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Protein composition in ancient wheats is determined by ploidy level

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ABSTRACT

Gluten and amylase/trypsin-inhibitors (ATIs) are known triggers for wheat-related disorders. Ancient wheat species like einkorn, emmer, Khorasan wheat, Polish wheat and spelt are suggested to have fewer of these immunoreactive proteins compared to modern common wheat, because breeding for higher yields and increased resistance to plant diseases might have resulted in changes in the protein composition. Currently, there are only very few studies on the protein composition of ancient wheat species. This comparative study investigated the bread volume, protein content as well as gluten and ATI content and composition, along with the inhibitory activity in ten ancient wheat varieties cultivated under organic conditions. The varieties had different ploidy levels and were harvested in three consecutive years. The various analyses consistently concluded that differences in protein composition were associated with the different ploidy levels. This means that the results were similar for the diploid species (einkorn), the tetraploid species (emmer, durum wheat, Khorasan wheat and Polish wheat) and for the hexaploid species (spelt and common wheat), respectively. These results suggest that breeding has likely not increased the immunoreactive potential of wheat and ancient wheats are unlikely to provide improved tolerability for individuals with wheat-related disorders.

1. Introduction

Wheat is one of the earliest cultivated grains and belongs to the *Poaceae* family. The most widely used wheat species is common wheat (*Triticum aestivum* ssp. *aestivum*, hexaploid), which is often referred to as bread wheat because of its main use. In addition to common wheat, the "modern" wheats also include durum wheat (*T. turgidum* ssp. *durum*, tetraploid), which is mainly used for pasta production. Among the "ancient" wheats there are the hulled species einkorn (*T. monococcum*, diploid), emmer (*T. turgidum* ssp. *dicoccon*, tetraploid) and spelt (*T. aestivum* ssp. *spelta*, hexaploid). In addition, there are also numerous other ancient forms of wheat. Recently, Polish wheat (*T. turgidum* ssp. *polonicum*, tetraploid) has been discovered to be a promising species for breeding new wheat varieties (Bieńkowska et al., 2020). Another example is tetraploid Khorasan wheat (*T. turgidum* ssp. *turanicum*) (Khlestkina et al., 2006). It is considered to be a natural hybrid between *T. turgidum* ssp. *durum* and *T. turgidum* ssp. *polonicum*, but its origin and taxonomic classification are not entirely clear, because it also carries traits from further wheat species. The trademark Kamut® specifically refers to the US-registered Khorasan variety QK-77. Further, wheat species can be differentiated into modern varieties and landraces. Wheat landraces are populations that have developed over the years by natural or human selection (Zencrci et al., 2021). Although there is no universally valid definition for the term "landrace", a few characteristics are associated with it. These include historical origin, local adaptation, genetic diversity and an association with traditional farming systems (Camacho-Villa et al., 2005). Ancient wheat species and landraces have lower yields and taller plant heights, which leads to a higher risk of lodging, which made them unattractive for modern agriculture. This is why they were replaced by modern wheats (Denčić et al., 2000; Longin et al., 2016). Although the different protein composition in ancient wheat species resulted in poorer baking properties compared to modern common wheat (Geisslitz et al., 2018), selected common wheat landraces yielded similar bread volumes as modern varieties (Jahn et al., 2024). This is one of the reasons why research has focused on these varieties recently. Some studies suggest a lower immunoreactive potential of ancient wheat species (especially einkorn) (Afzal et al., 2023;

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Abbreviations

ALGL albumins and globulins.
ANOVA analysis of variance.
ATI amylase/trypsin-inhibitor.
GLIA/GLUT gliadin/glutenin ratio.
HMW-GS high-molecular-weight glutenin subunits.
LC-MS/MS liquid chromatography tandem mass spectrometry.
LMW-GS low-molecular-weight glutenin subunits.
PCA principal component analysis.
RP-HPLC reversed-phase high-performance liquid chromatography.

Di Stasio et al., 2020) and thus a better tolerability (Seidita et al., 2022).

Wheat proteins including gluten and non-gluten proteins have been identified as triggers of a variety of wheat-related disorders. Gluten is composed of two main protein fractions that are typically analyzed with gel electrophoresis and reversed-phase high-performance liquid chromatography (RP-HPLC). Gliadins are soluble in aqueous alcohols and can be divided into ω5-, ω1,2-, α- and γ-gliadins. The glutenin fraction is soluble in aqueous alcohols under disaggregating and reducing conditions and increased temperatures. It comprises high- (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) and also ωb-gliadins. Although they are named gliadins, they are found in the glutenin fraction, because one amino acid is replaced with a cysteine residue. Therefore, disulfide bonds can link them to other gluten proteins (Wieser et al., 1998, Wieser et al., 2022). The α- and ω-gliadins are considered to be the most immunoreactive fractions, playing a role in celiac disease and wheat allergy. However, γ-gliadins, HMW-GS and LMW-GS are also immunoreactive components in wheat (Kissing Kucek et al., 2015). Further, there are water- and salt-soluble proteins, the albumins and globulins (ALGL). They contain mostly metabolic proteins such as enzymes and enzyme-inhibitors including amylase/trypsin-inhibitors (ATIs) (Dupont and Altenbach, 2003). ATIs are the main allergens causing baker's asthma (Sander et al., 2011) and can activate the toll-like receptor 4 and therefore contribute to other wheat-related disorders (Junker et al., 2012; Kissing Kucek et al., 2015). ATIs have the capability to inhibit amylase and trypsin, resulting in incomplete digestion of food components. This can lead to intra- and extraintestinal symptoms.

There are only few studies that focus on immunoreactive proteins in different ancient wheat species. Geisslitz et al. (2018) analyzed the gluten composition of different wheat species and found significant differences especially between einkorn and the other four species (common wheat and durum wheat, emmer and spelt). Geisslitz et al. (2020) and Sielaff et al. (2021) found the highest ATI content in spelt followed by common wheat, emmer and durum wheat and a very low content in einkorn. In agreement with the quantitative results, Jahn et al. (2023) found no inhibitory activity in einkorn samples. Call et al. (2020) examined the same five wheat species (but different varieties) regarding the ATI and gluten content and found no evidence that one wheat species might be less immunoreactive.

The protein content and composition and some bread-making parameters of common wheat landraces have already been reported (Jahn et al., 2024), as well as the ATI content and distribution and the inhibitory activity (Jahn et al., 2025). However, a comparative study about the proteins of different ancient wheat species including Khorasan wheat and Polish wheat cultivated together under organic conditions is missing so far. The aim of the present study was to analyze the protein, gluten and ATI content and composition along with the inhibitory activity in ten ancient wheat varieties, which had a different genetic background (diploid, tetraploid, hexaploid) and were harvested in three consecutive years (2021–2023). This allows an assessment whether ancient wheats may be beneficial for individuals with wheat-related disorders.

2. Materials and methods

2.1. Materials

Ten German varieties of different wheat species were cultivated at the Bavarian State Research Center for Agriculture LfL (Bayerische Landesanstalt für Landwirtschaft, Ruhstorf an der Rott, Germany) and harvested in three consecutive years (2021–2023) (Table 1). Two spelt (Babenhausener Zuchtvesen (BZV) and Muellers Gaiberger (MGA)), two emmer (Weihenstephan Emmer 1 (WEM1) and Weihenstephan Emmer 2 (WEM2)), one durum wheat (Winterdurum Sambadur (WDS)), one Khorasan wheat (KSW), one Polish wheat (POW) and three einkorn varieties (wild einkorn (WEK), Enkidu (ENK) and Terzino (TER)) were included. The two einkorn varieties ENK and TER were added after the first year of the trial. The cultivation was carried out under organic growing conditions without fertilization or any other treatment. The experimental layout with plot sizes of 2 × 4 m was a randomized complete block design (Latin rectangle) with three replications, which were harvested and milled together. The kernels were milled with a Quadrumat Junior (Brabender, Duisburg, Germany) to obtain type 550 flours (ash content of 0.51–0.63 % based on dry matter) according to the German flour classification system.

All chemicals were of analytical grade or higher and purchased from Carl Roth (Karlsruhe, Germany), VWR Chemicals (Radnor, PA, USA) and Thermo Fisher Scientific (Waltham, MA, USA). EnzChek Ultra Amylase Assay was obtained from Thermo Fisher Scientific. Trypsin (TPCK-treated, bovine pancreas) and porcine pancreas amylase (A3176) were from Sigma-Aldrich (St. Louis, MO, USA). Peptides were synthesized by GenScript (Piscataway, NJ, USA) as unlabeled peptides and as stable isotope labeled internal standards.

2.2. Crude protein content

The nitrogen content of the flours was determined in triplicate by the Dumas combustion method (ICC Standard No 167) using a Dumatherm nitrogen analyzer (Gerhardt Instruments, Königswinter, Germany). The crude protein content was calculated using a factor of 5.71.

2.3. Protein composition

ALGL, gliadins and glutenins were extracted from the flours and analyzed by RP-HPLC according to Jahn et al. (2024). The experiments were carried out in triplicate for each flour sample. The injection volumes were adjusted so that the resulting areas were in the middle range of the calibration. Typical injection volumes were: ALGL 20–30 μL, gliadins 5–10 μL, glutenins 20–50 μL. The content of ALGL, gliadins and glutenins was calculated using the corresponding total peak area. The ω5-gliadins, ω1,2-gliadins, α-gliadins and γ-gliadins were quantitated based on their percentage of the total peak area of gliadins. The HMW-GS, LMW-GS and ωb-gliadins were quantitated based on their percentage of the total peak area of glutenins.

2.4. Amylase/trypsin-inhibitor content

ATIs were extracted and measured with the stable isotope dilution analysis liquid chromatography tandem mass spectrometry (LC-MS/MS) method exactly as reported by Jahn et al. (2025). Each extraction step was carried out in triplicate. The measurement of the samples was performed in a randomly chosen order. Injection volumes were 2 μL for the samples and 10 μL for the response.

2.5. Amylase inhibitory activity against α-amylase

The α-amylase inhibitory activity of ATIs was measured with the

Table 1
Overview of wheat varieties and crude protein content of the flours. The values for crude protein content are given separately for the three harvest years and as mean of the three years. Some varieties were not available (n.a.) in every year. The values of the common wheat (CW) landraces were already reported by Jahn et al. (2024) and are included for comparison as the averaged value of all samples (n = 14).

Varieties	Abbreviation	Species	Ploidy level	Protein content [%]			
				2021	**2022**	**2023**	**Mean**
Common wheat landraces (n = 14)	CW	*T. aestivum* ssp. *aestivum*	hexaploid	9.0	11.5	8.9	9.8
Babenhausener Zuchtvesen	BZV	*T. aestivum* ssp. *spelta*	hexaploid	10.5	14.7	10.3	11.8
Muellers Gaiberger	MGA	*T. aestivum* ssp. *spelta*	hexaploid	8.2	11.7	8.0	9.3
Weihenstephan Emmer 1	WEM1	*T. turgidum* ssp. *dicoccon*	tetraploid	14.0	12.9	10.2	12.4
Weihenstephan Emmer 2	WEM2	*T. turgidum* ssp. *dicoccon*	tetraploid	14.1	11.8	7.8	11.2
Winterdurum Sambadur	WDS	*T. turgidum* ssp. *durum*	tetraploid	7.3	14.7	8.2	10.1
Khorasan wheat	KSW	*T. turgidum* ssp. *turanicum*	tetraploid	12.9	12.9	12.6	12.8
Polish wheat	POW	*T. turgidum* ssp. *polonicum*	tetraploid	n.a.	11.1	14.0	12.6
Wild einkorn	WEK	*T. monococcum*	diploid	13.5	11.8	n.a.	12.7
Enkidu	ENK	*T. monococcum*	diploid	n.a.	10.9	9.8	10.3
Terzino	TER	*T. monococcum*	diploid	n.a.	11.2	10.2	10.7

same method used by Jahn et al. (2025) using the EnzCheck Ultra Amylase Assay Kit followed by fluorescence measurement at an excitation wavelength of 485 nm and an emission wavelength of 515 nm as a continuous determination. All samples were extracted and measured in triplicate. Dilution factors of the samples were between 20 and 200 to ensure linear product formation.

2.6. Baking tests

The baking tests were performed as reported in Jahn et al. (2024). The bread volume was determined by the rapeseed displacement method. For certain varieties, baking trials could not be performed because of a shortage of harvest material.

2.7. Statistical analysis

Microsoft Office Excel 2016 (Microsoft Corporation, Seattle, WA, USA) was used for the calculation of mean values and standard deviations. OriginPro 2023 (OriginLab, Northampton, MA, USA) was used for statistical evaluation. Pearson correlation analysis was performed to identify linear relations between parameters. Correlation coefficients (r) were defined as follows: $\pm 0.54 < r \leq \pm 0.67$: weak correlation; $\pm 0.67 < r \leq \pm 0.78$: medium correlation; $\pm 0.78 < r \leq \pm 1.00$: strong correlation (Thanhaeuser et al., 2014). Analyses of variance (ANOVAs) were performed with Tukey's test ($p < 0.05$). Significant differences between the ploidy level of the varieties were identified by one-way ANOVA. The influence of harvest year and ploidy level was analyzed by two-way ANOVA. The common wheat samples were included in all two-way ANOVAs. Principal component analysis (PCA) was applied to reduce data dimensionality and identify relations between the analyzed parameters.

3. Results and discussion

3.1. Crude protein content

The crude protein content of all samples from all three harvest years and the respective mean is displayed in Table 1. The single contents varied between 7.3 % (WDS in 2021) and 14.7 % (BZV and WDS in 2022). Of all the varieties, which were harvested in all three years, the spelt varieties BZV and MGA and the durum wheat variety WDS had the highest protein content in 2022. The averaged content of all common wheat landraces that were part of the same field trial reported by Jahn et al. (2024) was also highest in 2022 (11.5 % compared to 9.0 % in 2021 and 8.9 % in 2023). Both emmer varieties (WEM1 and WEM2) had the highest crude protein content in 2021 (14.0 % and 14.1 %). POW, ENK, TER and WEK were only harvested in two years. The variety POW had a higher protein content in 2023 (14.0 %) than in 2022 (11.1 %). The einkorn samples ENK and TER had a higher protein content in 2022 (10.9 % and 11.2 %) than in 2023 (9.8 % and 10.2 %). WEK had a higher protein content in 2021 (13.5 %) than in 2022 (11.8 %). The protein content for KSW was consistent across the three years with 12.9 % in both 2021 and 2022 and 12.6 % in 2023. With 12.8 %, this variety also had the highest mean protein content. The other tetraploid varieties had similar mean contents: POW had 12.6 %, WEM1 12.4 %, WEM2 11.2 % and WDS 10.1 %. The einkorn varieties ENK (10.3 %) and TER (10.7 %) had a slightly lower protein content than WEK (12.7 %). The spelt sample MGA had the lowest mean protein content (9.3 %), similar to the one of the common wheat landraces analyzed earlier (9.8 %) (Jahn et al., 2024). The other spelt sample (BZV) had a mean value of 11.8 %. A two-way ANOVA showed that neither the ploidy level ($F = 1.51$; $p = 0.245$) nor the harvest year ($F = 3.30$, $p = 0.058$) had a significant influence on the crude protein content.

Geisslitz et al. (2018) also analyzed eight varieties each of common and durum wheat, spelt, emmer and einkorn regarding their protein content and composition. The crude protein content was in the same range as our results. The durum wheat varieties had the highest content (13.0–15.5 %). The content of common wheat was between 9.3 % and 13.3 % and that of spelt between 10.8 % and 16.1 %. Emmer and einkorn varieties had contents of 11.2–12.4 % and 11.6–13.9 %, respectively. Call et al. (2020) also found similar contents for these varieties. They analyzed 33 common wheat, six durum wheat, nine spelt, four emmer and seven einkorn varieties. The highest contents were reported for einkorn (15.5–19.8 %), emmer (14.9–16.9 %) and spelt (14.9–18.3 %). The protein content of durum wheat was between 12.7 % and 16.4 %, that of common wheat between 11.9 % and 16.8 %. Bieńkowska et al. (2020) analyzed 17 varieties of Polish wheat that had a mean protein content of 16.6 %. Di Loreto et al. (2017) analyzed Khorasan varieties which were grown between 1989 and 2012. The crude protein contents of the 21 varieties ranged between 12.3 % and 23.7 %. Compared to those studies our values were lower. One explanation for this might be the lack of fertilization, because the cultivation was carried out under organic growing conditions.

3.2. Albumins and globulins, gliadins and glutenins

Since there were no major differences between the three harvest years (Table S1+2) and to see differences between the varieties regardless of environmental factors, the protein composition is displayed as the mean of all three harvest years (Fig. 1). The ALGL proportion was similar for hexaploid, tetraploid and diploid species. It varied from 15.3 % (BZV) to 20.1 % (WEM2). The gliadins ranged from 60.2 % (MGA) to 78.5 % (POW). The tetraploid variety POW had the highest gliadin proportion, followed by the three diploid varieties TER (75.5 %), ENK (72.0 %) and WEK (70.9 %). All the other tetraploid varieties had slightly higher gliadin proportions (65.4–70.4 %) compared to the hexaploid ones (60.2–66.0 %). For the glutenins, it was the other way round. The glutenin proportions of the hexaploid species

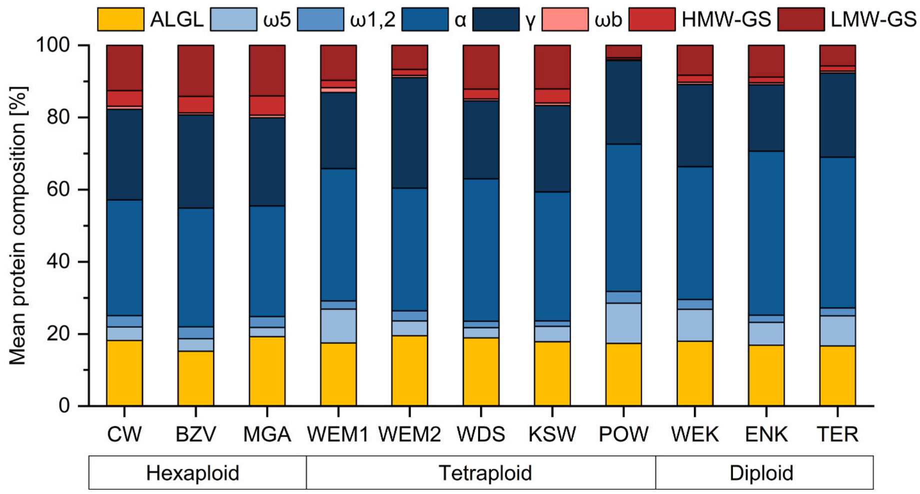

Fig. 1. Protein composition as mean of all three harvest years. Protein fractions are displayed relative to the total protein content (sum of all fractions) and include albumins/globulins (ALGL), gliadins and glutenins. Gliadins are displayed as ω5-gliadins (ω5), ω1,2-gliadins (ω1,2), α-gliadins (α) and γ-gliadins (γ). Glutenins include ωb-gliadins (ωb), high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS). Fourteen common wheat (CW) landraces were analyzed by Jahn et al. (2024) and are displayed as mean of all varieties. Abbreviations for the varieties can be found in Table 1.

were higher (17.4–20.4 %) than those of the diploid (7.8–11.1 %) and tetraploid (4.1–16.7 %) ones. Within the tetraploid species, the variety POW stood out again with the lowest glutenin proportion (4.1 %). Geisslitz et al. (2018) also found an increasing gliadin and decreasing glutenin proportion from the hexaploid to the tetraploid and diploid varieties.

There was no significant influence of ploidy level ($F = 0.64$; $p = 0.534$) or harvest year ($F = 1.78$, $p = 0.190$) on the proportion of ALGL. For gliadins, the effect of ploidy level was significant ($F = 6.70$; $p = 0.005$), while that of the harvest year was not ($F = 1.44$; $p = 0.257$). For glutenins, both the ploidy level ($F = 8.18$; $p = 0.002$) and the harvest year ($F = 4.60$; $p = 0.020$) had a significant effect.

3.3. Gluten protein types

The gluten composition was examined more closely (Table S2). The proportions of α-gliadins ranged from 30.4 % (MGA) to 45.3 % (ENK). The diploid species had the highest proportions followed by the tetraploid ones and the hexaploid ones with the lowest proportions. The share of γ-gliadins ranged from 18.3 % (ENK) to 29.9 % (WEM2) and was similar for all groups. The proportion of ω5-gliadins had values of 2.6 % (MGA) to 11.4 % (POW). The landrace POW had the highest proportion of ω5-gliadins, but the diploid varieties and the variety WEM2 also had higher values (6.4–9.2 %) than the rest of the varieties (2.6–4.3 %). The ω1,2-gliadins constituted the lowest share of all the gliadin protein types (1.5–3.3 %). Similar results were obtained by Geisslitz et al. (2018). They also found decreasing α-gliadin proportions from the diploid species to the tetraploid and hexaploid ones. The γ-gliadins were also lowest in the einkorn and highest in the emmer varieties. Furthermore, the ω5-gliadins were also highest in the einkorn varieties.

The LMW-GS had the largest proportion and the greatest range of the glutenins. It varied between 3.4 % (POW) and 14,4 % (MGA). The proportion of ωb-gliadins ranged from 0.2 % (POW) to 1,2 % (WEM1) and that of HMW-GS from 0.6 % (POW) to 5.3 % (MGA). The HMW-GS had the highest proportions in the hexaploid species. This was also observed by Geisslitz et al. (2018).

There was no significant influence of the harvest year on the proportions of any of the gluten protein types ($F \leq 2.57$; $p \geq 0.097$), except for the HMW-GS ($F = 5.34$; $p = 0.012$) and the ωb-gliadins ($F = 6.91$; $p = 0,004$). The effect of the ploidy level was also not significant for γ-gliadins, ωb-gliadins and LMW-GS ($F \leq 3.19$; $p \geq 0.059$), whereas it was significant for α-gliadins ($F = 14.46$; $p < 0.001$), ω5-gliadins ($F = 4.84$; $p = 0.017$), ω1,2-gliadins ($F = 4.73$; $p = 0.019$) and HMW-GS ($F = 14.25$; $p < 0.001$). Therefore, the gluten protein composition differed primarily according to the genetic background.

3.4. Gliadin/glutenin ratio and bread volume

The hexaploid species had the lowest gliadin/glutenin ratio (GLIA/GLUT) (Table S3). The spelt varieties BZV and MGA had mean values of 3.7 and 3.0, similar to the average of the 14 common wheat landraces with 3.7. The tetraploid varieties KSW and WDS had slightly higher mean GLIA/GLUT (4.6 and 5.3). WEM1 and WEM2 had mean values of 7.1 and 7.5 and, therefore, lay in the same range as the diploid einkorn samples (6.7, 7.0 and 9.9). POW had the highest GLIA/GLUT with a mean value of 19.1. These results are in accordance with those of Geisslitz et al. (2018). The common wheat and spelt varieties had the lowest ratios (2.0–3.2 and 2.8–4.0) followed by durum wheat (2.2–5.3), emmer (3.6–6.7) and einkorn (4.2–12.0). Ozuna and Barro (2018) also found GLIA/GLUT in the same range as ours. In their study, the hexaploid species had mean ratios of 2.3–3.6, tetraploid species of 2.5–6.7 and einkorn had the highest ratio with 7.8. The ratio of 4.7 reported for the Polish wheat varieties was around four times lower than the ratio in our variety POW. There was no significant influence of ploidy level ($F = 3.30$; $p = 0.057$) or harvest year ($F = 0.61$, $p = 0.555$) on GLIA/GLUT.

The highest bread volumes were obtained by the hexaploid species common wheat (628 mL) and spelt (588 and 615 mL), followed by the tetraploid species (385–530 mL), with KSW having the highest mean bread volume (530 mL) (Table S4). The lowest volumes were achieved by the diploid einkorn species with 345–400 mL. A correlation analysis revealed no correlation between the GLIA/GLUT and bread volume ($r = -0.40$), but the correlation diagram shows a clustering of the hexaploid and diploid species (Fig. 2).

3.5. Amylase/trypsin-inhibitor content

The total ATI content of all samples from the three harvest years varied between 0.6 mg/g (WEK, 2021 and ENK, 2022) and 9.5 mg/g (BZV, 2022) (Fig. 3A, Table S5A). All samples except the einkorn varieties (WEK, ENK and TER) and the tetraploid variety POW had the highest ATI content in 2022.

The spelt variety BZV had the highest ATI content in all three years (2021: 6.9 mg/g; 2022: 9.5 mg/g, 2023:6.5 mg/g), resulting also in the

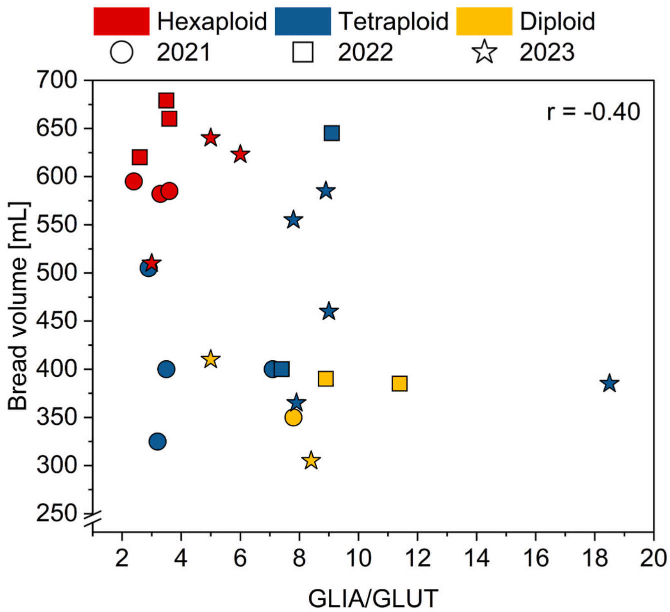

Fig. 2. Correlation diagram of bread volume and gliadin/glutenin ratio (GLIA/GLUT). The Pearson correlation coefficient (r) indicated no correlation between the two parameters. All included varieties can be found in Table S4. For some varieties no baking trials could be performed due to a shortage of harvest material.

highest mean value (7.6 mg/g). The other spelt sample MGA had the second highest mean content with 6.6 mg/g. The averaged content of all 14 common wheat landraces analyzed by Jahn et al. (2025) was 6.9 mg/g and thus between the mean content of the two spelt varieties. The hexaploid species therefore had the highest ATI content. KSW and WEM2 also had relatively high mean contents (5.9 mg/g and 6.0 mg/g) compared to WEM1 (4.4 mg/g). Since the contents for WEM1 and WEM2 were similar in 2022 (7.6 mg/g and 7.4 mg/g) and equal in 2023 (4.8 mg/g), the difference arose due to the low content of WEM1 in 2021 (0.8 mg/g), which is more similar to the values of the einkorn varieties ENK (0.6 mg/g in 2022 and 1.1 mg/g in 2023) and TER (0.9 mg/g in 2022 and 2023), as well as the content of WEK in 2021 (0.6 mg/g). The content of WEK 2022 (6.5 mg/g) was more than ten times higher compared to its content in 2021 and therefore the mean content was 3.6 mg/g, which was in the same range as those of WEM1 (4.4 mg/g), WDS (4.6 mg/g) and POW (3.3 mg/g). For POW, there were also large differences in the contents between the two harvest years. While it was 0.9 mg/g in 2022, it was more than six times higher in 2023 with 5.9 mg/g. A two-way ANOVA indicated that the ploidy level ($F = 13.28$; $p < 0.001$) had a bigger significant influence on the ATI content than the harvest year ($F = 3.57$; $p = 0.047$).

The proportions of ATI based on the crude protein content were between 0.4 % (WEK in 2021) and 7.5 % (MGA in 2022) and followed the same pattern as the absolute content. They were more consistent over the different years (Table S5B), because the crude protein was also the highest in 2022 for almost all samples (Table 1).

As ATIs are part of the ALGL, the ATI proportions based on the content of ALGL were also investigated. Because the ALGL contents were comparable (15.3–20.1 mg/g) and the ATI contents varied greatly within all samples, the ATI proportion based on ALGL also showed a wide range. The two einkorn varieties ENK and TER had the lowest proportions of around 5 %. POW, WEK and WEM1 had proportions of 13.5–17.4 %, followed by WDS, KSW and WEM2 with values of 22.3–26.1 %. The hexaploid species spelt and the common wheat samples had the highest proportions, ranging from 35.3 % to 38.1 %.

Geisslitz et al. (2020) analyzed eight varieties of common and durum wheat, emmer, spelt and einkorn. The contents were in a similar range as ours with 0.2 mg/g to 6.6 mg/g. They also found spelt to be the species with the highest ATI content, followed by common wheat and emmer and then durum wheat, which was confirmed by another study (Sielaff et al., 2021). The contents of the einkorn varieties were also the lowest with around 0.2 mg/g. Common wheat and spelt varieties also had the highest content in the study by Call et al. (2020), followed by emmer, durum wheat and einkorn. These studies, including ours, did not find any evidence that ancient wheat varieties contain less ATIs compared to landraces or modern wheat varieties.

3.6. Amylase/trypsin-inhibitor distribution

The distribution of the different ATIs was similar within each of the hexaploid, tetraploid and diploid varieties, respectively (Fig. 4, Tables S6–S17). The 14 hexaploid common wheat landraces reported by Jahn et al. (2025) had a comparable ATI distribution to the two hexaploid spelt varieties in all three years. The ATIs 0.19 and CM3 had the highest proportions with around 50 % in total. CM17, CM16, 0.28 and CM2 each contributed around 10 % to the total ATIs. CM1 and 0.53 contributed to about 5 % each, while CMX1/2/3, WCI, WASI and WTI made up the last 10 %.

The tetraploid varieties WEM1 (in 2022 and 2023), WEM2, WDS and POW (in 2023) had a similar ATI distribution, respectively. Compared to the hexaploid species, the proportions of CM3 and 0.19 were lower ranging from around 30–40 %. The proportions of CM17 and CM1 were also lower with around 5 % and 1 %. In contrast, the proportions of CM16 and CM2 were higher with around 20–25 %, respectively. The proportion of 0.53 was twice as high with around 10 %. CMX1/2/3, WCI, WASI and WTI had a similar proportion (ca. 10 %) in the tetraploid and the hexaploid species.

All in all, the variation in ATI distribution between the three harvest years was much greater in the tetraploid species than in any of the hexaploid ones. In 2021, the variety KSW had similar proportions to all the other tetraploid varieties, but in 2022 and 2023, the ATI composition was more similar to the hexaploid varieties. The variety WEM1 was also noticeable in 2021, where it had a much higher proportion of 0.19 compared to the other two years and therefore a lower proportion of CM3. Further, the proportions of CM16, 0.28 and CM2 were lower, whereas the proportions of CM17, CM1, CMX1/2/3 and WASI were much higher compared to 2022 and 2023. The variety POW in 2022 had a similar distribution to the variety WEM1 in 2021 and therefore also showed a different ATI distribution compared to 2023. These two varieties in these particular years had a higher proportion of CMX1/2/3 and WASI, which is characteristic for einkorn varieties (Geisslitz et al., 2020). The proportions of the aforementioned ATIs were also higher in the three diploid einkorn varieties (WEK, ENK, TER) compared to the other varieties in our study. The WTI had mean proportions of 1.4–1.7 % and the CMX1/2/3 of 13.2–15.2 %. There were also differences within the einkorn species. The two varieties ENK and TER had a similar ATI composition. The ATIs 0.19 and CM3 together accounted for around 40 %. CM17 and CM16 contributed about 10 % each, while the proportions of 0.28 and CM2 were a bit lower. The proportions of CM1 were similar to those of the hexaploid species with around 4 %, while 0.53 was not detectable in the two varieties. Around 15 % belonged to the CMX1/2/3 and 5–10 % to the WCI. The remaining 5 % consisted of WTI and WASI. The main difference to the variety WEK was that 0.53 was present in both years. In contrast, the proportion of this ATI was below the limit of detection in ENK and TER. However, the ATI distribution of WEK also differed a lot between the two harvest years. In 2022, the distribution was similar to the tetraploid variety KSW in 2022 and 2023. In 2023, it was similar to the hexaploid varieties spelt and common wheat. In general, the samples which differed a lot in the ATI distribution in one harvest year (WEM1 2021, POW, 2022; WEK, 2022) also had differences in the total ATI content in this specific year. The reasons for this variation are unknown and require further investigation.

Geisslitz et al. (2020) also found that the hexaploid species common

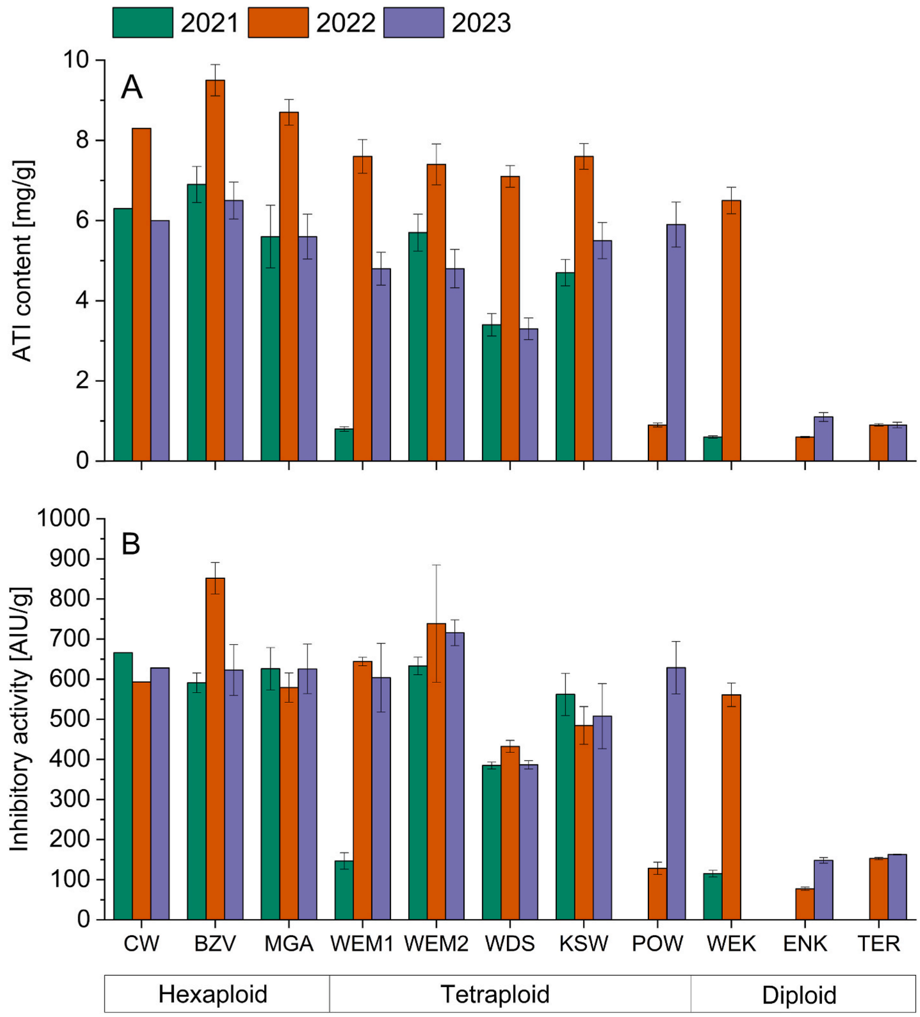

Fig. 3. Amylase/trypsin-inhibitor (ATI) content (A) and inhibitory activity against α-amylase (B) of all samples from all harvest years. Fourteen common wheat (CW) landraces were analyzed by Jahn et al. (2025) and are displayed as mean of all varieties. Abbreviations for the varieties can be found in Table 1.

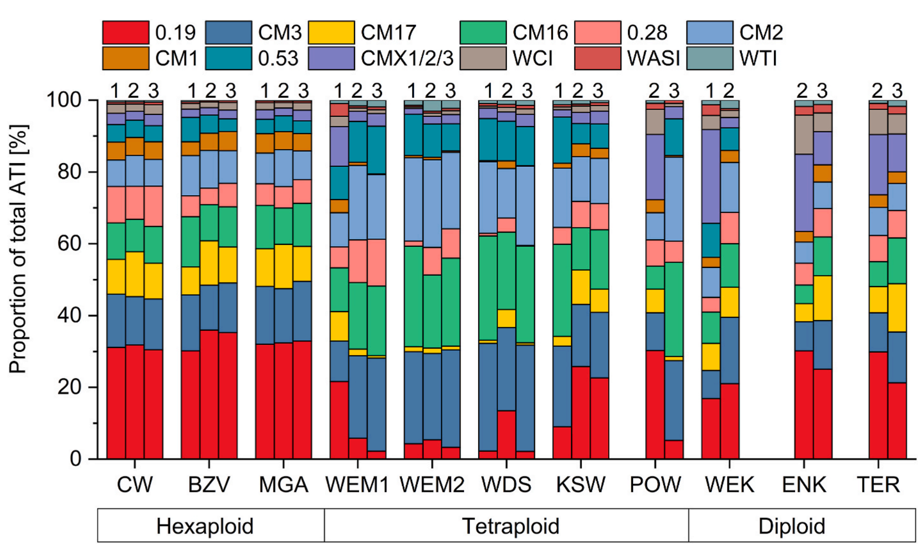

Fig. 4. Amylase/trypsin-inhibitor (ATI) proportions based on the total ATI content of all samples from all harvest years. 1: year 2021; 2: year 2022; 3: year 2023; CM: chloroform/methanol; WASI: wheat amylase subtilisin inhibitor; WCI: wheat chymotrypsin inhibitor; WTI: wheat trypsin inhibitor. Fourteen common wheat (CW) landraces were analyzed by Jahn et al. (2025) and are displayed as average of all varieties. Abbreviations for the varieties can be found in Table 1.

wheat and spelt and the tetraploid species durum wheat and emmer had a similar distribution of ATIs, respectively. Although the contents were lower than in the other species (e.g., for 0.19 around 10-fold higher in the hexaploid species) we were able to quantitate all ATIs, except for 0.53 in all einkorn varieties. In contrast to our study, Geisslitz et al. (2020) only detected CMX1/2/3 and WASI in all einkorn varieties. They also found that the varieties had similar distributions at different locations. Simonetti et al. (2022a) used ten wheat genotypes for gene sequencing of four representative ATI (WMAI, WDAI, WTAI-CM3, CMX) genes. They confirmed that these varieties can be assigned to diploid, tetraploid or hexaploid species according to the distribution of the different ATIs. All in all, the studies (including ours) concluded that it was possible to differentiate between the ploidy levels on the basis of the ATI composition.

3.7. Amylase inhibitory activity

The inhibitory activity against porcine pancreas α-amylase was strongly dependent on the variety and ranged from 77 to 852 AIU/g, resulting in mean values over all three years ranging from 113 to 696 AIU/g (Fig. 3B–Table S18). Here as well, a two-way ANOVA indicated that the ploidy level ($F = 11.28$; $p < 0.001$) had a more pronounced and significant influence on the inhibitory activity than the harvest year ($F = 0.42$; $p = 0.663$).

The lowest mean inhibitory activity was measured in the two einkorn varieties ENK and TER. ENK had activities of 77 AIU/g (2022) and 148 AIU/g (2023), resulting in a mean activity of 113 AIU/g. For TER, these activities were 153 AIU/g (2022) and 163 AIU/g (2023), which led to a mean activity of 158 AIU/g. There were three other varieties with similar activities, but only in one of the harvest years. The emmer variety WEM1 and the einkorn variety WEK had an activity of 147 AIU/g and 115 AIU/g in 2021, respectively. For WEM1 in the other two years these values were 644 AIU/g (2022) and 604 AIU/g (2023), respectively. The inhibitory activity for WEK in 2022 was 561 AIU/g. The last variety with a low activity was POW in 2022 (128 AIU/g). In 2023, the activity was 629 AIU/g. This resulted in similar mean values of 465 AIU/g for WEM1, 338 AIU/g for WEK and 379 AIU/g for POW.

The highest activity with 852 AIU/g was found for the spelt variety BZV in 2022. In 2021 (591 AIU/g) and 2023 (623 AIU/g) the inhibitory activities were slightly lower, resulting in a mean value of 688 AIU/g. The mean value of the other spelt sample (MGA) was similar (610 AIU/g). This variety had activities of 626 AIU/g (2021 and 2023) and 579 AIU/g (2022) in the three harvest years. Therefore, the spelt samples had activities in the same range as those of the hexaploid common wheat samples analyzed by Jahn et al. (2025) (mean of all three years: 629 AIU/g). The tetraploid varieties KSW and WDS had similar activities in all three years, resulting in mean values of 518 AIU/g and 401 AIU/g. The emmer variety WEM2 had the highest mean activity with 696 AIU/g.

Similar results were obtained by several other studies. In our earlier study (Jahn et al., 2023), we found that the hexaploid species spelt and common wheat had the highest inhibitory activities followed by the tetraploid species emmer and durum wheat. However, there were few (emmer) varieties which showed similar activities to the hexaploid species. No activities were found in the einkorn samples. Although Gélinas and Gagnon (2018) used a different method for determining the inhibitory activity, their results are in accordance with ours, showing similar activities for Khorasan, emmer, durum wheat, spelt and common wheat samples. Simonetti et al. (2022b) used yet another assay, but still obtained similar results. They analyzed varieties of common and durum wheat, spelt, einkorn, emmer and Khorasan harvested in 3 years at two different locations each. Landraces and modern varieties were included in the sample set. In their study, the inhibitory activity was strongly dependent on the harvest year, the location and the variety. No trend could be observed except that the hexaploid varieties showed the highest and einkorn the lowest activities. Zevallos et al. (2017) investigated the bioactivity of ATIs in murine and human cell lines. They found that emmer, einkorn, spelt and Khorasan had a lower ATI inflammatory activity than modern common wheat. All in all, these studies on the inhibitory activity agree with the results from the other analyses, that the differences between the ancient wheats is associated with the ploidy levels.

3.8. Principal component analysis

A principal component analysis (PCA) was carried out with all data of all years to summarize our findings of the different analyses (Fig. 5). PC1 and PC2 explained 61.6 % of the total variance. The first PC was predominantly influenced by the ATI proportion, as well as the proportions of gliadins, ω5-and α-gliadins, while the second one was associated with the proportion of LMW-GS and ωb-gliadins. The varieties were distributed equally in all directions. Certain trends were noticed for the different ploidy levels. All hexaploid species were clustered in the

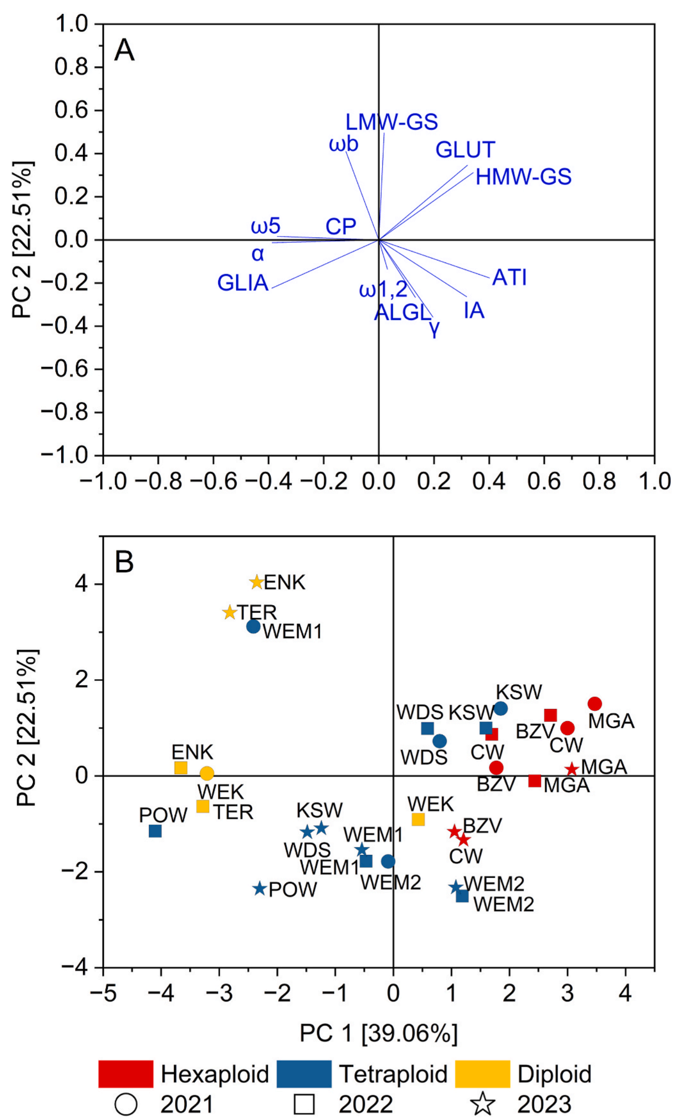

Fig. 5. Loadings plot (A) and scores plot (B) of the principal component analysis (PCA) of all samples. Included are crude protein (CP) content, inhibitory activity (IA), proportions of amylase/trypsin-inhibitors (ATI), albumins and globulins (ALGL), gliadins (GLIA), glutenins (GLUT), α-gliadins (α), γ-gliadins (γ), ω5-gliadins (ω5), ωb-gliadins (ωb), ω1,2-gliadins (ω1,2), high- (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS). Abbreviations for the varieties can be found in Table 1.

center of the positive side of PC1. Almost all tetraploid species were clustered at the bottom in the center of the negative side of PC2, with a few exceptions. WDS and KSW from 2021 to 2022 were in a separate cluster on the positive axis of PC1 and PC2, close to the hexaploid species. Further, the variety WEM1 from 2021 was found next to the einkorn varieties ENK and TER from 2023. A little farther away were the einkorn varieties ENK and TER from 2022 and WEK from 2021, leaving the variety WEK from 2022 in the cluster of the hexaploid species. Some trends could be observed for the different harvest years as well. For example, the spelt variety MGA and the emmer variety WEM2 were in similar spots in all three years. Taken together, it was also visible in the PCA that three varieties stood out in some analyses (WEM1 2021, WEK 2022 and POW 2022).

The variable loadings were also distributed equally in all directions. It was noticeable that the ones of the glutenins and HMW-GS pointed in the same direction, revealing a strong correlation ($r = 0.83$) (Fig. S1). A medium correlation was found for the proportion of gliadins and α-gliadins ($r = 0.74$). Moreover, the loadings of the ATI content and the inhibitory activity pointed in the same direction. Pearson correlation analysis of the two parameters ATI content and inhibitory activity resulted in a correlation coefficient of $r = 0.90$, which indicates a strong correlation. This is in contrast to the findings of Jahn et al. (2023) and (2025), where no correlation could be found. However, there are some possible explanations for this discrepancy. Compared to the study of 2025, where only common wheat varieties were analyzed, this study included different wheat varieties, resulting in a much broader range of values. Compared to the study of 2023, this sample set was a lot smaller, which is a limitation of this study. Another weakness of this study is that there were only few varieties available for each wheat species.

4. Conclusion

The aim of this study was to find out, if ancient wheat species grown under organic cultivation might be beneficial for individuals with wheat-related disorders. Therefore, the protein, gluten and ATI content and composition as well as the inhibitory activity were analyzed in ancient wheats of different ploidy levels of three consecutive harvest years. In addition, the bread volume was determined. The different analyses all came to the same conclusion that the differences in protein composition were attributed to the ploidy level. These findings suggest that ancient wheats are unlikely to offer improved tolerability for individuals with wheat-related disorders. We identified a few varieties that stood out, especially in terms of ATI distribution, but these characteristics only occurred in one year and therefore seem to be dependent on environmental factors. Nevertheless, ancient wheats can serve as a valuable alternative to modern varieties, particularly in organic farming, as they provide genetic diversity, can be grown more sustainably and are well-suited to regional conditions. Especially spelt might be interesting for specialty products. Like common wheat, spelt is hexaploid and our analyses showed that the spelt varieties were similar to the common wheat samples analyzed previously.

Compliance with ethics requirements

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

CRediT authorship contribution statement

Nora Jahn: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ulla Konradl:** Writing – review & editing, Resources. **Klaus Fleissner:** Writing – review & editing, Resources. **Sabrina Geisslitz:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition. **Katharina A. Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Informed consent

Not applicable.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2025.104185.

Data availability

The processed data required to reproduce the above findings are available within the manuscript and its supplement. Mass spectrometry data are publicly available on Panorama Public (https://panoramaweb.org/619dLa.url).

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