compared to both barley and malt as well as a nutritionally favorable amino acid composition. The major constituent of the BSG proteome was B3-Hordein, constituting more than 30 % (mol/mol) of the BSG protein. Moreover, a large proportion (> 45 % (mol/mol)) of the BSG protein are classified as potential allergens and antinutritional factors. This emphasizes the need for care during downstream processing of BSG to produce safe and functional food ingredients, while also providing protein-level insights for the development of targeted protein extraction and hydrolysis strategies.

1. Introduction

Brewer's spent grain (BSG) is the major side-stream from beer production, representing up to 85 % of the total waste from the brewing industry (Devnani et al., 2023). With an annual production of around 200 billion liters of beer from the global brewing industry, around 40 million tons of BSG are generated (Devnani et al., 2023; Hejna, 2021). A substantial BSG oversupply in combination with BSG being highly susceptible to rapid microbial contamination reduces its value (Bjerregaard et al., 2019). Consequently, most BSG has typically been used in cattle feed or end up in landfills (Bjerregaard et al., 2019; Wen et al., 2019). Particularly in the last decade, the applicability of BSG in production of e.g. energy, bioethanol, and paper has been explored (Mussatto, 2014). Moreover, BSG has been explored for higher value applications such as substrate for solid-state fungal fermentation, to not only enrich protein and improve functionality (Canedo et al., 2016; Lock et al., 2023; Moirangthem et al., 2025; Rusbjerg-Weberskov et al., 2025), but also for microbial production of valuable compounds such as enzymes, organic acids and bioplastics (Xie et al., 2024). BSG has even been used as e.g. a biosorbent for wastewater treatment, a more sustainable filler in bio-based composites and bricks, as well as a bio-fertilizer (Bianco et al., 2024; Dancker et al., 2025). Nevertheless, as BSG is a side-stream from brewing and therefore considered food-grade, the desire for its application and incorporation in food products has consistently increased

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within the last decade (Naibaho et al., 2024). Several research groups and companies are successfully incorporating BSG and BSG flour as an ingredient in products such as bakery items and snacks (Moirangthem et al., 2025; Nyhan et al., 2023; Saberian et al., 2024). Unfortunately, BSG inclusion levels are typically below 20 % due to a lack of compatible techno-functional and sensory properties, resulting in a negative impact on e.g. texture and colour, ultimately leading to consumer rejection (Lynch et al., 2016; Nyhan et al., 2023; Saberian et al., 2024). One of the main reasons is the high content of residual fiber (30–50 % *w*/w) (Ikram et al., 2017; Lynch et al., 2016), of which up to 50 % is constituted by indigestible fibers such as arabinoxylan (Steiner et al., 2015), limiting direct use of BSG as an ingredient. One strategy to improve functionality and applicability of BSG, is to enrich protein and reduce fiber content, through downstream processes (Bjerregaard et al., 2019; Wen et al., 2019). In this way, the typical protein content of 19–30 % (*w*/w) in BSG (Ikram et al., 2017; Lynch et al., 2016) can be enriched up to 85 % (w/w) (Vieira et al., 2014). Extraction of almost all the protein is also possible (Connolly et al., 2013; Qin et al., 2018). However, achieving functional protein in high yields and purity is not simple and usually requires rather harsh treatments, including acids, organic solvents, and elevated temperatures, which further impair the techno-functionality, in particular the solubility, of the BSG protein (Devnani et al., 2023; Rommi et al., 2018; Wen et al., 2019). Recently, the use of emerging green technologies for protein extraction from BSG was demonstrated (Mikkelsen et al., 2025). While this improved overall protein yields and content as well as functionality compared to an alkaline extraction, techno-functionality of the extracts was not remarkable. The impact of the extraction method on protein functionality corroborates recent studies on BSG (Chin et al., 2024).

One way to improve BSG techno-functionality, thereby valorizing it as a food ingredient is through enzymatic hydrolysis. In fact, enzymatic treatment has been shown to not only increase protein yield from BSG compared to alkaline extraction (Abeynayake et al., 2022a; Niemi et al., 2013), but also to enhance the functional and bioactive properties relative to conventionally extracted BSG proteins (Naibaho et al., 2024; Vieira et al., 2022; Wen et al., 2019; Yalçın et al., 2008; Zhang et al., 2025). BSG hydrolysates have shown various functionalities in vitro such as emulsifying (Vieira et al., 2022) and antioxidant activity (Abeynayake et al., 2022b). Previously, potent antioxidant peptides from barley glutelins (i.e. hordein) have been identified following enzymatic hydrolysis with Alcalase (Xia et al., 2012). The peptides from hydrolysis of BSG may not only have a single functionality but may represent multifunctional ingredients with potential health benefits (Aguilar-Toalá et al., 2024; Naik et al., 2024). BSG hydrolysates have not only shown potential in vitro but were also found to display potential health benefit when incorporated in e.g. muffins (Bazsefidpar et al., 2024). Moreover, combining enzymatic hydrolysis with emerging green extraction methods has also been shown to improve both yields and functionality (Ikram et al., 2020). Hitherto, BSG hydrolysis is almost exclusively performed using the conventional "black box" trial-and-error approach, where a range of enzymes and hydrolysis conditions are applied and the resulting hydrolysates are screened for activity and functionality. While this approach undoubtedly has facilitated the development of BSG hydrolysates with superior properties than BSG and BSG flours, little attention is given to the peptide-level composition which ultimately governs bulk functionality. This also explains why hydrolysis has not always been found to improve functionality (Zhao et al., 2025).

Recent advances in prediction of peptide-level properties, such as antioxidant activity (Olsen et al., 2020) and emulsification (García-Moreno et al., 2020a; García-Moreno et al., 2020b), have facilitated a fundamentally different approach in hydrolysate production. The approach is governed by mass spectrometry (MS)-based proteomics combined with bioinformatic data analysis and prediction of peptide functionality using artificial intelligence (García-Moreno et al., 2023). This has led to the discovery of many novel functional and bioactive peptides (García-Moreno et al., 2021; Varona et al., 2023; Yesiltas et al., 2022; Yesiltas et al., 2023) and has also illustrated the potential for valorization of several sustainable protein sources (Deka and Saikia, 2023; Gregersen Echers et al., 2022; Yesiltas et al., 2021). The concept was recently demonstrated in potatoes (Gregersen Echers et al., 2023), yielding hydrolysates with improved surface-active properties in vitro that were furthermore able to improve stability of perishable fish oil through encapsulation (Bjørlie et al., 2023). But to adapt such a strategy for BSG, a detailed and quantitative characterization of the proteome is required.

BSG is the side-stream from the initial phase of beer brewing prior to fermentation (Schulz et al., 2018). Barley seeds are allowed to partially germinate during malting, which upregulates a range of hydrolases, facilitating the degradation of both proteins and carbohydrates within the seed endosperm storage (Blanco et al., 2014). Following milling, malted barley is infused with hot water during mashing, where solubilized protein and starch are degraded to peptides and free amino acids (AAs) as well as fermentable sugars, respectively. The resulting liquid phase (wort) is separated from the brewer's spent grains residue, boiled with hops and fermented to produce beer. While many barley proteins ultimately remain in the final beer product (Schulz et al., 2018), a substantial amount of protein is retained in the underutilized BSG. Barley, as well as the proteome dynamics during malting and mashing, have previously been investigated to understand the underlying enzymatic machinery of these processes (Bahmani et al., 2021a; Colgrave et al., 2013; Kerr et al., 2023; Liu et al., 2022; Mahalingam, 2018). Such insight can enable optimization of both process parameters but also in cultivar selection and development, where key enzymes can be enriched and/or optimized to facilitate a more efficient process (Jin et al., 2014; Nájera-Torres et al., 2022; Qin et al., 2021). But to date, most focus has been on the wort and final beer product (Schulz et al., 2018). Very limited effort has been made to describe the protein-level changes during mashing and how this determines the final protein composition in BSG. This knowledge gap hinders the development of targeted processing strategies to valorize this massive side stream.

In this work, we present a deep characterization of the industrial barley "Planet", its corresponding malted form, commonly used in Danish brewing, followed by the production of representative BSG using a conventional mashing procedure. These streams of the pre-fermentation brewing process were initially compared using a range of different methods to investigate bulk changes. Using bottom-up proteomics, we investigate the protein-level changes at each stage of the process, dive into the BSG proteome to identify the most abundant proteins and use this insight to evaluate the potential benefits and challenges of using BSG as a food ingredient through in silico analysis. This provides the basis for developing targeted processes for BSG processing in terms of both protein extraction and enzymatic hydrolysis.

2. Materials and methods

2.1. Production of brewer's spent grains

Brewer's spent grains (BSG) were produced by conventional mashing, as previously described (Mikkelsen et al., 2025). Briefly, 50 g of malted barley (*Hordeum vulgare*, var. Planet) (Viking malt, Vordingborg, Denmark) was milled using a 0.7 mm DLFU disc mill (Bühler, Skovlunde, Denmark). The grist was added to 150 mL 0.3 mM $CaSO_4$ (in distilled water (dH_2O)) pre-heated to 65 °C in a mashing bath (Locher Labor + Technik GmbH, Berching, Germany) and rested for 50 min. Subsequently, the temperature was increased at a rate of 1 °C/min to 74 °C and rested for 10 min. After addition of 150 mL dH_2O (pre-heated to 74 °C), the mash was rested for an additional 10 min before being cooled to 25 °C and subsequently, 100 mL dH_2O was added for a final grist-to-water ratio of 1:8. The wort was removed by first running the mash through a common strainer and then through Whatman prepleated 320 mm filter paper (Cytiva, Marlborough, USA). The first filtered 100

mL was reused to wash the BSG and returned. All retained BSG was pooled and dried overnight at 60 °C.

2.2. Crude protein characterization

The produced BSG, along with the malted barley, was analyzed to establish crude characteristics of the streams. Unprocessed barley was obtained from the same batch and variety as the malted barley used for BSG production (Viking malt, Vordingborg, Denmark) and used for comparative purposes and to obtain insights on protein-level changes during malting.

2.2.1. Dry matter

Dry matter was determined gravimetrically. 2 g of sample was weighed in pre-weighed and dried beakers and placed in a heating cabinet at 105 °C for 24 h. The samples were cooled in a desiccator and weighed. All samples were measured in triplicates.

2.2.2. Crude protein content

The crude protein (CP) content was estimated based on nitrogen (N) content by DUMAS combustion using a Rapid Max N exceed (Elementar UK Ltd., Handforth, England). A nitrogen-to-protein conversion factor of 6.25 was used to calculate protein content. All determinations were performed in triplicate.

2.2.3. Amino acid analysis

Amino acid composition was determined based on the method described by Ghelichi et al. (Ghelichi et al., 2024). Briefly, approximately 30 mg of sample was hydrolyzed with 6 M HCl at 110 °C for 18 h. Samples were cooled and filtered through 0.22 μm cellulose acetate syringe filters and subsequently pH was adjusted and diluted with 200 mM potassium hydroxide and 100 mM ammonium acetate. Amino acid composition was analyzed with LC-MS (Agilent 1100 series, LC/MSD Trap) with a BioZen Glycan LC Column. Glutamine, asparagine, tryptophan and free cysteine cannot be determined using this method. Glutamine and asparagine are converted into glutamic acid and aspartic acid, respectively, and are reported as a combined sum. Disulfide-bound cysteine (cystine) is not degraded and is included in the analysis. Measurements were carried out in triplicate.

2.2.4. SDS-PAGE

One-dimensional SDS-PAGE was performed under reducing conditions, as previously described (Gregersen et al., 2021). Briefly, analysis was conducted using SurePAGE 4–20 % polyacrylamide gels (Genscript, USA) and a Tris-MOPS SDS Running Buffer system (Genscript). For solid samples, aliquots of cryogenically milled grains were mixed with 4× SDS sample buffer (50 mM Tris pH 6.8, 2 % SDS, 10 % glycerol, 0.02 % bromophenol blue, 12.4 mM EDTA, and 50 mM DTT) to a protein concentration of 4 mg/mL (based on CP) and subsequently incubated for 5 min at 95 °C. Afterwards, samples were allowed to cool, briefly centrifuged to sediment particles, and the supernatant was recovered for analysis. For size estimation, 5 μL PIERCE Unstained Protein MW Marker was loaded. Electrophoresis was carried out at 150 V for 40–50 min until the dye front reached the bottom of the gel. Gels were subsequently stained using Coomassie blue and imaged on a ChemDoc MP imaging System (BioRad, USA).

2.3. Proteomics analysis

To characterize the protein-level composition of the different biomasses, they were investigated using bottom-up proteomics (BUP). Prior to analysis, grain samples (barley and malt) were cryogenically milled using a mortar and pestle with liquid nitrogen. Milling was continued with addition of liquid nitrogen until a fine and homogenous flour was obtained. Cryo-milling was also performed on BSG to investigate any potential benefits in terms of protein extractability. Milled grains and BSG (milled and crude) were lyophilized to ensure a completely dry starting material prior to further processing.

2.3.1. Protein extraction optimization

To optimize the sample preparation protocol in relation to protein extraction efficiency, the effect of combining focused ultrasound with the commercial iST for plant tissue kit (PreOmics, Germany) was initially investigated. For each sample, an amount corresponding to 800 μg protein based on CP was weighed into Protein LoBind tubes (Eppendorf, Germany) and 1 mL iST "Lyse" buffer was added. Tubes were vortexed and placed on a pre-heated Thermomixer (Eppendorf) and incubated at 95 °C for 5 min at 1000 rpm. The solutions, and as much undissolved biomass as possible, were transferred to individual 1 mL AFA tubes (Covaris, USA) and the protein was extracted using a M220 focused ultrasonicator (Covaris). All samples were extracted through 0, 1, 2, 4, and 8 cycles of the protein extraction protocol specified by the manufacturer (peak incident power of 75 W, a duty factor of 10 %, 200 cycles per burst, and 180 s per cycle at 6 °C). After each of the indicated number of cycles, aliquots of the extracts were transferred to new LoBind tubes and subjected to a secondary round of heat incubation on the thermomixer using the same conditions. Extraction efficiency was subsequently evaluated by SDS-PAGE analysis. To ensure comparability, SDS-PAGE analysis was performed using the same sample load of 30 μL, corresponding to 24 μg protein (based on CP analysis, assuming full protein extraction). Samples were mixed with 4× sample buffer with 200 mM DTT in a 3:1 ratio, and subsequently prepared and analyzed as described above for solid samples.

2.3.2. Sample preparation for mass spectrometry

Prior to analysis by mass spectrometry, protein was extracted based on the optimized parameters. For grain samples (barley and malt), two AFA extraction cycles were applied, while for both BSG samples, one cycle was performed. After extraction and additional heat incubation to ensure full protein denaturation, disulfide reduction, and thiol carbamidomethylation, extracted protein was digested and desalted, as described by the kit manufacturer. Briefly, 100 μL of the extract (corresponding to 80 μg) was mixed with 50 μL resuspended protease (Trypsin/LysC) and incubated for three hours on a thermomixer at 37 °C and 500 rpm. Subsequently, the digested protein was transferred to iST cartridges and subjected to three rounds of washing (including "Wash 0" for plant tissue), two rounds of elution, and finally dried in a SpeedVac concentrator (Thermo-Fisher Scientific, USA). When dry, samples were resuspended in 70 μL "LC Load" and the peptide concentration was estimated by microvolume UV-spectroscopy (A280) using an SDS-11 FX (Denovix, USA) at standard settings (1 Abs = 1 mg/mL) and diluted to reach a final concentration around 0.2 μg/μL. Samples were stored at −18 °C until analysis. All extractions were performed in triplicate.

2.3.3. Bottom-up proteomics by LC-MS/MS

Shotgun BUP was performed on an EASY nLC-1200 ultra-high-performance liquid chromatography system (Thermo) with ESI coupled to a Q Exactive HF tandem mass spectrometer (Thermo). For each sample replicate, 1 μg digest was loaded on a PEPMAP trap column (75 μm × 2 cm, C18, 3 μm, 100 Å) followed by reversed-phase separation using a PEPMAP analytical column (75 μm × 50 cm, C18, 2 μm, 100 Å). The mobile phase (solvent A (0.1 %(*V*/V) formic acid) and solvent B (80 % (V/V) acetonitrile, 0.1 %(V/V) formic acid)), was introduced through a stepwise gradient from 5 % to 100 % solvent B over 60 min. The analysis was performed using full MS/ddMS2 Top20 data-dependent acquisition with an MS1 scan range of 300–1600 *m/z*, positive polarity, and a default charge of 2. The MS1 resolution was set to 60,000, while the dd-MS2 resolution was set to 15,000. AGC target and maximum injection time were set to 1e5 and 45 ms, respectively. The isolation window was defined as 1.2 m/z, collisional energy as 28 eV, and the dynamic exclusion window as 20.0 s. Peptide match was set as "preferred" and "exclude isotopes" was enabled.

2.3.4. Raw data processing

Raw LC-MS/MS data was processed with MaxQuant v.2.2.0.0 (Tyanova et al., 2016). The data was searched against the Ensembl Plants (Kersey et al., 2018) *Hordeum vulgare cv. Morex* IBSC_v2 database (Lee et al., 2020). Methionine oxidation and protein N-terminal methylation were used as variable modifications while cysteine carbamidomethylation was included as a fixed modification. Data was analyzed using tryptic in silico digestion, allowing up to two missed cleavages. Minimum peptide size was defined as 7 amino acids and maximum peptide mass as 4600 Da. A false discovery rate of 1 % was employed on both peptide- and protein-level. Match between runs and dependent peptides were enabled to boost identification rates.

The mass spectrometry proteomics data have been deposited into the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD062029 and https://doi.org/10.6019/PXD062029.

2.3.5. Downstream data analysis

Based on the incomplete protein annotation in the IBSC_v2 database, the nature of identified proteins was determined by BLAST analysis using Uniprot (The UniProt Consortium, 2017). Briefly, the sequences of identified proteins were extracted from the IBSC_v2 protein fasta into a custom fasta file using a custom Python script (v.3.8.8). Similarly, subproteome fasta files (e.g. differential or abundant proteins) were constructed for subsequent BLAST analysis. BLAST hits were evaluated by identity, and e-value with a preference given for proteins in a Uniprot barley proteome (UP001057469 or UP001177060). If no high identity and low e-value hits originated from a barley proteome, preference was given to proteins with a specifically annotated name (i.e. avoiding "Uncharacterized" and "Predicted" proteins) from closely related cereal species. For proteins annotated as "Uncharacterized" or "Predicted", the protein with the highest homology and distinct annotation was used to indicate protein function.

2.3.5.1. Inter-sample comparative analysis. For inter-sample comparison using label-free MaxLFQ (Cox et al., 2014) data, Mass Dynamics 2.0 (Quaglieri et al., 2022) was employed. Missing values were imputed using the built-in "missing not at random (MNAR)" algorithm with a mean position factor of 1.8 and a standard deviation factor of 0.3. For pair-wise differential analysis, proteins were considered significantly and differentially abundant if the adjusted p-value was lower than 0.05 (i.e. false discovery rate (FDR) < 5 %) and the fold change ratio was larger than two (i.e. log2(FC) > 1). Data was subsequently visualized in volcano plots using the threshold values for significance and fold change. For hierarchical sample and protein clustering in heatmap representation, a Euclidean distance of 4 and a row-wise Z-score normalization was applied. Only proteins identified as differential by the built-in ANOVA analysis were included in the heatmap representation.

2.3.5.2. Intra-sample relative quantification. For intra-sample quantitative analysis, intensity-based absolute quantification (iBAQ) (Schwanhüusser et al., 2011) was employed using the MaxQuant built-in option. The iBAQ algorithm uses the number of proteotypic peptides (6–35 amino acids of length) within a given protein to normalize for both length and compatibility with tryptic BUP. Initially, all false positive and contaminant proteins were filtered. Next, proteins were filtered based on the requirement that a protein must be quantitatively identified in at least two replicates of at least one sample. To obtain the relative molar protein-level abundance, iBAQ intensities were normalized to the sum of iBAQ intensities within a given sample, as previously described (Danner Aakjaer Pedersen et al., 2025). This provides the relative iBAQ (riBAQ), which can be considered an approximation of relative molar abundance or the abundance of individual proteins relative to all proteins within a given sample in % (mol/mol). To further segment identified proteins and focus on only the most abundant proteins, filters of 0.5 % riBAQ and 1 % riBAQ were applied. Statistical analysis of intra-sample quantification was performed as described below. To visualize protein-level enrichment and depletion during mashing, two approaches were applied for abundant proteins (riBAQ >0.5 % in any stream). First, the mean riBAQ (in%) for each stream was log2 transformed to reduce the dynamic range of values and better visualize differences. Secondly, a fold-change (FC) analysis between malt and BSG was performed. Here, the FC was calculated as the difference in mean riBAQ between BSG and malt divided by the mean riBAQ in BSG for each protein group. As such, a positive FC indicates enrichment in BSG while a negative FC indicates depletion.

2.3.5.3. Subcellular localization. Using the custom fasta with only identified proteins, subcellular localization was predicted using DeepLoc 2.0 (https://services.healthtech.dtu.dk/services/DeepLoc-2.0/) (Thumuluri et al., 2022). While DeepLoc potentially provides multiple probable subcellular origins, only the compartment with the highest probability was used to categorize proteins. Within each predicted compartment, the riBAQ of all associated proteins were summarized to obtain the grand distribution of proteins based on subcellular origin.

2.4. Statistical analysis

Data from crude protein characterization and intra-sample relative quantification by riBAQ (including subcellular localization) was analyzed to identify statistical differences using GraphPad Prism (v.10.0.2, build 232). Comparison of means from the different streams was performed using ordinary two-way ANOVA and Tukey's multiple comparison testing, with a 95 % confidence interval. Assumptions included Gaussian distribution of residuals, equal standard deviations between groups of unmatched replicates, and a single pooled variance. All p-values are reported as adjusted p-values to account for multiple comparisons.

3. Results and discussion

3.1. Characterization of protein content and amino acid composition

Based on initial characterization (Fig. 1), the barley and malted barley were overall found to be more comparable to each other than to BSG. While barley was found to have significantly lower ($p < 0.0001$) DM content than both malt and BSG (Fig. 1A, left), the crude protein (CP) content increased significantly ($p < 0.001$) during each step of the BSG production process (Fig. 1A, right), almost doubling from barley (9.0 % DM basis) to BSG (17.4 % DM basis), reflecting a more efficient extraction of non-protein constituents (e.g. fermentable carbohydrates) during mashing. This finding agrees with previous studies (Jaeger et al., 2021a; Karlsen and Skov, 2022; Lynch et al., 2016) and highlights the potential of BSG as protein-rich side-stream. We also compared protein content estimation using Dumas (N*6.25) and conventional amino acid analysis (AAA) in an unpaired t-test (Fig. S1). Here we found that while the nitrogen-based method slightly overestimated (5 %, 14 %, and 10 % for barley, malt and BSG, respectively) protein content compared to AAA (8.5 %, 9.2 %, and 15.8 % (DM basis) for barley, malt and BSG, respectively), there were no significant differences at 95 % confidence level. This indicates that while not significant, using the Jones' N-to-protein conversion factor of 6.25 (Jones, 1931) may overestimate protein in all streams, and ideally all streams should have a unique and lower conversion factor. Based on the AAA, this factor would be 5.94, 5.48, and 5.68 for barley, malt, and BSG respectively. This is in agreement with other studies, where the suggested conversion factor for barley ranges from 5.45 (Mariotti et al., 2008) to 5.83 (Bjerregaard et al., 2019). Comparing protein content based on AAA by one-way ANOVA, the difference between barley and malt becomes non-

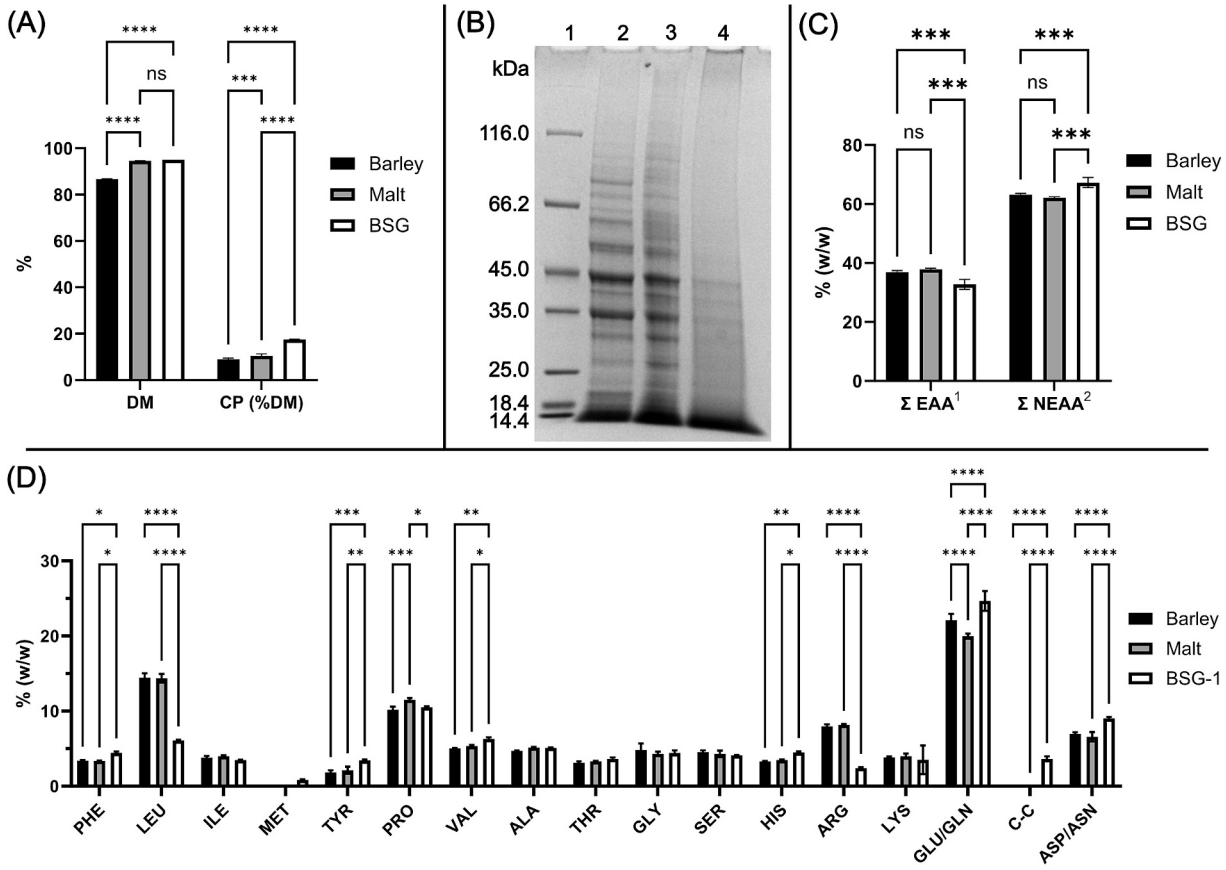

Fig. 1. Initial characterization of the crude barley, malt and BSG. A) Dry matter (DM) and crude protein (CP) content. B) Reducing SDS-PAGE analysis of barley (2), malted barley (3), and the produced BSG (4). All samples were analyzed in crude form by directly solubilizing cryo-milled samples in SDS-sample buffer. Molecular weight marker (1) mass (in kDa) is indicated. C) Sum of essential amino acids (Σ EAA) and non-essential amino acids (Σ NEAA) as per D) Amino acid analysis showing the distribution of individual amino acids as relative abundance (% *w*/w). Compositional analysis is indicated as means with the standard deviation ($n = 3$). Statistical analysis is performed as two-way ANOVA with significance level (from adjusted *p*-values) indicated by "ns" ($p > 0.05$), "*" ($p \leq 0.05$), "**" ($p \leq 0.01$), "***" ($p \leq 0.001$), and "****" ($p \leq 0.0001$). For the amino acid analysis (D), non-significant differences are not shown for simplicity. Numerical data can be found in Table S1.

significant (Fig. S1), further substantiating the similarity of barley and malted barley on the bulk protein level.

The greater similarity between barley and malt, compared to BSG, is also reflected in both the protein profile by SDS-PAGE analysis (Fig. 1B) and the amino acid (AA) composition (Fig. 1C, D). Differences in visible protein bands and intensities are observed between barley and malt, particularly above 50 kDa, but the overall profile remains rather similar. In contrast, BSG was devoid of most bands and only a few of the prominent bands (particularly in the range from 35 kDa to 45 kDa) were retained after mashing. In previous studies, prominent bands in this region have been ascribed to B-hordeins and embryo globulins (Bi et al., 2018a; Celus et al., 2006). Based on the amino acid profile, only the relative (% *w*/w) proportion of Pro and Glu/Gln differed significantly between barley and malt (Fig. 1D), and therefore the overall content of essential and non-essential amino acids (EAA and NEAA) showed no significant difference (Fig. 1C). In contrast, BSG showed a slight but significant ($p < 0.001$) decrease in EAA; from 38 to 39 % in barley and malt, to 33 % in BSG. While this represents a somewhat lower EAA content than e.g. milk protein (38 %), it is still substantially higher than other plant-based proteins like oat (21 %), wheat (22 %), and soy (27 %), and more comparable to egg (32 %) and casein (34 %), representing typical animal-based proteins (Gorissen et al., 2018). Moreover, the EAA content was well above the proportion of 27 % presented as the WHO/FAO/UNU amino acid requirements (WHO/FAO/UNU, 2007).

While the content of branched-chain AAs (BCAAs) Leu (6.1 %) and Ile (3.5 %) was lower in BSG than the comparable levels in barley and malt (approximately 14.4 % and 3.9 %, respectively), it still exceeds the WHO/FAO/UNU requirements of 5.9 % and 3.0 %, respectively. In contrast, the Val content was significantly enriched in BSG compared to barley and malt and a content of 6.3 % is far beyond the required 3.9 %. As such, BSG can be considered sufficient as a source of BCAAs. Only Lys (3.5 %) and Met (0.83 %) were below the required levels, as BSG was also found rich in aromatic AAs. As Met is commonly considered in combination with Cys, the cystine (C—C) content of 3.6 % in BSG also suggests there are sufficient sulphur-containing AAs. As such, only Lys can be considered a limiting AA in terms of nutritional quality based on our analysis. Ultimately, these findings show that BSG may indeed be considered a promising source of nutritional protein for use in foods. They also emphasize that more effort and resources should be allocated towards finding better use of this by-product. Not only for nutritional purposes, but also for improving both economic and environmental sustainability of the brewing industry by converting waste into value.

3.2. *Optimization of protein extraction for proteomic analysis*

To gain deeper insights on the protein-level composition, we performed LC-MS/MS-based BUP. However, as the samples represent complex flours and because protein may still be retained in the recalcitrant plant matrix, thereby limiting accessibility, we initially optimized protein extractability. While the employed iST kit is specifically developed for proteomics analysis of plant tissues (Cun et al., 2024), previous analyses have indicated that thermal and chemical treatment

alone may not be sufficient to facilitate complete and representative protein extraction (Berni et al., 2023; Lin et al., 2024). However, the combination of the kit with physical disruption methods, such as ultrasonication, has been shown to improve representative protein recovery (Danner Aakjaer Pedersen et al., 2025). Consequently, we attempted to combine the thermochemical lysis and extraction from the kit with adaptive focus ultrasound.

By subjecting all samples to multiple rounds of ultrasonication, we were able to evaluate extraction efficiency and protein quality by SDS-PAGE (Fig. 2). Application of other conventional quantitative protein assays (such as absorbance at 280 nm), and fluorochrome-based methods (such as Qubit), have previously been shown to be incompatible with the lysis buffer from the iST kit, due to either coextraction of non-proteogenic UV-active species or buffer incompatibility (Danner Aakjaer Pedersen et al., 2025). Consequently, SDS-PAGE analysis was applied for both qualitative and quantitative evaluation. From this analysis, it was evident that extraction from milled grain samples (barley and malt) benefited greatly from the application of focused ultrasound (Fig. 2A). It was also evident that two extraction cycles appeared sufficient for both flours (Fig. 2A, lanes 4 and 9) and that excessive treatment with up to eight cycles (Fig. 2A lanes 6 and 11) had a detrimental effect, particularly for higher MW proteins. BSG (Fig. 2B) did not appear to benefit from ultrasonication beyond one extraction cycle (Fig. 2B, lanes 3 and 9), but compared to no ultrasonication (Fig. 2B, lanes 2 and 8), a clear improvement was observed. Based on SDS-PAGE analysis, no apparent differences in the protein composition between crude and cryo-milled BSG were observed. The decreased requirement for mechanical force during extraction from BSG, compared to grain samples, is likely directly linked to the additional processing and breakdown of the recalcitrant matrix during mashing. Consequently, two cycles were employed for milled grain samples and one cycle for both BSG samples prior to BUP analysis. Compared to the SDS-PAGE analysis of the crude samples (Fig. 1B), the extracts displayed a high level of similarity in the protein profile, underlining the applicability of combining the iST kit and focused ultrasound for representative protein extraction.

3.3. Overall protein-level changes during BSG production

Using the optimized protocols for sample preparation, we identified a total of 967 barley protein groups across all samples by LC-MS/MS-based BUP, following the removal of common contaminants and false positives. From these 967 protein IDs, 706 were quantitatively identified in at least two replicates from unprocessed barley, while 857 were found in the malted barley samples. In BSG, 647 protein groups were identified in at least two replicates from the crude sample, while 604 protein groups were reproducibly quantified in the cryo-milled sample. Across all samples, 945 protein groups (98 %) were reproducibly found in at least one sample. Nearly half of the proteins (454) were identified in at least two replicates of all samples, while 660 proteins were shared between barley and malt (Fig. 3A). The proportion of proteins reproducibly identified in malt but not in barley (200) was substantially larger than the reverse (49). 196 proteins found in malt were not identified reproducibly in any BSG sample. These observations illustrate a dynamic process from barley to malt and to BSG.

The qualitative differences were also reflected when analyzing the overall replicate correlation (Fig. 3B) and experimental variability (Fig. 3C). Replicates between each sample displayed good overall correlation above 0.85 with grain samples generally showing higher replicate correlation (~0.92). This difference may be ascribed to the higher number of protein identifications in these samples and a potentially more homogenous nature compared to BSG. Comparing BSG samples with and without cryo-milling showed comparable correlations as replicates within each group. This indicates minimal effect of the milling. As expected, BSG replicates had a substantially higher correlation with malt replicates than barley replicates, as the BSG was produced from the malt and because malting represents the biological germination process of the barley grains. These observations were also reflected when investigating the overall variability in the dataset using principal component analysis (PCA) (Fig. 3C). Here, the two BSG samples clustered nicely and have a longer distance (but in the same direction) from barley than does malt along the first principal component. In contrast, barley and BSG were closely spaced along PC2, while malt differs substantially. This indicates that differences between samples are governed by several underlying distributions, which was also corroborated by Scree analysis (Fig. S2), where PC1 and PC2 were found to explain only 24 % and 14 % of the total variability, respectively. Overall, this analysis nicely illustrates the dynamic process of moving from barley to BSG through malting and mashing. These dynamics are also evident when considering the 272 differentially expressed/abundant proteins by ANOVA across the different samples (28.8 % of reproducibly identified proteins), showing distinct clustering on the protein level (Fig. 3C).

3.4. Malting reduces abundance of stress-related proteins and induces enzymatic storage depletion

Malting of barley represents the biological process of germination. As such, this process is also expected to be reflected on the protein-level through significant changes in expression patterns. Through a

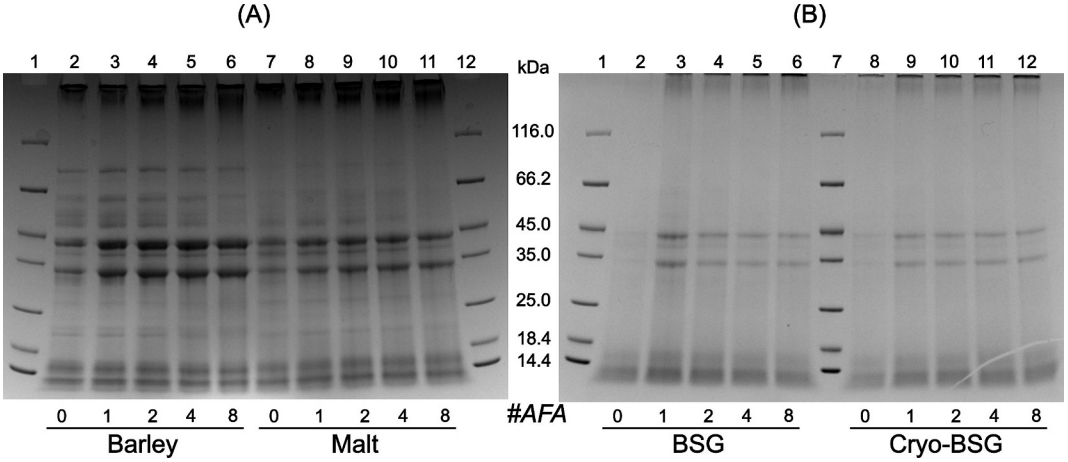

Fig. 2. Evaluation of protein extractability by reducing SDS-PAGE analysis. Gels are shown for (A) barley (lane 2–6) and malt (lane 7–11) as well as for (B) BSG (lane 2–6) and Cryo-BSG (lane 8–12). Protein was extracted using the iST plant tissue kit and in combination with 0, 1, 2, 4, and 8 AFA ultrasonication cycles (as indicated below each lane). As molecular weight marker, Pierce unstained marker was used (lanes A1, A12, B1, and B7) and mass (in kDa) is indicated.

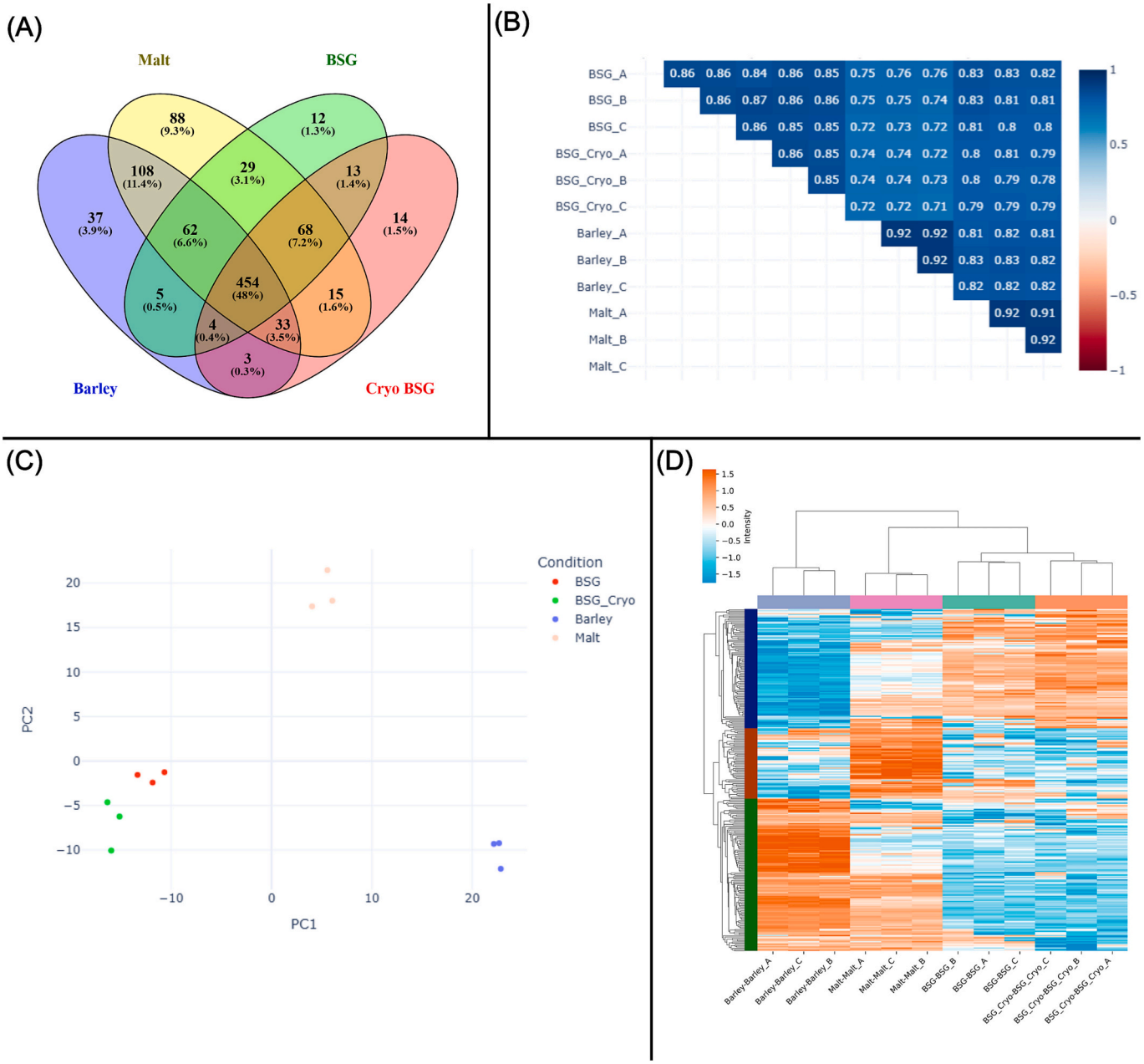

Fig. 3. Overview of LC-MS/MS proteomics analysis. (A) Venn diagram showing overlap of reproducibly identified proteins (i.e. identified in at least two of three replicates) across the four samples showing both the numerical and relative (in %) intersect. (B) Pearson correlation matrix showing the overall qualitative and quantitative reproducibility between individual replicates and samples from anti-correlation (red, −1), to no correlation (white, 0), and to perfect correlation (blue, 1). Pearson correlation coefficients were based on MaxLFQ intensity data after imputation of missing values in MassDynamics. (C) Principal component analysis to show replicate-wise sample similarity and clustering according to overall qualitative and quantitative variability. Individual sample replicates are depicted based on the two first principal components, together explaining 38 % of the total variability (see Scree plot in Fig. S2). (D) Heatmap of differentially abundant proteins by ANOVA analysis in MassDynamics. Data is depicted as z-score standardized MaxLFQ intensities by row (protein group) and clustered using a Euclidian distance of 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differential expression analysis, we indeed found 27 proteins to be significantly ($p < 0.05$) downregulated (FC > 2) in malt while 69 proteins were found to be significantly upregulated based on normalized LFQ intensities (Fig. 4A). As the proteome is suboptimally annotated, we performed BLAST analysis against the Uniprot reference proteome. Overall, we found strong BLAST hits with identities above 90 % (the majority at 100 %) and e-values below 10^{-50} (Table S2). Among the 27 downregulated proteins in malt, we predominantly found proteins previously associated with germination. For instance, we found multiple late embryogenesis abundant (LEA) proteins, which have been shown to be involved with seed adaptation to particularly cold stress (Zan et al., 2020). That these proteins are found to be depleted in the malted barley is not surprising as they have previously been shown to accumulate under certain stages of embryo development (Dehaye et al., 1997) and disappear after germination (Wilhelm and Thomashow, 1993). Similarly, other stress-related proteins such as the universal stress protein USP7364 were also found depleted in malt. Reduction in stress-related proteins may be considered a direct consequence of seeds transitioning from prolonged cold storage under dehydration to the germination phase, thereby entering a more normalized plant life cycle (Luo et al., 2023). Several seed maturation proteins and fumarase, which are linked with dormancy seed maintenance and metabolism (Grafahrend-Belau

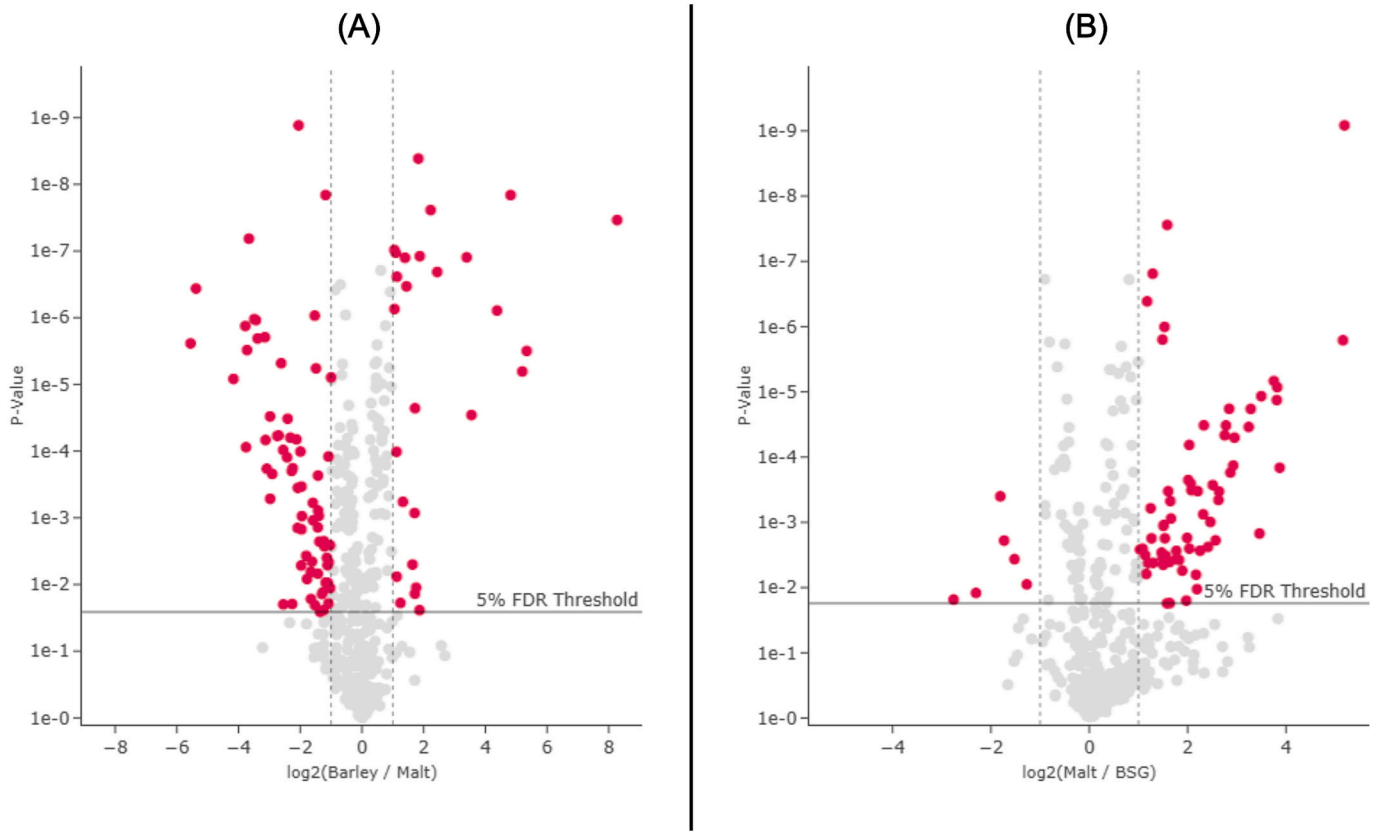

Fig. 4. Volcano-plots from pairwise differential analysis of protein LFQ intensity for barley vs. malt (A) and malt vs. BSG (B). Highlighted proteins (in red) were found to be significantly ($p < 0.05$) and substantially (FC > 2) differentially abundant in the pairwise analysis in MassDynamics. An overview of differentially abundant proteins for barley vs. malt can be found in Table S3 while differentially abundant proteins from malt vs. BSG can be found in Table S4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2009; Kushwaha et al., 2012), were also found to be depleted. Furthermore, a large variety of depleted proteins are known to be involved in seed energy and nutrient storage such as subtilisin-chymotrypsin inhibitors, alpha-amylase inhibitors, hordeins, oleosin, and cupin-type embryo globulins (Gorjanović, 2009; Graham, 2008; Østergaard et al., 2002; Shewry and Halford, 2002; Tanner et al., 2019). Together, these findings clearly indicate how the seeds deactivate the dormant and inhibitory machinery and start consuming stored nutrients to enter the germination process.

The upregulated subproteome in malt (69 proteins) is dominated by enzymes involved in various cellular pathways (Table S2). Not surprisingly, several isoforms of α-amylase were found to be upregulated. Additional carbohydrate degrading enzymes like β-mannosidase, α-glucosidase, β-glucanase, and chitinase, as well as other central enzymes in carbohydrate metabolism, such as malate synthase and Aldose 1-epimerase, were also found significantly ($p < 0.05$) and substantially (FC > 2) upregulated. Upregulation of carbohydrate degrading enzymes during malting has previously been demonstrated on both protein- (Bahmani et al., 2021a) and RNA-level (Vinje et al., 2021), as the seeds start to germinate and require liberation of stored nutrients. In addition to carbohydrate-related enzymes, a wide range of proteases were also found to be upregulated. This is also a direct consequence of the germination, where the seed storage proteins are degraded to facilitate initial plant growth (Schmitt et al., 2013). This biological process is reflected in several other upregulated proteins such as lipoxygenases (Viswanath et al., 2020) and dehydrin, (Hassan et al., 2021) as well as many other proteins involved in the central cellular machinery. In fact, the most upregulated protein is a histone, which further reflects the development of the seed embryo and initiation of growth.

The dramatic increase in enzymatic activity and initial plant development also induces increased oxidative stress on the seed (Ma et al., 2016). This calls for protective measures to be activated against e.g. the increased formation of reactive oxygen species (ROS). Consequently, enzymes protecting against ROS, such as superoxide dismutase and peroxidase were also found to be upregulated during malting. Overall, these findings are in agreement with earlier studies on protein-level changes during barley seed germination and the malting process in brewing (Bahmani et al., 2021b; Osama et al., 2021; Qin et al., 2021). As such, this analysis functions as a built-in quality control of the analytical pipeline.

3.5. *Selective enrichment and depletion during mashing and BSG isolation*

Similarly to the pairwise analysis of barley and malt (Fig. 4A), significant protein-level differences were also found when comparing malt and BSG by LFQ intensities (Fig. 4B). Here, 66 proteins were found to be significantly ($p < 0.05$) depleted (FC > 2) in the BSG while only six were found to be significantly enriched. When comparing malt and BSG, it is important to refrain from the up−/downregulation terminology and rather describe enrichment and depletion, as mashing represents a physical processing of the malt, whereas malting represents a biological process within the barley seeds. As described in relation to investigating the malting process, we performed BLAST analysis for differential proteins found after mashing. Overall, we found strong BLAST hits with identities above 90 % (the majority at 100 %) and e-values below 10^{-50} (Table S3). Among the most substantially depleted proteins were infection defense-related such as hordoindoline-B1, α-hordothionin, and thaumatin as well as stress-related proteins such as dehydrin, a LEA protein (Zaidi et al., 2024), and heat-shock proteins. The depletion of defense-related proteins may be considered an advantage for the use of

BSG in foods, as e.g. thionin are linked with ubiquitous toxicity (Johnson et al., 2005), while thaumatin is linked with increased allergenic risk (Breiteneder, 2004). Similarly, expansin (Sampedro and Cosgrove, 2005) and non-specific lipid transfer proteins (Breiteneder and Radauer, 2004a) are known allergens and were also depleted in BSG. Moreover, a range of different proteins identified as protease and amylase inhibitors, such as cystatin Hv-CPI5 and several bifunctional inhibitor/plant lipid transfer proteins, were depleted in BSG. This can be considered a highly beneficial change in the protein composition, as these inhibitors constitute anti-nutritional factors (Samtiya et al., 2020) and potential allergens (Breiteneder and Radauer, 2004a), thereby increasing the safe applicability of BSG in foods. Of the six enriched proteins in BSG, only glutathione S-transferase has previously been associated with allergy and has been classified as a minor allergen in birch pollen (Deifl et al., 2014). Moreover, the abundance is below 0.05 % (Table S1) and we therefore do not consider this particular protein problematic.

The selective depletion of proteins during mashing and subsequent removal of the wort is not only relevant on the protein-level but also on the bulk level. This is also illustrated from significant ($p < 0.001$) differences in the EAA/NEAA content between BSG and both barley and malt, while no significant difference was observed between the latter two (Fig. 1C). Moreover, this is also reflected when considering the cellular origin of the identified proteins considered as a whole (Fig. 5A). In barley, around 60 % (mol/mol) of the total protein (by riBAQ) is predicted to be extracellular. This is significantly ($p < 0.0001$) reduced in malt to 55 % but also significantly reduced ($p < 0.0001$) when comparing to BSG (51 %) (Fig. S3). This indicates a continued depletion of the relative proportion of extracellular protein, while the relative share of cytoplasmic, nuclear, and plastid proteins significantly increase. In general, depletion of extracellular protein correlates well with the finding that the majority of depleted proteins identified from pairwise analysis (Fig. 4B) are of extracellular origin (Table S4). This indicates that extracellular proteins, to some extent, are either degraded during mashing or end up in the wort due to their highly accessible and soluble nature. This finding corroborates earlier studies showing the dynamic process of endogenous proteolysis during mashing (Kerr et al., 2021), to which extracellular proteins would be more readily available.

To obtain further insights and understanding hereof, it is relevant to consider absolute abundance changes on the single protein level over the full process. For this purpose, we determined the molar abundance at the single-protein level using riBAQ for proteins constituting a substantial amount of the total protein in any of the analyzed samples (Fig. 5B). The most abundant proteins (riBAQ >0.5 %) across all samples, B3-Hordein and Serpin-Z4, together constituting 36–43 % of the

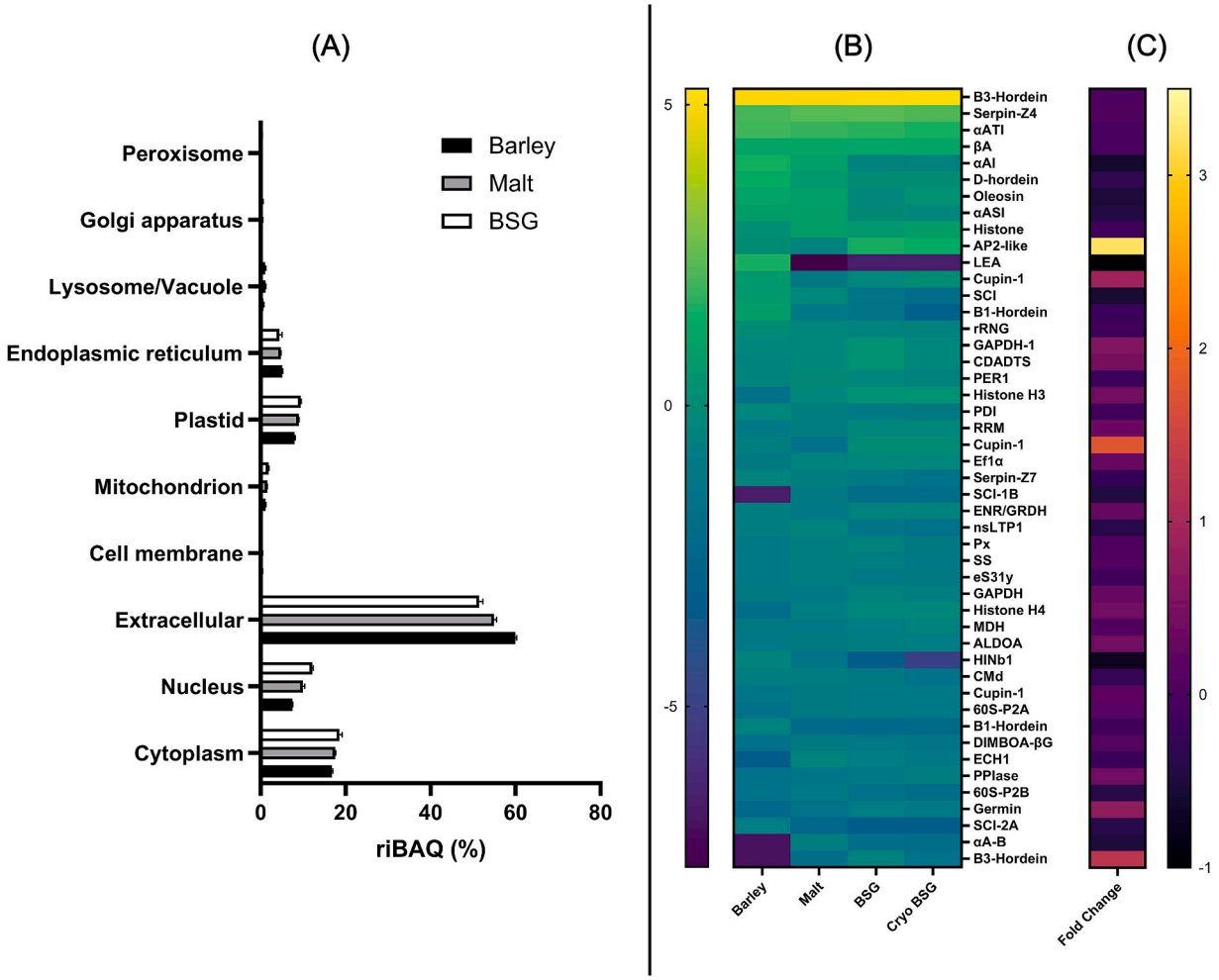

Fig. 5. Relative molar abundance on subcellular and single protein level. (A) Distribution of subcellular localization as predicted by DeepLoc 2.0. Each compartment represents the sum riBAQ-based molar abundance for all proteins which has bas been ascribed hereto based on maximum probability. (B) Abundance distribution of the most abundant proteins across all samples (mean riBAQ >0.5 % in any sample). Protein abundance is depicted as the log2 transform of the relative molar abundance (by riBAQ) and colour coded from low (blue) to high (yellow) abundance. Proteins are given using short names as defined in Table S6. (C) Protein-level fold change (by riBAQ) of BSG vs. malt to visualize enrichment/depletion after mashing. Proteins are shown from highly depleted (black) to highly enriched (yellow) in BSG and aligned with the protein names in panel B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

total protein, show quite comparable levels of abundance in all samples. However, the LEA protein found abundant in barley (3.3 %) was absent in downstream samples. This correlates with findings from the pairwise analysis of barley and malt (Fig. 4A). Several other proteins such as α-amylase inhibitor (αAI), D-hordein, oleosin, and subtilisin-chymotrypsin inhibitor (SCI) show a gradual decrease in abundance along the process and not only between individual process points, as previously described.

Interestingly, two proteins showed an opposite trend and became enriched and highly abundant in BSG. AP2-like factor was enriched threefold from barley (1.0 % riBAQ) to BSG (3.0 % riBAQ). This despite showing a minor depletion in malt (0.72 % riBAQ). However, this protein was only identified from one peptide across all samples and received a low Andromeda score (6.1). Therefore, this protein identification is associated with uncertainty (Table S1). Cupin-type 1 embryo globulin had an abundance of 0.61 % in barley, which decreased to 0.35 % in malt but became enriched in BSG at 0.96 %. These changes are also reflected by a fold change (FC) analysis between BSG and malt, where the two proteins obtained an FC of 3.2 and 1.8, respectively, based on molar abundance (Fig. 5C). Histone H3 showed a gradual increase from 0.31 % in barley, to 0.86 % in malt, and to 1.2 % in BSG. As such the BSG-to-malt FC is more subtle (0.39) but it may still be regarded as a highly abundant and enriched protein in BSG. The enrichment of a cupin-like protein may be disadvantageous as these are generally considered major allergens (Breiteneder and Radauer, 2004b), and require additional processing to reduce allergenic potential of BSG as food ingredient.

When evaluating the effect of BSG cryo-milling, we found that this did not entail an overall positive effect as anticipated. Firstly, there was no real difference in the protein profile by SDS-PAGE when compared to the crude BSG (Fig. 1B, 2B). Moreover, the number of reproducibly identified proteins actually dropped by 7 % from 647 in the crude BSG to 604 in the cryo-milled BSG. While the overall differences from differential analysis by ANOVA (Fig. 3D) and pairwise analysis (Fig. S4) were minimal, the overall loss of protein identifications and introduction of variance between BSG and cryo-milled BSG (Fig. 3C, 5B) indicates that the cryo-milling did not improve the analytical output. In fact, the additional processing steps may have introduced selective losses, which is why the analytical outcome may not fully reflect the BSG and could be considered somewhat detrimental instead. The fact that no additional improvement was obtained from BSG cryo-milling may be a result of the BSG already being quite heavily processed and can be considered equivalent to a residual grain flour. This finding facilitates easier and faster sample preparation for BSG proteomics analysis.

3.6. *Highly abundant proteins in BSG: Risk factors or potential targets for downstream processing?*

With the aim of identifying highly abundant proteins that could be potential targets for downstream processing and BSG valorization, we further investigated the most abundant proteins (> 1 % riBAQ) in BSG (Table 1). While BSG was found to be depleted in many proteins with antinutritional and allergenic potential, it is also clear that several of the most abundant proteins in BSG are still associated with risk factors for safe ingestion in foods. In a previous proteomic study of BSG (Bi et al., 2018b), many of the same proteins were identified as abundant. However, this study quantified protein abundance by relative LFQ intensity, thereby normalizing data between samples and not within the individual samples as done with riBAQ. This also entails that the relative protein distribution in that study did not reflect the molar distribution but rather the relative signal distribution, where the abundance of larger proteins was artificially inflated. Nevertheless, the MW of the most abundant proteins, in particular B3-Hordein, Serpin-Z4, and alpha-amylase/trypsin inhibitor (αATI), corresponded well with bands observed by SDS-PAGE analysis of BSG (Fig. 1B, 2B). This indicates that these proteins remained intact after mashing, while the substantial increase in a diffuse smear in the low MW region of BSG compared to both barley and malt, could indicate that some proteins may be partially digested in the process.

Hordeins, including the abundant B-Hordein and D-Hordein, are prolamins and thereby insoluble in water (Evans and Bamforth, 2009). In addition to their high natural abundance in barley, their insolubility makes it logical that residual hordeins end up in the solid BSG fraction after mashing. Hordeins are homologues to wheat gluten proteins (gliadins/glutelins), and hence also considered major allergens (Breiteneder and Radauer, 2004b). Being rich in glutamine and proline (Jaeger et al., 2021b), the high hordein content not only in BSG, but in all analyzed samples is a major driver of the high content of these AAs. Serpin-Z4 is considered a part of the albumin superfamily and is known to elicit serine protease inhibition. As such it is classified as an antinutritional factor (Huang et al., 2023). Earlier studies found that serpin-Z4 survives the entire brewing process and is in fact positively correlated with beer foaming and considered a marker of foam stability (Evans et al., 1999). Moreover, Serpin-Z4 was also found to survive gastrointestinal digestion in ruminant models (Huang et al., 2023), which further highlights its limited direct nutritional value. Similar results were found for αATI (Huang et al., 2023). Both Serpins and αATIs are known grain allergens (Breiteneder and Radauer, 2004b; Tatham and Shewry, 2008). In addition, Glyceraldehyde-3-phosphate dehydrogenase 1 (GADPH) is a known wheat allergen affiliated with Baker's Asthma (Sander et al., 2011). For the remaining highly abundant proteins, we were only able to find literature on their specific cellular function but no reports of cytotoxicity, antinutritional effect, or allergenic potential.

Table 1
Overview of abundant (> 1 %) BSG proteins by relative molar abundance (riBAQ). Table shows the ISBC_v2 AC#, the protein name (as inferred by BLAST analysis in Uniprot), short name, mean riBAQ (%) in BSG, molecular weight (MW) in kDa, the class and/or protein superfamily (if relevant), and know association with inhibitory potential against protein and/or carbohydrate digestive enzymes as well as allergenic potential based on protein superfamily or homology as indicated in literature (indicated by "+").

ISBC_v2 AC#	Protein	Short name	riBAQ	MW (kDa)	Protein class/ superfamily	Inhibitor	Allergen
HORVU1Hr1G001020.2	B3-Hordein	B-Hordein	32.2 %	30	Prolamin		+
HORVU4Hr1G013480.1	Serpin-Z4	Serpin-Z4	5.2 %	43	Albumin/Serpin	+	+
HORVU4Hr1G081660.1	Alpha-amylase/trypsin inhibitor	αATI	3.4 %	16	Prolamin	+	+
HORVU3Hr1G004480.36	AP2-like factor	LEA	3.0 %	47	Transcription factor		
HORVU4Hr1G089510.2	Beta-amylase	βA	2.2 %	60	Hydrolase		+
HORVU6Hr1G087190.1	Histone	Histone	1.4 %	16	Histone		
HORVU7Hr1G074690.1	Glyceraldehyde-3-phosphate dehydrogenase 1	GAPDH	1.2 %	36	Oxidoreductase		+
HORVU2Hr1G079560.1	Histone H3	Histone H3	1.2 %	15	Histone		
HORVU3Hr1G090190.1	Cysteine-dependent adenosine diphosphate thiazole synthase	CDADTS	1.2 %	36	Transferase		
HORVU1Hr1G066650.18	D-hordein	D-Hordein	1.0 %	80	Prolamin		+

Ultimately, and based on only the most abundant proteins, more than 45 % (mol/mol) of the BSG proteins are either associated with allergenicity or considered antinutritional. The use of BSG as a food ingredient already automatically results in allergen labelling in foods, since it comes from barley, which is well known to contain the allergenic hordeins. However, the findings highlight the complicated nature of using byproducts such as BSG as ingredients, since other allergens and antinutritional factors are not presently considered by industry and regulators. As such, further processing done by industry, which may be conducted to produce protein isolate fractions, may also up concentrate other poorly considered and understood allergens and antinutritional proteins. This also substantiates the importance of novel food regulations. Nevertheless, targeting these proteins for downstream processing by hydrolysis should be further explored as a possibility to reduce allergenicity or antinutritional properties, or to promote desired functionality, such as antioxidant or emulsification.

4. Conclusions

BSG was found to have a significantly higher protein content (17.4 %) than barley and malt (9.0 % and 10.5 %, respectively), albeit still modest in proportion for use as a protein-based food ingredient. Through bottom-up proteomics, we identified very distinct and significant protein-level changes between the three stages of the brewing process. Malting was found to reduce levels of stress related proteins while facilitating increased nutrient use by reducing endogenous enzyme inhibitors and enriching active enzymes. As a result of the mashing process and subsequent wort removal, the BSG was found to be depleted in a wide range of proteins also covering additional stress-related proteins and amylase/protease inhibitors. This finding correlated with an overall reduction of proteins of extracellular origin in BSG. While this reduction is beneficial in terms of food safety, a large proportion of BSG (> 45 %) is still constituted by proteins with known antinutritional and allergenic properties, of which hordeins represent the majority. Nevertheless, BSG was shown to have a good amino acid profile, with only Lys falling slightly below the recommended proportion of essential amino acids. As such, BSG can be considered a high-potential source of protein-based ingredients, but care is required in industrial downstream processing to alleviate potential limitations in applicability based on the high content of proteins with potential allergenicity and antinutritional effects meanwhile improving the subpar functional properties associated with BSG.

CRediT authorship contribution statement

Simon Gregersen Echers: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Rasmus Kranold Mikkelsen:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Naim Abdul-Khalek:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Lucas Sales Queiroz:** Writing – review & editing, Validation, Methodology. **Timothy John Hobley:** Writing – review & editing, Methodology, Conceptualization. **Benjamin L. Schulz:** Writing – review & editing, Conceptualization. **Michael Toft Overgaard:** Writing – review & editing, Funding acquisition, Conceptualization. **Charlotte Jacobsen:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Betül Yesiltas:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare no conflicting or competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2025.104277.

Data availability

The mass spectrometry proteomics data have been deposited into the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD062029 and https://doi.org/10.6019/PXD062029. Numerical data for crude characterization as well as MaxQuant output data and downstream analysis thereof is available in the supplementary material.

References

Abeynayake, R., Zhang, S., Yang, W., & Chen, L. (2022). Development of antioxidant peptides from brewers’ spent grain proteins. *LWT, Academic Press, 158*, Article 113162. https://doi.org/10.1016/J.LWT.2022.113162

Aguilar-Toalá, J. E., Cruz-Narváez, Y., Quintanar-Guerrero, D., Liceaga, A. M., & Zambrano-Zaragoza, M. L. (2024). In silico bioactivity analysis of peptide fractions derived from brewer’s spent grain hydrolysates. *International Journal of Food Science and Technology, Oxford Academic, 59*, 2804–2815. https://doi.org/10.1111/IJFS.17044

Bahmani, M., O’Lone, C. E., Juhász, A., Nye-Wood, M., Dunn, H., Edwards, I. B., & Colgrave, M. L. (2021). Application of mass spectrometry-based proteomics to barley research. *Journal of Agricultural and Food Chemistry, American Chemical Society, 69*, 8591–8609. https://doi.org/10.1021/ACS.JAFC.1C01871/ASSET/IMAGES/LARGE/JF1C01871_0002.JPEG

Bazsefidpar, N., Ghandehari Yazdi, A. P., Karimi, A., Yahyavi, M., Amini, M., Ahmadi Gavlighi, H., & Simal-Gandara, J. (2024). Brewers spent grain protein hydrolysate as a functional ingredient for muffins: Antioxidant, antidiabetic, and sensory evaluation. *Food Chemistry, Elsevier, 435*, Article 137565. https://doi.org/10.1016/J.FOODCHEM.2023.137565

Berni, R., Leclercq, C. C., Roux, P., Hausman, J. F., Renaut, J., & Guerriero, G. (2023). A molecular study of Italian ryegrass grown on Martian regolith simulant. *Science of The Total Environment, Elsevier, 854*, Article 158774. https://doi.org/10.1016/J.SCITOTENV.2022.158774

Bi, X., Ye, L., Lau, A., Kok, Y. J., Zheng, L., Ng, D., … Muller, J. (2018). Proteomic profiling of barley spent grains guides enzymatic Solubilization of the remaining proteins. *Applied Microbiology and Biotechnology, Springer Verlag, 102*, 4159–4170. https://doi.org/10.1007/S00253-018-8886-8/FIGURES/3

Bianco, A., Melito, S., Garau, M., Giannini, V., Zara, G., Assandri, D., … Budroni, M. (2024). The potential use of brewers’ spent grain-based substrates as horticultural bio-fertilizers. *Frontiers in Sustainable Food Systems, Frontiers Media SA, 8*, 1404914. https://doi.org/10.3389/FSUFS.2024.1404914/BIBTEX

Bjerregaard, M. F., Charalampidis, A., Frøding, R., Shetty, R., Pastell, H., Jacobsen, C., … Hobley, T. J. (2019). Processing of brewing by-products to give food ingredient streams. *European Food Research and Technology, Springer Verlag, 245*, 545–558. https://doi.org/10.1007/S00217-018-03224-6/FIGURES/5

Bjørlie, M., Yesiltas, B., García-Moreno, P. J., Espejo-Carpio, F. J., Rahmani-Manglano, N. E., Guadix, E. M., … Jacobsen, C. (2023). Bioinformatically predicted emulsifying peptides and potato protein hydrolysate improves the oxidative stability of microencapsulated fish oil. *Food Chemistry Advances, Elsevier, 3*, Article 100441. https://doi.org/10.1016/J.FOCHA.2023.100441

Blanco, C. A., Caballero, I., Barrios, R., & Rojas, A. (2014). Innovations in the brewing industry: Light beer. *International Journal of Food Sciences and Nutrition, Informa UK Ltd, 65*, 655–660. https://doi.org/10.3109/09637486.2014.893285

Breiteneder, H. (2004). Thaumatin-like proteins – a new family of pollen and fruit allergens. *Allergy, John Wiley & Sons, Ltd, 59*, 479–481. https://doi.org/10.1046/J.1398-9995.2003.00421.X

Breiteneder, H., & Radauer, C. (2004a). A classification of plant food allergens. *Journal of Allergy and Clinical Immunology, Mosby Inc., 113*, 821–830. https://doi.org/10.1016/J.JACI.2004.01.779/ASSET/F3E25BD8-CD50-4738-BF1C-2D5C27DB6695/MAIN.ASSETS/GR3.JPG

Breiteneder, H., & Radauer, C. (2004b). A classification of plant food allergens. *Journal of Allergy and Clinical Immunology, Mosby, 113*, 821–830. https://doi.org/10.1016/J.JACI.2004.01.779

Canedo, M. S., de Paula, F. G., da Silva, F. A., & Vendruscolo, F. (2016). Protein enrichment of brewery spent grain from Rhizopus Oligosporus by solid-state fermentation. *Bioprocess and Biosystems Engineering, Springer Verlag, 39*, 1105–1113. https://doi.org/10.1007/S00449-016-1587-8/FIGURES/6

Celus, I., Brijs, K., & Delcour, J. A. (2006). The effects of malting and mashing on barley protein extractability. *Journal of Cereal Science, Academic Press, 44*, 203–211. https://doi.org/10.1016/J.JCS.2006.06.003

Chin, Y. L., Keppler, J. K., Dinani, S. T., Chen, W. N., & Boom, R. (2024). Brewers' spent grain proteins: The extraction method determines the functional properties. *Innovative Food Science & Emerging Technologies, Elsevier, 94*, Article 103666. https://doi.org/10.1016/J.IFSET.2024.103666

Colgrave, M. L., Goswami, H., Howitt, C. A., & Tanner, G. J. (2013). Proteomics as a tool to understand the complexity of beer. *Food Research International, Elsevier, 54*, 1001–1012. https://doi.org/10.1016/J.FOODRES.2012.09.043

Connolly, A., Piggott, C. O., & Fitzgerald, R. J. (2013). Characterisation of protein-rich isolates and Antioxidative phenolic extracts from pale and black brewers' spent grain. *International Journal of Food Science and Technology, Oxford Academic, 48*, 1670–1681. https://doi.org/10.1111/IJFS.12137

Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N., & Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular and Cellular Proteomics, American Society for Biochemistry and Molecular Biology Inc., 13*, 2513–2526. https://doi.org/10.1074/MCP.M113.031591/ATTACHMENT/B414BAF4-20AE-46D2-BB85-A06FBF3A11C3/MMC1.ZIP

Cun, Z., Li, X., Zhang, J. Y., Hong, J., Gao, L. L., Yang, J., … Chen, J. W. (2024). Identification of candidate genes and residues for improving nitrogen use efficiency in the N-sensitive medicinal plant Panax Notoginseng. *BMC Plant Biology, BioMed Central Ltd, 24*, 1–20. https://doi.org/10.1186/S12870-024-04768-4/FIGURES/12

Dancker, P., Glas, K., & Gastl, M. (2025). Potential utilisation methods for brewer's spent grain: A review. *International Journal of Food Science and Technology, Oxford Academic, 60*, 22. https://doi.org/10.1093/IJFOOD/VVAE022

Danner Aakjaer Pedersen, K., Thopholm Andersen, L., Heiselberg, M., Agerskov Brigsted, C., Lyngs Støvring, F., Mailund Mikkelsen, L., Albrekt Hansen, S., Enrico Rusbjerg-Weberskov, C., Lübeck, M., & Gregersen Echers, S. (2025). Identifying Endogenous Proteins of Perennial Ryegrass (*Lolium Perenne*) with Ex Vivo Antioxidant Activity. In *Proteomes 2025, Vol. 13, Page 8, Multidisciplinary Digital Publishing Institute, **13**, 8*. https://doi.org/10.3390/PROTEOMES13010008

Dehaye, L., Duval, M., Viguier, D., Yaxley, J., & Job, D. (1997). Cloning and expression of the pea gene encoding SBP65, a seed-specific biotinylated protein. *Plant Molecular Biology, Springer, 35*, 605–621. https://doi.org/10.1023/A:1005836405211/METRICS

Deifl, S., Zwicker, C., Vejvar, E., Kitzmüller, C., Gadermaier, G., Nagl, B., … Bohle, B. (2014). Glutathione-S-transferase: A minor allergen in birch pollen due to limited release from hydrated pollen. *PLOS ONE, Public Library of Science, 9*, Article e109075. https://doi.org/10.1371/JOURNAL.PONE.0109075

Deka, A., & Saikia, V. (2023). Prediction of ACE-inhibitory and antioxidant peptides from storage proteins of edible seeds and nuts using in silico approaches. *Sustainable Food Proteins, John Wiley & Sons, Ltd, 1*, 84–98. https://doi.org/10.1002/SFP2.1013

Devnani, B., Moran, G. C., & Grossmann, L. (2023). Extraction, composition, functionality, and utilization of brewer's spent grain protein in food formulations. *Foods 2023, 12*, 1543. Multidisciplinary Digital Publishing Institute, 12, 1543 https://doi.org/10.3390/FOODS12071543.

Evans, D. E., & Bamforth, C. W. (2009). *Beer foam: Achieving a suitable head* (pp. 1–60). Beer: A Quality Perspective, Academic Press. https://doi.org/10.1016/B978-0-12-669201-3.00001-4

Evans, D. E., Sheehan, M. C., & Stewart, D. C. (1999). The impact of malt derived proteins on beer foam quality. Part II: The influence of malt foam-positive proteins and non-starch polysaccharides on beer foam quality. *Journal of the Institute of Brewing, John Wiley & Sons, Ltd, 105*, 171–178. https://doi.org/10.1002/J.2050-0416.1999.TB00016.X

García-Moreno, P. J., Jacobsen, C., Marcatili, P., Gregersen, S., Overgaard, M. T., Andersen, M. L., … Hansen, E. B. (2020a). Emulsifying peptides from potato protein predicted by bioinformatics: Stabilization of fish oil-in-water emulsions. *Food Hydrocolloids, Elsevier B.V., 101*, Article 105529. https://doi.org/10.1016/j.foodhyd.2019.105529

García-Moreno, P. J., Gregersen, S., Nedamani, E. R., Olsen, T. H., Marcatili, P., Overgaard, M. T., … Jacobsen, C. (2020b). Identification of emulsifier potato peptides by bioinformatics: Application to Omega-3 delivery emulsions and release from potato industry side streams. *Scientific Reports, Nature Research, 10*, 690. https://doi.org/10.1038/s41598-019-57229-6

García-Moreno, P. J., Yang, J., Gregersen, S., Jones, N. C., Berton-Carabin, C. C., Sagis, L. M. C., … Jacobsen, C. (2021). The structure, viscoelasticity and charge of potato peptides adsorbed at the oil-water Interface determine the physicochemical stability of fish oil-in-water emulsions. *Food Hydrocolloids, Elsevier, 115*, Article 106605. https://linkinghub.elsevier.com/retrieve/pii/S0268005X21000217.

García-Moreno, P. J., Yesiltas, B., Gregersen Echers, S., Marcatili, P., Overgaard, M. T., Hansen, E. B., & Jacobsen, C. (2023). Recent advances in the production of emulsifying peptides with the aid of proteomics and bioinformatics. *Current Opinion in Food Science, Elsevier, 51*, Article 101039. https://doi.org/10.1016/J.COFS.2023.101039

Ghelichi, S., Sørensen, A. D. M., Hajfathalian, M., & Jacobsen, C. (2024). Effect of post-extraction Ultrasonication on compositional features and antioxidant activities of enzymatic/alkaline extracts of Palmaria Palmata. In *, 22. Marine Drugs 2024, Vol. 22, Page 179, Multidisciplinary Digital Publishing Institute* (p. 179). https://doi.org/10.3390/MD22040179

Gorissen, S. H. M., Crombag, J. J. R., Senden, J. M. G., Waterval, W. A. H., Bierau, J., Verdijk, L. B., & van Loon, L. J. C. (2018). Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino Acids, Springer-Verlag Wien, 50*, 1685–1695. https://doi.org/10.1007/S00726-018-2640-5/TABLES/2

Gorjanović, S. (2009). A review: Biological and technological functions of barley seed pathogenesis-related proteins (PRs). *Journal of the Institute of Brewing, John Wiley & Sons, Ltd, 115*, 334–360. https://doi.org/10.1002/J.2050-0416.2009.TB00389.X

Grafahrend-Belau, E., Schreiber, F., Koschützki, D., & Junker, B. H. (2009). Flux balance analysis of barley seeds: A computational approach to study systemic properties of central metabolism. *Plant Physiology, Oxford Academic, 149*, 585–598. https://doi.org/10.1104/PP.108.129635

Graham, I. A. (2008). Seed storage oil mobilization. *Annual Review of Plant Biology, Annual Reviews, 59*, 115–142. https://doi.org/10.1146/ANNUREV.ARPLANT.59.032607.092938/CITE/REFWORKS

Gregersen Echers, S., Abdul-Khalek, N., Mikkelsen, R. K., Holdt, S. L., Jacobsen, C., Hansen, E. B., … Overgaard, M. T. (2022). Is Gigartina a potential source of food protein and functional peptide-based ingredients? Evaluating an industrial, pilot-scale extract by proteomics and bioinformatics. *Future Foods, Elsevier, 6*, Article 100189. https://doi.org/10.1016/J.FUFO.2022.100189

Gregersen Echers, S., Jafarpour, A., Yesiltas, B., García-Moreno, P. J., Greve-Poulsen, M., Hansen, D. K., … Hansen, E. B. (2023). Targeted hydrolysis of native potato protein: A novel workflow for obtaining hydrolysates with improved interfacial properties. *Food Hydrocolloids, Elsevier, 137*, Article 108299. https://doi.org/10.1016/J.FOODHYD.2022.108299

Gregersen, S., Kongsted, A.-S. H., Nielsen, R. B., Hansen, S. S., Lau, F. A., Rasmussen, J. B., … Jacobsen, C. (2021). Enzymatic extraction improves intracellular protein recovery from the industrial carrageenan seaweed Eucheuma Denticulatum revealed by quantitative, subcellular protein profiling: A high potential source of functional food ingredients. *Food Chemistry: X, 12*, Article 100137. https://doi.org/10.1016/J.FOCHX.2021.100137

Hassan, M. J., Geng, W., Zeng, W., Raza, M. A., Khan, I., Iqbal, M. Z., … Li, Z. (2021). Diethyl Aminoethyl Hexanoate priming ameliorates seed germination via involvement in hormonal changes, osmotic adjustment, and Dehydrins accumulation in white clover under drought stress. *Frontiers in Plant Science, Frontiers Media S.A., 12*, Article 709187. https://doi.org/10.3389/FPLS.2021.709187/BIBTEX

Hejna, A. (2021). More than just a beer—the potential applications of by-products from beer manufacturing in polymer technology. *Emergent materials 2021 5:3. Springer, 5*, 765–783. https://doi.org/10.1007/S42247-021-00304-4

Huang, Y., Jonsson, N. N., McLaughlin, M., Burchmore, R., Johnson, P. C. D., Jones, R. O., … Eckersall, P. D. (2023). Quantitative TMT-based proteomics revealing host, dietary and microbial proteins in bovine Faeces including barley serpin Z4, a prominent component in the head of beer. *Journal of Proteomics, Elsevier, 285*, Article 104941. https://doi.org/10.1016/J.JPROT.2023.104941

Ikram, S., Huang, L. Y., Zhang, H., Wang, J., & Yin, M. (2017). Composition and nutrient value proposition of brewers spent grain. *Journal of Food Science, John Wiley & Sons, Ltd, 82*, 2232–2242. https://doi.org/10.1111/1750-3841.13794

Ikram, S., Zhang, H., Ahmed, M. S., & Wang, J. (2020). Ultrasonic pretreatment improved the antioxidant potential of enzymatic protein hydrolysates from Highland barley brewer's spent grain (BSG). *Journal of Food Science, John Wiley & Sons, Ltd, 85*, 1045–1059. https://doi.org/10.1111/1750-3841.15063

Jaeger, A., Zannini, E., Sahin, A. W., & Arendt, E. K. (2021). Barley protein properties, extraction and applications, with a focus on brewers' spent grain protein. In *Foods 2021, Vol. 10, Page 1389, multidisciplinary digital publishing institute, **10**, 1389*. https://doi.org/10.3390/FOODS10061389

Jin, Z., Cai, G. L., Li, X. M., Gao, F., Yang, J. J., Lu, J., & Dong, J. J. (2014). Comparative proteomic analysis of Green malts between barley (Hordeum Vulgare) cultivars. *Food Chemistry, Elsevier, 151*, 266–270. https://doi.org/10.1016/J.FOODCHEM.2013.11.065

Johnson, K. A., Kim, E., Teeter, M. M., Suh, S. W., & Stec, B. (2005). Crystal structure of α-Hordothionin at 1.9 Å resolution. *FEBS Letters, John Wiley & Sons, Ltd, 579*, 2301–2306. https://doi.org/10.1016/J.FEBSLET.2004.12.100

Jones, D. B. (1931). *Factors for converting percentages of nitrogen in foods and feeds into percentages of protein*. US Department of Agriculture.

Karlsen, F., & Skov, P. V. (2022). Review – Potentials and limitations of Utilising brewer's spent grain as a protein source in aquaculture feeds. *Journal of Cleaner Production, Elsevier, 357*, Article 131986. https://doi.org/10.1016/J.JCLEPRO.2022.131986

Kerr, E. D., Caboche, C. H., Josh, P., & Schulz, B. L. (2021). Benchtop Micro-mashing: High-throughput, robust, experimental beer brewing. In *, 11. Scientific Reports 2021 11:1, Nature Publishing Group* (pp. 1–10). https://doi.org/10.1038/s41598-020-80442-7

Kerr, E. D., Fox, G. P., & Schulz, B. L. (2023). Proteomics and metabolomics reveal that an abundant α-glucosidase drives Sorghum Fermentability for beer brewing. *Journal of Proteome Research, American Chemical Society, 22*, 3596–3606. https://doi.org/10.1021/ACS.JPROTEOME.3C00436/ASSET/IMAGES/LARGE/PR3C00436_0006.JPEG

Kersey, P. J., Allen, J. E., Allot, A., Barba, M., Boddu, S., Bolt, B. J., … Yates, A. (2018). Ensembl genomes 2018: An integrated omics infrastructure for non-vertebrate species. *Nucleic Acids Research, Oxford Academic, 46*, D802–D808. https://doi.org/10.1093/NAR/GKX1011

Kushwaha, R., Lloyd, T. D., Schäfermeyer, K. R., Kumar, S., & Downie, A. B. (2012). Identification of late embryogenesis abundant (LEA) protein putative interactors using phage display. *International journal of molecular sciences 2012, Vol. 13, pages*

6582-6603. Molecular Diversity Preservation International, 13, 6582–6603. https://doi.org/10.3390/IJMS13066582

Lee, S., Lee, T., Yang, S., & Lee, I. (2020). BarleyNet: A network-based functional omics analysis server for cultivated barley, Hordeum Vulgare L. *Frontiers in Plant Science, Frontiers Media S.A., 11*, Article 510185. https://doi.org/10.3389/FPLS.2020.00098/BIBTEX

Lin, Z., Schaefer, K., Lui, I., Yao, Z., Fossati, A., Swaney, D. L., … Wells, J. A. (2024). Multiscale photocatalytic proximity labeling reveals cell surface neighbors on and between cells. *Science (New York, N.Y.), American Association for the Advancement of Science, 385*, Article eadl5763. https://doi.org/10.1126/SCIENCE.ADL5763/SUPPL_FILE/SCIENCE.ADL5763_MDAR_REPRODUCIBILITY_CHECKLIST.PDF

Liu, S., Kerr, E. D., Pegg, C. L., & Schulz, B. L. (2022). Proteomics and glycoproteomics of beer and wine. *PROTEOMICS, John Wiley & Sons, Ltd, 22*, 2100329. https://doi.org/10.1002/PMIC.202100329

Lock, T. J., Siau, Mah, H., Zee, & Lai, W. Z. (2023). Versatile applications of brewer's spent grain: Solid-state fermentation and nutritional added value. *Applied biochemistry and biotechnology 2023 196:8. Springer, 196*, 5508–5532. https://doi.org/10.1007/S12010-023-04769-3

Luo, D., Wu, Z., Bai, Q., Zhang, Y., Huang, M., Huang, Y., & Li, X. (2023). Universal stress proteins: From gene to function. *International Journal of Molecular Sciences 2023, Vol. 24, Page 4725, Multidisciplinary Digital Publishing Institute, 24*, Article 4725. https://doi.org/10.3390/IJMS24054725

Lynch, K. M., Steffen, E. J., & Arendt, E. K. (2016). Brewers' spent grain: A review with an emphasis on food and health. *Journal of the Institute of Brewing, John Wiley & Sons, Ltd, 122*, 553–568. https://doi.org/10.1002/JIB.363

Ma, Z., Marsolais, F., Bykova, N. V., & Igamberdiev, A. U. (2016). Nitric oxide and reactive oxygen species mediate metabolic changes in barley seed embryo during germination. *Frontiers in Plant Science, Frontiers Research Foundation, 7*, Article 179637. https://doi.org/10.3389/FPLS.2016.00138/BIBTEX

Mahalingam, R. (2018). Temporal analyses of barley malting stages using shotgun proteomics. *PROTEOMICS, John Wiley & Sons, Ltd, 18*, 1800025. https://doi.org/10.1002/PMIC.201800025

Mariotti, F., Tomé, D., & Mirand, P. P. (2008). Converting nitrogen into protein—Beyond 6.25 and Jones' factors. *Critical Reviews in Food Science and Nutrition, Taylor & Francis Group, 48*, 177–184. https://doi.org/10.1080/10408390701279749

Mikkelsen, R. K., Queiroz, L. S., Gregersen Echers, S., Hobley, T. T., … Yesiltas, B. (2025). Extracting proteins from brewers' spent grain using emerging technologies: Evaluating efficiency and use as emulsifier*, 3. Sustainable Food Proteins*. John Wiley & Sons, Ltd, Article e70016. https://doi.org/10.1002/SFP2.70016

Moirangthem, K., Knaapila, A., Lee, Y., Sandell, M., Skibinska, I., Kilcawley, K. N., … Coda, R. (2025). Tailored bioprocessing of brewers' spent grain for the development of upcycled plant-based Spoonable snacks. *Future Foods, Elsevier, 11*, Article 100621. https://doi.org/10.1016/J.FUFO.2025.100621

Mussatto, S. I. (2014). Brewer's spent grain: A valuable feedstock for industrial applications. *Journal of the Science of Food and Agriculture, John Wiley & Sons, Ltd, 94*, 1264–1275. https://doi.org/10.1002/JSFA.6486

Naibaho, J., Korzeniowska, M., Sitanggang, A. B., Lu, Y., & Julianti, E. (2024). Brewers' spent grain as a food ingredient: Techno-processing properties, Nutrition, acceptability, and market. *Trends in Food Science & Technology, Elsevier, 152*, Article 104685. https://doi.org/10.1016/J.TIFS.2024.104685

Naik, A. S., Brugiere, M., Dunne, K., Sorohan, M., Grasa, L., & Javed, B. (2024). Protease hydrolysed brewers' spent grain bioactive extracts for applications in Human health. *Systems Microbiology and Biomanufacturing, Springer Nature, 4*, 1062–1075. https://doi.org/10.1007/S43393-024-00241-3/FIGURES/4

Nájera-Torres, E., Bernal-Gracida, L. A., González-Solís, A., Schulte-Sasse, M., Castañón-Suárez, C., Juárez-Díaz, J. A., … Cruz-García, F. (2022). Proteolytic activities and profiles as useful traits to select barley cultivars for beer production. *Journal of Food Biochemistry, John Wiley & Sons, Ltd, 46*, Article e14094. https://doi.org/10.1111/JFBC.14094

Niemi, P., Martins, D., Buchert, J., & Faulds, C. B. (2013). Pre-hydrolysis with Carbohydrases facilitates the release of protein from brewer's spent grain. *Bioresource Technology, Elsevier, 136*, 529–534. https://doi.org/10.1016/J.BIORTECH.2013.03.076

Nyhan, L., Sahin, A. W., Schmitz, H. H., Siegel, J. B., & Arendt, E. K. (2023). Brewers' spent grain: An unprecedented opportunity to develop sustainable plant-based Nutrition ingredients addressing global malnutrition challenges. *Journal of Agricultural and Food Chemistry, American Chemical Society, 71*, 10543–10564. https://doi.org/10.1021/ACS.JAFC.3C02489/ASSET/IMAGES/LARGE/JF3C02489_0005.JPEG

Olsen, T. H., Yesiltas, B., Marin, F. I., Pertseva, M., García-Moreno, P. J., Gregersen, S., … Marcatili, P. (2020). AnOxPePred: Using deep learning for the prediction of Antioxidative properties of peptides. *Scientific Reports, Nature Research, 10*, 21471. https://doi.org/10.1038/s41598-020-78319-w

Osama, S. K., Kerr, E. D., Yousif, A. M., Phung, T. K., Kelly, A. M., Fox, G. P., & Schulz, B. L. (2021). Proteomics reveals commitment to germination in barley seeds is marked by loss of stress response proteins and mobilisation of nutrient reservoirs. *Journal of Proteomics, Elsevier, 242*, Article 104221. https://doi.org/10.1016/J.JPROT.2021.104221

Østergaard, O., Melchior, S., Roepstorff, P., & Svensson, B. (2002). Initial proteome analysis of mature barley seeds and malt. *Proteomics, 2*, 733–739. https://doi.org/10.1002/1615-9861(200206)2:6<733::aid-prot733>3.0.co;2-e

Perez-Riverol, Y., Bai, J., Bandla, C., García-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S., … Vizcaíno, J. A. (2022). The PRIDE database resources in 2022: A hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Research, Oxford Academic, 50*, D543–D552. https://doi.org/10.1093/NAR/GKAB1038

Qin, F., Johansen, A. Z., & Mussatto, S. I. (2018). Evaluation of different pretreatment strategies for protein extraction from brewer's spent grains. *Industrial Crops and Products, Elsevier, 125*, 443–453. https://doi.org/10.1016/J.INDCROP.2018.09.017

Qin, Q., Liu, J., Hu, S., Dong, J., Yu, J., Fang, L., … Wang, L. (2021). Comparative proteomic analysis of different barley cultivars during seed germination. *Journal of Cereal Science, Academic Press, 102*, Article 103357. https://doi.org/10.1016/J.JCS.2021.103357

Quaglieri, A., Bloom, J., Triantafyllidis, A., Green, B., Condina, M. R., Ngov, P. B., … Webb, A. I. (2022). Mass dynamics 2.0: An improved modular web-based platform for accelerated proteomics insight generation and decision making. In *bioRxiv, cold Spring Harbor Laboratory, 2022.12.12.517480*. https://doi.org/10.1101/2022.12.12.517480

Rommi, K., Niemi, P., Kemppainen, K., & Kruus, K. (2018). Impact of thermochemical pre-treatment and carbohydrate and protein hydrolyzing enzyme treatment on fractionation of protein and lignin from brewer's spent grain. *Journal of Cereal Science, Academic Press, 79*, 168–173. https://doi.org/10.1016/J.JCS.2017.10.005

Rusbjerg-Weberskov, C. E., Foley, J. D., Yang, L., Terp, M., Gregersen Echers, S., Orlien, V., & Lübeck, M. (2025). Combined Rhizopus Oryzae fermentation and protein extraction of brewer's spent grain improves protein functionality. *Food and Bioprocess Technology, 18*.

Saberian, H., Ghandehari Yazdi, A. P., Nejatian, M., Bazsefidpar, N., Mohammadian, A. H., Rahmati, M., … Jafari, S. M. (2024). Brewers' spent grain as a functional ingredient in bakery, pasta, and cereal-based products. *Future Foods, Elsevier, 10*, Article 100479. https://doi.org/10.1016/J.FUFO.2024.100479

Sampedro, J., & Cosgrove, D. J. (2005). The Expansin superfamily. *Genome Biology, BioMed Central, 6*, 1–11. https://doi.org/10.1186/GB-2005-6-12-242/FIGURES/8

Samtiya, M., Aluko, R. E., & Dhewa, T. (2020). Plant food anti-nutritional factors and their reduction strategies: An overview. *Food production, processing and Nutrition 2020 2:1. BioMed Central, 2*, 1–14. https://doi.org/10.1186/S43014-020-0020-5

Sander, I., Rozynek, P., Rihs, H. P., Van Kampen, V., Chew, F. T., Lee, W. S., … Raulf-Heimsoth, M. (2011). Multiple wheat flour allergens and cross-reactive carbohydrate determinants bind IgE in baker's asthma. *Allergy, John Wiley & Sons, Ltd, 66*, 1208–1215. https://doi.org/10.1111/J.1398-9995.2011.02636.X

Schmitt, M. R., Skadsen, R. W., & Budde, A. D. (2013). Protein mobilization and malting-specific proteinase expression during barley germination. *Journal of Cereal Science, Academic Press, 58*, 324–332. https://doi.org/10.1016/J.JCS.2013.05.007

Schulz, B. L., Phung, T. K., Bruschi, M., Janusz, A., Stewart, J., Meehan, J., … Vickers, C. E. (2018). Process proteomics of beer reveals a dynamic proteome with extensive modifications. *Journal of Proteome Research, American Chemical Society, 17*, 1647–1653. https://doi.org/10.1021/ACS.JPROTEOME.7B00907/ASSET/IMAGES/LARGE/PR-2017-009078_0006.JPEG

Schwanhüusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., … Selbach, M. (2011). Global quantification of mammalian gene expression control. *Nature, Nature Publishing Group, 473*, 337–342. https://doi.org/10.1038/nature10098

Shewry, P. R., & Halford, N. G. (2002). Cereal seed storage proteins: Structures, properties and role in grain utilization. *Journal of Experimental Botany, Oxford Academic, 53*, 947–958. https://doi.org/10.1093/JEXBOT/53.370.947

Steiner, J., Procopio, S., & Becker, T. (2015). Brewer's spent grain: Source of value-added polysaccharides for the food industry in reference to the health claims. *European Food Research and Technology, Springer Verlag, 241*, 303–315. https://doi.org/10.1007/S00217-015-2461-7/TABLES/3

Tanner, G. J., Colgrave, M. L., Blundell, M. J., Howitt, C. A., & Bacic, A. (2019). Hordein accumulation in developing barley grains. *Frontiers in Plant Science, Frontiers Media S. A., 10*, Article 443201. https://doi.org/10.3389/FPLS.2019.00649/BIBTEX

Tatham, A. S., & Shewry, P. R. (2008). Allergens to wheat and related cereals. *Clinical & Experimental Allergy, John Wiley & Sons, Ltd, 38*, 1712–1726. https://doi.org/10.1111/J.1365-2222.2008.03101.X

The UniProt Consortium. (2017). UniProt: The universal protein knowledgebase. *Nucleic Acids Research, 45*, D158–D169. https://doi.org/10.1093/nar/gkw1099

Thumuluri, V., Almagro Armenteros, J. J., Johansen, A. R., Nielsen, H., & Winther, O. (2022). DeepLoc 2.0: Multi-label subcellular localization prediction using protein language models. *Nucleic Acids Research, Oxford Academic, 50*, W228–W234. https://doi.org/10.1093/NAR/GKAC278

Tyanova, S., Temu, T., & Cox, J. (2016). The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nature Protocols, Nature Publishing Group, 11*, 2301–2319. https://doi.org/10.1038/nprot.2016.136

Varona, E., García-Moreno, P. J., Gregersen Echers, S., Olsen, T. H., Marcatili, P., Guardiola, F., … Yesiltas, B. (2023). Antioxidant peptides from alternative sources reduce lipid oxidation in 5% fish oil-in-water emulsions (PH 4) and fish oil-enriched mayonnaise. *Food Chemistry, Elsevier, 426*, Article 136498. https://doi.org/10.1016/J.FOODCHEM.2023.136498

Vieira, E., Rocha, M. A. M., Coelho, E., Pinho, O., Saraiva, J. A., Ferreira, I. M. P. L. V. O., & Coimbra, M. A. (2014). Valuation of brewer's spent grain using a fully recyclable integrated process for extraction of proteins and Arabinoxylans. *Industrial Crops and Products, Elsevier, 52*, 136–143. https://doi.org/10.1016/J.INDCROP.2013.10.012

Vieira, M. C., Brandelli, A., & Thys, R. C. S. (2022). Evaluation of the technological functional properties and antioxidant activity of protein hydrolysate obtained from brewers' spent grain. *Journal of Food Processing and Preservation, John Wiley and Sons Inc, 46*, Article e16638. https://doi.org/10.1111/JFPP.16638;WGROUP:STRING:PUBLICATION

Vinje, M. A., Henson, C. A., Duke, S. H., Simmons, C. H., Le, K., Hall, E., & Hirsch, C. D. (2021). Description and functional analysis of the transcriptome from malting barley. *Genomics, Academic Press, 113*, 3310–3324. https://doi.org/10.1016/J.YGENO.2021.07.011

Viswanath, K. K., Varakumar, P., Pamuru, R. R., Basha, S. J., Mehta, S., & Rao, A. D. (2020). Plant lipoxygenases and their role in plant physiology. *Journal of Plant*

Biology, Springer, 63, 83–95. https://doi.org/10.1007/S12374-020-09241-X/FIGURES/3

Wen, C., Zhang, J., Duan, Y., Zhang, H., & Ma, H. (2019). A Mini-review on brewer's spent grain protein: Isolation, physicochemical properties, application of protein, and functional properties of hydrolysates. *Journal of Food Science, John Wiley & Sons, Ltd, 84*, 3330–3340. https://doi.org/10.1111/1750-3841.14906

WHO/FAO/UNU. (2007). *PROTEIN AND AMINO ACID REQUIREMENTS IN HUMAN NUTRITION*. Geneva: World Healh Organization. https://apps.who.int/iris/bitstream/handle/10665/43411/WHO_TRS_935_eng.pdf?sequence=1&isAllowed=y.

Wilhelm, K. S., & Thomashow, M. F. (1993). Arabidopsis Thaliana Cor15b, an apparent homologue of Cor15a, is strongly responsive to cold and ABA, but not drought. *Plant Molecular Biology, Kluwer Academic Publishers, 23*, 1073–1077. https://doi.org/10.1007/BF00021822/METRICS

Xia, Y., Bamdad, F., Gänzle, M., & Chen, L. (2012). Fractionation and characterization of antioxidant peptides derived from barley Glutelin by enzymatic hydrolysis. *Food Chemistry, Elsevier, 134*, 1509–1518. https://doi.org/10.1016/J.FOODCHEM.2012.03.063

Xie, Z., Dan, M., Zhao, G., & Wang, D. (2024). Recent advances in microbial high-value utilization of brewer's spent grain. *Bioresource Technology, Elsevier, 408*, Article 131197. https://doi.org/10.1016/J.BIORTECH.2024.131197

Yalçın, E., Çelik, S., & İbanoğlu, E. (2008). Foaming properties of barley protein isolates and hydrolysates. *European Food Research and Technology, Springer Verlag, 226*, 967–974. https://doi.org/10.1007/S00217-007-0618-8/FIGURES/10

Yesiltas, B., Gregersen, S., Lægsgaard, L., Brinch, M. L., Olsen, T. H., Marcatili, P., … García-Moreno, P. J. (2021). Emulsifier peptides derived from seaweed, Methanotrophic Bacteria, and potato proteins identified by quantitative proteomics and bioinformatics. *Food Chemistry, Elsevier, 362*, Article 130217. https://doi.org/10.1016/J.FOODCHEM.2021.130217

Yesiltas, B., García-Moreno, P. J., Gregersen, S., Olsen, T. H., Jones, N. C., Hoffmann, S. V., … Jacobsen, C. (2022). Antioxidant peptides derived from potato, seaweed, microbial and spinach proteins: Oxidative stability of 5% fish oil-in-water emulsions. *Food Chemistry, Elsevier, 385*, Article 132699. https://doi.org/10.1016/J.FOODCHEM.2022.132699

Yesiltas, B., Soria Caindec, A. M., García-Moreno, P. J., Echers, S. G., Olsen, T. H., Jones, N. C., … Jacobsen, C. (2023). Physical and oxidative stability of fish oil-in-water emulsions stabilized with emulsifier peptides derived from seaweed, Methanotrophic Bacteria and potato proteins. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, Elsevier, 663*, Article 131069. https://doi.org/10.1016/J.COLSURFA.2023.131069

Zaidi, I., Hanin, M., Saidi, M. N., Soltani, N., & Brini, F. (2024). The barley Dehydrin 4 and stress tolerance: From gene to function. *Environmental and Experimental Botany, Elsevier, 224*, Article 105802. https://doi.org/10.1016/J.ENVEXPBOT.2024.105802

Zan, T., Li, L., Li, J., Zhang, L., & Li, X. (2020). Genome-wide identification and characterization of late embryogenesis abundant protein-encoding gene family in wheat: Evolution and expression profiles during development and stress. *Gene, Elsevier, 736*, Article 144422. https://doi.org/10.1016/J.GENE.2020.144422

Zhang, J., Perez-Gavilan, A., & Cunha Neves, A. (2025). Determination of functionalities of proteins and their corresponding hydrolysates from brewers' spent grain. *Journal of the American Society of Brewing Chemists, Taylor and Francis Ltd., 83*, 203–214. https://doi.org/10.1080/03610470.2024.2411650;REQUESTEDJOURNAL:JOURNAL:UJBC20;PAGE:STRING:ARTICLE/CHAPTER

Zhao, X., Cui, C., & Sun-Waterhouse, D. (2025). Air classification modifies the susceptibility of brewer's spent grain to enzymatic hydrolysis and the protein content and functional properties of derived hydrolysates. *Journal of Cereal Science, Academic Press, 124*, Article 104203. https://doi.org/10.1016/J.JCS.2025.104203