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Effect of sourdough fermentation of wheat flours on quantities and pro-inflammatory bioactivities of amylase/trypsin-inhibitors

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1. Introduction

Wheat is a vital staple food for human nutrition that provides energy, carbohydrates, dietary fiber, proteins, vitamins and minerals (Shewry, 2009; Wieser et al., 2023). However, the consumption of wheat products may also cause wheat-related disorders, including celiac disease, wheat allergy and non-celiac wheat sensitivity (NCWS), which together may affect up to 15% of wheat consuming populations (Catassi et al., 2017). NCWS describes inflammatory reactions to (non-gluten) wheat proteins that were recently defined as a wheat induced type 2 allergy detectable as intestinal barrier defect using diagnostic confocal laser endomicroscopy (Fritscher-Ravens et al., 2014; Fritscher-Ravens et al., 2019; Gjini et al., 2023) and a major trigger of irritable bowel syndrome, and as ATI (wheat amylase/trypsin-inhibitor) sensitivity. While type 2 allergy causes clinically delayed intestinal symptoms, ATIs have been identified as activators of the Toll-like receptor 4 (TLR4) complex expressed on monocytes, macrophages and dendritic cells of the intestinal mucosa (Junker et al., 2012). ATIs are a family of 17 structurally related non-gluten wheat proteins that are highly resistant to gastrointestinal peptidases and temperatures used for most food products made of wheat flours. Importantly, ingested wheat or purified ATIs promote intestinal and extra-intestinal inflammation in several mouse models of inflammatory, metabolic, degenerative and especially autoimmune diseases (Bellinghausen et al., 2019; Caminero et al., 2019; Junker et al., 2012; Pickert et al., 2020; Zevallos et al., 2017; Zevallos et al., 2019; Zevallos et al., 2023). These findings were recently also confirmed in controlled clinical studies of patients with familial Mediterranean fever, ulcerative colitis and primary sclerosing cholangitis, multiple sclerosis and metabolic dysfunction associated steatotic liver disease (MASLD) (Armandi et al., 2024; Carroccio et al., 2020; Engel et al., 2023; Liwinski et al., 2023). ATIs that contain 120–154 amino acids exist as several subspecies, such as mono-, di- and tetramers, the latter belonging to chloroform-methanol soluble (CM) ATIs (Altenbach et al., 2011). ATIs 0.19 and 0.53 are usually dimeric, while monomeric ATIs belong to the 0.28 subspecies. The secondary structure of wheat ATIs is dominated by five intramolecular disulfide bonds that endow them with high stability, and of stretches of alpha helices and beta barrels. ATIs play a role in pest resistance and possibly in wheat

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maturation (Ryan, 1990). Apart from their more recently discovered TLR4 activating properties, ATIs CM3, CM16 and 0.28 have been identified as inhalative allergens in Baker's asthma (Sanchez-Monge et al., 1992; Tundo et al., 2018).

Sourdough fermentation is used to improve taste, flavor and quality of bread, prolongs shelf life, results in higher loaf volume, and improves texture and nutritional quality. Sourdough bread consumption in rats improved glucose and insulin regulation, mineral absorption, and lipid profiles (Gil-Cardoso et al., 2021). In contrast to straight dough processes with added baker's yeast, sourdough fermentation degraded CM3 ATI (Huang et al., 2020) and reduced total ATI content by up to 41%, as measured via HPLC (Boakye et al., 2022). However, these methods cannot demonstrate loss of ATI secondary structure and of TLR4-stimulating bioactivity. In contrast, Western blotting to show the disappearance of intact ATI proteins is a more reliable but still insufficient surrogate of a loss of immunogenicity. Here, we compared a broad range of sourdough fermentations using different wheat flours to evaluate their effectiveness in reducing ATI-induced TLR4-stimulating bioactivity, and employed objective measures, including ATI-subspecies–specific Western blotting, to rigorously assess ATI degradation.

2. Results

Eight different types of sourdough and one chemically acidified control with the use of starter cultures were prepared with different wheat varieties such as spelt, emmer and common bread wheat (Table S1). The impact of fermentation of wheat flours was characterized for the content of specific ATIs using mass spectrometry (LC-MS/MS) and Western blotting (WB), and correlated with TLR4 stimulating ATI bioactivity. General protein composition was assessed using qualitative and semi-quantitative SDS-PAGE, quantitative RP-HPLC and LC-MS/MS.

2.1. Protein fractions and types during sourdough fermentation

Albumin/globulin, gliadin and glutenin fractions were quantified via RP-HPLC as shown in Fig. 1 and Fig. S1. Wheat, spelt, and emmer protein fractions as a sum each remained stable (≤8% variation) (Table S2). Emmer contained more gliadins and fewer glutenins than wheat/spelt (Fig. 1A). Protein proportions were unchanged in chemically acidified dough (Fig. 1B). Relative proportions of albumins/globulins increased (37–65% at 24 h, 72–99% at 48 h, 92–118% at 72 h), while the proportion of gliadins/glutenins decreased. Wheat glutenins were reduced by up to 66% in (BRS) and 59% (ML), gliadins by 14% and 5%, respectively. After 72 h, reductions of gliadins and glutenins in spelt were 20% and 51%, and in emmer 33% and 17%, respectively (Fig. 1A).

Among gliadins, the relative proportion of ω5-gliadins increased by 69%, and that of γ-gliadins decreased by 70% in wheat), while α- and ω1,2-gliadin shifts were inconsistent. High-molecular-weight (HMW) and low-molecular-weight (LMW) glutenin subunits (GS) decreased by 69% in wheat, by 57% in spelt, and only by 21% in emmer. Shorter fermentation (≤30 h) in three different sourdoughs (*Fl. sanfranciscensis 1/2*, *L. pontis*, and *M. humilis*) led to less pronounced changes but followed the same pattern of an increase of albumins/globulins and a decrease of γ-gliadins, LMW- and HMW-GS (Fig. 1G-J). This confirms that multi-strain lactic acid bacteria (LAB) and yeast might promote more effective protein degradation than single strains. At 0 h, doughs with Böcker and Mailänder sourdough starters, both containing bacteria and yeast, matched their unfermented flours (Figs. 2C–F). Changes of sourdough protein composition became obvious after 7 h or 18 h of fermentation (BRS and ML sourdough, respectively).

2.2. Protein profiles during sourdough fermentation

2.2.1. Albumins/globulins

SDS-PAGE analysis under reduced conditions of albumin/globulin fractions in wheat, spelt, and emmer flours showed four major bands at 54, 31, 27, and 14 kDa which served as crude markers of overall protein degradation (Fig. S2A). Spelt and emmer had an additional 37 kDa band, weakly visible in wheat, while wheat and spelt had a unique 13 kDa band absent in emmer. The chemically acidified control dough retained the wheat flour band pattern with no changes over time.

Sourdough fermentation altered band patterns depending on microorganism type, fermentation time, and flour type (Fig. S2B–E). All wheat sourdoughs showed the 54 kDa band disappearing, while 27 and 13 kDa bands intensified. The 31 kDa band that contain farinins and other proteins stabilized by intramolecular disulfide bonds remained unchanged across wheat, spelt, and emmer. The 13 kDa band shows the ATIs (Huang et al., 2020).

2.2.2. Gliadins

SDS-PAGE of gliadin fractions showed expected patterns, with 62 and 75 kDa bands (ω5- and ω1,2-gliadins) and multiple α- and γ-gliadin bands between 30 and 45 kDa (Fig. S3A). Emmer and spelt exhibited similar patterns but with variations in ω-gliadins. The chemically acidified control dough showed no changes over time.

Sourdough fermentation caused gliadin band blurring, indicating protein solubilization and partial hydrolysis (Fig. S3B–E) (55, 56). Wheat sourdoughs fermented with BRS and ML sourdoughs showed more pronounced changes (Fig. S3B) than those with single LAB or yeast strains (Fig. S3D, E). Emmer and spelt sourdoughs exhibited more degradation, with ω-gliadins disappearing after 18 h and α- and γ-gliadins showing partial breakdown (Fig. S3C).

2.2.3. Glutenins

The glutenin fraction showed HMW-GS bands (85–125 kDa) and LMW-GS bands (30–50 kDa) (Fig. S4A). The chemically acidified control dough showed no changes over time. Sourdough fermentation resulted in increasing HMW-GS and LMW-GS degradation over time (Fig. S4B–E), more pronounced than in the albumin/globulin and gliadin fractions. BRS and ML sourdough (Fig. S4A) degraded glutenins more effectively within 18 h compared to single LAB or yeast fermentation (Fig. S4 D, E).

LC-MS/MS Detectable Changes of Amylase/Trypsin Inhibitor (ATI) Signatures During Sourdough Fermentation.

ATI quantification by LC-MS/MS used 20 marker peptides as described in Methods and by Jahn et al. (Jahn et al., 2023). The pattern of ATI species in tetraploid emmer differed from hexaploid wheat including spelt (Fig. 2A). Emmer had a higher proportion of CM2, CM3, and CM16 (74%) than 0.19, 0.28, and 0.53 (19%), whereas wheat and spelt had a more balanced distribution (40% and 39% vs. 50% and 63%). Minor ATIs (WASI, CMX1/2/3, WCI, WTI) comprised 10% in wheat, 7% in emmer, and 5% in spelt. Fermentation and microbial type had no impact on ATI distribution, except for a minor shift in emmer for BRS sourdough at 72 h (CM3 downregulated 5%, CM16 upregulated 5%) (Fig. 2F).

The total ATI content (sum of 0.19, 0.28, 0.53, CM1–CM17, WASI, CMX1/2/3, WCI, WTI) was highest in spelt (6.9 mg/g), followed by hexaploid wheat (5.2 mg/g) and tetraploid emmer (4.5 mg/g) (Fig. S5A). ATI levels in sourdoughs decreased slightly in spelt (5.7–6.3 mg/g) and emmer (3.9–4.2 mg/g), with the lowest levels at the longest fermentation times (spelt: 24 h, emmer: 72 h) (Fig. S5B). In wheat, ATI remained stable with BRS and increased slightly with ML sourdoughs. LAB- or *M. humilis*-fermented wheat sourdoughs showed minimal changes, with significant differences for *Fl. sanfranciscensis 1* (18 h, 24 h) and L. *pontis* (30*h*) (Fig. S5C). The control and *M. humilis* doughs remained constant, except for *M. humilis* (slight increase) at 18 h.

2.3. Impact of fermentation on the TLR4 activation potential of amylase-trypsin inhibitors

ATI bioactivity was determined in eight different types of sourdough and one chemically acidified control after quantitative extraction and

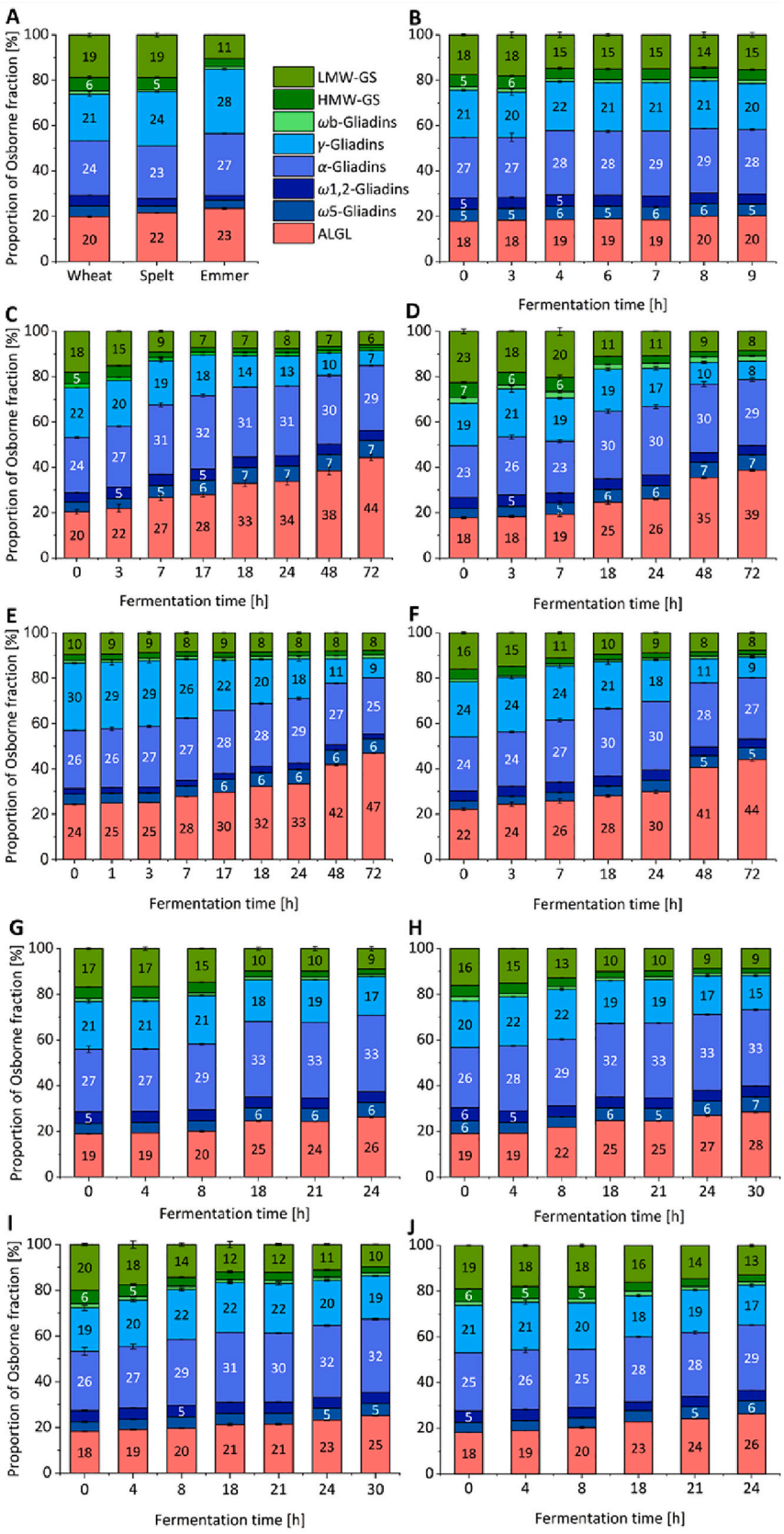

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Fig. 1. Protein composition of modern hexaploid wheat, spelt and emmer flours before and after sourdough fermentations between 1 and 72 h. The Osborne fractions (albumin/globulin, gliadin, and glutenin) were extracted from the flours and sourdough samples stepwise with salt solution (albumins/globulins), 60% ethanol (gliadins), and a glutenin extraction solution (50% 2-propanol, TRIS, urea, DTT) as described by Wieser et al. (Wieser et al., 1998). The fractions were then filtered (0.45 μm) and separated using a SIL-40C HPLC system with a YMC Triart Bio C18 column. Separation was achieved with a gradient of trifluoroacetic acid (TFA) in water and acetonitrile, with detection at 210 nm. Quantitation was performed via external calibration using PWG-gliadin (2.5 mg/mL) as described by the Prolamin Working Group. Wheat, spelt and emmer flours (A), chemically acidified control (B), wheat sourdough with Böcker Reinzuchtsauerteig starter (W + BRS) (C), wheat sourdough with Mailänder "Le Chef" starter (W + ML) (D), spelt sourdough with BRS (S + BRS) (E), emmer sourdough with BRS (E + BRS) (F), wheat sourdough with *Fructilactobacillus sanfranciscensis 1* (W + FlS1) (G) and with *Fructilactobacillus sanfranciscensis 2* (W + FlS2) (H) and wheat sourdough with *Limosilactobacillus pontis* (W + LP) (I) and with *Maudiozyma humilis* (W + MH) (J). LMW-GS: low-molecular-weight glutenin subunit, HMW-GS: high-molecular-weight glutenin subunit, ωb-gliadins: glutenin-bound ω-gliadins, ALGL: albumins and globulins.

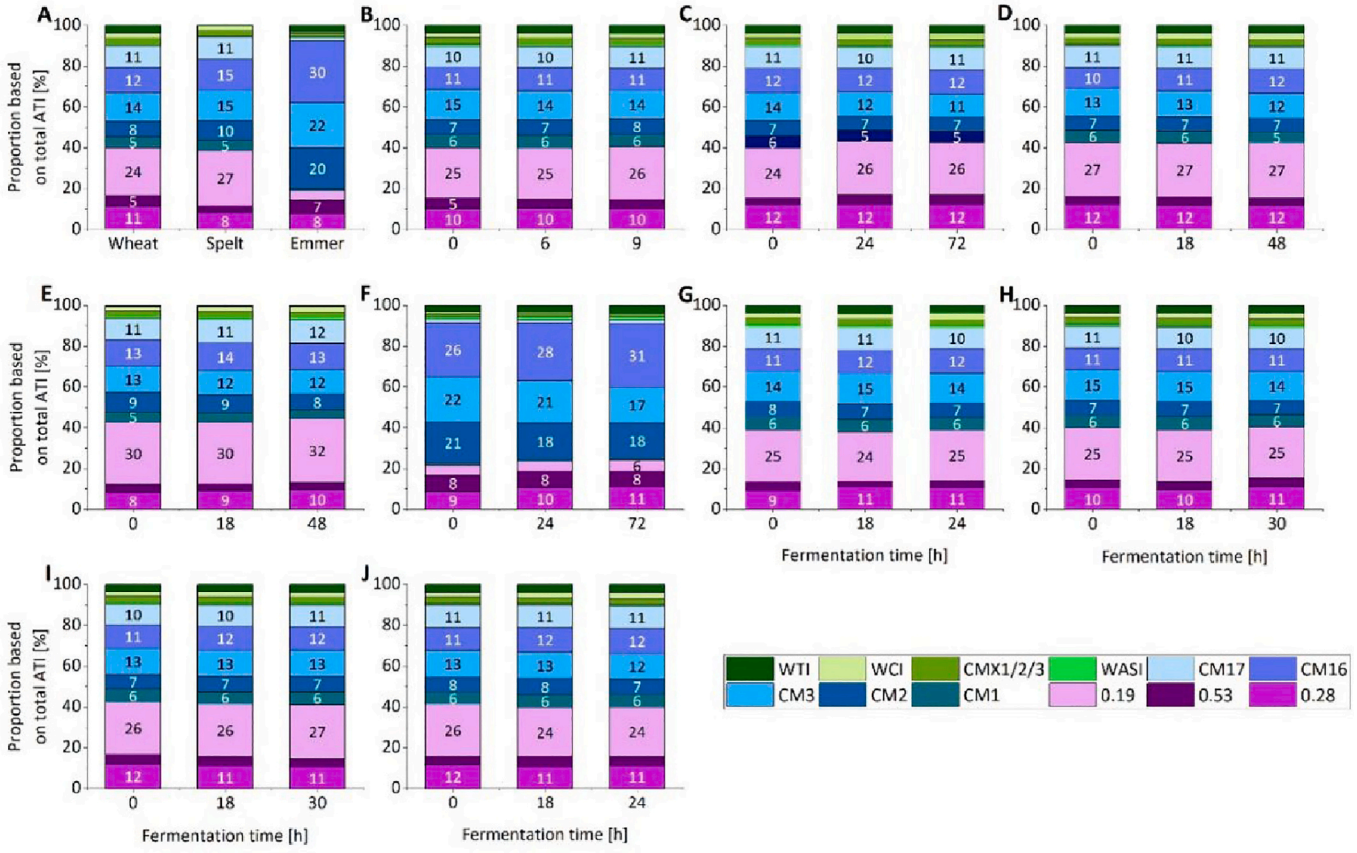

Fig. 2. Proportion of ATI subspecies in unfermented and fermented flours of different wheat species. ATIs were extracted from 50 mg samples using ammonium bicarbonate buffer and quantified by LC-MS/MS with SIDA using peptides, as described by Geisslitz et al. and Jahn et al. (Geisslitz et al., 2020; Jahn et al., 2023). Amylase/trypsin inhibitor types relative to the ATI total content in wheat, spelt and emmer flours and sourdough samples taken at different fermentation times from 0 h up to 72 h. Wheat, spelt and emmer flours (A), chemically acidified control (B), wheat sourdough with Böcker Reinzuchtsauerteig starter (W + BRS) (C), wheat sourdough with Mailänder "Le Chef" starter (W + ML) (D), spelt sourdough with BRS (S + BRS) (E), emmer sourdough with BRS (E + BRS) (F), wheat sourdough with *Fructilactobacillus sanfranciscensis 1* (W + FlS1) (G) and with *Fructilactobacillus sanfranciscensis 2* (W + FlS2) (H) and wheat sourdough with *Limosilactobacillus pontis* (W + LP) (I) and with *Maudiozyma humilis* (W + MH) (J). CM: Chloroform/Methanol-soluble tetrameric ATIs, WASI: Wheat Amylase Subtilisin Inhibitor, WTI: Wheat Trypsin Inhibitor, 0.19, 0.28 and 0.53: dimeric ATIs named after their electrophoretic mobility.

using our cell-based dual reporter assay, including hexaploid modern wheat ($n = 48$), tetraploid emmer ($n = 9$), and hexaploid spelt ($n = 7$) detailed in Methods (Table S1), and the impact of fermentation conditions on ATI digestibility and immune stimulatory is shown in Table S3, Fig. 3. Fermentation with BRS sourdough showed a time dependent reduction of ATI bioactivities up to 24 h in hexaploid spelt and modern wheat, while bioactivity remained unaffected in tetraploid emmer. Fermentation with ML sourdough also reduced ATI bioactivity in hexaploid wheat up to 24 h though less efficiently (Fig. 3A). ATI bioactivity was significantly reduced through fermentation with *Fl. sanfranciscensis II (Fl-SII)* and L. *pontis*, with the exception of modern wheat fermented with *Fl. sanfranciscensis I (Fl-SI)* at 10 h, where activity started to decrease only after 10 h (Fig. 3B). Similarly, acidification, with or without microorganisms, resulted in a time-dependent decline in ATI bioactivity (Fig. 3C).

2.4. Effect of fermentation on amylase-trypsin inhibitors as assessed by quantitative western blotting

Fig. 4 shows Western blots of ATI extracts from modern wheat fermented under different conditions (listed in Table S4). Modern wheat with both sourdough starters BRS and ML showed decreased ATI subspecies 0.19, CM2, CM3 and CM16 (Fig. 4). Interestingly, 0.19, CM2 and CM3 were significantly reduced with sourdough fermentation by all microorganisms, with and without acidification, while CM2 was partly degraded to a lower molecular weight monomeric fragment as shown in Figs. 4A and S8.

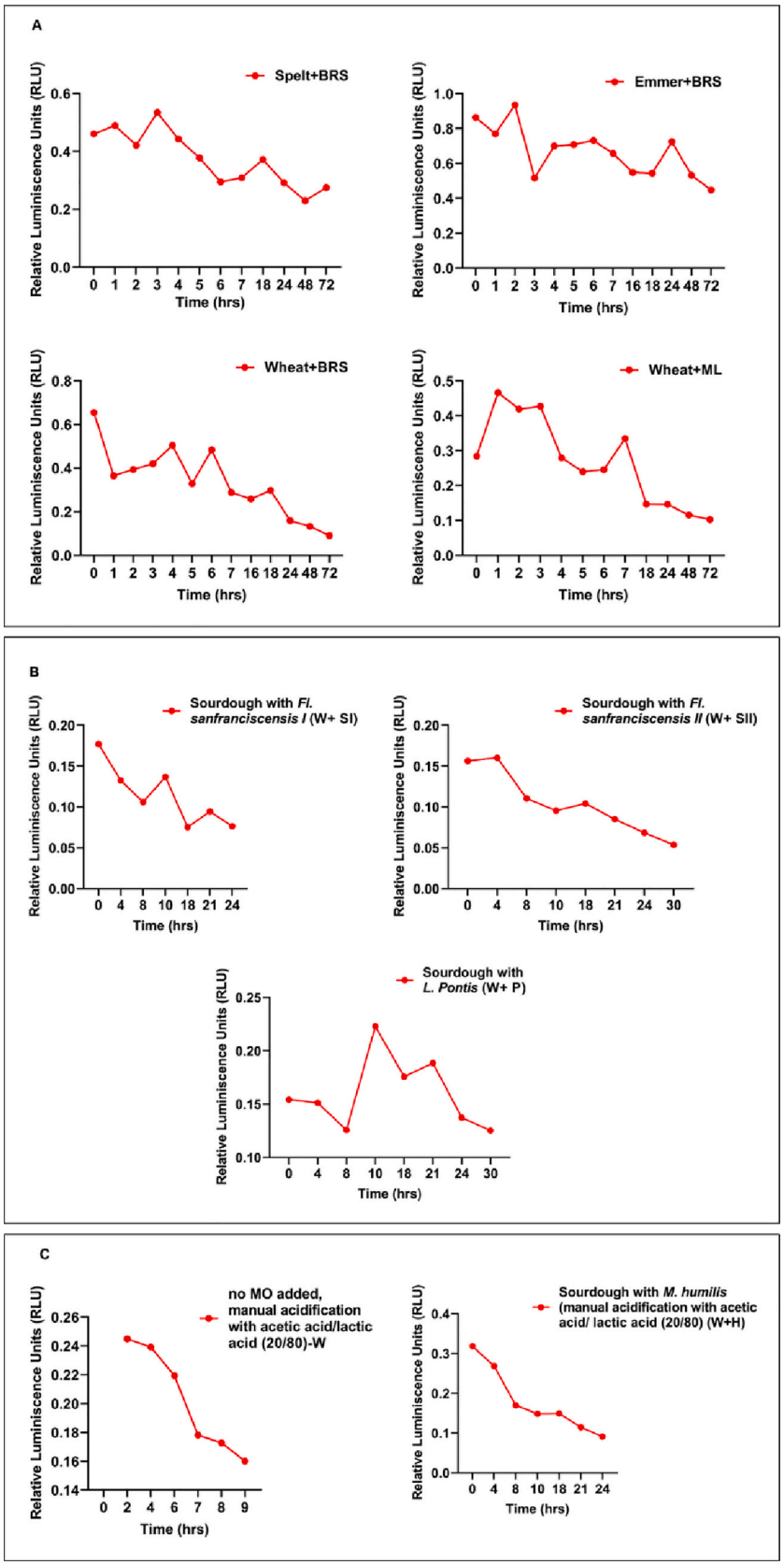

Fig. 3. TLR4 stimulating ATI bioactivities of sourdough fermented wheat flours tested using our HeLa TLR4 dual reporter cells. A- ATI bioactivities: with BRS (Böcker Reinzuchtsauerteig) and ML (Mailänder "Le Chef" sourdough) sourdough. B- ATI bioactivities: with *Fructilactobacillus sanfranciscensis 1* (W + SI), *Fl Sanfranciscensis II* (W + SII), *Limosilactobacillus pontis* (W + P). C- ATI bioactivities: with manual acidification but without microorganisms (MO) compared to with microorganism *Maudiozyma humilis* (W + H). Bioactivity was tested in duplicates for each sample.

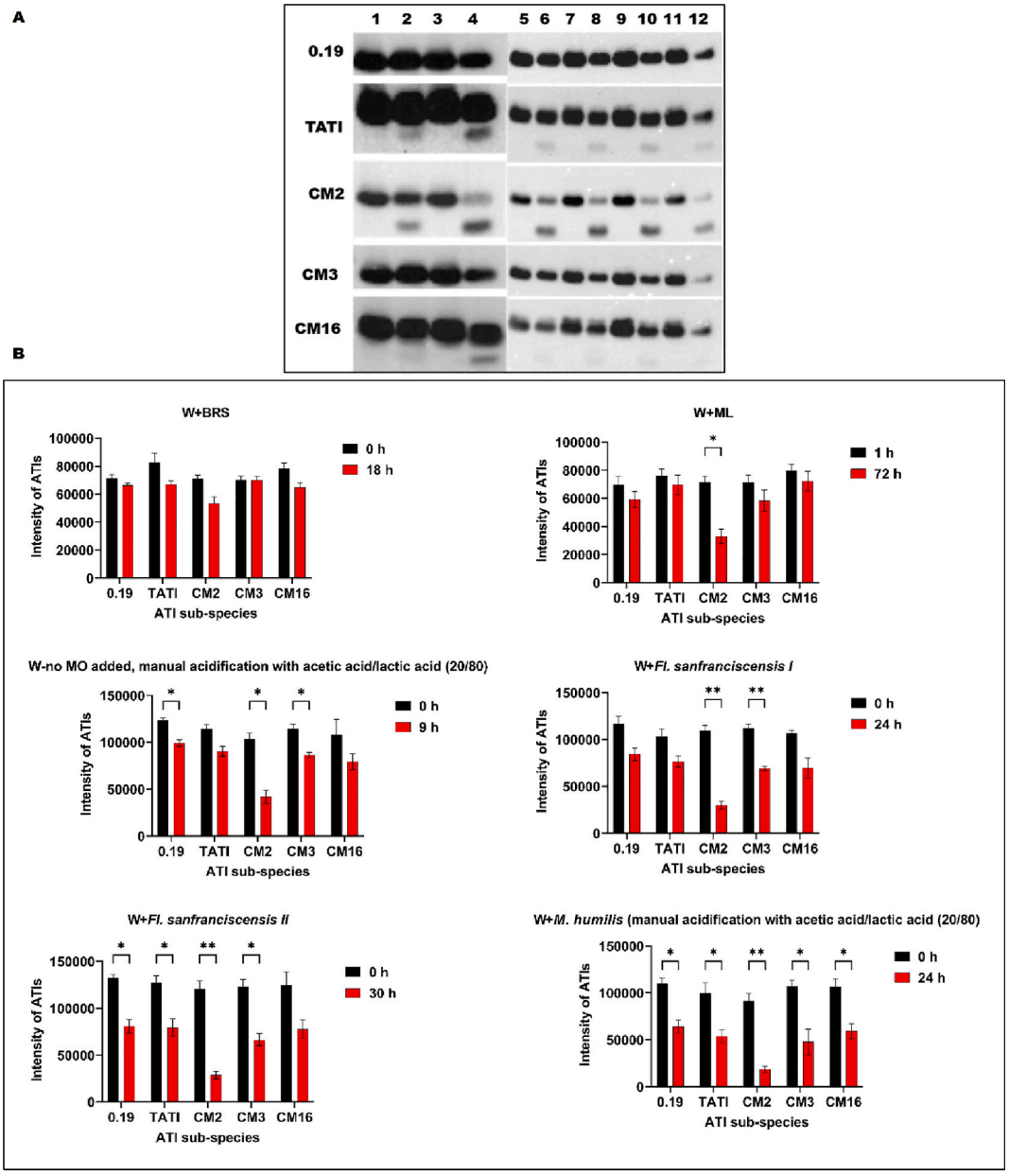

Fig. 4. Western blot analysis of ATI subspecies after different sourdough fermentations of modern hexaploid wheat flour. Quantitative ATI extraction was normalized to 1 g of flour. 20 μL of each sample was loaded onto gel for comparison of intensities of signals generated by ATI peptide specific antibodies via western blotting. (A) Lanes 1,2: W + BRS- 0 h and 18 h; 3,4- W + ML-1 h and 72 h; 5, 6-W- no Mo added 0 h and 9 h; 7,8- W + F1 SI 0 h and 24 h; 9,10- W + F1 SII 0 h and 30 h; 11,12- W + MH 0 h and 24 h; (B) respective band intensities of ATI subtypes analyzed by WB and quantified by ImageJ software. BRS: Böcker Reinzuchtsauerteig sourdough, ML: Mailänder le Chef sourdough. W + FlS1: wheat + *Fructilactobacillus sanfranciscensis 1*, W + FlS2: wheat+ *Fructilactobacillus sanfranciscensis II*, W + MH: *Maudiozyma humilis*. MO: Microorganisms. h: hours of fermentation. For individual Western blots, refer to Supplementary Fig. S8.

2.5. Effect of pH on protein composition, ATI content, and bioactivity

Initial pH was higher in LAB/yeast wheat sourdoughs (5.9–6.0) than those with BRS or ML (5.4–5.7) (Fig. S6, Table S5). Acidification began after 1.5 h in BRS sourdough but took 6 h in ML sourdough. Using BRS, emmer reached a pH <4.0 in 6 h, earlier than wheat (9–13 h) and spelt (8.5 h). *L. pontis* acidified after 12 h (pH <4.0 in 22 h). *Fl. sanfranciscensis —I* and *II* acidified in 3 h (pH <4.0 in 10 h). Protein changes aligned with acidification: with BRS wheat took 3 h, and ML took 7–18 h to acidify. Using BRS, glutenin reduction was greatest in wheat (down −66%), low in spelt (down −20%), and smallest in emmer (down −17%). Evolution of gliadin reduction was the opposite (14% for wheat, 20% for spelt, and 33% for emmer) (Fig. 1 and Table S2).

2.6. Effect of acidification on digestion of ATIs and their TLR4 stimulating bioactivity

Visualization of total proteins of the crude ATI fraction from hexaploid modern wheat is presented in Fig. S9A, showing no notable differences between time 0 h and 48 h of incubation at pH 4.0. When the HPLC purified and crude ATI fraction subjected to these incubations were subjected to quantitative Western blot analysis for ATI subtypes (Fig. S9B), no significant impact of acidic pH alone was found, except for a slight increases of CM3 and CM16 monomer ATIs after 48 h. TLR4 stimulating ATI bioactivities were also unaffected as shown in Fig. S9C, indicating that acidification does not alter the stability or bioactivity, in contrast to acidic enzymes in sourdough as shown in Figs. 3 and 4.

2.7. Effect of Aspergillus niger proline endopeptidase (AN-PEP) on ATI digestibility and bioactivity

The crude ATI fraction was digested with the acidic peptidase AN-PEP under defined conditions at several time points (0 h, 24 h, 48 h and 72 h) as described in Methods. Fig. S10 shows SDS PAGE analysis, Western blot analysis and bioactivity upon treatment with an enzyme to substrate ratio of 1:100 compared to controls without enzyme. SDS PAGE (Fig. S10A) and Western blot analysis (Fig. S10B and C) revealed increased formation of monomeric from oligomeric ATIs and further solubilization of mostly insoluble material, leading to higher band intensity and a slight increase in bioactivity (Fig. S10D). At higher enzyme concentration (1,50), no major changes were observed in between ATIs analyzed via SDS PAGE (Fig. S11A), and WB (Fig. S11B) at 0 h and 24 h along with only a minor decrease in bioactivity at 24 h as shown in Fig. S11C, suggesting that both the acidic environment and natural cereal endopeptidases may act synergistically on ATIs along with sourdough proteases.

3. Discussion

Sourdough fermentation is gaining popularity for its health benefits, complex flavors, and natural process. Different strains of lactic acid bacteria (LAB) and fermentation conditions influence its flavor profile, with rye fermentations producing more intense volatiles than wheat (Ravyts & De Vuyst, 2011). Mixed starter cultures of yeast and LAB improve sourdough bread quality, texture, and shelf life (Xu et al., 2019). The microbiota composition is influenced by fermentation duration, temperature, and pH. L. *sanfranciscensis* and yeast thrive under various conditions, while L. *reuteri* and L. *johnsonii* prefer higher temperatures (Vogelmann & Hertel, 2011). During sourdough fermentation, HMW-GS are broken down, and endogenous cereal proteolytic activity is significantly enhanced as shown before (Loponen et al., 2004). While these results can be relevant for patients with celiac disease, ATIs of gluten-containing cereals have been implicated as drivers of chronic inflammation via stimulation of intestinal TLR4, especially of autoimmunity and metabolic diseases (Bellinghausen et al., 2019; Caminero et al., 2019; Junker et al., 2012; Pickert et al., 2020; Zevallos et al., 2017; Zevallos et al., 2019; Zevallos et al., 2023). Here, a better understanding of the impact of dough fermentation on ATI degradation and especially inflammatory bioactivity could aid in producing wheat flours with reduced or even absent TLR4-stimulating ATIs.

Huang et al. showed that sourdough fermentation reduces CM-ATI tetramers, as assessed by size exclusion chromatography, in line with decreased pro-inflammatory activity, as determined via cytokine release by THP-1 monocytes, while mere yeast fermentation preserved both CM-tetramer quantity and bioactivity (Huang et al., 2020). Here we show that sourdough fermentation with *Fl. sanfranciscensis* and L. *pontis* significantly reduced ATI bioactivity in modern wheat. In general, proteolysis in sourdough is not significantly mediated by *Lactobacillus sanfranciscensis*, as this strain lacks extracellular protease activity which was reported for the strain DSM 20451. Here, protein degradation was attributed to flour-derived aspartic proteases, while the bacterium contributed to amino acid metabolism through peptide uptake systems (Opp and DtpT) and intracellular peptidases (PepN, PepC, PepT, PepR) (Vermeulen et al., 2005). In this vein, others showed that also *F. Sanfranciscensis*, present in our BRS sourdough, does not express extracellular protease activity (Vogelmann & Hertel, 2011). BRS is a rye-based starter culture which could explain that it showed higher degradative efficacy in common hexaploid wheat due to its adaption on more similar complex genome. *M. humilis* yeast recycles amino acids released during fermentation with a limited direct impact on protein breakdown but again has prominent intracellular protease activity (Gänzle et al., 2008). Finally, through acidification during sourdough fermentation, the bacteria activate cereal endogenous proteases, enhancing overall protein degradation, in line with our findings that acidification alone or combined with microorganisms caused a time-dependent decrease of bioactivity in all wheats (Fig. 3). Minervini F et al. (Minervini et al., 2015) showed that genera such as Lactobacillus, Lactococcus, Streptococcus, and Enterococcus are consistently associated with wheat tissues and transferred to flour during processing. Their abundance is influenced by environmental factors like water availability and temperature, which also shift during dough fermentation. Notably, *Lactobacillus plantarum* persists across wheat developmental stages, supporting its activation under sourdough conditions.

Unexpectedly, ATI quantities remained unaffected when determined by LS-MS/MS based on specific tryptic marker peptides. However, when ATI subspecies degradation was assessed by quantitative WB analysis (Figs. 4A, B and S8), wheat fermented with BRS and ML starters, showed a time-dependent proteolytic degradation of ATI subtypes 0.19, CM2, CM3 and CM16. Thus, CM2 was partly degraded including a lower molecular weight fragment, and total CM3 was significantly reduced by approximately 50%, while LC-MS/MS detected only a minor reduction of CM3 peptides, and also no significant reduction of total ATI content (Table S6). These findings suggest that LC-MS/MS for ATIs does not reflect proteolytic inactivation of ATI biological (TLR4-stimulating) activity, since fragment patterns of the small peptides may well remain unchanged with fermentation, while the protein secondary structures that mediate biological activities are destroyed. We present bioactivity as a function of ATIs based on Western blot analysis, which distinguishes intact from degraded protein species, whereas LC-MS/MS quantifies prototypic peptides, resulting from intact or degraded target proteins. LC-MS/MS alone therefore does not reflect (intact) protein functionality. In contrast, quantitative Western blotting allows characterization of oligomeric vs monomeric intact proteins, which are closely linked to protein functionality, which here is supplemented with the purely functional TLR4 stimulation bioassay. This analytical distinction is critical for interpreting the observed discrepancies compared to analyses based on LC-MS/MS alone.

Unfermented spelt had a higher ATI content than wheat and emmer, consistent with previous studies (Geisslitz et al., 2020; Jahn et al., 2023). The higher gliadin-to-glutenin ratio in emmer (Fig. 1A) is well known (Geisslitz et al., 2019). Changes in total ATI content as determined by LC-MS/MS were small, which may be due to some effect of extractability and analytical uncertainty. Distribution of protein fractions at 0 h (Fig. 2C-F) confirmed that dough preparation and mixing had no effect. Extractability of total protein can vary based on gluten quality, with some studies showing reduced protein extraction from dough (Wang et al., 2020).

Prolonged fermentation degraded proteins, mainly gluten, as shown by SDS-PAGE and RP-HPLC. Partial hydrolysis of α- and γ-gliadins was observed in SDS-PAGE, while RP-HPLC indicated only γ-gliadin degradation, which can be explained by peak overlap/incomplete fractionation according to solubility (Lexhaller et al., 2019). SDS-PAGE showed a reduction in ω-gliadins, but RP-HPLC suggested constant or increased levels, which may be due to better extractability of ω-gliadins that can be bound covalently to glutenins (Wang et al., 2020). Gliadin and glutenin degradation likely increased albumin/globulin solubility, and SDS-PAGE revealed disassembly of tetrameric ATI into dimeric and monomeric forms, which was not observed in RP-HPLC and LC-MS/MS. The observed increased albumin/globulin content with longer fermentation suggests structural breakdown of the gluten framework.

No clear correlation could be observed between ATI content and bioactivity, though more research is needed. ATI content of unfermented cereals, as determined by LC-MS/MS, followed emmer < wheat < spelt, while bioactivity ranked spelt < wheat < emmer, indicating that ancient wheat species may not be better tolerable for patients with ATI-induced NCWS. The higher CM-type ATI content of emmer may explain this discrepancy. Since only one flour per species was analyzed, these rankings may not represent the species overall. We previously showed that ancient wheats such as emmer and spelt have higher levels of ATIs compared to modern bread wheat (Geisslitz et al., 2020), while diversity

of ATI subtypes is higher in modern wheat (Sielaff et al., 2021), highlighting marked species-specific differences in ATI content (Geisslitz et al., 2019). Tomić et al. (Tomić et al., 2023) reported higher proteolytic activity in wheat sourdough than in emmer and spelt sourdoughs, while we observed the strongest pH drop (correlating with proteolytic activity) in emmer. Accordingly, ATI bioactivity decreased consistently with lower pH values as shown in Fig. S7. Variability in ATI bioactivity during the first 7 h was likely due to insufficient dough homogeneity, stabilizing after 18 h. The strongest reduction occurred after 18 h for all sourdoughs except for L. *pontis*, which acidified later. The other LAB strains were most active in the first 18 h, reflected by a steady pH drop and ATI bioactivity reduction. Different proteolytic activities and pH dynamics likely explain these variations. It appears that certain cereal proteases become activated and act synergistically in the digestion of ATIs. This is supported by our results with both the HPLC-purified and the crude ATI-enriched fractions, where natural proteases from sourdough were absent. Under these conditions, no major differences in bioactivity or ATI digestibility were observed on prolonged incubations at acidic pH (Fig. S9), unlike the pronounced impact of the sourdough's acidic environment on ATIs shown in Figs. 3, 4, and S7. Our data suggest that acidification activated flour-resident enzymes, while added acidic endopeptidase AN-PEP which effectively degrades gluten exerted only mild proteolytic effects. Digestion at pH 3.8 and a 1:100 enzyme to substrate ratio generated monomeric from oligomeric ATI forms, slightly enhancing bioactivity up to 72 h (Fig. S10), whereas a higher ratio (1,50) led to minor degradation of monomeric ATIs and a slight decrease in bioactivity after 24 h (Fig. S11). More research is needed, especially given the limited flour samples analyzable per species in this study. Therefore, an effective large-scale reduction of proinflammatory activity related to wheat ATI proteins will require a multidimensional approach integrating targeted genetic/molecular breeding, environmental control, and optimized fermentation to yield wheat varieties and wheat products with truly reduced disease-promoting properties. How far specific lactic acid bacterial strains may have significant ATI-degrading capability requires further study. In summary, our data suggest that generally long-time sourdough fermentation of wheat flours leads to healthier wheat products with reduced inflammatory properties. Sourdough fermentation therefore has a great potential to reduce the TLR4 activating and allergenic potential of ATIs, especially in modern hexaploid wheats. Thus, optimized sourdough fermentations will support the production of hypoallergenic, “gut-friendly” and healthy baked goods, in line with the growing consumer demand for “Clean Label” natural, long-fermented, additive-free bakery products. Future strategies to reduce ATI bioactivity in wheat-based products may involve the optimization of processing conditions, the selection of suitable wheat genotypes, and the development of targeted fermentation or other complementary approaches. A better understanding of how these factors influence ATI bioactivity could support the development of wheat products with reduced inflammatory potential and improved suitability for individuals with chronic inflammatory diseases.

4. Methods

4.1. Sourdough fermentation of wheat flours

Eight different types of sourdough were prepared from different wheat varieties such as spelt, emmer and common modern wheat was performed using starter cultures, Böcker Reinzuchtsauerteig starter (BRS) (Fig. 3A and Fig. 4) and Mailänder “Le Chef” starter (ML) (Fig. 3A and Fig. 4). Böcker Reinzuchtsauerteig is a natural, backslopped sourdough cultivated at constant conditions containing a mixture of lactic acid bacteria (LAB) and yeasts. In comparison, Le Chef is different in terms of numbers and composition of LAB and yeasts with a higher proportion of yeasts and it is suitable for mildly acidified baked goods from wheat. The single strains of LAB and yeast constitute the main microorganisms in the sourdough starters. Fermentation was also performed using different microorganisms such as three strains of LAB (Fl. sanfranciscensis I, Fl. sanfranciscensis II, *L. pontis*) (Fig. 3B and Fig. 4) and one strain of yeast (*M. humilis*) (Fig. 3C and Fig. 4) at several time points. In addition, manual acidification in the presence of the yeast strain, *M. humilis* on common modern wheat was also performed. One chemically acidified wheat dough without addition of microorganisms served as a control. All sourdough starters and microorganisms were provided by all Ernst Böcker GmbH (Minden, Germany). Table S1 shows the abbreviations, flours, microorganisms, recipes, fermentation times and Table S5 shows final pH values. All doughs were incubated at a relative humidity of 80%. The pH of all sourdoughs was recorded for at least 17 h by a digital pH conductivity meter (WTW GmbH, Weilheim, Germany). Prior to analysis, all doughs were freeze-dried and the resulting powder was manually homogenized using mortar and pestle.

4.2. Protein extraction for SDS PAGE analysis, RP-HPLC analysis and LC/MS analysis

The proteins in the flours and sourdough samples were extracted following a stepwise procedure to obtain the albumin/globulin, gliadin and glutenin fractions according to Wieser et al. (Wieser et al., 1998). In brief, 1 mL of salt solution (0.4 mol/L NaCl in 0.067 mol/L Na2PO4/KH2PO4 (pH 7.6)) was added to 100 mg of the samples, followed by vortex mixing for 2 min, magnetic stirring for 10 min and centrifugation (3550 ×*g*, 25 min, 22 °C). The extraction was repeated once and both supernatants were combined and made up to 2 mL (albumins/globulins). Then, 0.5 mL of 60% aqueous ethanol (v + v) were added to the residue and the same steps were carried out as before. The extraction was repeated two times and the three supernatants were combined and made up to 2 mL (gliadins). Last, 1 mL of glutenin extraction solution (50% (*v*/v) 2-propanol +0.1 mol/L Tris-HCl (pH 7.5) + 12 g urea +10 mg/mL dithiothreitol (DTT)) was added to the residue under argon atmosphere, followed by vortex mixing for 2 min, magnetic stirring for 30 min at 60 °C in a water bath and centrifugation (3550 ×*g*, 25 min, 22 °C). The extraction was repeated once and both supernatants were combined and made up to 2 mL (glutenins).

4.3. Targeted liquid chromatography tandem mass spectrometry (LC-MS/MS)

The content of ATIs was analyzed by LC-MS/MS as described by Geisslitz et al. (Geisslitz et al., 2020) and Jahn et al. (Jahn et al., 2023). In brief, for sample preparation, 50 mg of the samples were extracted twice with ammonium bicarbonate solution (0.5 mL, 50 mmol/L, pH 7.8) for 30 min at 22 °C. The suspensions were centrifuged (3550 ×*g*, 25 min, 22 °C) and the two supernatants were combined. The extracts were evaporated to dryness at 37 °C and 8 mbar and stored at −20 °C until analysis. The dried extracts were reconstituted in 640 µL of Tris-HCl (0.5 mol/L, pH 8.5)/1-propanol (1 + 1, v + v). A mixture of 20 [13C]/[15 N]-labeled peptides as internal standards (50 µL) was added for stable isotope dilution analysis (SIDA). After reduction with tris(2-carboxyethyl) phosphine, alkylation with chloroacetamide and solvent removal, the proteins were hydrolyzed with trypsin. The reaction was stopped with 2 µL of TFA. Then, the solution was evaporated to dryness at 37 °C and 8 mbar and reconstituted in 1 mL of acetonitrile/0.1% formic acid (FA) (2 + 98, v + v) and filtered (0.45 µm, Whatman, Global Life Sciences Solutions, Marlborough, MA, USA).

The peptides were analyzed using a Vanquish UHPLC system coupled to a Q Exactive Plus Orbitrap (ThermoFisher Scientific, Waltham, MA, USA) exactly as described in Jahn et al. (Jahn et al., 2023) and content of ATI was calculated as described therein. Briefly, two peptides per protein were selected to account for potential post-translational modifications, except for 0.53 (only one unique peptide vs. 0.19), CM1 and CM17 (low intensities), and CMX1/2/3 (no additional peptides), resulting in 20 marker peptides (P1–P20) for quantifying 13 wheat ATIs.

4.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).

For SDS-PAGE, the proteins were precipitated by adding ice-cold acetone at a ratio of 1:4 (*v*/v) to 200 μL of the albumin/globulin or gliadin fractions and 300 μL of the glutenin fraction. The solution was stored at −20 °C for 16 h followed by centrifugation (10,000 ×*g*, 10 min, 22 °C). The supernatant was discarded. Then, 600 μL of acetone were added to the protein pellet and after mixing, the solution was centrifuged again using the same conditions as before. The pellet was solubilized in 500 μL of extraction buffer (293.3 mmol/L sucrose, 246.4 mmol/L Tris, 69.4 mmol/L SDS, 0.51 mmol/L EDTA, 0.22 mmol/L Brilliant Blue G-250, 0.177 mmol/L phenol red, 0.105 mmol/L HCl, 50 mmol/L DTT, pH 8.5) diluted with bidist. Water (1 + 1, v + v), vortex mixed for 30 min at 22 °C and shaken for 10 min at 60 °C followed by centrifugation (2370 ×*g*, 5 min, 20 °C). The proteins were separated under reducing conditions on a NuPAGE 4–12% Bis-Tris gradient gel (Thermo Fisher Scientific, Darmstadt, Germany) using MES running buffer (50 mmol/L MES, 50 mmol/L Tris, 3.5 mmol/L SDS, 1 mmol/L EDTA, pH 7.7) for albumins/globulins and MOPS running buffer (50 mmol/L MOPS, 50 mmol/L Tris, 3.5 mmol/L SDS, 1 mmol/L EDTA, pH 7.7) for gliadins and glutenins according to Lagrain et al. (Lagrain et al., 2012) and Xhaferaj et al. (Xhaferaj et al., 2023). PageRuler Unstained Protein Ladder (Thermo Fisher Scientific) was used as a molecular weight (MW) marker from 200 kDa to 10 kDa.

4.5. Reversed-phase high performance liquid chromatography (RP-HPLC)

The albumin/globulin, gliadin and glutenin extracts were filtered (0.45 μm, WICOM, Heppenheim, Germany) and separated using a SIL-40C HPLC (Shimadzu, Nakagyo-ku, Kyoto, Japan) and the following parameters: stationary phase: YMC Triart Bio C18, 150 mm × 2.1 mm, 3 μm (YMC Europe, Dinslaken, Germany); mobile phase A: 0.1% trifluoroacetic acid (TFA) in bidist. Water and B: 0.1% TFA in acetonitrile; flow rate: 0.4 mL/min; column temperature: 60 °C; injection volume: 15 μL for albumins/globulins, 10 μL for gliadins and 20 μL for glutenins; gradient for albumins/globulins: 0–0.4 min, 0% B; 0.5 min, 20% B; 8 min, 60% B; 8.1 min, 90% B; 8.1–13 min, 90% B; 13.1–27 min 0% B and gradient for gliadins and glutenins: 0–0.4 min, 5% B; 0.5 min, 30% B; 16 min, 60% B; 16.1–22.1 min, 90% B; 22.2 min, 5% B; 22.2–30 min, 5% B; detection: UV absorbance at 210 nm. The proteins were quantified using external calibration with Prolamin Working Group (PWG)-gliadin (distributed by Working group for Grain Research, Detmold, Germany) (2.5 mg/mL) as described earlier (van Eckert et al., 2006).

4.6. Determination of ATI bioactivity from unfermented and sourdough fermented wheat flours

1 g wheat flour (freeze dried after fermentation) was extracted with 5 mL of 10 mmol/L Tris, 0.5 mol/L NaCl, pH 7.8, in two consecutive extractions each and both supernatants were then pooled, according to our published method, with slight modifications (Zevallos et al., 2017; Zevallos et al., 2023; Ziegler et al., 2020). The HeLa TLR4 Dual Luciferase Reporter Cell Line was generated and used for bioactivity measurements as described by us (Lagrain et al., 2012). Briefly, 20,000 HeLa-TLR4 dual reporter cells per well were seeded in 100 μL of DMEM + Hygromycin (125 μg/mL) in 96-well flat-bottomed wells. After leaving overnight to adhere the cells to the bottom of the wells, 5 μL of ATI extract in 100 μL DMEM was added in duplicates per well and cells were stimulated for 7 h. LPS (0.5–2 ng/100 μL) was served as positive control while plain medium served as a negative control. Common off- the shelf modern hexaploid wheat extract was set as an internal standard. Later, cells were washed with PBS and 10 μL/well passive lysis buffer was added to lyse the cells via freeze thawing. 50 μL of LAR II solution was added to lysed cells and 40 μL of that was transferred to a white plate to initially read for the Firefly luminescence signal, that measures the total no of cells using a Tecan Reader. This was followed by 40 μL addition of Stop & Glo buffer that reads the Renilla luciferase signal, which measures the TLR4 activation. The normalized TLR4 activity was calculated by the dividing the TLR4-driven Renilla luciferase signal with constitutive Firefly luciferase signal that yields normalized TLR4 stimulating bioactivities which were further normalized to LPS (positive control) driven TLR4 stimulating activity. ATI bioactivities were then calculated according to the common wheat extract standard curve set to 3 mg of bioactive ATI protein per g of the dried standard wheat flour.

4.7. Quantitative Western blotting of ATI subspecies

Western blotting was performed using peptide specific rabbit antibodies directed to ATIs 0.19, CM3, CM16 and CM2, and to rabbit antibodies to total ATIs (mainly lacking CM-subtypes). Antibodies were raised to immunogenic ATI-subtype specific epitopes and affinity purified using the respective peptide (Biomatik, USA) as shown in Table S7.

High titer, specificity and lack of cross-reactivity with other ATI subspecies was confirmed by ELISA. Primary antibodies were used at 1:3000 dilution and goat anti -rabbit (IgG) HRP-conjugated secondary antibody (ab205718, Abcam, Germany) diluted 1:20,000. ATI bands were visualized using a ChemiDocTM XRS+ Imaging system using a chemiluminescence and a colorimetric setting for the standard.

4.8. Acidification of HPLC purified and Crude ATI fractions from commercial hexaploid wheat flour

An HPLC-enriched ATI fraction (50 μL) was diluted to 1 mL in 0.01 M acetic acid (pH 4), and incubated for 0 or 48 h (500 μL each) at 28 °C. Samples were subsequently freeze-dried, lyophilized, and re-dissolved in 25 μL of PBS. Western blotting was performed with 5 μL of sample per lane, while bioactivity was evaluated using 20 μL in HeLa-TLR4 dual reporter cells.

The crude ATI fraction, obtained by 1.8 M ammonium sulfate precipitation, dialysis, and lyophilization, was processed similarly using 100 μL starting material and resuspended to 50 μL per time point (0 h and 48 h) of acidic incubation. SDS-PAGE and Western blotting were performed with 10 μL, and bioactivity was assessed using 20 μL in HeLa-TLR4 dual reporter cells.

4.9. Digestion of ATIs with AN-PEP proline-specific endopeptidase

The crude ATI fraction was subjected to enzymatic treatment using Aspergillus niger endopeptidase, AN-PEP (Clarex, DSM, Netherlands) in 0.01 M acetic acid pH 3.8 at 20 °C. The effect of the enzyme on ATI digestibility and bioactivity was evaluated at multiple time points (0 h, 24 h, 48 h and 72 h) using an enzyme-to-substrate ratio of 1:100. In a separate experiment, a higher enzyme concentration (1,50) was tested, with samples analyzed at 0 h and 24 h. After incubation at 20 °C, samples (10 μL) were analyzed by SDS-PAGE and western blotting to assess changes in ATI protein profiles, including formation of monomeric from oligomeric forms. Band intensities were compared relative to untreated controls. In parallel, TLR4-stimulating bioactivity of treated and control samples (5 and 10 μL) was evaluated using the HeLa-TLR4 dual reporter cell assay to determine functional changes induced by enzymatic digestion.

4.10. Data analysis

Means and standard deviations were calculated in Excel. Two sample *t*-test were conducted with Origin (Origin Pro 2023, OriginLab Corporation) at a significance level of least $p \leq 0.05$.

5. Conclusion

Sourdough fermentation significantly reduced ATI bioactivity. It also

promoted partial degradation of gliadins and glutenins, increasing albumin/globulin fractions. While LC-MS/MS suggested a stable total ATI content, SDS-PAGE and especially Western blot analyses revealed ATI proteolytic fragmentation, particularly a decrease in CM3 and CM2 tetrameric ATIs in modern wheat. Degradation correlated with dough acidification. Sourdough fermentation also decreased ATI-induced pro-inflammatory activity. These findings are particularly important for patients that experience (ATI-induced) worsening of their autoimmune and metabolic inflammatory diseases. Optimizing fermentation processes using the presented technologies should further improve tolerability and health related properties of wheat-based products.

CRediT authorship contribution statement

Manjusha Neerukonda: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Sabrina Geisslitz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Sarah Jöstl:** Writing – review & editing, Formal analysis, Data curation. **Rebecca Lingenfelder:** Formal analysis, Data curation. **Sibylle Neufang:** Formal analysis, Data curation. **Sandra Koch:** Formal analysis, Data curation. **Klajdi Begaj:** Formal analysis, Data curation. **Ernesto Bockamp:** Writing – review & editing, Formal analysis. **Markus Jörg Brandt:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Formal analysis, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Detlef Schuppan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.150662.

Data availability

Data will be made available on request.

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