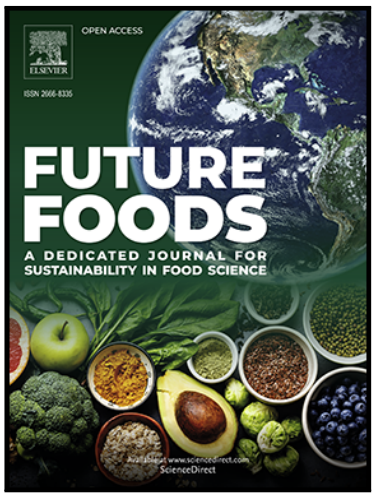

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Comparative analysis of amino acid and bioactive peptide profiles during time-resolved fermentation of wheat and stout brewer's spent grain by *Lactiplantibacillus plantarum*

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Abstract

Brewers' spent grain (BSG), a protein- and fibre-rich brewing by-product, contains bioactive compounds, yet techniques to fully recover and utilise these bioactive fractions remain underdeveloped. This study investigated the proteolytic transformation of two BSG types, non-roasted wheat and roasted stout BSG, during 10 days of fermentation with *Lactiplantibacillus plantarum*. Discovery and targeted LC-MS/MS proteomics were used to characterise protein abundance, peptide release, and free amino acid profiles throughout fermentation. Proteomic analysis identified 503 proteins in wheat BSG and 347 in stout BSG. Wheat BSG generated higher peptide diversity than stout BSG (1,227 vs. 436 peptides), with peptide peaking on Day 4, whereas stout BSG showed delayed peptide release, primarily during Days 7–9, indicating substrate-dependent proteolytic dynamics. Targeted quantification of 28 peptides revealed distinct temporal patterns for predicted antimicrobial and antihypertensive activities. Antimicrobial peptides peaked during mid-fermentation in both BSG types, while antihypertensive peptides peaked earlier in wheat BSG and later in stout BSG. Peptides containing partial wheat allergy and coeliac disease-related epitope motifs were detected predominantly in wheat BSG and accumulated throughout fermentation, whereas such motifs were largely absent in stout BSG. This suggests that intensive thermal processing associated with stout production may reduce the prevalence of safety-relevant epitope-containing protein regions. Free amino acid profiling revealed higher hydrophobic amino acid concentrations in stout BSG (0.98 vs. 0.61 mg/100 g protein) and charge-related inter-substrate differences. These findings demonstrate that BSG type strongly influences proteolytic outcomes and

support substrate-specific fermentation strategies for the targeted and safe valorisation of brewing by-products.

Keywords: Brewers' spent grain; *Lactiplantibacillus plantarum*; Peptide profiling; Food allergen epitopes; Bioactive peptides

1. Introduction

Brewer's spent grain (BSG), a major by-product of beer production, is attracting growing attention for its abundant availability and nutritional composition. Rich in proteins, fibres, and phenolic compounds, BSG represents a substrate with a potential for the development of bioactive ingredients. Although BSG is primarily used in animal feeds or discarded in landfills (Agrawal et al., 2023), it may offer substantial potential for recovery and valorisation within the food sector.

However, BSG is not a uniform material, and its composition is strongly influenced by brewing conditions, malt processing, and grain formulation. Barley is the primary cereal used in brewing, while adjuncts such as wheat and oats are commonly incorporated to modify beer characteristics, and different beer styles require distinct malting and processing conditions. During mashing, soluble proteins are released into the wort, whereas less-soluble proteins remain in the spent grain matrix. In addition, the formation of disulfide bonds promotes protein–protein crosslinking and the development of high molecular weight aggregates. These protein networks can associate with carbohydrates, leading to protein–carbohydrate complexes that limit extractability, thereby making subsequent hydrolysis important for protein recovery and utilisation (Chin et al., 2024; Stringari et al., 2025). Consequently, wheat- and stout-derived beers are expected to generate BSG with distinct protein profiles due to differences in formulation and processing (Fadimu et al., 2023). In particular, stout production involves extensively roasted malts to achieve its characteristic colour and flavour, and such high-temperature processing can induce protein denaturation and chemical modifications that may influence subsequent protein hydrolysis and fermentation behaviour. These differences suggest that BSG derived from different beer styles may respond differently to downstream bioprocessing, yet comparative studies addressing this variability remain limited.

Recent research has explored enzymatic hydrolysis as a means to convert BSG into functional food ingredients or peptide-rich products. For instance, Báez et al. (2021) used Alcalase to hydrolyse BSG and incorporated it into bread formulations as flour replacement, achieving comparable physicochemical properties to conventional bread with an improved antioxidant activity. Similarly, Abeynayake et al. (2022) demonstrated that a combination of Alcalase and Flavourzyme enzymes can enhance radical scavenging activity of the resulting peptides, while Kriisa et al. (2022) reported that treatment of BSG with Protamex enzymes, alone or with Flavourzyme, increased the solubility of BSG hydrolysates without altering sensory qualities.

Collectively, these studies highlight the potential of enzymatic treatments in producing value-added ingredients from BSG.

While the use of enzymatic treatments to valorise BSG is promising, commercial enzyme preparations are typically substrate-specific, require tightly controlled reaction conditions, and can lose activity within the variable matrix environment of solid BSG, making large-scale application costly and technically demanding (Dancker et al., 2025). As a more sustainable alternative, microbial fermentation using lactic acid bacteria (LAB) has gained attention as an environmentally friendly approach to BSG valorisation. LAB can produce their own proteinases in situ, self-sustain under fermentation conditions, and are inherently compatible with solid-state cereal matrices, offering practical advantages for food-grade applications at scale. Several LAB fermentation of BSG has been investigated for distinct valorisation goals; for example, Pejin et al. (2017) demonstrated that *Lactobacillus rhamnosus* fermentation of BSG hydrolysate could serve as a low-cost feedstock for industrial lactic acid production, while Shetty et al. (2023) showed that a two-step fermentation of BSG liquid using *Lactiplantibacillus plantarum (Lb. plantarum)* followed by propionic acid-producing bacteria yielded an organic acid-enriched, clean-label food ingredient with extended shelf life. Similarly, Qamar et al. (2024) reported that fermentation of BSG with *Lb. plantarum* and *Lactobacillus reuteri* increased soluble protein content, total flavonoid content, total polyphenol content, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activities compared with unfermented BSG. Despite these advances, the proteolytic potential of LAB fermentation for bioactive peptide generation from intact BSG substrates remains comparatively underexplored.

LABs possess a proteolytic system, serving as a mechanism to generate an essential source of nitrogen and carbon for LAB growth and metabolism (Kieliszek et al., 2021). The proteolytic system of LAB fermentation encompasses a series of enzymatic steps that degrade proteins into peptides and amino acids, which can be used to replace the use of commercial enzyme. This process begins extracellularly, where cell-envelope proteinases located on the surface of LAB cell walls cleave intact proteins into smaller fragments, such as peptides (Ji et al., 2021). These peptides are then transported inside the bacterial cells by oligopeptide permeases and further cleaved into peptide fragments or free amino acids, which facilitate the bacteria's nutrient uptake (Ji et al., 2021). This capability is not only essential for their survival, as it provides free amino acids and a nitrogen source for bacterial growth, but also significantly influences the flavour and texture of the final food product (Venegas-Ortega et al., 2019). Beyond these roles, LAB fermentation of plant-derived substrates such as amaranth has been shown to generate peptides with antioxidant, antihypertensive, and antimicrobial activities, highlighting the broader health-promoting potential of this approach (Singh, 2025).

Among LAB species, the capabilities of *Lactiplantibacillus plantarum (Lb. plantarum)*, formerly known as *Lactobacillus plantarum*, on plant-based food fermentation have been frequently reported due to its versatile metabolic capabilities and robustness in various environments (Yilmaz et al., 2022). This is because its genome encodes a range of stress-related proteins that enable it to survive and thrive in multiple niches and harsh conditions (Corsetti & Settanni, 2007; Yilmaz et al., 2022). This genetic adaptability facilitates its effective utilisation

of diverse plant-derived substrates, enhancing the overall fermentation process and the quality of the resulting products. A study conducted by Verni et al. (2020) on BSG fermented with *Lb. plantarum* reported that the small molecular weight fraction (<3 kDa) exhibited higher antioxidant activity than the larger fractions; the authors also identified five peptides across all active fractions. The biofunctional potential of such fermentation-derived peptides depends not only on their generation during fermentation but also on key physicochemical properties, including molecular size, net charge, and hydrophobicity, all of which are known to significantly influence gastrointestinal stability and intestinal absorption (Shen & Matsui, 2019). Shen and Matsui (2019) noted that short hydrophobic peptides are generally more resistant to brush-border peptidase activity and more amenable to transport across the intestinal epithelium via the peptide transporter 1 (PepT1). Although the assessment of bioavailability and absorption lies beyond the scope of the present study, these physicochemical characteristics are worth noting as they provide important context for the interpretation of the peptide profiles generated during fermentation.

Despite growing interest in peptide generation using LAB fermentation, key knowledge gaps remain. Specifically, current literature provides limited understanding of how BSG from different brewing processes responds to *Lb. plantarum* proteolysis, including: (i) the lack of comparative analysis of proteolysis and peptide generation between BSG types that differ in grain composition and thermal history, (ii) the absence of time-resolved screening and mapping of peptide formation and turnover during fermentation, and (iii) limited examination of safety-relevant gluten/allergen and coeliac-disease epitope dynamics, particularly in BSG derived from roasted malts, where brewing-related thermal processing may modify or mask epitope-containing regions and thereby influence the release of sequences relevant to coeliac disease and food allergy during fermentation. As a result, the unique research value of controlled LAB fermentation has not been adequately linked to the raw-material characteristics of BSG (roasting treatment, cereal composition) that likely affect the fermentation outcomes. We hypothesised that substrate characteristics, particularly grain formulation and roasting history, govern proteolytic accessibility during *Lb. plantarum* fermentation, resulting in substrate-dependent differences in bioactive peptide generation, free amino acid profiles, and allergen epitope dynamics.

Therefore, this study aims to address these gaps by applying a systematic, time-resolved fermentation approach using *Lb. plantarum* to two BSG types derived from distinct brewing processes (wheat- and stout-BSG). Using discovery proteomics, peptide profiling, targeted peptide quantification, and free amino acid analysis, this work provides a systematic 10-day insight into protein degradation and peptide generation. In addition, peptides were screened for the presence of allergen- and coeliac-disease-related epitopes to provide a safety-relevant perspective on fermentation outcomes. By explicitly integrating substrate characteristics (grain formulation and thermal processing history) with fermentation behaviour, this study investigates how brewing-derived differences in BSG influence *Lb. plantarum*-driven proteolysis and the resulting peptide and free amino acid profiles.

2. Materials and methods

The overall experimental workflow is illustrated in Figure 1. Briefly, two types of BSG, wheat- and stout-beer derived, were produced from distinct beer formulations, fermented with *Lb. plantarum* over a 10-day period under controlled anaerobic conditions, and subjected to an analytical pipeline. This includes microbial growth and pH monitoring, discovery proteomics for protein and peptide identification, targeted multiple reaction monitoring for peptide quantification, and free amino acid profiling. Each component is described in detail in the subsections below.

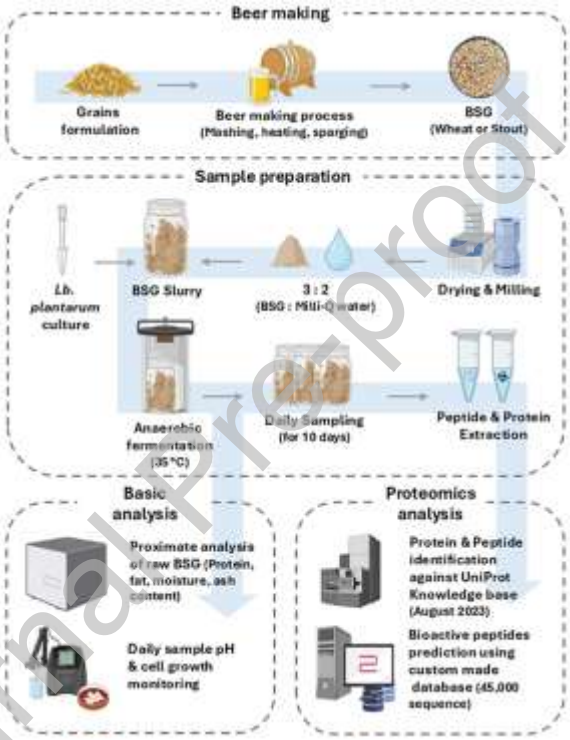

Figure 1. Overview of the experimental workflow for the fermentation of wheat- and stout-beer derived brewers' spent grain (BSG) with *Lactiplantibacillus plantarum* and the subsequent analytical pipeline for protein identification, peptide profiling, bioactive peptide prediction, and free amino acid analysis.

2.1. Chemicals and reagents

All reagents and solvents used were of analytical grade. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), urea, 1,4-dithiothreitol (DTT), 2-iodoacetamide, ammonium bicarbonate, dimethyl sulfoxide (DMSO), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, sodium tetraborate decahydrate (borax), amino acid standards (No. A9906), D4-alanine, formic acid, methanol and acetonitrile were purchased from Sigma Aldrich (Darmstadt, Germany). Sequencing grade trypsin was purchased from Invitro Technologies

(Auckland, New Zealand). Difco™ *Lactobacilli* MRS broth, GasPak™ and agar were purchased from Fort Richard (Auckland, New Zealand).

2.2. Generation of BSG

Two groups of BSG were used in this study: wheat beer BSG (WB) produced from Hefeweizen blonde wheat beer, and stout beer BSG (SB) from vanilla and cocoa milk stout. The two beers were produced for teaching purposes using commercially purchased grains (Brewshop, Hamilton, New Zealand) and malts in the food laboratory of the School of Science, Auckland University of Technology, New Zealand. Briefly, the ingredients of each beer (approx. 2.5 kg of grains per batch) were mashed and then heated at 67 °C for 60 min, followed by sparging with hot water at 75 °C. Afterwards, the produced liquid wort (approx. 10 L) was used for beer fermentation, and the BSG samples were collected, freeze-dried, ground into powder, and stored in vacuum bags at –20 °C for further experiments. Notably, the ingredients used to make the two beers included: i) for wheat beer: Pilsner malt (Simpsons Malt, Northumberland, United Kingdom; 75%), white wheat malt (Brewshop, Hamilton, New Zealand; 7.5%), Munich malt (Weyermann® Specialty Malts, Bamberg, Germany; 8.5%), and dextrin malt (Simpsons Malt, Northumberland, United Kingdom; 8.5%); ii) for stout beer (all malts sourced from Gladfield Malts, Canterbury, NZ): chocolate malt (7%), eclipse malt (7%), ale malt (62%), toffee malt (10%), and flaked oats (Brewshop, Hamilton, New Zealand; 14%).

2.3. Proximate analysis of BSG

The proximate analysis of BSG powder (prior to fermentation), including moisture, ash, protein, and fat content, were conducted using standardised methods AOAC 934.01, 942.05, 2001.11, and 2003.05 (AOAC International, 2019). The content of carbohydrate was determined by the differential method, calculated by subtracting the sum of percentages of moisture, protein, fat, and ash from 100.

2.4. Fermentation of BSG

BSG (wheat or stout) was mixed with sterile Milli-Q water in a ratio of 3:2 (BSG powder:water), to prepare 100 g sample, and inoculated with *Lb. plantarum.*. The fermentation experiments were conducted following the method described by Zhao et al. (2022). Briefly, 100 mL of MRS broth was inoculated with 1 mL of the stock culture of *Lb. plantarum* (School of Science, Auckland University of Technology, New Zealand) with an approximate bacterial suspension of 9.0×10^8 CFU/mL (3.0 McFarland standard) stored in 20% glycerol. After overnight incubation at 35 °C, 1 mL of the broth was added to inoculate the BSG sample. For the control, 1 mL of sterile Milli-Q was added in place of the broth. The BSG samples were incubated at 35 °C in a GasPak™ anaerobic jar, and samples were collected every 24 h over a

10-day duration. The incubation temperature was selected to support the sustained metabolic and proteolytic activity of *Lb. plantarum* in cereal-based matrices, while remaining within the reported optimal growth range for this species as reported by Zhao et al. (2022) and Shu et al. (2015). The 10-day fermentation period was chosen to enable time-resolved observation of early, mid, and late fermentation phases, allowing assessment of peptide generation, turnover, and free amino acid dynamics. In this study, fermentation parameters (strain, inoculum level, temperature, and duration) were intentionally kept constant so that differences in proteolytic outcomes could be attributed to substrate characteristics, grain formulation and thermal processing history, rather than to variation in fermentation conditions. The two brewing relevant substrates were also selected for practical experimental reasons. All treatments had to be fermented at the same time in one synchronised run. At each time point, samples were destructively collected and analysed for viable cell counts and pH in biological triplicate, alongside preparation for several downstream analyses.

2.5. Microbial growth and pH measurement

The microbial growth was monitored by the plate count method. Briefly, 1 g of the sample was homogenised with 9 mL of peptone water to obtain a 10^{-1} dilution, followed by preparation of a serial 10-fold dilution series as required. Then, 0.1 mL of each dilution was spread-plated on an MRS agar plate, followed by incubation in an anaerobic jar at 35 °C for 24 h. Colony-forming units (CFU) were calculated based on plates containing 30–300 colonies. Gram staining was performed on samples collected during the first three days of fermentation to verify culture purity, confirming *Lb. plantarum* as the dominant organism with no morphological evidence of contamination. Colony morphology on MRS agar was additionally monitored throughout the full 10-day period as a complementary check. The pH of both fermented and control samples was measured at each fermentation day using a calibrated pH meter. All experiments were conducted in triplicate.

2.6. Amino acid and peptide extraction

Free amino acids and peptides generated from the fermentation were extracted using an in-house optimised procedure with 60% acetonitrile. The solvent was selected based on its established ability to precipitate large proteins while retaining smaller peptides in solution, and its compatibility with downstream LC-MS analysis, as previously demonstrated in peptide extraction workflows (Liu et al., 2026). The samples were mixed with 60% acetonitrile at a 1:10 ratio (w/v), followed by 30 min ultrasonication. For amino acid analysis (Section 2.7), samples were held 30 min further on ice, followed by centrifugation at 10,000× g for 15 min at 4 °C. The supernatant was stored at –20 °C prior to use for derivatisation with AccQ•Tag reagent before LC-MS analysis. For peptide analysis (Section 2.9), the samples were centrifuged at 10,000× g for 30 min at 10 °C, then the supernatant was filtered using 10 kDa

molecular weight cut-off (MWCO) filters (Millipore, Bayswater, VIC, Australia), followed by centrifugation at 10,000× g for 30 min at 10 °C. The filtrate was collected, lyophilised, and stored at –20 °C until further analysis by LC-MS.

2.7. Amino acid analysis

Amino acids in the supernatant obtained above were derivatised before LC-MS analysis using the method described by Roland et al. (2024). Briefly, 40 µL of the supernatant was mixed with an equal volume of methanol containing 10 mg/L of d4-alanine internal standard. The mixture was vortexed and centrifuged at 10,000× g for 5 min at 4 °C. The supernatant was derivatised using AccQ•Tag reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, made up of 2.8 mg in 1 mL acetonitrile. The derivatisation reaction mixture included 70 µL of borate buffer, 10 µL of sample, standard or blank, and 10 µL of AccQ•Tag reagent. The mixture was incubated for 15 min at 55 °C followed by the addition of 400 µL of 90% formic acid, and the acidified mixture was injected into LC-MS.

The LC-MS system consisted of an Agilent 1260 Infinity Quaternary LC system (Santa Clara, CA, USA), connected to a 6420 Triple-Quadrupole Mass Spectrometer (Triple Q-MS) with an electrospray ionisation source. A Kinetex Evo C18 column (2.1 × 150 mm, 1.7 µm; Phenomenex, Torrance, CA, USA) was operated at 25 °C with a flow rate of 0.25 mL/min. The mobile phases used were 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B), with the following gradient program: 0–8 min: 95% A, 8–13 min: 90% A, 13–16.5 min: 85% A, 16.5–18.5 min: 20% A, 18.5–29 min: 95% A. The MS ionisation source conditions were set as follows: capillary voltage of 4 kV, a drying gas temperature of 300 °C, a drying gas flow rate of 10 L/min, and a nebuliser pressure of 30 psi. The quantitative analysis was conducted in positive ion mode using multiple reaction monitoring (MRM). Data analysis was carried out with MassHunter Qualitative Analysis version 10.0 and MassHunter Quantitative Analysis version 10.1 (Agilent Technologies, Santa Clara, CA, USA).

2.8. Protein digestion

The powdered BSG (5 mg) was dissolved in 125 µL of 8 M urea, 0.1 M tris-HCl and 2% DTT (w/v). Twenty-five µL of the BSG solution was mixed with 75 µL of 8 M urea, 0.1 M tris-HCl and 2% DTT buffer, then transferred onto 10 kDa molecular weight cut-off (MWCO) filters (Millipore, Bayswater, VIC, Australia) for trypsin digestion using filter-aided sample preparation (FASP) described by Tahmasian et al. (2022) with some modifications. The protein extracts were washed twice using 100 µL of a buffer containing 8 M urea and 0.1 M Tris-HCl (pH 8.2). After centrifugation of the filters at 20,000× g for 10 min, 100 µL of 25 mM iodoacetamide (made up of the urea and tris-HCl buffer) was added to the filters. The protein solutions were alkylated by incubation of the samples in the dark at room temperature (20 °C)

for 20 min. The samples were centrifuged (20,000× g, 10 min) and washed twice with 100 µL of the urea and tris-HCl buffer to remove excess iodoacetamide. The buffer was replaced with 50 mM ammonium bicarbonate (pH 8.4) by centrifugation (20,000× g, 10 min); this step was repeated twice. Subsequently, 190 µL of 0.02 µg/µL sequencing-grade trypsin (Promega) (made up of 50 mM ammonium bicarbonate) was added to the filters, and the samples were incubated overnight at 37 °C. The digested peptide mixture was collected by centrifugation (20,000× g, 10 min, 10 °C) using fresh centrifuge tubes. Subsequently, 200 µL of 0.1% formic acid was added to wash the filters and acidify the digests, and the combined filtrates were evaporated to dryness using a freeze drier. The dried digests were stored at –80 °C before LC-MS/MS analysis.

2.9. Protein and peptide analysis

2.9.1. Data-dependent acquisition (DDA)-MS for BSG's protein and fermented BSG's peptide profiling

The tryptic peptides were reconstituted in 100 µL of 0.1% formic acid, and the samples were spiked 1:10 with iRT reference peptide solution (1 pmol; Biognosys, Zurich, Switzerland). The DDA analysis was performed on the pooled samples of technical replicates and following a method described by Colgrave et al. (2017). Briefly, samples (1 µL) were injected onto an Ekspert nanoLC 415 (Eksigent, Dublin, CA, United States) liquid chromatography system coupled with a TripleTOF 6600 (SCIEX, Redwood City, CA, United States), and desalted on a polar C18 ProteCol trap column (Trajan; 3 µm Particle Size ×300 Å Pore Size, 10 mm × 300 µm ID) using a flow rate of 10 µL/min of 0.1% formic acid for 3 min. Protein digests or peptides were separated on a ChromXP C18 (3 µm, 120 Å, 150 mm × 0.3 mm) column at 30 °C. The LC system delivered buffer A (5% DMSO, 0.1% formic acid) and buffer B (5% DMSO, 0.1% formic acid, 90% acetonitrile) at total flow rate of 5 µL/min with a separation gradient of 5–45% B over a period of 40 min, followed by 45–90% B for 5 min, a 5 min hold at 90% B, followed by return to 5% B over 1 min, and re-equilibration for 11 min. The eluent from the analytical column was directed to IonDrive Turbo V ion source with 50 µm electrode (SCIEX, Redwood City, CA, United States). The ion spray voltage was set to 5,500 V, the curtain gas was set to 30 psi, the heated interface was set to 100 °C, and ion source gases 1 and 2 (GS1 and GS2) were set to 18 and 20 psi, respectively. The MS was operated in DDA top 30 mode with MS1 scans operated between 350 and 1250 m/z with 0.25 s accumulation time with data-dependent acquisition, where the top 30 most intense precursors with selection criteria of charge state between 2 and 5 and intensity greater than 150 counts per second were selected for collision-induced fragmentation. Product ion spectra were acquired over the mass range of 100–1800 m/z with an accumulation time of 0.05 s. Manufacturer-derived rolling collision energy (CE) and a collision energy spread (CES) of 5 V were set for optimum peptide fragmentation. Dynamic exclusion was enabled to exclude precursor ions after two occurrences within a 15 s interval and a mass tolerance of 100 ppm; peaks within 4 Da of the precursor.

The raw DDA data files were searched using ProteinPilot 5.0.3 software (SCIEX, Redwood City, CA, United States) against a combined database consisting of *Triticum aestivum* and *Hordeum vulgare* protein sequences downloaded from UniProt Knowledgebase (August 2023), concatenated with translated gene models of the *Avena sativa* cv. Sang reference genome (Kamal et al., 2022), cRAP proteins (https://www.thegpm.org/crap/), and *Lactobacillus* proteins identified in a pilot study. The final database size included 210,981 protein sequences. The search effort was set as "Thorough ID" with iodoacetamide as a cysteine alkylation agent and trypsin as a digestion enzyme for BSG protein identification, and no iodoacetamide and trypsin settings for fermented BSGs' peptide identification; TripleTOF 6600 was selected for the instrument type, and biological modifications were enabled for ID focus. The false discovery rate (FDR) was set at a 1% global FDR cut-off. Protein identifications were aligned across group files using the SCIEX Protein Alignment Template v3.002p.

2.9.2. Prediction of potential bioactive peptides and epitopes associated with coeliac disease (CD) and food allergy

The identified peptides from the DDA runs were scanned against a custom-made bioactive peptide library consisting of over 45,000 bioactive peptides. In addition, CD-specific HLA-DQ epitopes were retrieved from published resources (Sollid et al., 2012; Sollid et al., 2020) and food allergy-related linear epitopes were downloaded from the IEDB (https://www.iedb.org) analysis resource and used for epitope mapping using CLC Genomics Workbench v22 (Qiagen, Aarhus, Denmark). Bioactive peptides were mapped with a minimum of 80% sequence similarity to explore potential bioactive peptide isoforms, while CD and food allergy epitopes were mapped with 100% sequence match.

2.9.3. Peptide quantification using multiple reaction monitoring (MRM)

Peptide selection for MRM

The peptides identified at 1% global FDR confidence level from the combined database searching in ProteinPilot were imported into Skyline software (v19.1.0.193) as an ion library, and a multiple reaction monitoring (MRM) targeted assay was designed by selecting the peptides of interest, the precursor ion and transitions that were observed in the ion library. The selected transitions were compiled into a set of MRM methods, with 50–60 transitions each. The MRM methods were then applied to pooled samples from the fermented BSG collected at time points 0, 2, 4, 6, 8 and 10 days of fermentation using an LC-MS system consisting of an Exion LC-30AD UHPLC system (SCIEX, Redwood City, CA, United States) coupled to a 6500+ QTRAP MS (SCIEX, Redwood City, CA, United States). A Kinetex C18 column (100 × 210 µm, 1.7 µm particle size and 100 Å pore size) heated to 60 °C was used. A flow rate of 0.4 mL/min was used, with a binary gradient 5 to 45% B over 10.2 min, 45 to 80% B in 0.8 min, 1 min hold at 80% B, return to 5% B in 0.1 min and 2.9 min re-equilibration was used. Solvent A was aqueous 0.1% FA, and solvent B was 90% acetonitrile and 0.1% FA in 9.9% Milli-Q water. Data was acquired in positive ion mode with the ion source temperature and the

ion spray voltage floating set at 500 °C and 5,500 V, respectively. The Skyline software was utilised for peak integration (MacLean et al., 2010), and for each precursor trialled the four most intense transitions showing no interference were identified and retained. The retention time (RT) of each precursor was then used to schedule the transitions, and a single scheduled MRM (sMRM) method with a cycle time of 0.3 s was exported into Analyst (SCIEX, Redwood City, CA, United States). In total, 28 peptides that exhibited the highest signal intensity and the lowest technical variability were selected for the quantification of bioactive peptides in fermented BSGs.

MRM acquisition

The sMRM method was then applied to three biological replicates from the *Lb. plantarum* fermented samples, at treatment time points: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days, while the control samples were at time points: 0, 2, 4, 6, 8, and 10 days. The analysis was performed in batches, with randomised sample order to minimise the impact of batch effects on the results. A quality control sample composed of 1 mg/mL BSA tryptic digestion spiked with iRT peptides (1:10) was run before and after each batch to assess instrument performance. All sMRM peaks were inspected manually to ensure correct peak detection, signal-to-noise (S/N) >5, and accurate integration was performed manually in Skyline (MacCoss Lab, University of Washington, Seattle, WA, USA).

Data processing and statistical analysis

The UpSet plot of quantitative data (binary value transform) was generated by the UpSet module within UpSetR Shiny App (Khan & Mathelier, 2017), where all peptides released from fermented BSG were presented. For targeted analysis, peptide abundance data (peak areas) were exported from Skyline, and the batch effect was corrected by using the "Remove batch effect" function within the Limma R package using the batch information and the sample injection order as the covariate (Ritchie et al., 2015). The technical variation across the triplicates was assessed by examining the coefficient of variation (CV) for each peptide. The differences in MRM peak areas among the two BSG types and across different fermentation days for the 28 quantified bioactive peptides were analysed using two-way ANOVA, where BSG types and fermentation days were used as factors and statistical significances were defined as $p \leq 0.05$. The free amino acid profiles at day 10 (the end of the fermentation period) were analysed using one-way ANOVA followed by Tukey's Honest Significant Difference test for the two BSG groups' comparisons. All statistical analyses of the MRM peak areas and free amino acid profile at Day 10 were performed using Minitab version 21.3 (Minitab, LLC, Pennsylvania, USA) with data expressed as mean ± standard deviation (SD) from three replicates per sample ($p < 0.05$). A heatmap of the free amino acids was generated by

MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/), and the Morpheus website (https://software.broainstitute.org/morpheus) was used to visualise peptides' abundance.

3. Results and discussion

3.1. Proximate composition of BSG

The protein content remaining in brewers' spent grain (BSG) after the brewing process remained relatively high, at 15.37% for WB and 16.91% for SB (Table 1). Although the difference between the two BSG types was not statistically significant ($p > 0.05$), SB exhibited a slightly higher protein content. These values are still considered high for a brewing by-product and indicate that a substantial fraction of grain protein is retained in BSG following wort extraction. This reinforces the potential of BSG as a protein-rich substrate that can be further utilised in food, feed, or fermentation-based applications.

The protein levels observed in this study are consistent with those reported in the literature, confirming that the values obtained are within the expected compositional range for BSG. Devnani et al. (2023) reported comparable protein contents for BSG, while Mussatto and Roberto (2006), Xiros et al. (2008), and Jay et al. (2008) reported protein contents of 15.2%, 14.2%, and 15–17%, respectively. The agreement with these studies supports the reliability of the current results and suggests that variations in raw material or brewing conditions do not substantially diminish the protein fraction of BSG.

As expected for a cereal-derived by-product, carbohydrates constituted the major component of both WB and SB BSG, accounting for 74.9% and 73.8%, respectively (Table 1). However, it should be noted that carbohydrate values were calculated by difference and may therefore include other carbohydrate components such as lignin, certain hemicelluloses, and residual minerals (Wang et al., 2024). This methodological limitation is well recognised and likely contributes to the variability in carbohydrate contents reported across studies. For example, Sobukola et al. (2013) reported 60.64% of carbohydrate content, excluding the crude fibre (9.19%). Yitayew et al. (2022) further demonstrated that excluding crude fibre and mineral fractions can substantially reduce the calculated carbohydrate content. Conversely, the values observed in the present study are comparable to those reported by Adeniran et al. (2010), who reported a 79.9% carbohydrate content in BSG.

Although these carbohydrate fractions may not represent readily digestible carbohydrates in the conventional sense, they remain functionally relevant. Structural polysaccharides and associated components present in BSG can still serve as fermentable or metabolizable substrates for microorganisms such as *Lb. plantarum*, supporting their use as fermentation media despite the complex nature of the carbohydrate fraction.

Minor differences were observed in other proximate components, with WB containing slightly higher fat and ash contents than SB. However, these variations were not statistically significant and were not considered critical to the objectives of the present study.

Table 1. Proximate composition (%) of wheat brewers' spent grain (WB) and stout brewers' spent grain (SB). Data are presented as means ± standard deviation (SD) of triplicate measurements (n = 3). Superscript letters (a–e) indicate statistically significant differences; values sharing the same letter are not significantly different ($p > 0.05$).

BSG	Protein	Moisture	Ash	Fat	Carbohydrate
WB	15.37 ± 0 [a]	3.51 ± 0.41 [b]	2.75 ± 0.01 [c]	4.93 ± 0.19 [d]	74.9 ± 1.79 [e]
SB	16.91 ± 1.26 [a]	2.53 ± 0.29 [b]	1.99 ± 0.01 [c]	4.79 ± 0.77 [d]	73.78 ± 1.52 [e]

3.2. Microbial growth and pH value

As depicted in Figure 2A, the pH of all samples at the beginning of the fermentation period was approximately 6. A sharp decline in pH to below 4 was observed on the second day for both BSG types, after which the pH of FSB remained relatively stable at approximately 3.7 throughout the fermentation period, while FWB showed a slight increase from approximately 3.75 on Day 3 to 4.2 by Day 10. In contrast, the control samples without starter culture (Figure S1) exhibited a more gradual and comparatively slower decline in pH, approximately 50% less than that observed in the *Lb. plantarum*-treated samples. The rapid acidification observed in the inoculated samples reflects efficient fermentation by *Lb. plantarum*, while the slower pH decline in the controls provides a clear comparison of bacterial performance.

The pH behaviour observed in the present study is consistent with previous findings reported by Ye et al. (2008), who investigated the use of *Lb. plantarum* as a starter culture for preserving a food waste mixture predominantly composed of rice, noodles, and animal off-cuts. In samples inoculated with 2% *Lb. plantarum* broth, the authors reported a rapid decrease in pH to approximately 4 within one day, followed by stabilisation throughout the 14-day fermentation period. Ye et al. (2008) suggested that this rapid acidification effectively inhibited the growth of enterobacteria and extended the preservation of the substrate, supporting the fermentation efficiency observed in the current study.

Bacterial growth patterns (Figure 2B) provided further confirmation of the observed pH trends. The initial bacterial populations differed between the two BSG groups, with fermented wheat BSG (FWB) exhibiting a higher initial cell count of nearly 8 log CFU/g, whereas fermented stout BSG (FSB) started at approximately 6 log CFU/g. Despite these differences, both groups reached peak cell counts of approximately 10 log CFU/g on the second day of fermentation, coinciding with the sharp decline in pH observed at the same time point. This temporal alignment confirms that the rapid acidification was driven by active microbial growth and metabolism during the early stages of fermentation.

Towards the end of the fermentation period, FWB maintained a relatively higher viable cell count compared to FSB (7.46 vs. 6.59 log CFU/g, respectively). This difference may be associated with the higher terminal pH observed in FWB compared to FSB (4.3 vs. 3.7,

respectively). The lower pH in FSB may have inhibited bacterial survival, as excessive accumulation of organic acids can disrupt intracellular pH homeostasis and lead to growth cessation (Papadimitriou et al., 2016). The slight pH increase observed in FWB during late fermentation likely reflects a progressive reduction in lactic acid production as fermentable carbohydrates became limiting, combined with the accumulating buffering effect of amino acids released through ongoing proteolysis**.** The free amino acids produced during the fermentation of FWB (discussed in Section 4), including histidine, tyrosine, aspartic acid, glutamic acid, and arginine, may have contributed to an increased buffering capacity, thereby moderating pH changes. These amino acids can accept or donate protons in response to pH changes, helping to stabilise the fermentation environment (Herrington & Kellogg, 2021; Iyengar et al., 2022; Valéry et al., 2015).

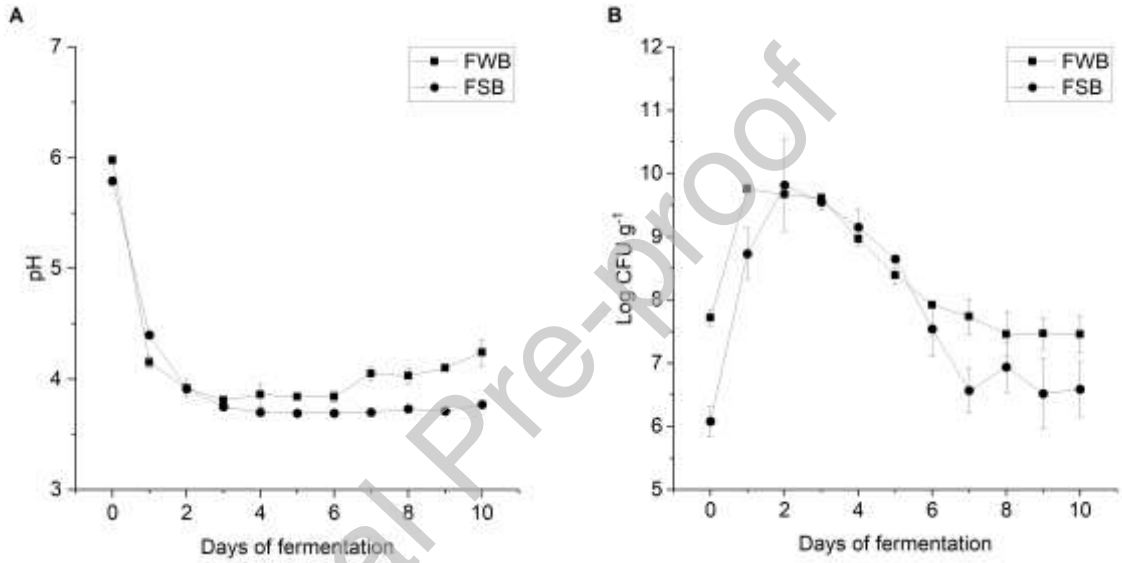

Figure 2. Changes in pH and viable cell counts during fermentation of wheat brewers' spent grain (FWB) and stout brewers' spent grain (FSB) over a 10-day period. **(A)** Viable cell counts (log CFU/g). **(B)** pH values of fermented samples. Data are presented as means ± standard deviation of triplicate measurements (n = 3).

3.3. Proteolysis of BSG

3.3.1. Protein identification

Discovery proteomics showed that the BSG proteome retained after brewing is strongly shaped by the grain formulation and processing severity, particularly roasting. A total of 503 and 347 unique proteins were identified in WB and SB, respectively (Figure 3). Across both BSG types, barley was the dominant source, contributing 384 proteins (76.3%) in WB and 178 proteins (51.3%) in SB, with wheat contributing the second highest number of proteins (110 in WB and 66 in SB). Importantly, the inclusion of oat flakes in stout beer formulations (Section 2.2) was clearly reflected in the proteome, with a tenfold increase in oat protein identifications in SB

compared to WB (103 vs. 9 proteins), indicating that ingredient inputs are preserved as detectable signatures in the residual spent grain.

The predominance of barley proteins is consistent with prior LC-MS/MS datasets showing that barley storage proteins, particularly B-, C-, and D-hordeins, were among the most abundant grain proteins detected in brewing-related matrices (Gregersen Echers et al., 2025). In agreement, B-, C-, and D-hordeins were detected in the present study (Table S1A and S1B). While wheat is often included in certain beer styles (e.g., Hefeweizen and Witbier) to improve foam quality, largely through proteins such as lipid transfer proteins (LTPs) and protein Z, along with polysaccharides such as beta-glucans (Hu et al., 2019), the presence of a relatively high number of "wheat" proteins in SB despite the absence of wheat in the formulation likely reflects analytical constraints in cereal proteomics. Specifically, storage proteins from wheat, barley, and oats share homologous sequences and peptides, which can complicate confident assignment at the cereal-species level and may lead to apparent wheat identifications due to sequence homology (Schalk et al., 2017). This interpretation is consistent with the observed strong barley and oat signals in SB and highlights the need to interpret taxonomic attribution cautiously when cereals share conserved protein families.

Beyond formulation effects, the lower total number of identified proteins in SB relative to WB likely reflects the more intensive thermal processing associated with stout production. Stout beer production involves roasting grains at high temperatures to generate the characteristic dark colour and roasted flavour profile, and these conditions can induce protein denaturation (Bischof & He, 2006), potentially reducing the solubility and extractability of native grain proteins. In particular, dry-heat roasting can promote protein polymerisation and incorporation into disulfide-bonded or otherwise cross-linked networks via thiol oxidation, which reduces solubility and detectability and may leave a smaller subset of heat-stable proteins identifiable by LC-MS/MS identifiable (Gregersen Echers et al., 2025; He et al., 2025; Pietsch et al., 2017). Therefore, the reduced protein identifications in SB likely reflect not only ingredient composition, but also the chemical consequences of roast intensity on protein recovery and analytical visibility.

The proteomic profile further highlighted the distinction between proteins that are retained in BSG. In the present dataset (Table S1A & B), BSG was dominated by proteins typically considered less soluble, including dehydrins, oleosins, glutenins, gliadins, and enzymes. Storage proteins such as barley hordeins and wheat glutenins/gliadins are generally less soluble due to their complex structures and high molecular weight (Biesiekierski, 2017; Tanner et al., 2019) and thus are likely retained in the spent grain fraction after lautering. In contrast, more soluble proteins, such as protein Z and LTPs, are solubilised into the beer during lautering and contribute to sensory and physical properties such as foam stability (Hu et al., 2019). In summary, these results show that, regardless of beer type, BSG retains a characteristic protein profile enriched in less-soluble grain proteins.

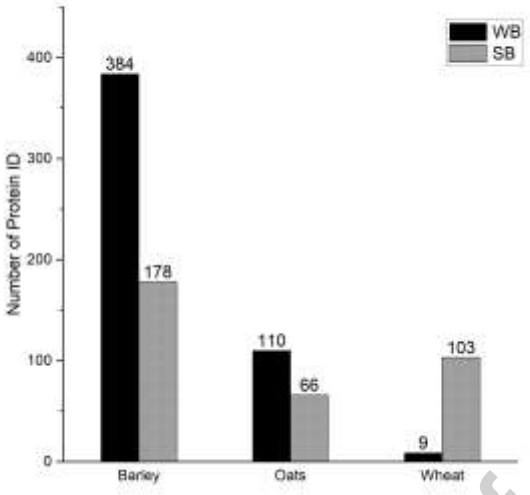

Figure 3. The bar chart showing unique proteins identified in wheat and stout BSG (WB and SB, respectively) by discovery proteomics using a custom barley–oat–wheat protein database.

3.3.2. Peptide profile

The impact of lactic acid bacteria (LAB) on brewers' spent grain (BSG), particularly their proteolytic activity, was investigated by peptide profiling of fermented BSG extracts using LC-MS/MS in data-dependent acquisition (DDA) mode. This approach enabled the identification of peptides generated over the 10-day fermentation period and allowed comparison of bacterial hydrolysis between the two BSG types. LC-MS/MS analysis identified a total of 1,663 peptides across all fermented BSG samples, with 1,227 peptides detected in fermented wheat BSG (FWB; Table S1A) and 436 peptides detected in fermented stout BSG (FSB; Table S1B). Among these, 52 peptides were common to both BSG types (Tables S1A and S1B), indicating largely distinct peptide profiles following fermentation.

Peptide generation dynamics differed significantly between the two BSG types. In FWB, the number of detected peptides increased rapidly from 49 peptides on Day 0 to 137 peptides on Day 1, reaching a maximum on Day 4 with 162 peptides, of which 28 were unique (Figure 4A). As illustrated by the UpSet plot, this early increase was primarily driven by the appearance of day-specific (unique) peptides, reflecting rapid and dynamic proteolysis during the initial fermentation stages. Following this peak, peptide numbers declined steadily, reaching a minimum of 33 peptides on Day 9, before increasing again to 88 peptides on Day 10. In summary, the late stage of the fermentation in FWB were characterised by a reduction in both unique and shared peptides, suggesting peptide turnover and/or degradation rather than continued accumulation.

In contrast, FSB exhibited a substantially lower and delayed peptide release throughout fermentation, with peptide numbers increasing gradually from 24 peptides on Day 0, to 66 peptides on Day 9, of which 12 peptides were unique (Figure 4B). Unlike FWB, where peptide

abundance increased sharply within the first day, FSB showed a mid-to-late fermentation increase, beginning around Day 4 and reaching the highest peptide numbers on Days 7 and 9, with 64 and 66 peptides detected, respectively. The UpSet plot analysis indicates that peptide accumulation in FSB was dominated by peptides shared across multiple time points rather than by large numbers of unique peptides, suggesting slower but more sustained proteolytic release.

Despite ongoing fermentation, a subset of peptides persisted over extended periods, suggesting structural stability or limited accessibility to further bacterial hydrolysis. In FWB, six peptides were consistently detected from Day 1 to Day 10 (Figure 4A), including IPQQPQQPFPLQPHQP (C-hordein, #125), PQQPGQGQQPGQRQP (D-hordein, #207), PYVDPMAPLPRSGP (beta-amylase, #223), and three closely related D-hordein-derived peptides: TVSPHQGQQTTVSPHQG, TVSPHQGQQTTVSPHQGQ, and VSPHQGQQTTVSPHQGQ (#391, #392, and #424, respectively) (Table S1A). A similar pattern was observed in FSB, where four peptides originating from oat 12S seed storage globulins, DVNNNANQLEPR (#19), DVNNNANQLEPRQ (#20), QSFTPWQSSRQGGL (#102), and VEHQAYQPIQSQEGQSTQ (#139), were consistently detected from Day 3 to Day 10 (Figure 4B; Table S1B). The persistence of these peptides across multiple fermentation days, as reflected by the intersection structure of the UpSet plots, suggests that they were either generated early at relatively high abundance or were less susceptible to further *L. plantarum*-mediated proteolysis.

Overall, the higher peptide diversity in FWB than that in FSB likely reflects differences in grain pre-processing and protein accessibility. Wheat-based brewing grains are generally subjected to milder thermal treatments than the extensively roasted grains used in stout production, which are processed to generate the characteristic dark colour and roasted flavour. Although these grains were commercially purchased and detailed information processing conditions were not disclosed, literature reports indicate that roasting temperatures for brewing grains range from approximately 120 °C to 210 °C, with roasting times between 10 and 50 min depending on the desired flavour profile and process conditions (Coghe et al., 2006; Lasekan et al., 2010; Parr et al., 2021). High temperature and prolonged roasting time can induce chemical changes in proteins, including Maillard reaction, aggregation, and oxidation, which may alter amino acid side chains or N-terminal residues and reduce the accessibility of protease cleavage sites. Such modifications could limit LAB proteolytic activity and peptide release during fermentation (Poojary & Lund, 2022). For example, Deng et al. (2017) noted that glycation significantly alters protein hydrolysis by lysine- and arginine-specific proteases, such as trypsin, by preventing cleavage at modified residues and reducing the maximal degree of hydrolysis. However, in the present study, no definitive conclusions can be drawn regarding the specific chemical modifications present in FSB proteins. Further investigation using targeted LC-MS/MS approaches to identify and quantify post-translationally modified proteins would be required to confirm the role of roasting-induced modifications in shaping peptide release during fermentation.

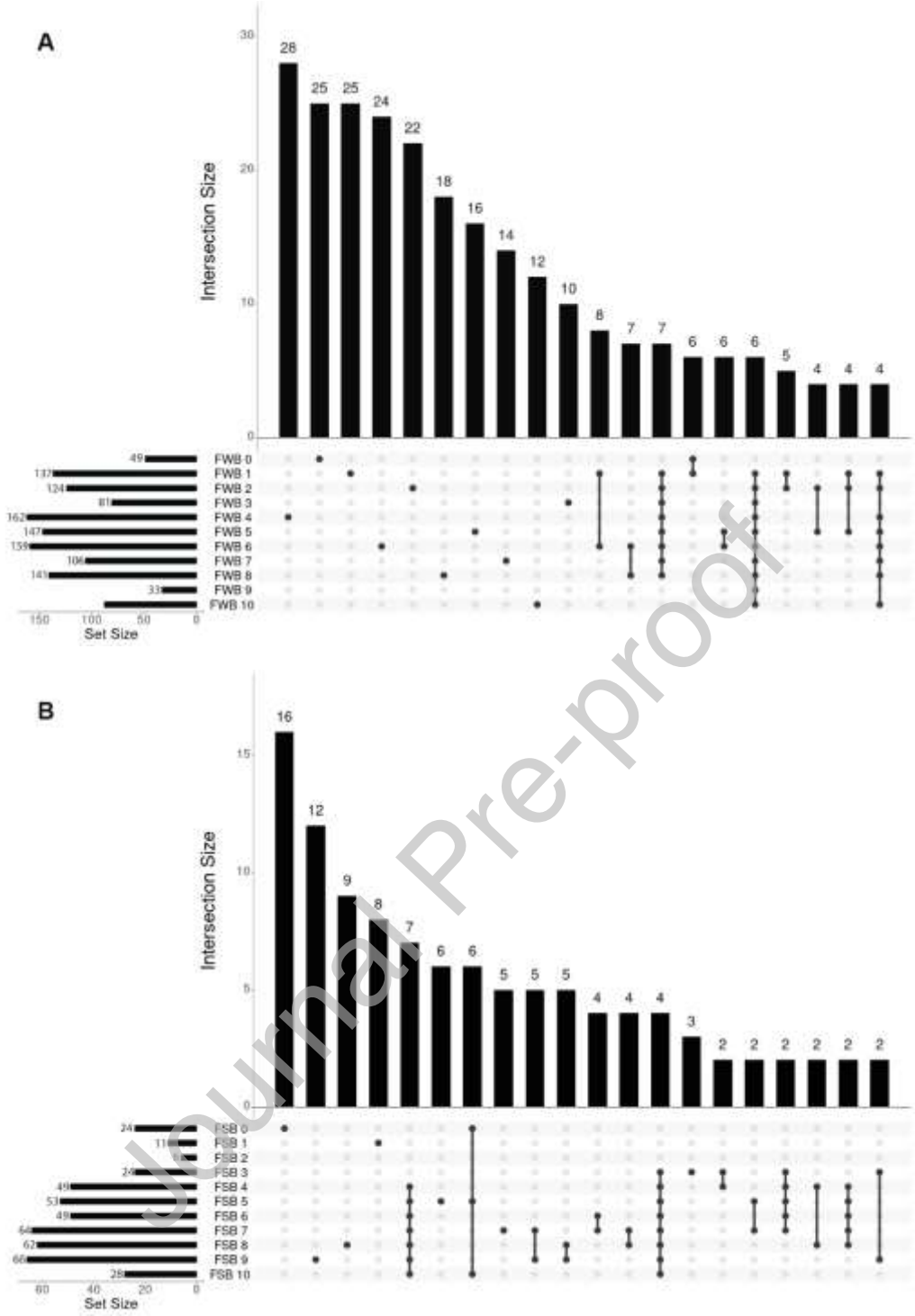

Figure 4. UpSet plots showing the number of peptides identified in **(A)** fermented wheat brewers' spent grain (FWB) and **(B)** fermented stout brewers' spent grain (FSB) over a 10-day fermentation period at 35 °C. Numbers (0–10) represent fermentation days. The ***Set Size*** bars indicate the total number of peptides detected on each day, while the ***Intersection Size*** bars represent the number of peptides unique to a single day (single dots) or shared across multiple days (dots connected by lines).

3.3.3. Peptides with potential bioactive properties

Peptides released during BSG fermentation were further annotated for reported or predicted bioactive properties, including antihypertensive, antimicrobial, and antibacterial activities (Figure 5). Overall, FWB generated a greater diversity of peptides associated with potential bioactivities than FSB throughout the fermentation period. Early fermentation stages showed distinct differences between the two BSG types. From Day 0 to Day 1, FWB exhibited a range of peptides annotated with antihypertensive, antimicrobial, and antibacterial activities, whereas FSB showed a shift from antimicrobial and antibacterial peptides at Day 0 to predominantly antihypertensive peptides by Day 1, with antihypertensive annotations accounting for 100% of the detected bioactivities at that time point. In addition, a higher number of peptides associated with predicted hormonal activities were detected in FSB, warranting further investigation into hormone-related peptides and their possible association with different brewing processes.

As fermentation progressed, the number of peptides annotated with potential bioactive properties in FWB gradually declined, accompanied by a relative increase in peptides containing wheat allergy (WA) and coeliac disease (CD) epitopes. Distinct profiles of intact WA and CD epitopes were observed between FWB and FSB, with a higher number of such epitopes detected in FWB across the fermentation period (Figure 5). At Day 0, approximately 20% of the peptides detected in FWB contained intact WA and CD epitopes, whereas no intact WA or CD epitopes were identified in peptides from FSB. During fermentation, the proportion of intact WA epitopes increased in FWB, while these epitopes remained undetected in FSB samples.

The observed differences in WA and CD epitope profiles between FWB and FSB may be attributed to the thermal treatments applied during stout malt production. High-temperature processing associated with stout beer production may modify or eliminate WA and CD regions within protein sequences (Fadimu et al., 2023), thereby reducing their accessibility to enzymatic digestion during the lautering process (Steiner et al., 2011). Consistent with this interpretation, Rao et al. (2023), employing enzyme-linked immunosorbent assay (ELISA), reported that thermal treatments such as steaming (100 °C) and baking (200 °C) significantly reduced the allergenicity of wheat matrices, with baking (higher temperatures) resulting in more pronounced reductions in allergenic potential. Together, these findings suggest that differences in brewing grain processing may influence the types and proportions of peptides released during fermentation, including those associated with potential bioactive properties and allergenicity.

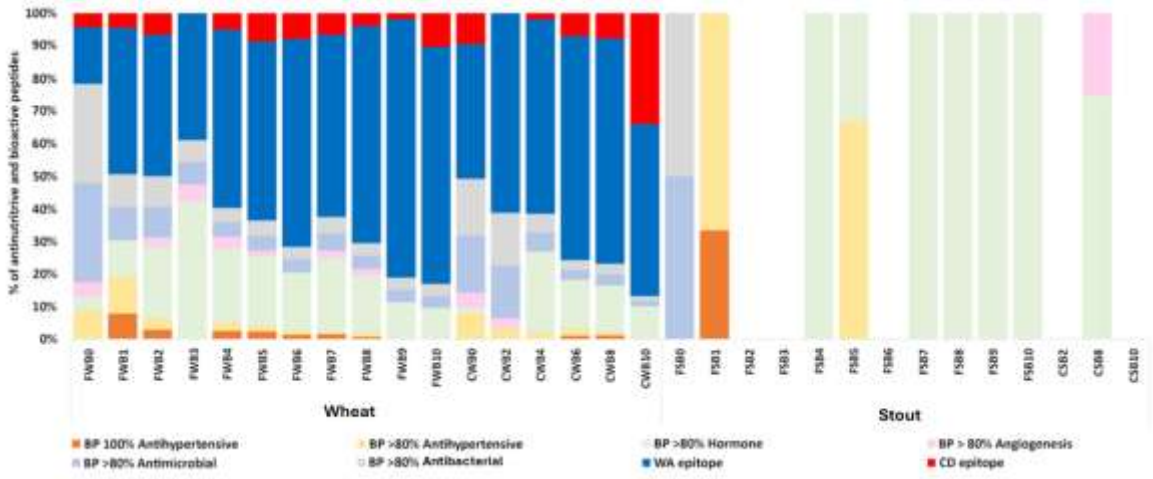

Figure 5. Distribution of peptides annotated with potential bioactive and antinutritive properties in fermented wheat brewers' spent grain (FWB), fermented stout brewers' spent grain (FSB), and their corresponding control samples (CWB and CSB) over a 10-day fermentation period. Numbers indicate the fermentation day.

3.3.4. Peptide quantification

Targeted multiple reaction monitoring (MRM) was used to quantify the temporal abundance of 28 selected peptides across the 10-day fermentation period. This approach enabled comparison of peptide accumulation and persistence between FWB and FSB and provided quantitative support for the peptide release patterns observed in the discovery analysis.

Figure 6 shows that peptides annotated as having predicted bioactivities (e.g., antimicrobial and antihypertensive) as well as peptides corresponding to allergen epitopes were predominantly released during fermentation with *Lb. plantarum*, as evidenced by higher abundances in fermented samples (F0–10) compared with the non-inoculated controls (C0–10). Among the quantified peptides, two peptides annotated with predicted antimicrobial activity, GQRGGGGGYGGGGGGYGGGGGGY and GQRGGGGGYGGGGGGYGGGGGGYG, both originating from a single protein (accession F2CR90), exhibited similar trends in both BSG types (Figure 6; Table S2A). In FWB, the abundance of these peptides increased during mid-fermentation, reaching a maximum on Day 6 before declining by Day 10. In FSB, these peptides showed a gradual increase, plateauing from Day 6 to Day 8, peaking on Day 9, and subsequently decreasing by Day 10.

Peptides annotated with antihypertensive activity and containing the amino acid motifs GGVIPN and GGVLPN were detected at higher relative abundances than the antimicrobial peptides and exhibited distinct patterns between the two BSG types. In FWB, these peptides showed their highest abundance early in fermentation, peaking on Day 2. The relative abundances on Days 1 and 2 were significantly higher than those observed at later stages of fermentation ($p < 0.05$), followed by a progressive decline to a minimum by Day 10 ($p < 0.05$; Figure 6; Table S2B). In contrast, FSB exhibited a delayed accumulation of these peptides, with higher abundances observed during the mid-fermentation phase (Days 3–7) and a peak on

Day 5. Although a decline was observed after this peak, the reduction was not statistically significant ($p > 0.05$; Table S2B).

Peptides annotated with potential bioactivity motifs previously associated with angiotensin-I converting enzyme (ACE) inhibition were also detected. The peptide GGVIPN, identified among the quantified peptides (Figure 6; Table S2A), has been reported as an ACE-inhibitory peptide and is predominantly associated with wheat-derived cereal proteins (Polya, 2003). In addition, peptides such as GGVLPN and C-hordein-derived peptides including LPLQP and IPLQP, previously reported to exhibit ACE- and DPP-IV-inhibitory activities (Cermeño et al., 2019), were also detected in the present dataset (Tables S1A and S1B), indicating that *Lb. plantarum* fermentation released multiple motifs associated with reported bioactivities. Notably, these short hydrophobic peptides (GGVIPN, GGVLPN, LPLQP, and IPLQP) also possess structural characteristics that are generally associated with greater gastrointestinal stability and intestinal permeability (Amigo & Hernández-Ledesma, 2020; Shen & Matsui, 2019; Wang et al., 2020), suggesting that the peptides generated in this study may warrant further investigation for bioavailability and *in vivo* efficacy.

3.3.5 Dynamics of wheat allergy and coeliac disease epitopes during fermentation

As described in Section 2.9.2, the detection and mapping of wheat allergy (WA) and coeliac disease (CD)–related epitope motifs were performed entirely *in silico*, by mapping DDA-MS/MS–identified peptides against CD-specific HLA-DQ epitopes (Sollid et al., 2012; 2020) and food allergy–related linear epitopes from the Immune Epitope Database (IEDB) using CLC Genomics Workbench v22 with 100% sequence match stringency; no immunological assays were performed in this study.

The partial allergen epitopes containing the motif sequences PQQPFP and QQPGQ displayed distinct abundance patterns throughout fermentation (Figure 6; Tables S2C and S2D). In FWB, the abundance of PQQPFP increased steadily from Day 0 to Day 7 before decreasing by Day 10. In FSB, PQQPFP exhibited a more gradual and sustained increase, reaching its highest abundance on Day 9. While both BSG types showed an overall increasing trend for PQQPFP, the QQPGQ peptide exhibited contrasting behaviour. In FWB, QQPGQ abundance peaked early on Day 2 and then gradually declined, whereas in FSB, QQPGQ increased progressively to a maximum on Day 5 before decreasing thereafter.

These partial epitope motifs originate from cereal storage proteins, including γ- and ω-gliadins in wheat and γ-hordeins in barley, and are closely associated with known wheat allergy epitopes (Matsuo et al., 2005). Their detection in fermented BSG therefore reflects the persistence of cereal storage protein-derived sequences during fermentation. Their detection in fermented BSG therefore reflects the persistence of cereal storage protein-derived sequences during fermentation. As discussed in Section 3.3.1, wheat and barley accounted for 43.3% and 36.6% of protein identifications in FWB and 43.7% and 28.3% in FSB, respectively, which may explain the prevalence of cereal-derived peptides detected during the fermentation.

Beyond their detection, the temporal dynamics of these motifs carry additional relevance from a food safety perspective. The sustained or increasing abundance of PQQPFP in FWB from Day 0 through Day 7 indicates that *Lb. plantarum* fermentation did not reduce, but in fact promoted the release of epitope-containing sequences over time. This observation is notable given that fermentation is frequently proposed as a strategy for reducing gluten-related allergenicity in cereal matrices (Rizzello et al., 2007; Hernández-Figueroa et al., 2025). The present data suggest that the extent of epitope reduction is highly substrate-dependent, and that fermentation of wheat-derived BSG under the conditions tested here may not be sufficient to substantially diminish WA- and CD-relevant sequences. In contrast, the negligible detection of these motifs in FSB across the fermentation period further supports roasting, rather than LAB fermentation per se, as the more effective determinant of epitope reduction in the current experimental system.

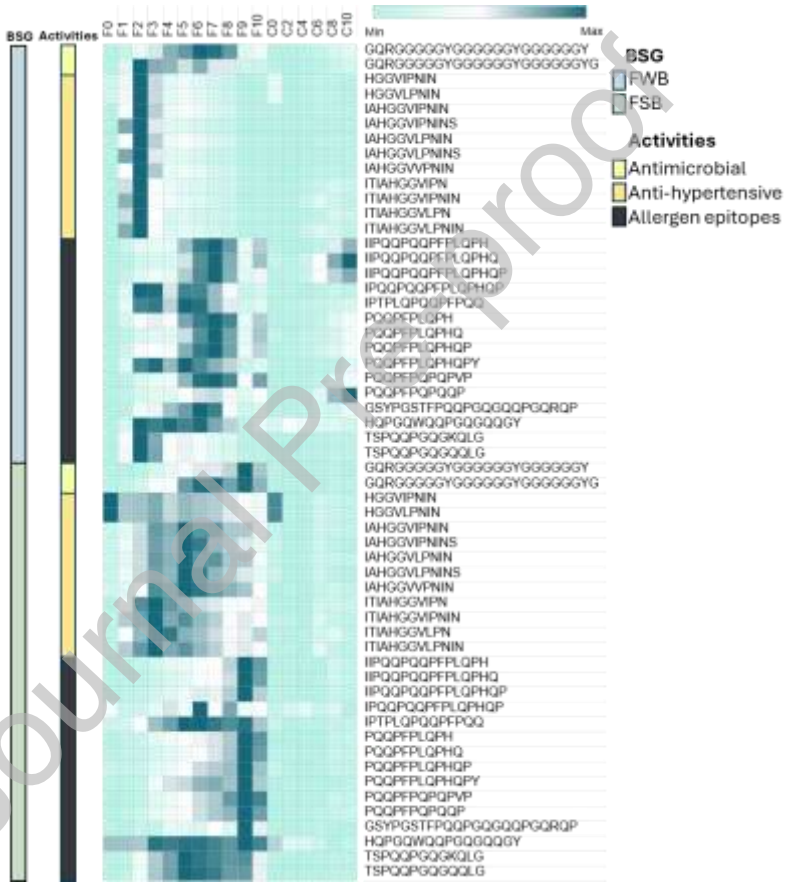

Figure 6. Heat map showing the relative abundance (peak area) of 28 quantified peptides in fermented wheat brewers' spent grain (FWB), fermented stout brewers' spent grain (FSB), and control samples over a 10-day fermentation period. Each cell represented the means of triplicates (n = 3). Samples are denoted by **F** for fermented and **C** for control, with the accompanying number indicating the fermentation day. Fermented samples were collected daily, whereas control samples were collected every two days.

4. Free amino acid content

Free amino acids were grouped into proteogenic amino acids (PAAs; the 20 amino acids released from proteins) and non-proteogenic amino acids (NPAAs; not directly incorporated into proteins). Profiling PAAs provides insight into the extent and selectivity of *Lb. plantarum* proteolysis in BSG during fermentation, while NPAAs provide complementary information on microbial metabolism and biochemical transformations occurring in the matrix.

Figure 7 summarises the concentration patterns of PAAs and NPAAs identified in the two fermented BSG groups, and their corresponding controls (without starter culture). In both FWB and FSB, the total content of PAAs in fermented samples was generally higher than that in the control samples, indicating that inoculation with *Lb. plantarum* promoted protein hydrolysis and/or amino acid release during fermentation (Figure 7). Across the 10-day period, PAA concentrations showed an overall increasing tendency in both fermented BSGs, with L-glutamic acid consistently being the most abundant amino acid. In addition, increases in L-proline, L-leucine, L-valine, L-phenylalanine, L-alanine, and L-lysine were observed across fermented samples in both BSG types (Figure 7), consistent with progressive release of free amino acids as fermentation proceeded.

A notable late-stage feature in Figure 7 is that, despite lower PAA levels during the early stages of fermentation, the control samples exceeded the PAA concentrations of the *Lb. plantarum*-fermented samples by the end of the fermentation period. Importantly, this does not indicate superior proteolytic efficiency in the control but rather reflects fundamental differences between controlled starter-driven fermentation and unregulated background microbial activity. In the *Lb. plantarum*-inoculated samples, free amino acid levels were shaped by both release and subsequent utilisation, as amino acids liberated through proteolysis may be assimilated as nitrogen sources or redirected into other metabolic pathways as fermentation progresses. This dynamic turnover is characteristic of defined LAB fermentations and reflects active metabolic regulation rather than simple accumulation.

In contrast, the control samples represent an open system in which amino acid accumulation likely reflects a different balance of processes, including slower endogenous enzyme activity and heterogeneous background microflora, in the absence of a defined starter culture. While such conditions may lead to elevated free amino acid levels at later stages, they are inherently variable and mechanistically difficult to interpret. The use of *Lb. plantarum* therefore remains critical to this study, as it enables reproducible fermentation behaviour, clearer attribution of proteolytic and metabolic trends, and meaningful comparison between substrates. Consequently, the higher late-stage PAA levels observed in the control samples should be interpreted as a by-product of uncontrolled microbial and enzymatic activity rather than as evidence of improved fermentation performance.

Among the NPAAs, ethanolamine and γ-aminobutyric acid (GABA) were predominant, followed by L-citrulline and L-ornithine. The accumulation of these NPAAs in post-fermentation samples is consistent with active microbial metabolism, and several LAB species such as *Lactobacillus brevis*, *Lactococcus lactis*, *Lb. plantarum*, and *Streptococcus salivarius subsp. thermophilus*, are known to produce ornithine and GABA under appropriate

conditions (Hwang & Lee, 2018; Li & Cao, 2010). However, the specific pathways contributing to NPAA patterns in this study cannot be resolved from these data alone and would require targeted metabolic analysis. Notably, NPAA levels were highest in the control samples of both BSG types, where L-ornithine and L-citrulline were the most abundant, which may again reflect contributions from a broader background microflora in the absence of starter inoculation.

From a biochemical perspective, these NPAAs have been described in the literature as biologically relevant metabolites. Ethanolamine serves as a precursor of phosphatidylethanolamine, an essential membrane phospholipid involved in cellular structure and lipid metabolism (Zhou et al., 2018). L-citrulline participates in nitric oxide metabolism (Aguayo et al., 2021), GABA is a well-characterised microbial metabolite with neuromodulatory roles (Hepsomali et al., 2020), and L-ornithine is involved in nitrogen metabolism and ammonia detoxification pathways (Miyake et al., 2014). The detection of these compounds in fermented BSG highlights the metabolic complexity of the fermentation system; however, their presence alone does not imply physiological efficacy, and their bioavailability and functional relevance would require further investigation.

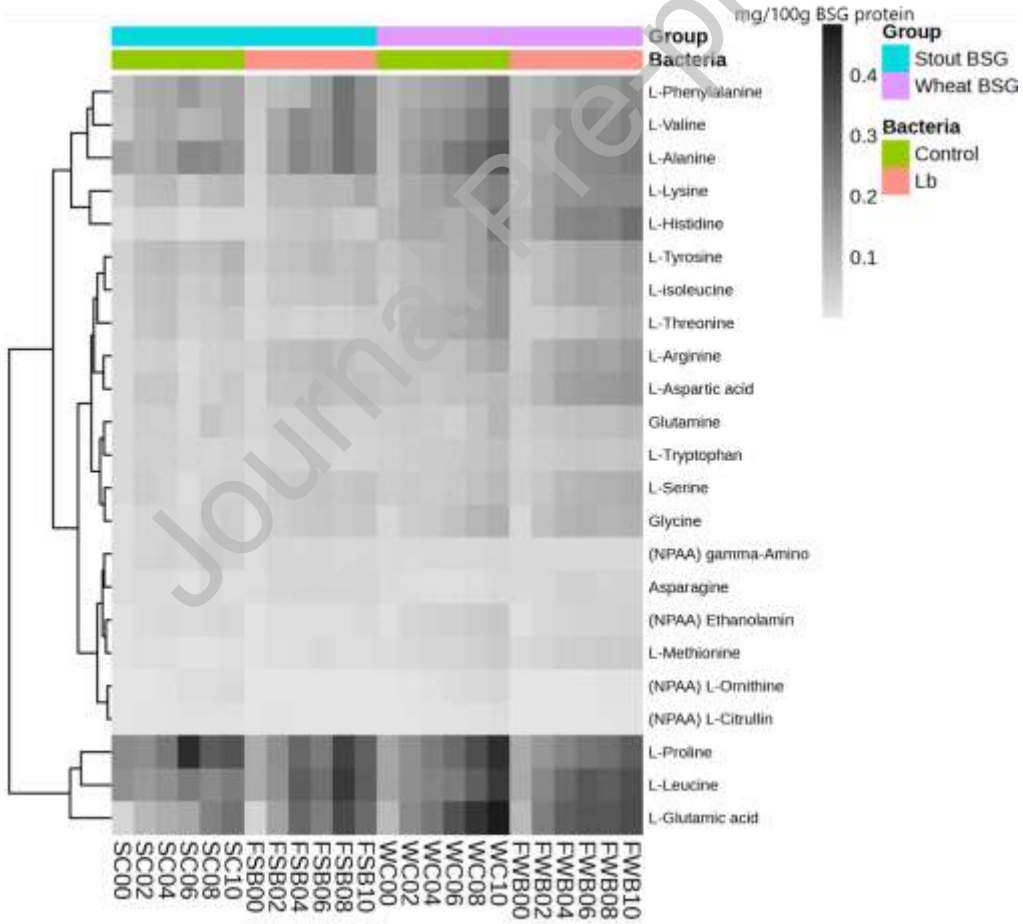

Figure 7. Heat-map and clustering analysis of free amino acid concentration (mg/100 g protein of BSG) during 10-day fermentation of wheat and stout beer's BSG. The variable in each row represents an amino acid, and each column represents the BSG group, time and bacteria

treatment, where "Lb" is *Lactobacillus plantarum-treated* and "control" is the control sample; "NPAA" is non-proteogenic amino acid; the uppercase letter "FWB, FSB" indicates the fermented wheat and stout beers' BSGs, respectively; the uppercase letter "WC, SC" indicates the control samples of wheat and stout beers' BSGs, respectively, whereas the number indicates the fermentation days. Each data point was measured in triplicate.

To further compare how *Lb. plantarum* fermentation influenced amino acid composition between substrates, Figure 8 shows the production of free proteogenic amino acids (PAAs) in FWB and FSB after 10 days of fermentation. Consistent with Figure 6, glutamic acid was the most abundant amino acid in both groups (0.259 vs. 0.250 mg/g protein, respectively), followed by leucine (0.219 vs. 0.185 mg/g protein, respectively) and proline (0.185 vs. 0.182 mg/g protein, respectively).

When amino acids were grouped by charge, aspartic acid and glutamic acid accounted for the highest concentrations among negatively charged amino acids in both FWB and FSB (aspartic acid: 0.108 vs. 0.052 mg/g protein; glutamic acid: 0.259 vs. 0.250 mg/g protein, respectively), with no significant differences observed between BSG groups ($p > 0.05$). In contrast, FWB contained significantly higher levels of positively charged amino acids compared to FSB, particularly arginine (0.113 vs. 0.023 mg/g protein) and histidine (0.167 vs. 0.023 mg/g protein, respectively; $p < 0.05$).

Although a higher proportion of acidic amino acids can contribute to a more negatively charged and acidic environment (Idrees et al., 2020), no significant differences were observed in the concentrations of negatively charged amino acids between FWB and FSB. Nevertheless, FSB exhibited a more acidic end-point (pH 3.77 on Day 10; Section 3.2), coinciding with a lower cell population relative to FWB. Rather than reflecting increased acid generation, this pH difference appears to be associated with reduced buffering capacity in FSB. Positively charged amino acids such as histidine and arginine can accept protons and contribute to pH buffering during fermentation (Herrington & Kellogg, 2021; Iyengar et al., 2022; Valéry et al., 2015). The significantly lower abundance of these amino acids in FSB therefore limits buffering against organic acid accumulation, resulting in a more pronounced pH decrease. In contrast, the higher buffering capacity of FWB likely contributed to its higher terminal pH and improved cell survival towards the end of fermentation (Section 3.2; Figure 2A–B).

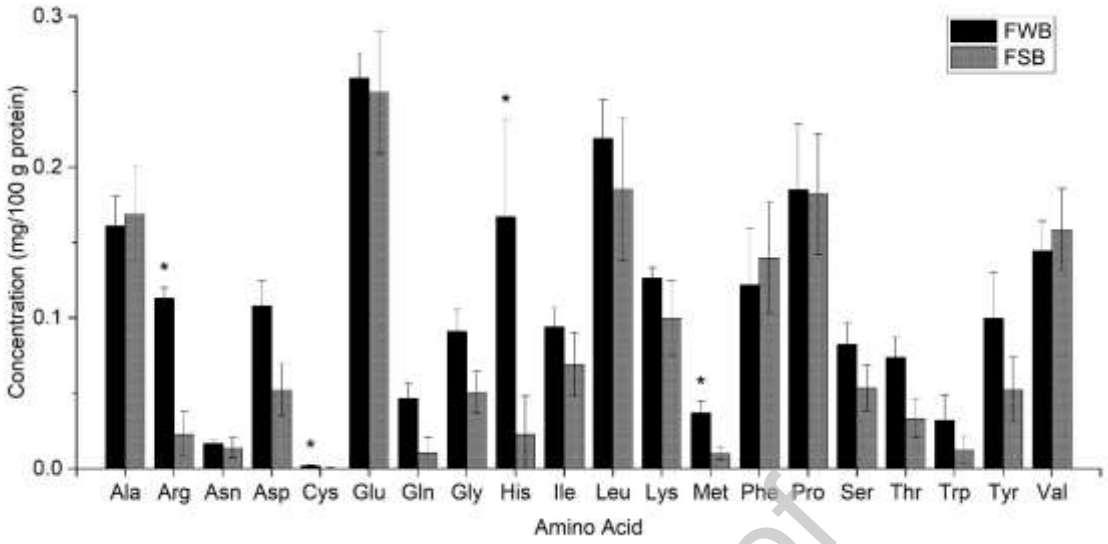

Figure 8. Concentrations of proteogenic amino acids were measured after 10 days of fermentation in fermented wheat brewers' spent grain (FWB) and fermented stout brewers' spent grain (FSB). Data are presented as means ± standard deviation of triplicate measurements (n = 3). Asterisks (*) indicate significant differences in individual amino acid concentrations between the two BSG types ($p < 0.05$).

5. Conclusion

This study investigated the biochemical changes occurring during the fermentation of brewers' spent grain (BSG) with the addition of *Lactiplantibacillus plantarum*, with a focus on peptide and amino acid profiles in wheat- and stout-derived BSG. Distinct fermentation behaviours were observed between the two substrates. Wheat BSG showed rapid peptide generation during the early to mid-stages of fermentation. In contrast, stout BSG exhibited a delayed and reduced peptide release.

Peptide profiling and targeted quantification revealed the formation of diverse peptides during fermentation, including peptides annotated with predicted antihypertensive and antimicrobial activities, as well as peptides containing epitopes associated with wheat allergy and coeliac disease. These allergenic epitopes were less prevalent in stout BSG, supporting the hypothesis that intensive thermal processing reduces the availability of allergen-related protein regions. However, the presence of peptides annotated with bioactive properties does not imply functional efficacy, and further validation is required.

Free amino acid analysis demonstrated substrate-dependent differences in amino acid release and composition during fermentation. Changes in the abundance and charge distribution of free amino acids between wheat and stout BSG were consistent with differences in fermentation dynamics, pH evolution, and microbial growth observed earlier in the study. Together, these

findings highlight how raw material processing and controlled lactic acid bacterial fermentation interact to shape peptide and amino acid outcomes in BSG.

Overall, this study was designed as a systematic, process-aware investigation to establish a controlled framework for understanding how brewing-derived differences in BSG influence *Lb. plantarum*-driven proteolysis over time. This framework provides a foundation for several important directions in future research. While a single LAB strain and fixed fermentation conditions were utilised to enable mechanistic comparison between substrates, future studies should extend this framework by evaluating different lactic acid bacteria, *in vitro* validation of the identified ACE- and DPP-IV-inhibitory peptides (GGVIPN, GGVLPN, LPLQP, and IPLQP) and cytotoxicity assessment of the fermented BSG extract. Furthermore, immunological confirmation of the *in silico* allergen and coeliac disease epitope findings, particularly the reduced epitope prevalence in stout BSG should be conducted. Lastly, evaluation of co-culture and mixed-strain LAB fermentation with different fermentation conditions should also be considered to broaden proteolytic cleavage diversity and further optimise bioactivity and allergen reduction.

CRediT authorship contribution statement

Minh Quoc Ha: Writing – review & editing, Writing – original draft, Formal analysis, Software, Conceptualization. **Angéla Juhász:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Teguh Santoso:** Writing – review & editing, Writing – original draft, Formal analysis, Software, Conceptualization. Software, Conceptualization. **Mitchell G. Nye-Wood:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Mathilde Berthevas:** Validation, Methodology, Investigation **Zhiyu Zhao:** Validation, Methodology. **Eileen Kitundu** Validation, Methodology. **Tony Chen:** Validation, Methodology. **Michelle L. Colgrave:** Writing – review & editing, Resources. **Thao T. Le:** Writing – review & editing, Supervision, Conceptualization, Resources, Project administration, Funding acquisition.

Data Availability Statement: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Supplementary Materials: The following supporting information can be downloaded at: www.xxxx.com

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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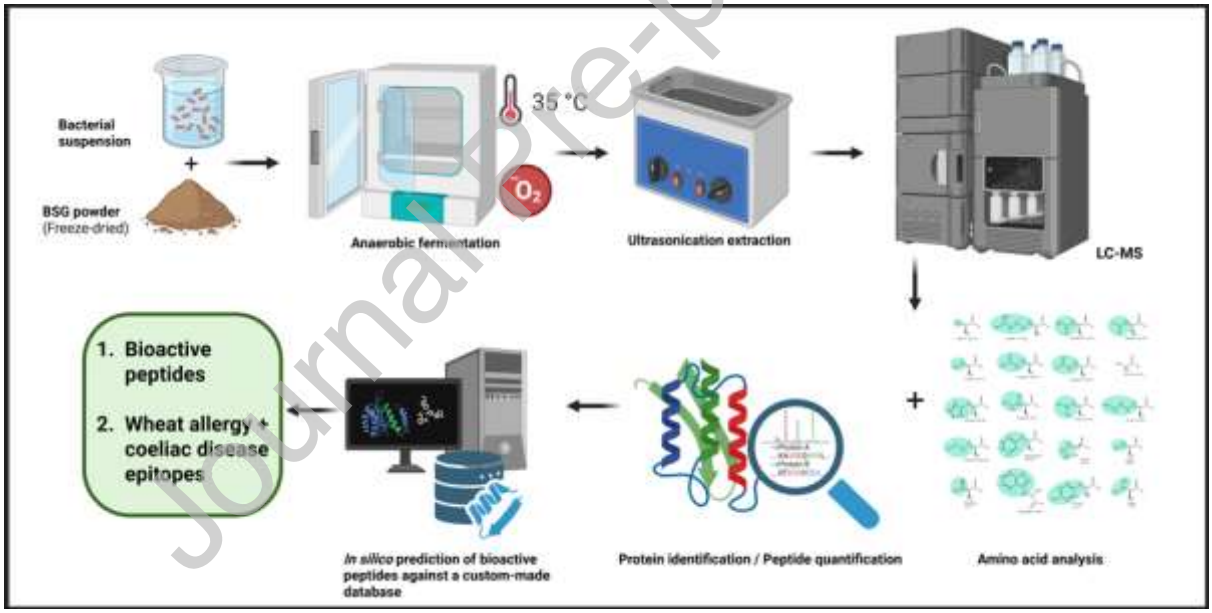
Ethics statement

Ethical approval was not required for this study, as it did not involve human participants or animal experimentation.

Graphical Abstract



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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