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Identification of celiac disease-active peptides in gluten-free barley beers by nanoLC-MS/MS

Eleonora Tissen [a,b,c], Sabrina Geisslitz [b], Barbara Maier [b], Katharina Anne Scherf [b,a,*]

[a] Technical University of Munich, TUM School of Life Sciences, Professorship of Food Biopolymer Systems, Lise-Meitner-Str. 34, 85354 Freising, Germany
[b] Leibniz Institute for Food Systems Biology at the Technical University of Munich, Lise-Meitner-Str. 34, 85354 Freising, Germany
[c] Department of Bioactive and Functional Food Chemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Adenauerring 20 a, 76131 Karlsruhe, Germany

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ABSTRACT

Barley beers may be rendered gluten-free by specialized brewing processes. One controversial issue is whether these beers contain residual gluten peptides carrying at least one celiac disease (CeD)-active epitope, because routine methods to assess whether the beer is gluten-free like competitive enzyme-linked immunosorbent assays (ELISA) may fail to detect potentially harmful peptides. We used untargeted nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) to identify CeD-active peptides in 21 gluten-free barley beers and four regular or carbohydrate-reduced beers. While the G12 ELISA confirmed gluten levels below 20 mg/kg in all gluten-free beers, the R5 ELISA detected gluten levels in four beers exceeding this threshold, highlighting method discrepancies. nanoLC-MS/MS identified 44 CeD-active peptides in seven gluten-free and four non-gluten-free beers, of which 17 CeD-active peptides had no ELISA epitope and therefore escape detection. Accurate quantitation of these peptides combined with *in vivo* toxicity assessment will be needed to provide guidance on clinical relevance.

1. Introduction

Gluten, a complex protein mixture found in wheat, barley, and rye, triggers a harmful immune response in individuals with celiac disease (CeD), leading to a range of severe health issues (Ludvigsson et al., 2013). The key driver of the disease is the activation of proinflammatory $CD4^+$ T cells by gluten-derived peptides. These peptides undergo deamidation by tissue transglutaminase and are defined as CeD-active peptides by the presence of at least one immunogenic epitope, typically consisting of a core nine-amino-acid sequence (Chlubnová et al., 2023; Sollid et al., 2020). These CeD-active peptides are presented by antigen-presenting cells via the HLA-DQ2 or HLA-DQ8 heterodimers to $CD4^+$ T cells in the lamina propria. This immune recognition triggers an inflammatory response, ultimately leading to damage of the intestinal mucosa and apoptosis of epithelial cells (Iversen & Sollid, 2023).

As the prevalence of CeD rises, ensuring the safety of gluten-free products is a significant challenge, particularly to accurately detect and quantitate gluten (Singh et al., 2018). Special food processing methods have been developed to partially hydrolyze gluten in wheat-, rye- or barley-based raw materials to render the final product gluten-free (gluten content $\leq$ 20 mg/kg), as laid down in Regulation (EU) No 828/2014 (2014). Other legislation outside the EU only allows "gluten-reduced" labels on products where the production is not based on inherently gluten-free materials (Cubero-Leon et al., 2024). Beer, typically made from barley malt, undergoes fermentation combined with additional physical treatments and/or enzymes that can crosslink or hydrolyze gluten proteins (Kerpes et al., 2017). Gluten-free beers made out of barley are frequently produced using prolyl endopeptidase (PEP), which cleaves at the C-terminus of proline residues (Guerdrum & Bamforth, 2012; Knorr et al., 2016). However, the brewing process together with the enzymatic treatment, may not entirely eliminate all CeD-active peptides in those gluten-free barley beers, and if CeD-active peptides are retained, they may exhibit immunogenic properties that are potentially clinically relevant (Cubero-Leon et al., 2024; Real et al., 2014).

The routine method used to assess regulatory compliance of foods containing fermented or partially hydrolyzed gluten is the R5 competitive enzyme-linked immunosorbent assay (ELISA) (Codex Alimentarius Commission, 2015). Such foods are especially problematic, as routine methods may not detect all CeD-active peptides (Scherf et al., 2021).

* Corresponding author at: Leibniz Institute for Food Systems Biology at the Technical University of Munich, Lise-Meitner-Str. 34, 85354 Freising, Germany.
E-mail address: k.scherf.leibniz-lsb@tum.de (K.A. Scherf).

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Using ELISA alone, the reliable and accurate detection and quantitation of gluten in partially hydrolyzed and fermented foods is challenging (Colgrave et al., 2017; Li et al., 2018). Therefore, complementary methods are needed to thoroughly examine gluten peptides in beer. Several research groups utilized liquid chromatography-tandem mass spectrometry (LC-MS/MS) to evaluate the effectiveness of PEP in breaking down gluten proteins into non-immunogenic peptides in beer (Akeroyd et al., 2016; Colgrave et al., 2017; Decloedt et al., 2024; Fiedler et al., 2018; Knorr et al., 2016; Panda et al., 2015a). However, the results partly conflicted because one study confirmed the degradation of all known gluten epitopes (Akeroyd et al., 2016),whereas another one reported the presence of CeD-active peptides carrying at least one complete epitope in PEP-treated beers (Fiedler et al., 2018). This is why Decloedt et al. (2024) suggested to supplement the ELISA analysis of gluten-free beers with LC-MS/MS analysis to get a more realistic picture of the potential risk for CeD patients due to the presence of residual CeD-active peptides. Further, previous studies have shown that gluten-reduced beer induced responses in sera of active CeD patients (Allred et al., 2017) and stimulation of T-cells from intestinal biopsies, although gluten was removed below the gluten-free threshold (Spada et al., 2020). This indicates that residual CeD-active peptides are still present in these products.

We, therefore, aimed to identify CeD-active peptides in commercially available gluten-free barley beers. The comprehensive proteomics approach by untargeted nanoLC-MS/MS focused on proteolytically resistant CeD-active peptides common to all barley-based beers. We hypothesized that currently used competitive ELISAs overlook important, potentially immunogenic gluten peptides in beers because they miss CeD-active peptides, which can be detected using proteomics approaches.

2. Materials and methods

2.1. Reagents

All chemicals were of analytical or higher grade and purchased from VWR Merck (Darmstadt, Germany), Fisher Scientific (Waltham, MA, USA), Carl Roth (Karlsruhe, Germany), or Sigma-Aldrich (Steinheim, Germany). Sera-Mag Carboxylate-Modified Magnetic Beads, hydrophilic and hydrophobic, were purchased from Cytiva (Marlborough, MA, USA).

Table 1
Sample set of different beers analyzed for the identification of celiac disease-active (CeD) peptides by untargeted nano-liquid chromatography-tandem mass spectrometry. The gluten content was analyzed with competitive R5 (R5c) and competitive G12 (G12c) enzyme-linked immunosorbent assays (ELISA). Values for gluten content ($n = 3$) are given as means ± standard deviation. Total numbers are given for identified peptides, gluten peptides, and peptides with at least one R5 epitope, G12 epitope, or CeD-active epitope.

ID	Type of beer	Grains	Gluten content by R5c ELISA) [mg/kg]	Gluten content by G12c ELISA [mg/kg]	No. of identified peptides	No. of identified gluten peptides	No. of identified peptides with R5 epitope	No. of identified peptides with G12 epitope	No. of identified peptides with CeD-active epitope
A	Rice beer GF	Rice	<LOQ	<LOQ	26	n.d.	n.d.	n.d.	n.d.
B	Light lager GF	Barley	33.1 ± 0.6	2.8 ± 0.5	55	9	4	n.d.	3
C	Lager GF	Barley, maize	13.9 ± 0.1	<LOQ	105	9	n.d.	n.d.	n.d.
D	Lager GF	Barley	14.1 ± 3.3	<LOQ	163	11	n.d.	n.d.	n.d.
E	Lager GF	Barley, rice	15.3 ± 2.1	<LOQ	95	10	n.d.	n.d.	n.d.
F	Lager GF, AF	Barley	14.6 ± 3.3	<LOQ	92	2	n.d.	n.d.	n.d.
G	Pilsner GF	Barley	29.2 ± 0.9	3.1 ± 0.2	231	82	33	3	21
H	Malt beer GF	Barley	27.9 ± 6.8	<LOQ	169	40	14	1	13
I	Ale GF	Barley	10.8 ± 0.6	<LOQ	165	10	n.d.	n.d.	n.d.
J	Ale GF	Barley	18.4 ± 3.2	<LOQ	74	2	n.d.	n.d.	n.d.
K	Ale GF	Barley	14.0 ± 2.6	<LOQ	81	4	n.d.	n.d.	1
L	Wheat beer GF	Barley, wheat	18.0 ± 5.6	<LOQ	155	42	3	n.d.	5
M	Lager GF	Barley, maize	16.4 ± 5.1	<LOQ	83	2	n.d.	n.d.	n.d.
N	Light lager GF	Barley	41.4 ± 2.2	<LOQ	143	15	1	n.d.	3
O	Wheat beer GF, AF	Barley	<LOQ	2.9 ± 0.9	109	28	n.d.	n.d.	n.d.
P	Lager GF, AF	Barley, maize	<LOQ	<LOQ	348	5	n.d.	n.d.	n.d.
Q	Ale GF	Barley	<LOQ	<LOQ	40	n.d.	n.d.	n.d.	n.d.
R	Blond Ale GF	Barley	<LOQ	<LOQ	53	n.d.	n.d.	n.d.	n.d.
S	Blond Ale GF	Barley	<LOQ	<LOQ	51	n.d.	n.d.	n.d.	n.d.
T	Ale GF	Barley	<LOQ	<LOQ	80	5	n.d.	n.d.	n.d.
U	Ale GF	Barley	<LOQ	<LOQ	126	10	n.d.	n.d.	n.d.
V	Lager GF	Barley	<LOQ	<LOQ	66	5	1	n.d.	1
W	Light beer	Barley, rice	70.7 ± 10.4	4.9 ± 1.2	214	110	38	3	32
X	Light lager	Barley	35.7 ± 3.1	3.0 ± 0.3	161	57	16	n.d.	14
Y	Ice beer	Barley	86.4 ± 3.5	3.2 ± 0.5	115	22	8	n.d.	13
Z	Common barley beer	Barley	64.4 ± 7.2	8.2 ± 0.3	251	42	9	2	10

GF: gluten-free; AF: alcohol-free; LOQ: limit of quantitation for the R5c ELISA is 10 mg/kg and for the G12c ELISA, it is 2.5 mg/kg; No.: number; n.d.: not detected.

2.2. Samples

A range of beers (single bottles, one batch) was selected, including different types of malt and brewing styles. They were chosen based on their advertised ingredients (barley malt) and gluten-free claims and purchased from international commercial vendors in 2021. The samples and their corresponding abbreviations are summarized in Table 1. Beer A is a gluten-free beer (negative control) based on the non-gluten-containing grain rice. Beer Z is a regularly brewed common barley beer and was selected as a positive control. Beers B to V are barley-based beers that are labeled gluten-free. Additionally, W and X were chosen as carbohydrate-reduced beers and Y as ice beer (undergoing some freezing during the process) to evaluate differences due to the brewing process. W, X, and Y are not labeled as gluten-free beers. Beers were degassed after purchase and stored in centrifuge tubes at −20 °C until analysis.

2.3. Enzyme-linked immunosorbent assay

The R5 Ridascreen Gliadin competitive ELISA kits (R5c ELISA) were purchased from R-Biopharm (Darmstadt, Germany). The GlutenTox ELISA Competitive G12 kits (G12c ELISA) were purchased from Hygiena (Camarillo, *CA*, USA). Samples were extracted and analyzed in triplicate exactly according to the manufacturers' instructions.

2.4. Single-pot, solid-phase-enhanced sample preparation

Two carboxylate-modified paramagnetic beads (hydrophilic and hydrophobic) were used for single-pot solid-phase-enhanced sample preparation (SP3). Before use, the two types of beads were combined in a ratio of 1:1 (v/v) and rinsed with water. After immobilizing the beads by incubation on a magnetic rack for 2 min, the supernatant was discarded, and beads were reconstituted in water at a concentration of 20 μg solids/μL or 50 μg solids/μL. The prepared bead mixes were stored at 4 °C. The SP3 digest was performed in triplicate for each beer according to the protocol by Sielaff et al. (2017) with slight adjustments. In brief, 50 μL of tris(hydroxymethyl)aminomethane-hydrochloride (TRIS-HCl, 0.5 mol/L, pH 8.5) was added to 250 μL of beer. Reduction and alkylation were performed by adding 35 μL of a combined solution containing tris (2-carboxyethyl)phosphine (TCEP, 0.1 mol/L in 0.5 mol/L TRIS-HCl, pH 8.5) and chloroacetamide (CAA, 0.4 mol/L in 0.5 mol/L TRIS-HCl, pH 8.5) and incubating for 1 h at 37 °C in the dark. The prepared bead mix (1–5 μL) was added to 80 μL of the reduced and alkylated beer to achieve a bead:protein ratio of 1:10. Before, the protein content of the beer samples was determined using the Pierce 660 nm Protein Assay (ThermoFisher Scientific, Darmstadt, Germany) according to the manufacturer's instructions. Detailed information regarding the Pierce Assay can be found in the Supplement (Figure S1). Afterward, acetonitrile was added to a final concentration of 96 % (v/v), and the samples were allowed to settle at 22 °C for 18 min. Subsequently, the beads were immobilized by incubation on a magnetic rack for 2 min. The supernatant was discarded, and the pellet was rinsed twice with acetonitrile, discarding the supernatant as the last step. Chymotrypsin hydrolysis (80–100 μL, enzyme:substrate ratio 1:25, in 0.05 mol/L ammonium bicarbonate, pH 8.0) was performed for 18 h at 37 °C in the dark. After overnight digestion, acetonitrile was added to reach a final concentration of 95 % (v/v). The supernatant was removed after mixing and immobilizing the beads on the magnetic rack. Peptides bound to the beads were eluted using 100–400 μL of 2.5 % (v/v) formic acid in water to achieve a final peptide concentration of approximately 30 ng/μL. The supernatant containing purified peptides was transferred into a fresh tube after immobilizing the beads on the magnetic rack and stored at −20 °C until analysis.

2.5. Liquid chromatography-mass spectrometry

Analysis was performed according to Geisslitz et al. (2022) with very slight modifications. An UltiMate 3000 RSLC nano system (ThermoFisher Scientific) was coupled to a Q Exactive Plus Orbitrap mass spectrometer (ThermoFisher Scientific). Peptide loading onto a trap column (Acclaim PepMap Neo 5 μm C18 300 μm × 5 mm nano-trap column; ThermoFisher Scientific) was performed for 5 min at a flow rate of 8 μL/min using water containing 2 % acetonitrile and 0.1 % formic acid. Subsequently, peptide separation was carried out on an analytical column (bioZen 2.6 μm Peptide XB-C18 Nano column, 250 mm × 0.075 mm, SecurityLink; Phenomenex, Torrance, *CA*, USA) under the following conditions: Solvent A consisted of 0.1 % formic acid in water (v/v); solvent B consisted of 0.1 % formic acid in acetonitrile (v/v). The flow rate was maintained at 300 nL/min at a column temperature of 40 °C using the following gradient: 2 % B from 0 to 5 min, increasing to 5 % B from 5 to 6 min, 5 % to 20 % B from 6 to 45 min, 20 % to 33 % B from 45 to 60 min, 33 % to 100 % B from 60 to 62 min, holding at 100 % B from 62 to 65 min, followed by a decrease to 2 % B from 65 to 66 min, and equilibration at 2 % B until 80 min. The injection volume was adjusted for each sample to achieve a total protein amount of 200 ng per injection volume. The eluate from the analytical column was ionized using a Nanospray Flex Series ion source (ThermoFisher Scientific) in positive electrospray ionization (ESI^+) mode. A source voltage of 1.8 kV at a capillary temperature of 250 °C and an S-lens level of 60 was applied. The Q Exactive Plus mass spectrometer operated in data-dependent acquisition (DDA) mode, selecting the 20 most intense precursor ions from each MS1 full scan for fragmentation with an isolation width of 2.0 *m/z* at 28 % normalized collision energy and default charge state of 2+. MS1 spectra were recorded in the Orbitrap within a mass range of *m/z* 360–1300 at a resolution of 70,000 (at *m/z* 200), with an automatic gain control (AGC) target of 3E6 and a maximum injection time of 50 ms. MS2 spectra were also acquired in the Orbitrap at a resolution of 17,500 for ions with charges ranging from 2+ to 7+, with an AGC target of 1E5, a maximum injection time of 50 ms, and a fixed first mass of *m/z* 140 and dynamic exclusion was set to 15 s. Data acquisition was performed with Xcalibur (ThermoFisher Scientific, version 4.2.47). The MS was calibrated weekly according to the manufacturer's standard external calibration procedure prior to analysis.

2.6. Peptide identification

Peptides were identified with the software MaxQuant (version 2.5.1.0) (Tyanova et al., 2016) by searching the MS raw data directly as input against two FASTA files of the UniProtKB (The UniProt Consortium, 2025) database containing all entries from *Hordeum vulgare* (taxonomy: [4513], 55,751 entries, downloaded November 2022) and *Triticum aestivum* (taxonomy: [4565], 152,266 results, downloaded August 2023). The parameters were set as follows: digestion mode: specific; enzyme: chymotrypsin; maximum missed cleavage sites: 2; variable modifications: oxidation (M), Acetyl (Protein N-term); fixed modification: Carbamidomethyl (C); minimum peptide length: 7; max. peptide mass: 4600 Da; 1 % peptide and protein false discovery rate. Match between runs was activated, and the matching time window was set to 0.4 min. Other parameters were set as default.

2.7. Data analysis & statistics

Data analysis was performed using the MaxQuant-generated peptides.txt output table with Excel. Peptides identified as potential contaminants or with an Andromeda search score of <40 were excluded (Norwig et al., 2024). In addition, peptides had to be identified in at least two of three technical replicates with an intensity greater than 0. The remaining peptides were classified as identified peptides. Afterward, peptides assigned to gliadins, glutenins, or hordeins using the fasta header were classified as identified gluten peptides. A BLAST (basic local alignment search tool, default settings with the UniProtKB reference proteomes and Swiss-Prot as targeted databases) and peptide search in the UniProtKB database was used for peptide sequences with

no fasta header named gliadin, glutenin, or hordein. If the presence of the peptide in gluten proteins was verified, the peptide was added manually to the number of identified gluten peptides. To identify peptides with R5 and G12 epitopes, all identified peptide sequences were filtered for the main epitope sequences recognized by the R5 (QQPFP, QQQFP, LQPFP, and QLPFP) or G12 (QPQLPY, QPQLPF, QPQLPL, and QPQQPY) monoclonal antibodies according to the literature (Kahlenberg et al., 2006; Morón et al., 2008). Furthermore, peptides were filtered to select sequences containing at least one complete CeD-active epitope according to Sollid et al. (2020) and Chlubnová et al. (2023). In the following the CeD-epitopes are named according to Sollid et al. (2020). Hor-, glia-, and glut- are the short terms denoting the gluten protein of origin: hordein (in barley), gliadin and glutenin (in wheat).

For visualization as a heatmap, all intensities of the identified peptides per sample replicate were summed up, and the relative intensity of every single peptide was normalized to the summed total intensity. The mean relative intensity of triplicates was used for heatmap visualization with Origin Pro 2016b (version 9.8.5.212) after log transformation (ln). Visualizing the number of peptides containing at least one CeD-active epitope, R5 epitope, G12 epitope, or sharing more than one epitope was performed with an UpSet Plot (Gadhave et al., 2019). Only peptides from beer samples that contained CeD-active epitopes were included for UpSet Plot visualization (samples B, G, H, K, L, N, V, W, X, Y, Z).

3. Results

3.1. R5 and G12 competitive ELISA

The content of partially hydrolyzed gluten in the 26 degassed beers was measured using the R5c and G12c ELISA kits. The R5c ELISA results showed that while some beers contained no detectable gluten, others exceeded the 20 mg/kg gluten threshold for gluten-free products, as defined by the Codex Alimentarius (Codex Alimentarius Commission, 2015). Among the 21 gluten-free barley beers, the gluten-free rice beer A and the gluten-free barley beers O to V had gluten levels below the limit of quantitation (LOQ: 10 mg/kg). Nine samples had gluten levels below the 20 mg/kg threshold, with values ranging between 10.8 and 18.4 mg/kg. Four gluten-free beers exceeded the 20 mg/kg threshold. The beers B, G, H, and N contained gluten levels of 27.9–41.4 mg/kg, and therefore, they do not meet the criteria for being labeled gluten-free. In addition, the common barley beer Z, the carbohydrate-reduced beers W and X, and the ice beer Y showed the highest gluten contents with 35.7–86.4 mg/kg. In comparison, the G12c ELISA results indicated that all beers had gluten levels below the 20 mg/kg gluten-free threshold. Nineteen of the 21 gluten-free beers had gluten levels below the LOQ (2.5 mg/kg). Beers B, G, W, X, Y and Z that exceeded the threshold with the R5c ELISA showed gluten levels with concentrations ranging from 2.8 to 8.2 mg/kg with the G12c ELISA.

3.2. Identification of gluten peptides with CeD-active epitopes

The total number of identified peptides varied between the different beer samples from 26 to 348 per sample. The lowest number of peptides was identified in the gluten-free rice beer A and the highest in beer P, an alcohol- and gluten-free lager beer made of barley and maize. The identified proteins in beer A were used for a BLAST search in the UniProtKB database (The UniProt Consortium, 2025), revealing a high sequence homology between some barley and wheat proteins and rice proteins. None of the identified peptides were gluten peptides (Table S1), but proteins like enzymes can be shared between different types of grains. For example, the identified UniprotKB accession A0A8I6Y2R8 in beer A, which is an RNA helicase in barley, showed 98.2 % similarity in the BLAST search to A0A0N7KMN9, an RNA helicase in rice. This explains the identification of peptides in beer A, although the proteomics data evaluation focused on barley and wheat peptides.

Twenty-two out of 26 beers contained between two and 110 gluten peptides derived from hordeins, glutenins, or gliadins. No gluten peptides were detected in beers A, Q, R, and S, which was in agreement with ELISA (<LOQ). Among the identified peptides, some contained epitopes recognized by the R5 monoclonal antibody. In ten beers, at least one peptide with R5 epitope was detected. Notably, the gluten-free beers N and V each contained one peptide with an R5 epitope, with beer N showing a gluten content of 41.4 mg/kg, as measured by the R5c ELISA. In beers B, L, X, Y, and Z, three to 16 peptides with R5 epitopes were found. All these beers showed a gluten content close to or above 20 mg/kg in the R5c ELISA. The highest numbers of peptides with R5 epitopes were identified in gluten-free beer G (33) and beer W (38), which also had a gluten content above 20 mg/kg. The other 16 beers had no detectable peptide sequences with R5 epitopes.

In contrast, beers G, W, and Z contained two to three peptides carrying G12 epitopes and a gluten content above the LOQ as determined by the G12c ELISA. Interestingly, beer H contained one peptide with a G12 epitope but showed no quantifiable gluten content in the G12c ELISA. Furthermore, beers B, O, X, and Y exhibited a gluten content of up to 3.2 mg/kg with the G12c ELISA despite having no detectable peptides with G12 epitopes. All other beers showed neither detectable peptides with G12 epitopes nor a quantifiable gluten content in the G12c ELISA.

The identified gluten peptide sequences were also filtered for CeD-active epitopes. Only peptides that contained an exact match of a complete CeD-active epitope were considered. One to 21 peptides with at least one CeD-active epitope were identified in beers B, G, H, K, L, N, and V, with the most identified CeD-active peptides in beer G (Table 1). All these barley-based beers are labeled gluten-free. In all non-gluten-free beers (W to Z), the number of CeD-active peptides ranged from ten to 32. Peptides with complete CeD-active sequences were absent in the remaining 15 gluten-free beers. 3.3 Comparison of peptides with CeD-active epitopes.

Fig. 1 shows the combined results of all beers containing at least one CeD-active peptide (beers B, G, H, K, L, N, V, W, X, Y, Z) in an UpSet Plot (Gadhave et al., 2019). In total, 195 different gluten peptides were identified. 121 gluten peptides contained neither R5, G12, or CeD-active epitopes, while 28 gluten peptides contained at least one R5 epitope. Both CeD-active and R5 epitopes were present in 23 peptides, while 17 peptides were identified with only CeD-active epitopes. Figure S2 shows another version of the UpSet plot with an additional G12 epitope (QPQQPF), since it was previously shown to be slightly recognized by the G12 antibody (Real et al., 2014). While here 48 peptides contained at least one G12 epitope, still 16 peptides were identified with only CeD-active epitopes. Table 2 shows all identified CeD-active peptides with the CeD-active epitopes. In total, 44 CeD-active peptides were identified in seven barley-based gluten-free beers (B, G, H, K, L, N, V) and four non-gluten-free beers (W, X, Y, Z). The identified peptides had lengths between twelve and 31 amino acids. Some peptides only contained one epitope sequence, all HLA-DQ2.5 restricted. The hordein-derived hor-3a epitope PIPQQPQPY occurred in 11 peptides, of which none contained an R5 or G12 epitope. The three peptides derived from glutenins with a glut-L2 or glut-L1 epitope also did not contain an R5 or G12 epitope. These two glut-epitopes only occurred in peptides that were identified in beer L, which is the only gluten-free beer that was brewed with barley and wheat. Nevertheless, gliadin-derived glia-epitopes ascribed to wheat gluten were present in some peptides, although these samples should only be barley-based. This is not unexpected as there is a high sequence homology between barley and wheat gluten, and immunogenicity studies have mainly focused on wheat gluten (Sollid et al., 2020).

All peptides with at least one glia or secalin-derived *sec* epitope also contained the R5 epitope QQPFP. The peptide PQQPQQPFPQPQLPFPQQSEQ with the glia-γ4c epitope was the only peptide with another R5 epitope (QLPFP). A G12 epitope (QPQLPF or QPQQPY) was identified in four peptides with glia and R5 epitope. All

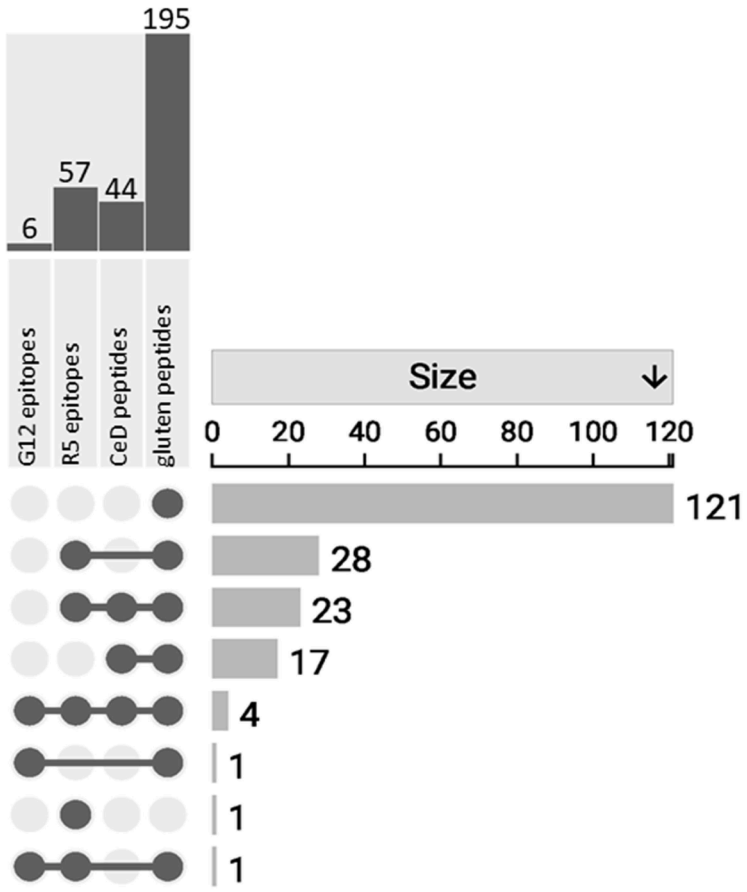

Fig. 1. UpSet Plot with numbers of identified gluten peptides, as well as peptides carrying at least one G12 epitope, R5 epitope or celiac disease-active epitope (CeD). The numbers represent all peptides present in the beers B, G, H, K, L, N, V, W, X, Y, Z that contained at least one peptide with CeD-active epitope identified by untargeted nano-liquid chromatography-tandem mass spectrometry.

glia-γ4c-containing peptides also contained the HLA-DQ8-restricted glia-γ1a epitope, because they share the same epitope sequence.

Fifteen peptides contained more than one CeD-active epitope, some of which overlapped. The peptide PLQPQQPFPQQPQQPFPQQPQQIIF had the most overlapping epitopes (glia-γ4e, glia-γ5, glia-γ4c, sec-3). This peptide was only identified in the two non-gluten-free beers, W and Z.

One peptide (PQQPQPFPQQPIPQQPQPY) was identified in all analyzed beers that contained CeD-active epitopes. In contrast, some peptides were only identified in one of the samples. The peptides PQQPQPFPQQPIPQQPQPYPQQPQPF and PQQPIPQQPQPY with hor-3a epitopes also occurred in four of seven gluten-free beers (G, H, L and N).

Since the work focused on the qualitative identification of peptides, no absolute quantitative statements can be made, only relative comparisons within one sample. For this, the intensities of the single peptides were compared to the summed intensity of all identified peptides within one sample. Fig. 2 shows a heatmap of ln-transformed relative intensities of all identified CeD-active peptides in the analyzed beer samples (beers B, G, H, K, L, N, V, W, X, Y, Z). It should be noted that the heatmap-based representation of relative peptide intensities serves solely as a qualitative visualization of peptide occurrence patterns. Due to the compositional nature of the data and the applied normalization strategy, no conclusions regarding absolute or inter-sample quantitative differences can be drawn from this analysis. Therefore, the comparison of intensities can only be made within one beer, not between different beers. Beers G and W contained the highest total numbers of identified CeD-active peptides, whereby CeD-active peptides had higher relative intensities in beer G than in beer W. Therefore, CeD-active peptides in beer G had a higher impact on the summed total intensity of all identified peptides than the CeD-active peptides in beer W and made up a larger fraction of the analyzed proteome in that beer. This is indicated in the heatmap for beers G and W, where fields for beer G are darker compared to beer W and accordingly have higher intensities. This figure enables, on the one hand, a visualization of CeD-active peptides, which are shared and present in relatively high abundance across different beers, and on the other hand, it internally ranks the CeD-active peptides within one sample. Nevertheless, absolute quantities are needed for the evaluation of the intensities and the resulting gluten content.

4. Discussion

This study identified 44 CeD-active peptides in barley-based beers, of which 29 were present in barley-based gluten-free beers. The commercially available gluten ELISA test kits for analyzing fermented products, such as beer, that may contain gluten peptides from wheat, barley, and rye are based on the recognition of specific epitopes by the R5 or G12 monoclonal antibody (Haas-Lauterbach et al., 2012). Gluten-free beers are produced using either naturally gluten-free cereals such as rice or technical solutions to reduce the level of gluten from the naturally gluten-containing cereals barley and wheat. This includes protein precipitation and/or enzymatic gluten hydrolysis (Watson et al., 2019). Therefore, beer A was expected to be gluten-free, because it was produced solely from naturally gluten-free rice. The higher gluten content in beers W to Z was also expected due to the lack of gluten-free labeling. However, the gluten content of beers B, G, H, and N declared gluten-free was higher than the threshold of 20 mg/kg according to the R5c ELISA and can consequently be a potential and unknown risk when consumed by CeD patients. According to the results of the G12c ELISA, all beers

Table 2
Peptide sequences of identified peptides with at least one celiac disease (CeD)-active epitope according to Sollid et al. (2020) after reversion of deamidation in different beers analyzed with untargeted nano-liquid chromatography-tandem mass spectrometry. Some peptides also contain epitope sequences that are recognized by the R5 (Kahlenberg et al., 2006) and/or G12 monoclonal antibody (Moron et al., 2008).

Peptide sequence	DQ2.5 restricted epitopes	DQ8 restricted epitopes	R5 epitopes	G12 epitopes	Identification in beer sample										
					B	G	H	K	L	N	V	W	X	Y	Z
SQQQQPPFSQQQQSPFSQQQQPPF	glut-L2								x						
SQQQPPFSQQQQPVLPQQPSF	glut-L1								x						
SQQQQPPFSQQQQSPF	glut-L2								x						
QPQQPFPQPQQPFPLQPQQPF	sec-1		QQPFP			x						x			
PLQPQQPFPQQPQQPFPQQPQQIIF	glia-γ4e, glia-γ5, glia-γ4c, sec-3	glia-γ1a	QQPFP (2x)									x			x
PQQPQQPFPQQPQQIIF	glia-γ4c, glia-γ5, sec-3	glia-γ1a	QQPFP									x			
PQQPQQPFPQPQQPF	glia-γ4c, sec-1*, hor −1*, glia-ω1*	glia-γ1a	QQPFP			x	x					x	x	x	
QPQQPFPQPQQPF	sec-1*, hor −1*		QQPFP			x						x	x		
PQQPQQPFPQPQQPFFPW	glia-γ4c, sec-1*, hor −1*, glia-ω1*, glia-ω2	glia-γ1a	QQPFP (2x)			x	x					x			
PQQPQQPQQPFPQPQQPF	glia-γ4c, sec-1*, hor −1*, glia-ω1*	glia-γ1a	QQPFP			x									
PQQQQPFPQPQQPQQPFPQPQQPQLPF	glia-γ4c	glia-γ1a	QQPFP	QPQLPF		x						x			
PQQPQQPFPQQSQQPFPQTQQPQQPF	glia-γ4c	glia-γ1a	QQPFP (2x)									x			
PQQPQQPFPQPQLPFPQQSEQ	glia-γ4c	glia-γ1a	QQPFP, QLPFP	QPQLPF								x			
PQQPQQPFPQTQQPQQPF	glia-γ4c	glia-γ1a	QQPFP											x	
PQQPQQPFPQPPQQPY	glia-γ4c	glia-γ1a	QQPFP		x										
PQQPQQPFPQTQQPKQPF	glia-γ4c	glia-γ1a	QQPFP				x								
PQQPQQPFPQPQQPQQPF	glia-γ4c	glia-γ1a	QQPFP			x	x					x			
PQQPQQPFPQTQQPKQPF	glia-γ4c	glia-γ1a	QQPFP			x						x			x
PQQPQQPFPQQPPF	glia-γ4c	glia-γ1a	QQPFP			x	x					x	x		
PQQPQQPFPQPQLPF	glia-γ4c	glia-γ1a	QQPFP	QPQLPF		x	x					x			x
QPQQPFPQPQEPIPQQPQQPFPQQPQQPF	glia-γ4c	glia-γ1a	QQPFP									x			
PQQPFPQQPQPY	glia-γ5		QQPFP									x	x		x
QPQQPFPQQPQQPFPQY	glia-γ5, glia-γ4c	glia-γ1a	QQPFP (2x)			x						x			
PQQPFPQQPQQPYPQRPQQPF	glia-γ5, glia-γ3	glia-γ1b	QQPFP	QPQQPY											x
PQQPQQPFPQQPPFW	glia-γ4c, glia-γ5	glia-γ1a	QQPFP			x									
PQQPQQPFPQQPQQPF	glia-γ4c, glia-γ5	glia-γ1a	QQPFP											x	
PLQPQQPFPQQPQQ	glia-γ5, glia-γ4e		QQPFP			x						x	x		
QPLPQQQPFPQQPQP	glia-γ5		QQPFP			x	x					x	x		
QPQQPFPQQPQQPF	glia-γ5		QQPFP			x						x			
PQQPFPQQPQQPF	glia-γ5		QQPFP				x								
QPQQPFPQQTIPQQPQPYPQQPQPY	hor-3b		QQPFP									x	x		
SQQPIPQQPQPYPQQPQPF	hor-3a											x			
YPQQPQPFPQQPIPQQPQPY	hor-3a					x						x	x		
PQQPQPYPQQPQPFPQQPIPQQPQPY	hor-3a											x			x
PQQPQPFPQQPIPQQPQPYPQQPQPF	hor-3a					x	x		x	x		x	x	x	x
PQQPQPFPQQPIPQQPQPY	hor-3a				x	x	x	x	x	x	x	x	x	x	x
PQQPIPQQPQPYPQQPQPYPQQPF	hor-3a, hor-3b											x		x	
PQQPIPQQPQPYPQQPQPY	hor-3a, hor-3b						x					x			
PQQPIPQQPQPYPQQPQPFSQQPIPQQPQPY	hor-3a (2x)					x						x			
PQQPIPQQPQPFPQQPQPFPQQPIPQQPQPY	hor-3a (2x)											x			
PQQPIPQQPQPYPQQPQPFPQQPIPQQPQPY	hor-3a (2x)											x			
PQQPIPQQPQPYPQQPQPFPQQPF	hor-3a											x			
PQQPIPQQPQPYPQQPQPF	hor-3a					x	x						x	x	x
PQQPIPQQPQPY	hor-3a				x	x	x			x		x	x		x

* Sequences are identical after reversion of deamidation, the following short terms only denote the proteins of origin. glut: glutenin; *sec*: secalin; hor: hordein; glia: gliadin.

were tested as gluten-free, as their gluten content was below 20 mg/kg. This shows the difference in reactivity of the R5 and G12 antibodies to the remaining gluten peptides in beer. It was already shown before that competitive ELISAs do not reliably detect and quantitate gluten in various wort and beer samples (Panda et al., 2015b). Additionally, the gluten contents in the same samples resulted in lower values with the G12c ELISA than with the R5c ELISA, which agrees with our results. It may be possible that the R5 antibody reacts with non-immunogenic peptides in partially hydrolyzed foods leading to a certain over-estimation of gluten content (Moreno et al., 2016). Thus, it is important to have a closer look at the peptide sequences, as the R5 monoclonal antibody preferably recognizes other peptide sequences (QQPFP, QQQFP, LQPFP, and QLPFP) (Kahlenberg et al., 2006) than the G12 monoclonal antibody (QPQLPY, QPQLPF, QPQLPL, and QPQQPY) (Morón et al., 2008). Both antibodies may also react to further peptide sequences with lower specificities. This shows systematic discrepancies between both methods and the need for more accurate non-immunoassay-based testing methods to evaluate the remaining gluten peptides in beer. Earlier work by Comino et al. (2013) has already demonstrated the need for combining LC- and MS/MS-based methods with immunoassays to detect CeD-active peptides in a wide range of beers.

The use of untargeted nanoLC-MS/MS allows the identification of peptide sequences present in beer and, subsequently, the presence of CeD-active epitopes to evaluate their immunoreactive potential. Based on the guidelines for risk assessment of allergenicity by the European Food Safety Authority, all identified peptides with a complete CeD-active epitope sequence were considered to be hazardous to CeD

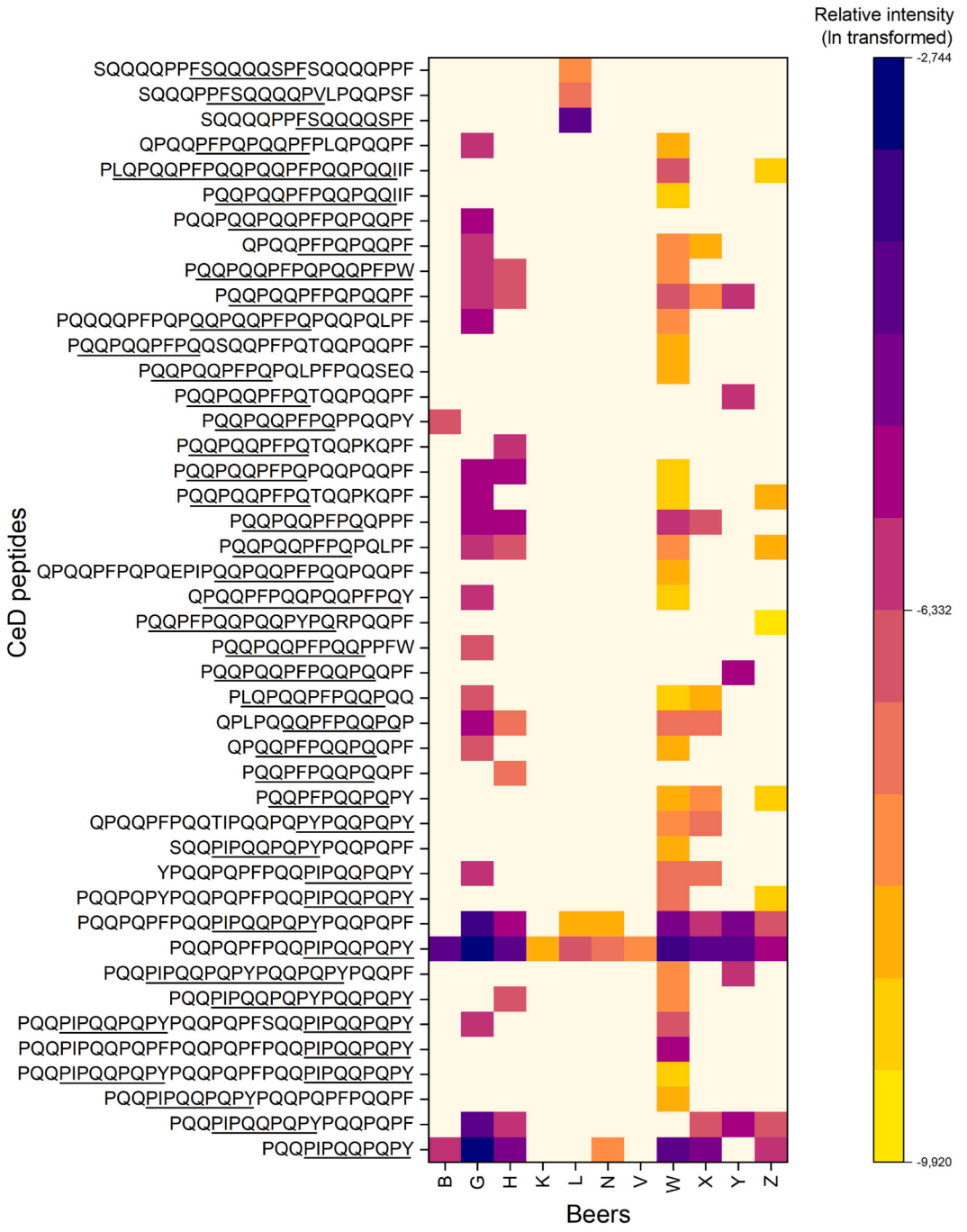

Fig. 2. Relative intensity of 44 peptides with at least one celiac disease-active (CeD) epitope in beers based on untargeted nano-liquid chromatography-tandem mass spectrometry analysis. CeD-active epitope sequences are underlined. The intensities of identified CeD peptides in one beer were normalized to the summed intensity of all identified peptides in one replicate of the beer, and mean values of all three normalized replicates were ln-transformed.

patients (Naegeli et al., 2017). We identified complete CeD-active epitopes in different gluten-free barley beers, which can, therefore, be classified as hazardous, as the immune system of CeD patients can recognize these peptides. Flanking residues that extend out of the HLA-DQ2/8 binding groove may also be necessary for binding (Sollid et al., 2020, 2012), but were not considered in this work. Nye-Wood et al. (2023) additionally identified peptides with CeD-active epitopes in three low-gluten beers that were processed based on silica and filtering steps to remove gluten. Other studies also evaluated this for PEP-treated beers compared to untreated reference beers (Colgrave et al., 2017; Decloedt et al., 2024; Watson et al., 2021). They detected peptides with incomplete CeD-active epitopes in the PEP-treated gluten-free beer and considered partial CeD-active sequences as potentially hazardous. For our work, we have explicitly focused on complete CeD-active sequences since their hazard for triggering immune responses is known and these were also shown to be present in PEP-treated gluten-free beers by others (Fiedler et al., 2018; Spada et al., 2020). Their work identified peptide sequences containing the glut-L1, glut-L2, glia-γ5, and hor-1 epitopes, which agrees with our findings. This broader epitope coverage is also supported by T cell–based studies. Tye-Din et al. (2010) demonstrated that although α-gliadin-derived peptides are immunodominant after wheat challenge, oral challenge with a mixture of wheat, barley, and rye predominantly induced T cell responses against ω-gliadin/-C-hordein-derived peptides with the glia-ω1 and glia-ω2 epitopes, which

were also present in our study. This suggests that immunodominance is not restricted to α-gliadins but extends to other prolamins, particularly hordeins, when multiple cereals are present. This concept is further supported by recent efforts to rank gluten-derived T cell epitopes according to their immunological relevance by integrating multiple steps of the CeD pathogenic pathway (Vriz et al., 2021). Using such an integrative approach, α-gliadins were ranked as most relevant, followed by ω-gliadins and hordeins, whereas γ-gliadins, avenins, secalins, and glutenins showed lower or comparable relevance. Importantly, this work highlighted that epitope characteristics, such as proline content and the contribution of flanking residues to HLA binding, strongly influence immunogenic potential, emphasizing that clinically relevant immunogenicity cannot be inferred from protein class alone. These findings underline that the immunogenic relevance of gluten peptides is driven by their epitope sequence rather than their protein class of origin, supporting the peptide-centric strategy applied in the present study. However, without quantitative information on peptide concentrations, the immunogenic potential of the detected CeD-active peptides cannot be linked to clinically relevant exposure levels. Future research should therefore focus on the generation of quantitative data and on subsequent biological assessments, such as *in vitro* or *in vivo* studies, to evaluate their potential clinical relevance for CeD patients.

Furthermore, peptide sequences in the different studies are not always similar, highlighting the complexity of partially hydrolyzed gluten and indicating the variation resulting from using different beers, brewing processes, sample preparation, and analysis methods. A lot of research on immunogenic peptides in cereals has been done by DDA based LC-MS/MS methods, where the most abundant ions from the eluting samples are selected for fragmentation and following analysis (Alves et al., 2019). This could potentially lead to a loss of information, as not all ions present in a sample are detected. In approaches using data independent acquisition (DIA), there is no previous selection of precursors or threshold of ion intensities, which also makes the data and data evaluation more complex. Current progress in combining different MS-based methodologies could additionally result in a more comprehensive and reproducible proteomic profiling and should be considered in the future as promising approach (Bromilow et al., 2017b; Victorio et al., 2018).

Variability among different brewing replicates was also observed in the analysis of peptides in wheat-gluten-incurred sorghum beer treated with PEP (Fiedler et al., 2018). This would change the profile of CeD-active peptides from lot to lot, making it more challenging to interpret the hazard of the analyzed sample. The variety of released peptides is influenced by both the nature of the raw materials and the brewing process. The necessary digestions for bottom-up untargeted proteomics analyses with enzymes like chymotrypsin and trypsin also influence the outcome of identified peptides (Colgrave et al., 2017). Especially when the suitability of a brewing process to fully hydrolyze all CeD-active peptides should be evaluated, a more comprehensive approach with and without additional enzymatic treatment during proteomics sample preparation could be feasible. Potential hydrolysis of immunogenic epitopes through chymotryptic or tryptic cleavage would thus be ruled out. The direct analysis of peptides present in the beer without chymotryptic hydrolysis could provide additional information about the frequency and efficiency of gluten degradation, as shown in previous studies (Colgrave et al., 2017, 2014). Due to intellectual property restrictions, we do not have information on the gluten-free brewing methods used on all of our samples. As a result, a systematic evaluation of specific methods used for gluten degradation or the efficiency of enzymatic hydrolysis was not possible, representing a limitation of the present study. Future research should therefore focus on controlled studies using well-defined brewing protocols to elucidate how specific processing parameters influence peptide profiles and to identify best practices for eliminating residual CeD-active peptides. At the same time, the data generated in this study highlight this gap in knowledge and provide a relevant basis for future targeted research, in which defined processing conditions can be systematically linked to the occurrence and absolute quantities of CeD-active peptides.

Furthermore, additional “unknown” CeD-active epitopes may be present, but their CeD-immunostimulatory effect has not yet been confirmed (Chlubnová et al., 2023). The identification of further CeD-active epitopes, particularly those specific to barley, therefore represents an important direction for future research to enable a more comprehensive assessment of gluten-derived peptides in beer. Another limitation relates to the protein databases used for peptide identification. The correct and complete detection of peptides requires a well-curated database, because the quality of the protein database influences the reliability of peptide and protein identifications. Although a curated genome is available for barley (The International Barley Genome Sequencing Consortium, 2012), the protein databases often lack comprehensive coverage or consistent annotation of highly repetitive and polymorphic gluten protein sequences (Daly et al., 2020). Future efforts should therefore focus on improving database completeness and annotation quality to reduce redundancy and misannotation for proteins, as risk evaluation requires a complete identification of CeD-active peptides (Bromilow et al., 2017a). Additionally, the *in vivo* effect of the identified CeD-active peptides on CeD patients is unclear, as their quantities, as well as further degradation in the human gastrointestinal tract, bioaccessibility, and bioactivity need to be considered as well.

Remarkably, 17 of 44 CeD-active peptides identified in all beers did not contain a recognition epitope of the R5c or G12c ELISA and would thus not be detected by these ELISAs. As expected, the gluten content determined by competitive ELISA in a beer was also not directly related to the number of identified CeD sequences. Beyond epitope presence or absence, antibody-based assays are additionally influenced by epitope-dependent binding affinities. Quantitative ELISA responses therefore reflect not only peptide abundance but also antibody specificity and affinity, commonly expressed as IC_{50} values determined for individual epitopes using synthetic reference peptides (Real et al., 2014). However, such IC_{50} values are assay- and antibody-specific and are typically established under defined conditions that do not reflect the complexity of partially hydrolyzed food matrices. Consequently, in this study, ELISA results were interpreted in the context of regulatory threshold assessment rather than epitope-level quantitative affinity differences. Previous studies have also shown discrepancies between ELISA and LC-MS/MS results, where especially hordein contents were explored (Colgrave et al., 2014; Nye-Wood et al., 2023; Tanner et al., 2013). LC-MS-based approaches offer several key advantages over immunoassays, particularly in the high-throughput analysis of complex and processed samples. They enable the simultaneous detection and differentiation of multiple peptides, including modified forms, with high sensitivity and specificity. Furthermore, MS allows for both targeted and untargeted approaches, making it suitable for a wide range of analytical objectives. The flexibility in LC-MS/MS configurations and detection strategies further enhances its versatility and adaptability to diverse sample types, including those subjected to extensive processing (Alves et al., 2019). Nevertheless, ELISA remains advantageous for rapid, cost-effective, and high-throughput screening, particularly when routine monitoring of gluten content is required. However, its dependence on antibody specificity limits its applicability to partially hydrolyzed gluten peptides, where LC-MS/MS provides the necessary resolution and analytical power. Therefore, the results of our study clearly demonstrate that commercially available gluten-free barley beers contain gluten peptides that are potentially hazardous for CeD patients and would be overlooked in ELISA analysis. We show that untargeted LC-MS/MS can be used to overcome current challenges in immunoassays-based gluten analysis. The methods established in this work were further used to identify proteolytically resistant CeD-active peptides in more samples containing partially hydrolyzed gluten (Tissen et al., 2026). The combined results of such identification could be further assessed by T cell proliferation assays with synthetic peptides or western blot analysis with sera of CeD

patients (Picariello et al., 2012). Further work based on the identified CeD-active peptides from this work was performed by developing a targeted LC-MS/MS method for the accurate quantitation of selected peptides (Tissen et al., 2025). In this way, untargeted MS serves as a discovery tool to identify relevant proteolytically resistant peptides, while targeted MS enables precise quantitation and monitoring. At this point, the qualitative identification of CeD-active peptides alone does not allow any risk assessment and cannot be used to estimate the clinical relevance to CeD patients. To achieve this, these results need to be complemented with quantitative data as well as *in vitro* and *in vivo* toxicity assessments.

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Data availability

The mass spectrometry untargeted proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD059719.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the first author used Microsoft 365 Copilot Chat in order to analyze paragraphs for refinement of phrasing, clarity, and consistency. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Ethical statement

This article does not contain any studies with human or animal subjects.

Supporting information description

Overview of peptides identified in beer A (Table S1). Calibration curve for protein determination by Pierce Assay (Figure S1). UpSet Plot with the numbers of identified peptides (Figure S2).

CRediT authorship contribution statement

Eleonora Tissen: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sabrina Geisslitz:** Writing – review & editing, Supervision, Conceptualization. **Barbara Maier:** Writing – review & editing, Supervision, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2026.101952.

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