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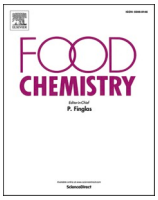

Conventional solid-state fermentation impacts the white lupin proteome reducing the abundance of allergenic peptides

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ABSTRACT

The demand for high-quality and sustainable protein sources is on the rise. Lupin is an emerging plant-based source of protein with health-enhancing properties; however, the allergenic potential of lupins limits their widespread adoption in food products. A combination of discovery and targeted quantitative proteome measurements was used to investigate the impact of solid-state fermentation induced by *Rhizopus oligosporus* on the proteome composition and allergenic protein abundances of white lupin seed. In total, 1,241 proteins were uniquely identified in the fermented sample. Moreover, the effectiveness of the solid-state fermentation in reducing the abundance of the tryptic peptides derived from white lupin allergens was demonstrated. Comparably, a greater decrease was noted for the major white lupin allergen based on β-conglutin peptide abundances. Hence, conventional solid-state fermentation processing can be beneficial for reducing the potential allergenicity of lupin-based foods. This finding will open new avenues for unlocking the potential of this under-utilised legume.

1. Introduction

Lupin is a grain legume from the *Lupinus* genus comprising approximately 200 species, wherein the major domesticated species are narrow-leafed lupin (NLL, *Lupinus angustifolius*), white lupin (*Lupinus albus*), pearl lupin (*Lupinus mutabilis*) and yellow lupin (*Lupinus luteus*) (Wolko, Clements, Naganowska, Nelson, & Yang, 2011). Lupin seeds are a sustainable plant-based source of food with unique nutritive, techno-functional, and health-promoting attributes. These seeds are one of the richest natural sources of protein and fibre and can be implemented in food products such as bakery and gluten-free goods, pasta and meat analogues to enhance their nutritional quality (Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2016). Despite their potential to contribute to food and nutritional security, lupin consumption as a food ingredient remains limited and stockfeed is the dominant end-use for this crop (White, Staines, & Staines, 2007). One constraint to large-scale adoption of this legume in food products, is because it is acknowledged as an allergen and is subjected to mandatory labelling on food products in many countries (Villa, Costa, & Mafra, 2020).

Allergic reaction to lupin-based food products may result due to the primary sensitisation to lupin proteins as well as the cross-reactions to other legume (such as soybean and peanut) allergens owing to the high sequence similarity of these proteins (Jappe & Vieths, 2010). Several protein families associated with lupin induced food allergy have been identified to date. This includes the globulin (α-, β-, and γ-conglutin) and albumin (δ-conglutin) protein families which account for the major lupin seed proteins as well as the non-specific lipid transfer protein (nsLTP), pathogenesis-related (PR)-10 and profilin minor protein fractions (Villa et al., 2020). The β-conglutin, a 7S globulin protein, is recognised as a major allergen from this legume and is included as Lup an 1 in the allergen list of the Allergen Nomenclature Subcommittee of the World Health Organization and International Union of Immunological Societies (WHO/IUIS; https://www.allergen.org/) (Goggin, Mir, Smith, Stuckey, & Smith, 2008). In addition, Lup an 3 of *L. angustifolius*

Abbreviations: LC-MS, Liquid chromatography-mass spectrometry; LC-MRM-MS, Liquid chromatography-multiple reaction monitoring-mass spectrometry; SSF, Solid-state fermentation; DTT, dithiothreitol; HCA, Hierarchical clustering analysis; GO, Gene ontology; IDA, Information dependent acquisition.

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belonging to nsLTP family and Lup a 5 of *L. albus* a profilin protein are the other allergens recognised by WHO/IUIS from this legume (Jappe et al., 2021).

Different food processing technologies such as physical treatment, heat treatment, proteolysis, and fermentation can lead to chemical and structural modifications of the substrate proteins and thereby alter their properties (Nikbakht Nasrabadi, Sedaghat Doost, & Mezzenga, 2021). Among these technologies, solid-state fermentation (SSF) is an economical and widely used method in the food industry, which has also been applied for enhancing the nutritional, sensorial and functional properties of legumes (Garrido-Galand, Asensio-Grau, Calvo-Lerma, Heredia, & Andrés, 2021). In addition, the fermentation process can effectively reduce food allergenicity, through inducing microbial proteolysis and degradation of allergens. This effect has been demonstrated for fermented soybean products through several studies (Meinlschmidt, Ueberham, Lehmann, Schweiggert-Weisz, & Eisner, 2016; Song, Frias, Martinez-Villaluenga, Vidal-Valdeverde, & de Mejia, 2008; Yang et al., 2018); however, the studies addressing the allergenicity reduction of fermented lupin products are at an earlier stage. Previously, the lactic acid fermentation of NLL protein isolate has been indicated to be insufficient for the effective degradation of lupin allergens based on SDS-PAGE analysis (Schlegel, Leidigkeit, Eisner, & Schweiggert-Weisz, 2019). Alternately, the combination of lactic acid fermentation and enzymatic digestion of NLL protein isolates was stated to be efficacious in degrading the Lup an 1 lupin major allergen (Schlegel, Lidzba, Ueberham, Eisner, & Schweiggert-Weisz, 2021).

Herein, the effect of SSF (carried out with *Rhizopus oligosporus*) on the proteome composition of white lupin seed (cv. Luxor) was qualitatively evaluated by means of discovery proteome analysis. This was followed by the quantitative assessment of the changes introduced by fermentation to the white lupin allergens across seven protein groups (α-, β-, δ- and γ-conglutin, nsLTP, profilins and PR-10). The relative quantitative evaluation was achieved by comparing the signal of tryptic peptides, specific to each allergenic protein group across the pre-fermented and fermented samples. The findings from this study are expected to provide further insight into the potency of the SSF technique in reducing the abundance of allergenic proteins in white lupin seed and enhance opportunities for the development of innovative hypoallergenic lupin-based products.

2. Materials and methods

2.1. Lupin samples

The samples of this study were provided by Eighth Day Foods (Coburg North, Victoria, Australia). These included: 1- milled raw white lupin (cv. Luxor) sample, 2- pre-fermented sample comprised of 38% (w/w) milled raw white lupin (cv. Luxor), 2.2% white rice vinegar, 0.7% *R. oligosporus* and 59% distilled water which was subjected to Eighth Day Foods' (Coburg North, VIC, Australia) proprietary hydrothermal treatment and 3- fermented product known as Lupreme® comprised of 38% white lupin cv. Luxor, 2.2% white rice vinegar, 0.7% *R. oligosporus* and 59% distilled water which was subjected to Eighth Day Foods' proprietary hydrothermal treatment and controlled fermentation (22–26 h) process. The samples were blast-chilled, vacuum packed and stored at −80 °C prior to freeze drying. Finally, a mixer mill (model MM400 Retsch, Germany) was used to grind the samples into a to a fine powder.

All reagents and solvents used for sample preparation and LC-MS analysis were analytical grade and obtained from ChemSupply (Gillman, SA, Australia) if not stated separately.

2.2. Sample defatting, protein extraction and protein concentration estimation

Approximately 20 mg of each flour sample was weighed into a protein lo-bind microtube (n = 4 technical replicates). For lipid removal, 200 μL of *n*-hexane was added, thoroughly vortexed and then further mixed using a thermoshaker (Thermo Fisher Scientific, VIC, Australia) (1,000 rpm, at 20 °C for 20 min). All samples were centrifuged, and the supernatant was removed (repeated two times). Throughout the experiment the centrifugation was performed at 20,800 × g for 15 min, unless specified.

The defatted samples were airdried in a fume hood and a urea-based protocol optimised for NLL samples (Tahmasian, Broadbent, et al., 2022) was used for extracting the proteins. Briefly, 800 μL of extraction buffer (8 M urea, 2% (w/v) DTT, 1% (v/v) protease inhibitor cocktail in 0.1 M Tris-HCl; pH 8.6) was added, the samples were vortexed until thoroughly combined and incubated in a thermoshaker (600 rpm) at 20 °C for 45 min. After centrifugation the protein concentration of the supernatant extracts were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, VIC, Australia).

2.3. Tryptic digestion by filter-aided sample preparation

The trypsin digestion of the proteins was achieved using a filter-aided sample preparation method (Wiśniewski, Zougman, Nagaraj, & Mann, 2009). For this, 30 μL of each extract was loaded on a 10 kDa molecular weight cut-off (MWCO) filter (Millipore, Bayswater, VIC, Australia) and washed twice through centrifugation with 200 μL of urea buffer (8 M urea in 0.1 M Tris-HCl, pH 8.4). Next, cysteine alkylation was achieved by addition of 100 μL of 25 mM iodoacetamide (IAM) and incubation in dark for 20 min at 20 °C. The excess IAM was removed through two consecutive wash/centrifugation steps using 100 μL of urea buffer, followed by buffer exchange by the addition of 200 μL of 100 mM ammonium bicarbonate (pH = 8.4) and centrifugation, repeated once more. At this point the filter units were transferred to clean collection tubes; 125 μL of 0.02 μg/μL sequencing grade trypsin (Promega, NSW, Australia) in 50 mM ammonium bicarbonate was added to each filter and the samples were incubated at 37 °C overnight (~18 h). Subsequently, the samples were centrifuged to collect the tryptic peptides and the filters were washed with 200 μL of 0.1% formic acid (FA) with centrifugation. The filtrates were evaporated to dryness in a vacuum centrifuge and stored at −80 °C until further analyses.

2.4. Discovery proteome measurement using information-dependant acquisition mass spectrometry (IDA-MS)

The digested peptides were reconstituted in 100 μL of 0.1% FA prior to injection (2 μL) onto an Ekspert nanoLC415 (Eksigent, CA, USA). At first, the peptide solution (2 μL) was desalted for 5 min on a polar C18 ProteCol trap (10 mm × 300 μm, 3 μm particle size and 300 Å pore size) at 10 μL/min flowrate of 0.1% FA. The peptides were separated on a ChromXP C18 (150 mm × 0.3 mm, 3 μm particle size and 120 Å pore size) column (30 °C) using 5 μL/min flowrate. The mobile phase solvent A was aqueous 5% DMSO and 0.1% FA, and solvent B was aqueous 5% DMSO, 90% acetonitrile and 0.1% FA. A linear gradient from 5 to 45% solvent B was applied over 40 min, followed by a 5 min linear gradient to 90% B and 5 min hold at 90% B, before returning to 5% B over a 1 min period and re-equilibration for 11 min. The information-dependant data acquisition was performed on a TripleTOF 6600 MS (SCIEX, CA, USA) system fitted with a DuoSpray ion source. The acquisition method parameters were as previously described (Colgrave et al., 2016). Briefly, a survey MS scan (350–1250 *m/z* with a 0.25 s accumulation time) was conducted, after which the MS/MS scans (100–1800 *m/z* with 0.05 s accumulation time) were acquired for the 30 most intense precursor ions with a charge state of 2 to 5 and intensity above 150 cps (exclusion for 15 s after two occurrences). The following parameters were applied for both scans: ion spray voltage of 5500 V, curtain, GS1 and GS2 gas flow of 25, 20 and 15 psi, respectively, source temperature of 150 °C and a delustering potential of 80 eV whilst enabling the rolling collision energy option for optimal peptide fragmentation.

2.5. Protein and peptide identification

To construct the database of this study, the UniProt *L. albus* protein sequence entries (68,773 sequences retrieved on 03/05/2022) were merged with the UniProt *Rhizopus* protein sequence entries (84,850 sequences retrieved on 17/07/2022) as well as the common repository of adventitious proteins. This was followed by the removal of the 100% identical sequences (including fragment sequences) from the combined database. ProteinPilot v5.0.3 software (SCIEX) was used to search the raw lupin sample IDA data files against the final database (comprising 133,794 sequences) (Shilov et al., 2007). The search parameters used were as follow: trypsin as the cleavage enzyme, TripleTOF 6600 for the instrument type, IAM as the cysteine alkylation agent, "Thorough ID" for the search effort and finally the ID focus was set on biological modifications. The false discovery rate (FDR) analysis was enabled (Tang, Shilov, & Seymour, 2008), and the identification yields at a 1% global FDR cut-off were reported.

To evaluate the differences in the digestion specificity of white lupin proteins across the pre-fermented and fermented samples, the IDA data files were also searched against the combined database by setting the enzyme to "none". The previous and next amino acids for each peptide were determined by mapping the lupin peptides identified through "no enzyme" searches on the protein sequences of the combined database (using the motif search tool in CLC Main Workbench v21.0.4 software). Subsequently, the frequencies of the cleavage between each pair of residues were calculated across the replicates of each sample. The level of the change in the digestion frequency because of fermentation was determined by calculating their fold change (FC) in the fermented and pre-fermented lupin sample groups. The results were visualised by means of Morpheus software (Broad Institute, Cambridge MA, USA; https://software.broadinstitute.org/morpheus/).

2.6. Target protein selection and computational allergenicity prediction

The proteins identified at 1% FDR from raw white lupin sample (1,561 proteins) were subjected to allergenicity prediction using AllerCatPro 2.0 web server (Nguyen, Krutz, Limviphuvadh, Lopata, Gerberick, & Maurer-Stroh, 2022). The sequences with >80% sequence identity (linear 80 aa window) with the known allergens and strong evidence (based on AllerCatPro model assessment) for allergenicity were considered as potential allergens. The additional putative allergens identified from this *in-silico* prediction analysis together with the previously reported white lupin conglutin sequences (Hufnagel et al., 2020) and allergens (Villa et al., 2020) identified at 1% FDR were included in the list of target proteins, which were evaluated further for the presence of potential immunogenic regions. For this, the known linear legume epitopes were retrieved from the Immune Epitope Database and Analysis Resource website (https://www.iedb.org/) and used to create a motif list, which was searched against the target proteins (100% accuracy) through the motif search tool within the CLC Main Workbench v21.0.4.

2.7. Multiple reaction monitoring (MRM) method development

The sequences of the target proteins were imported into Skyline v21.2 and subjected to *in-silico* tryptic digestion (MacLean et al., 2010). The ProteinPilot group file from the combined database searching of the raw sample (cv. Luxor) replicates were used as an input in Skyline to generate a BiblioSpec library. Firstly, for each protein, all the library-matched tryptic peptides (cut-off score: 0.95) with a length between 6 and 30 residues and no unusual modifications (allowing only carbamidomethylation of Cys, oxidation of Met, and pyroglutamate formation from *N*-terminal Gln residues, considering that no substantial fermentation induced changes were noted in the modification patterns of these peptides) were selected. Then for each precursor, the six most intense transitions were picked based on the MS/MS data retrieved from the spectral library and included in a preliminary MRM method. This unscheduled MRM assay was used to experimentally determine the peptide retention times and refine the MRM transition list, wherein a minimum of three intense transitions (threshold 1,000 counts) per peptide with matching elution profiles were retained in the final scheduled MRM method.

The specificity of each of the peptides to the target proteins were evaluated by mapping the peptides to the protein sequences of the combined database, using the motif search tool in CLC Main Workbench v21.0.4 software. The number of protein hits for each MRM peptide (with 100% sequence identity) was evaluated for determining the peptide specificity among the analysed allergenic protein groups.

2.8. Targeted quantitative protein measurement using MRM-mass spectrometry (MRM-MS)

The quantitative data acquisition for the pre-fermented and fermented samples was performed on an Exion AD UHPLC (Eksigent, CA, USA) coupled to a 6500 + QTRAP mass spectrometer (SCIEX, CA, USA) system, with minor modifications to a previously described method (Tahmasian, Juhász, Broadbent, Nye-Wood, Le, & Colgrave, 2022). Briefly, the reconstituted samples (2 μL) were injected onto a Kinetex C18 column (100 × 210 μm, 1.7 μm particle size and 100 Å pore size) and chromatographically separated at a flowrate of 0.4 mL/min by applying the following gradient: 5 to 45% B over a period of 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 (solvent A consisted of aqueous 0.1% FA, and solvent B consisted of aqueous 90% acetonitrile and 0.1% FA). MRM data acquisition was conducted in positive mode by applying a 5500 V ion spray voltage and 500 °C ion source temperature. The cycle time was set at 0.6 s and the MRM transitions were monitored within 60 s of their expected retention time. To monitor the instrument performance throughout the data acquisition, a quality control sample (1 μg/μL bovine serum albumin tryptic digest with 1 pmol/μL iRT peptides) was analysed initially and periodically after five experimental samples.

2.9. Quantitative data processing and statistical analysis

The quantitative data was integrated using Skyline software (MacLean et al., 2010), wherein the peak groups were reviewed manually to ensure correct peak detection, accurate integration boundaries and a signal-to-noise ratio > 5. The peptide peak areas were calculated by summing the XIC areas of the monitored three MRM transitions. The coefficient of variation across the replicates of pre-fermented and fermented samples (n = 4) was examined for technical variability evaluation of the peptide measurements, and those > 15% were excluded from the subsequent data processing.

The heatmap and the corresponding hierarchical cluster analysis (Pearson correlation) of the peptide level data (log_{10} transformed) were generated using Morpheus software (Broad Institute, Cambridge MA, USA; https://software.broadinstitute.org/morpheus/). Pairwise comparison of the sample groups was performed in MetaboAnalyst (version 5.0), wherein the median peptide peak areas (log_{10} transformed and Pareto scaled data) were compared and an unpaired *t* test was applied. The rest of the graphs used to visualise the quantitative data were generated using custom R scripts.

3. Results and discussion

SSF is a widely applied process used to enhance the nutritional, bioactive and functional attributes of food products (Chai, Voo, & Chen, 2020; De Villa, Roasa, Mine, & Tsao, 2021; Olukomaiya, Adiamo, Fernando, Mereddy, Li, & Sultanbawa, 2020). The previous reports on the potency of SSF in effective degradation of allergenic proteins (De Villa et al., 2021; Huang et al., 2019; Pi et al., 2021; Yang et al., 2018), offer opportunities to evaluate the potential of this cost-effective and traditional food processing method in addressing the allergenicity challenges

attributed to several emerging food sources (Kabasser et al., 2022; Mattison, He, Zhang, Dupre, & Lloyd, 2023). Herein, we have applied discovery and targeted quantitative proteomic approches for the extensive evaluation of the effect of SSF (carried out with *R. oligosporus*) on the proteome composition and the allergenic protein profiles of white lupin (cv. Luxor). The findings from this proteomic study can provide new avenues for exploitation of lupin seed as a food ingredient.

3.1. Protein estimation and identification of proteins and peptides from discovery proteome analysis

The protein concentration estimation results (Table S1) using the BCA assay revealed a higher protein concentration for the replicates of the pre-fermented sample (~12.2 mg/mL) compared to the raw white lupin sample extracts (~9.7 mg/mL). This observation can be explained by the compositional changes caused by the processing steps applied in the preparation of this sample, which can lead to degradation of starch or lipids and consequently result in a higher relative protein concentration (dry weight basis). On the other hand, the protein concentration of the fermented sample extracts (~8 mg/mL) was estimated to be lower than the raw and pre-fermented samples. The noted lower protein concentration for the fermented sample can be attributed to the microorganism metabolising the proteins as a source of energy or substrate for growth (Nkhata, Ayua, Kamau, & Shingiro, 2018), such that smaller peptides (with length < 3 amino acids) and single amino acids can be produced which are unable to react with the BCA reagent (biuret reaction) (Shen, 2019).

The combined database searching of the raw, pre-fermented and fermented replicates resulted in 1,561, 1,751 and 2,420 protein identifications at 1% FDR, respectively (Table S1). In addition, all the IDA datasets were subjected to a combined database search which resulted in identification of 3,113 proteins and was used to align the protein identification resulted from the three experimental groups. This qualitative comparison revealed that only 35.3% of the identified proteome was commonly detectable across all samples whilst 40% of the proteome was uniquely identified from the fermented samples (Fig. S1). Furthermore, a high degree of proteome sharedness (76.3%) was found for the raw and pre-fermented lupin samples (Fig. S1). The higher frequency of unique protein identifications from the fermented sample (~98% of which were assigned to *Rhizopus* species) relates to the active growth of the fungi and the proteins produced from this process. To attain functional insights, the proteins yielded exclusively from the fermented sample were subjected to Gene Ontology (GO) over-representation analysis (Supplementary data 2). The most pronounced over-represented biological processes were related to metabolic processes such as cellular nitrogen compound (GO:0034641), small molecule (GO:0044281) and organic substance metabolic (GO:0071704) processes. Additionally, biological processes related to generation of precursor metabolites and energy were noted to be significantly enriched, which included energy derivation by oxidation of organic compounds (GO:0015980), electron transport chain (GO:0022900), cellular respiration (GO:0045333), ATP metabolic process (GO:0046034) and carbohydrate derivative metabolic process (GO:1901135). The other abundant terms included alpha-amino acid (GO:1901606), aromatic amino acid family (GO:0009074) and proteasomal ubiquitin-independent protein (GO:0010499) catabolic processes. Regarding the molecular function, transporter activity (GO:0005215), acyl-CoA dehydrogenase activity (GO:0003995) and flavin adenine dinucleotide binding (GO:0050660) were among the significantly enriched terms. Meanwhile the significantly enriched cellular component terms were associated with intracellular non-membrane-bounded organelle (GO:0043228), intracellular membrane-bounded organelle (GO:0043231), extracellular matrix (GO:0031012) and protein-containing complex (GO:0032991). These results are indicative of the metabolic activity of *R. oligosporus* in the liberation of energy required for growth, proliferation, and biological functions.

3.2. Evaluation of the specificity of proteolytic degradation of white lupin proteins in fermented sample

The digestion specificity summaries provided in the tryptic database search reports (Fig. S2) showed that a higher percentage of peptides with unexpected cleavages (over-cleaved) was identified from the fermented sample (22.1%) in comparison to the pre-fermented sample (8.6%). The fermentation process involves production of many enzymes including proteases, which can subject the proteins to proteolytic degradation (Varzakas, 1998; Zhao, Sun-Waterhouse, Zhao, Zhao, Qiu, & Su, 2018). To obtain knowledge on the substrate specificity of the SSF proteases, the cleavage frequencies observed between each residue pair were determined from the enzyme-free peptide identifications and compared across the pre-fermented and fermented sample replicates (n = 4). The examined digestion frequencies revealed that for the lupin proteins the cleavage rates increased significantly in the fermented sample after valine (average FC = 5.6), leucine (average FC = 5.4), isoleucine (average FC = 6.3) and phenylalanine (average FC = 3.9) hydrophobic residues, wherein the presence of aspartic acid, glutamic acid and arginine on the carboxylic side of these residues negatively affected the preferential cleavage behaviour (Fig. 1). Additionally, across the fermented sample replicates the digestion rates after alanine (average FC = 3.6), methionine (average FC = 2.4), threonine (average FC = 3.6), tyrosine (average FC = 3) and tryptophan (average FC = 2.7) residues were found significantly elevated with a lower level of specificity. The observed cleavage behaviour was similar to that reported for proteinase K, a subtilisin-related serine protease, which exhibits broad specificity in cleaving the carboxylic sides of aliphatic and aromatic hydrophobic residues (Saenger, 2013). Previously, the expression of a serine protease with similar physicochemical properties to subtilisin family was determined during the fermentation of soybean protein-raffinose-phytate liquid medium with different *Rhizopus* species. This was in addition to the characterisation of the well-known *Rhizopus*-pepsin (an aspartic protease) from these culture filtrates (Heskamp & Barz, 1997).

3.3. Allergenic proteins and peptides selection for protein integrity evaluation

The discovery data from the raw white lupin sample (cv. Luxor) was used for development of a targeted MRM quantitative method. This method was applied to compare the abundance of the allergenic proteins between the pre-fermented and fermented lupin samples. The four major seed storage protein families in lupin, α-, β-, δ- and γ-conglutins are known to be associated with lupin sensitisation as well as cross-sensitisation with other legumes (Villa et al., 2020). Overall, 14 conglutin protein sequences were identified at 1% FDR from the raw sample (Table S2), including three α, five β, three δ and three γ protein sequences from their respective conglutin protein families. In addition, two non-specific lipid transfer proteins (nsLTP), two pathogenesis-related (PR)-10 s and three profilin protein sequences — with predicted allergenicity using AllerCatPro 2.0 — were identified from the raw sample (Fig. S3).

The abundance of these 21 putative allergenic proteins were compared across the pre-fermented and fermented lupin samples by monitoring 165 tryptic peptides (Fig. S3), by LC-MRM-MS analysis. The inclusion of several tryptic peptides specific to each allergen family in the targeted quantitative assay enables the comprehensive evaluation of the fermentation induced changes on different regions of these protein sequences. In brief, the changes driven by fermentation in the levels of the α-, β-, δ- and γ-conglutin, nsLTP, PR-10 and profilin proteins were assessed through 56, 63, 10, 20, 9, 3 and 4 specific peptides to these protein categories (Fig. S3).

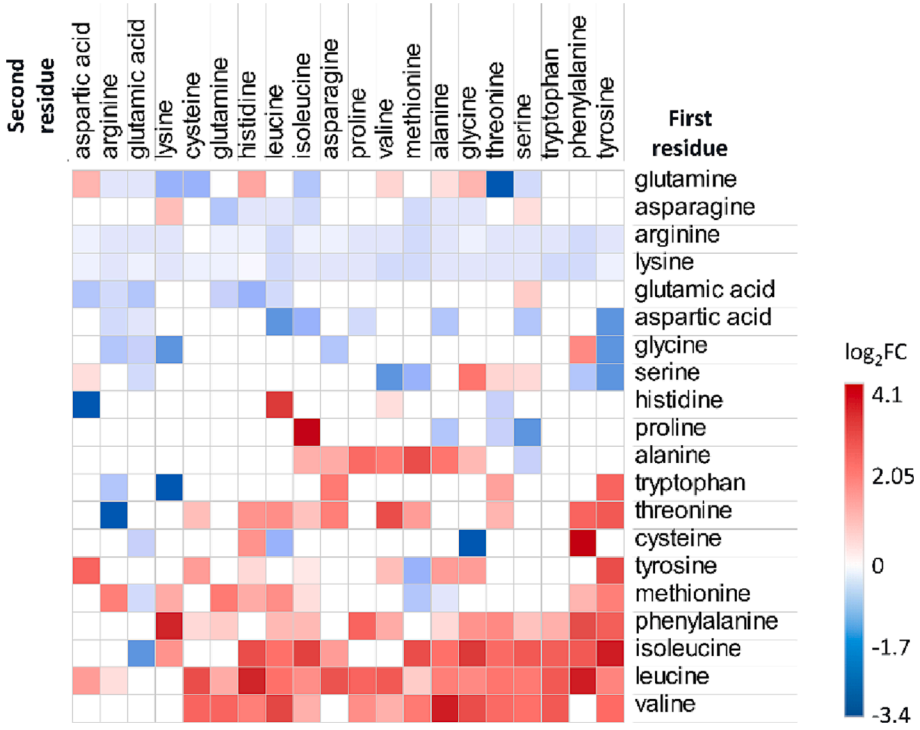

Fig. 1. The digestion specificity evaluation of white lupin proteins across fermented and pre-fermented samples. The cleavage frequencies between any pair of residues (first–second) are presented as a FC calculated from the pairwise comparison of these frequencies across fermented and pre-fermented samples. The heatmap depicts the $\log_2$ transformed FC values, where darker red and darker blue indicate the elevated and reduced cleavage rates in the fermented sample relative to the pre-fermented sample, respectively. For the insignificant changes ($p > 0.05$) a FC equal to one was considered. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Relative quantitative evaluation of the putative allergenic proteins across the pre-fermented and fermented samples

The tryptic peptides specific to each allergen family were monitored prior to- and post-fermentation to discern the changes in the abundance of white lupin allergenic proteins driven by fermentation. The heatmap and the corresponding hierarchical cluster analysis (HCA) of the $\log_{10}$-transformed peptide abundance data demonstrates the stratification of the pre-fermented and fermented sample replicates, where the relative abundance for most of the MRM peptides were measured at lower levels in the final fermented product (Fig. 2). The magnitude of abundance change for the white lupin allergen derived peptides was evaluated by calculating the ratio of the abundances (FC) measured after and before fermentation. Upon applying a *t* test, 96% of the tryptic peptides (159

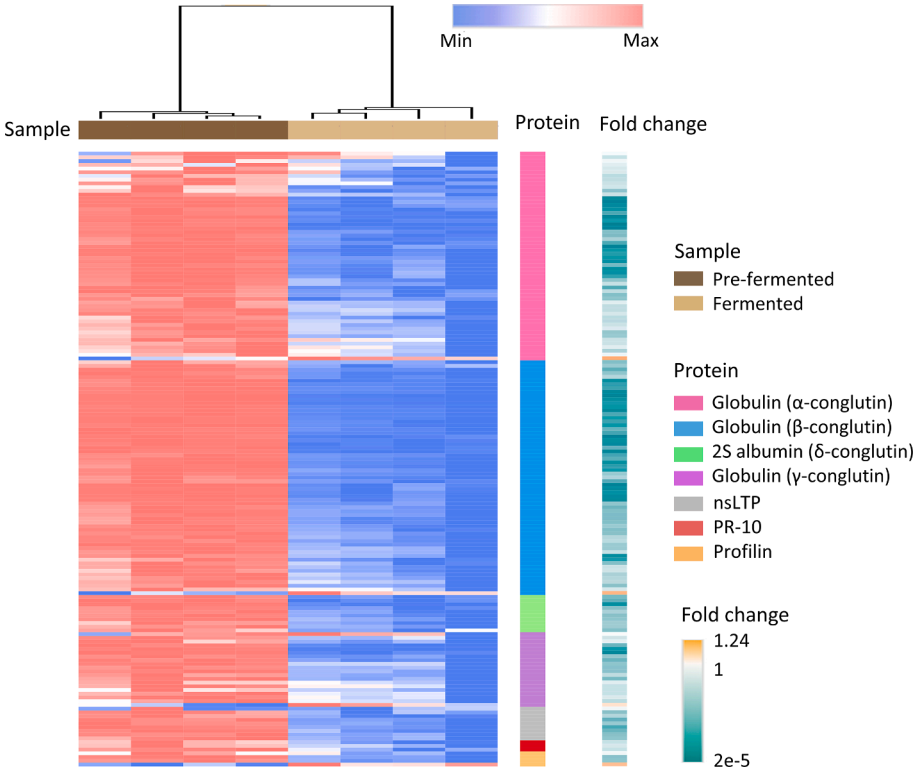

Fig. 2. The heatmap and the unsupervised HCA displaying the relative peak areas of the allergen derived peptides ($\log_{10}$ transformed) and the relationships of the sample groups. Each column corresponds to a sample replicate and each row represents a tryptic peptide, increasing brightness towards red indicates higher and blue indicates lower peptide responses. The protein family column depicts the protein origin of each peptide whilst the FC column indicates the magnitude of abundance change for each tryptic peptide in fermented sample relative to the pre-fermented sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

out of 165 peptides) were found to be significantly different between the fermented and pre-fermented experimental groups. This included 155 tryptic peptides that were measured with lower abundances in the fermented sample relative to the pre-fermented sample. Of these, 83 peptides showed a decrease greater than 50% (FC < 0.5) in the fermented product. This shows the potency of the fermentation process in degrading these peptide regions in the protein sequence, as observed as lower signal for these intact tryptic peptides through LC-MS analysis. From the significantly altered peptides, only four were measured at an elevated level in the fermented sample; however, the increase was less than 24% (FC < 1.24) relative to the pre-fermented product. This increase is possibly caused by SSF induced protein structural changes such as denaturation and/or hydrolysis of the neighbouring regions, which can potentially improve the liberation of these peptides when trypsin is applied.

The distribution of the peptide FCs for each putative allergenic protein group (Fig. 3a) revealed a greater reduction in the peptide levels corresponding to β-conglutin proteins, wherein the relative abundances of ~70% of the monitored tryptic peptides (45 out of 63 peptides) were measured at >50% reduction in signal in the fermented product. Previously, the combination of enzymatic hydrolysis and lactic acid fermentation was described as an effective means to degrade *L. angustifolius* β-conglutin Lup an 1 allergen (UniProt ID: B8Q5G0) (Schlegel et al., 2021). In addition, a greater level of liquid-state fermentation-induced degradation (observed by SDS-PAGE) and reduction in immunoreactivity (observed by sandwich ELISA and Western blot) was reported for the soybean β-conglycinin subunits (Meinlschmidt et al., 2016), which are homologous to lupin β-conglutin proteins (Taylor, Remington, Panda, Goodman, & Baumert, 2015). Tracing the peptide sequences with significant abundance changes on the allergenic protein sequences (Fig. 3b) revealed that the β-conglutin peptides with a greater change (>80% decrease or FC < 0.2) are mainly mapped to the first 200 N-terminal amino acids of these sequences (referred to as mobile *N*-terminal arm). This region — which is the distinctive feature of lupin β-conglutin proteins compared to the other legume vicilins (Jimenez-Lopez, 2020) — is predicted to encompass several immunogenic epitopes based on the *in silico* evaluation of the NLL β-conglutin sequences (Lima-Cabello, Robles-Bolivar, Alché, & Jimenez-Lopez, 2016).

The relative abundances of the α-conglutin peptides were also considerably reduced in the fermented product. As such, 23 out of the 56 monitored peptides were found to be decreased by more than 50% (FC < 0.5). The distribution of the FCs shows that the levels of some α-conglutin peptides were influenced more by the fermentation process (Fig. 3a). The physicochemical property evaluation of the substantially reduced α-conglutin peptides (decreased > 50%) compared to the peptides with a lesser degree of change (decreased < 30%) did not reveal any significant differences in the pI, aromaticity or GRAVY scores of these peptides. Hence, the observed differences might be due to the presence of disulfide bridges in the structure of α-conglutin proteins (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008), which can contribute to higher resistance of some protein regions to proteolytic degradation.

The fermentation changes exerted on the δ-conglutin and nsLTP peptides were notable by a decrease of greater than 40% (FC < 0.6) in the levels of 9 out of 10 (δ-conglutin) and 7 out of 9 (nsLTP) peptides evaluated for these proteins, respectively (Fig. 3a). Since the δ-conglutins are also known to have low digestibility properties (Ogura et al., 2013), the fermentation-driven extensive degradation of these proteins can be expected to positively impact the digestibility of the fermented Lupreme® product.

A lower degree of change was observed for the levels of γ-conglutin peptides post-fermentation (5 out of 20 peptides reduced by more than 50%) (Fig. 3a). Previously, the degradation resistance of white lupin γ-conglutins to multiple proteolytic enzymes has been demonstrated at pH values greater than 4. This resistance has been ascribed to the compact native conformation of these proteins, which makes the amino acid residues inaccessible by proteases (Capraro, Magni, Scarafoni, & Duranti, 2009). In addition, interactions of γ-conglutins with flavonoids have been suggested to be associated to insensitivity of these proteins to enzymatic hydrolysis (Czubiński, Siger, & Lampart-Szczapa, 2016). Finally, the relative abundances of the peptides monitored for the evaluation of PR-10 and profilin proteins were not found to be altered considerably by the commercial SSF; however, the extensive evaluation of these proteins was limited due to the paucity of suitable tryptic peptides for monitoring these proteins.

3.5. Evaluation of the immunogenic regions identified from white lupin putative allergens

To date, attempts have been made to predict conformational and linear epitopes from NLL lupin β-conglutin proteins using bioinformatic-based approaches (Lima-Cabello, Alché, & Jimenez-Lopez, 2019; Lima-Cabello et al., 2016). The conformational epitopes are known to denature easier during food processing treatments, whereas changes in the linear epitopes require manipulation of specific amino acid residues in the protein sequence (Rahaman, Vasiljevic, & Ramchandran, 2016). Herein, the presence of the experimentally confirmed linear legume epitopes retrieved from the IEDB immune epitope database was evaluated in the structure of the target proteins of this study. The *in-silico* epitope mapping analysis allowed the identification of potential immunogenic regions from α- and β-conglutin sequences, which comprise epitopes from Gly m 6 soybean allergen and Gly m 5 soybean and Ara h 1 peanut allergens, respectively. In addition, γ-conglutin regions including epitopes from soybean 7S basic globulin 2 allergen (UniProt ID: Q8RVH5) were discovered. The analysis also evidenced the presence of epitopes from soybean Gly m 4 allergen and peanut Ara h 5 and soybean Gly m 3 and allergens within the PR-10 and profilin protein sequences (Fig. S4), respectively. The alterations in the peak areas of the MRM-targeted peptides encompassing the conserved legume epitopes can be directly used for the quantitative evaluation of the effect of the commercial SSF on the regions of the proteins comprising sequences known to trigger food allergy. As an example, Fig. 4 depicts the 31 peptides used to monitor β-conglutin protein UniProt ID: A0A6AN4Q3 and the known linear epitopes found within this protein sequence. The two peptides, DQQSYESGFSR and NTLEATFNTR, which are specific to the β-conglutin family, include an epitope (FSRNTLE) from peanut Ara h 1 allergen. This epitope is conserved across all the white lupin β-conglutin sequences. These peptides were found at 44% and 28% lower levels in the fermented product, respectively. Similarly, the abundances of β-conglutin specific peptides LLGFGINADENQR and NFLAGSK, which encompass a conserved soybean Gly m 5 allergen epitope (RNFLA), were 93% and 76% lower after fermentation, respectively. In addition, the immunogenic region (Gly m 5 epitope AQPQQ) containing NQQQSYFANAQPQQQQQQQSEK peptide sequence, which is specific to this β-conglutin accession, was present at 85% lower levels in the fermented product. This example together with those included in Fig. S5 for α-conglutin, γ-conglutin, PR-10 and profilin protein sequences indicate the effectiveness of the SSF process used in the production of Lupreme® in disrupting the immune reactive regions, which are also potentially involved in cross-reactivity between lupin, soybean, and peanut.

4. Conclusion

In this study, the SSF driven changes in the proteome composition and levels of putative allergens in white lupin were evaluated by discovery and targeted quantitative LC-MS analyses. The qualitative evaluation of the detected proteins revealed the identification of 1,241 unique sequences (mainly assigned to *Rhizopus* species) from the fermented sample, the functional annotation of which indicated significant enrichment of GO terms mainly related to processes involving energy liberation. Furthermore, the assessment of the peptide identifications

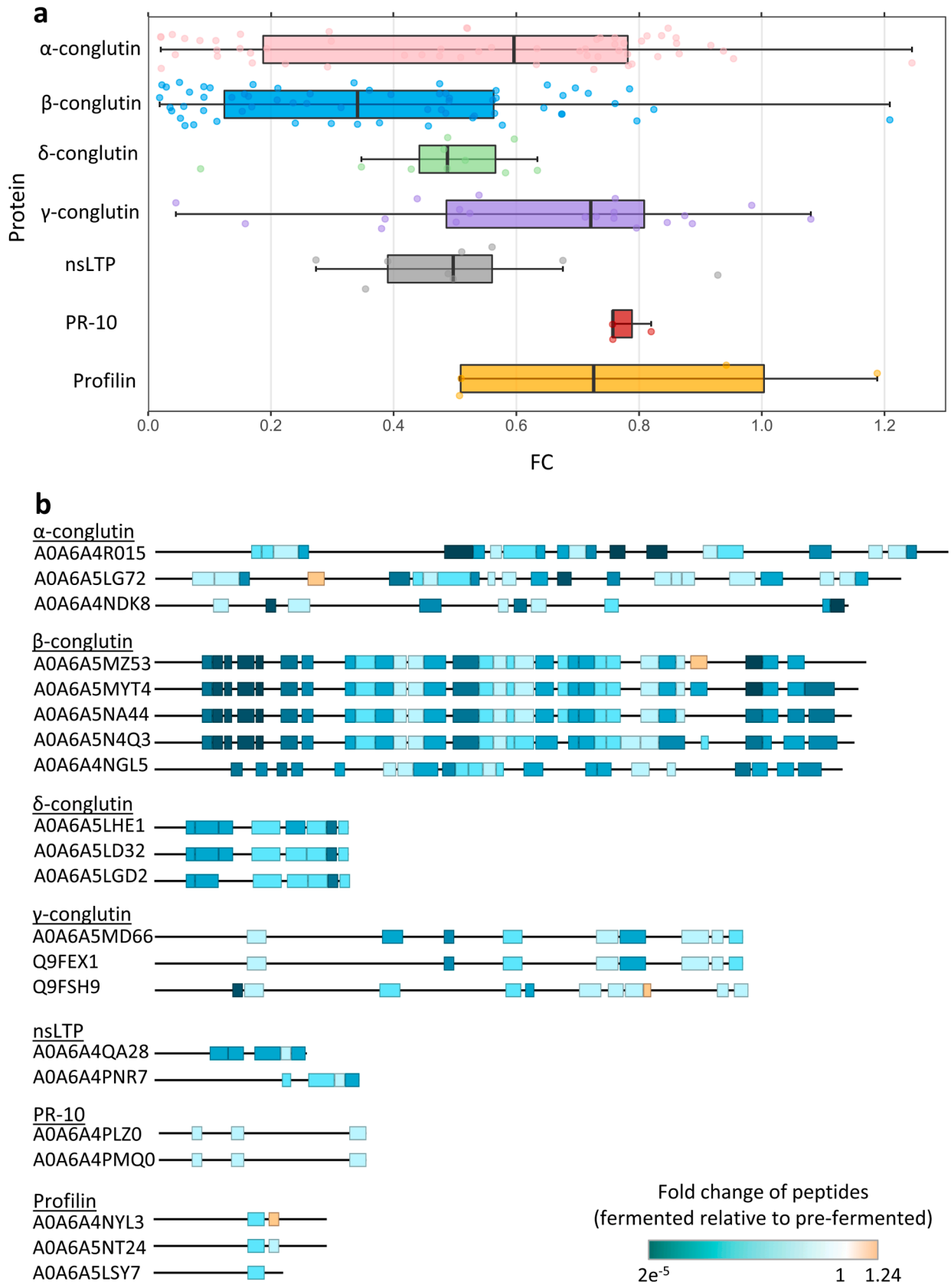

Fig. 3. Evaluation of the level of change in the abundances of the allergen derived peptides in the fermented sample relative to the pre-fermented sample. (a) The distribution of the FCs of the specific peptides monitored for each allergenic protein category. (b) Significantly altered ($p < 0.05$) peptides (coloured blocks) mapped on the target protein sequences, wherein the darker colour indicates tryptic peptide sequences showing a greater decrease in abundance because of SSF.

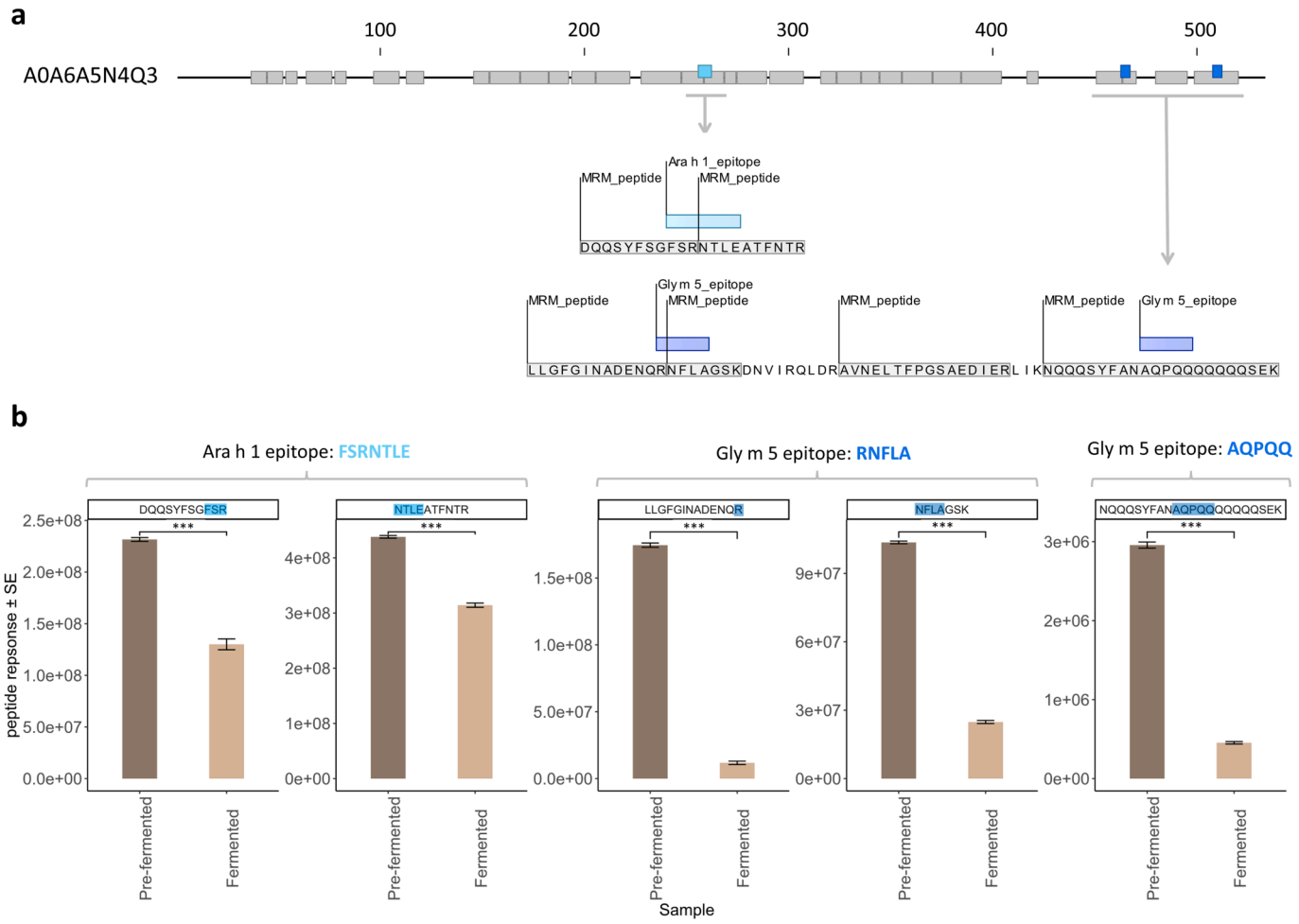

Fig. 4. The evaluation of the potential immunogenic region alterations induced by SSF, for β-conglutin protein with UniProt ID: A0A6AN4Q3. (a) The schematic representation of the MRM peptide sequences (grey blocks) and the known linear epitopes (blue blocks) from peanut Ara h 1 and soybean Gly m 5 allergens mapped on this protein sequence (100% identity). (b) Bar graphs showing the reduction of the peptides spanning the epitope regions comparing the pre-fermented and fermented samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

displayed an increased cleavage rate after the aliphatic and aromatic hydrophobic residues for lupin proteins in the fermented sample. Meanwhile, the comparison of the relative abundances of the allergen derived peptides across the pre-fermented and fermented samples revealed a significant decrease in the levels of 155 out of 165 monitored peptides resulting from commercial SSF process. In this regard, while tryptic peptides from all the examined allergenic protein groups were impacted, the peptide measurements for the lupin major allergens, β-conglutins, were affected more substantially. These findings show that SSF of white lupin seeds with *R. oligosporus* causes partial degradation of the allergenic proteins into peptides or/and constituent fragments. This can lead to alterations in the epitopes and consequently result in a decreased binding efficiency between allergen and IgE. The knowledge acquired through this study, demonstrates the efficiency of SSF for reducing the targets of allergenicity from within white lupin seeds. These findings can potentially be applied to support addressing the food allergy challenges involved with many other emerging protein sources. However, this process may require additional optimisation for the total elimination of the allergenic potential of these sources, which will need validation through clinical studies.

Author contribution

M.C. conceived the design of the study and provided guidance to A.T. through the sample preparation, data acquisition and processing steps; A.T. prepared the proteomics samples, developed the targeted MRM assay, processed the data and drafted the manuscript; R.D. provided research funding, processed the lupin products, provided guidance on the food treatment methods and experimental design; J.B. provided A.T. guidance to design the targeted MRM assay, develop scripts for statistical analysis and visualisation; A.J. provided A.T. guidance on sequence analysis, allergen prediction, epitope mapping and data visualisation; M. N. provided assistance with the sample preparation and data acquisition; All authors reviewed and approved the final version of the manuscript.

CRediT authorship contribution statement

Arineh Tahmasian: Methodology, Investigation, Formal analysis, Software, Visualization, Writing – original draft. **Roger Drew:** Conceptualization, Methodology, Resources, Writing – review & editing. **James A. Broadbent:** Supervision, Methodology, Software, Visualization, Data curation, Writing – review & editing. **Angéla Juhász:** Supervision, Methodology, Software, Visualization, Data curation, Writing – review & editing. **Mitchell Nye-Wood:** Methodology, Investigation, Writing – review & editing. **Michelle L. Colgrave:** Supervision, Conceptualization, Methodology, Visualization, Resources, Writing – review & editing.

Data availability

The datasets presented in this study have been uploaded to the CSIRO Data Access Portal. Data access link: https://doi.org/10.25919/kpss-3d87.

Declaration of Competing 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136622.

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